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The effect of binge alcohol consumption on the development of adolescent Sprague Dawley rat mandibles

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THE EFFECT OF BINGE ALCOHOL CONSUMPTION ON THE DEVELOPMENT OF ADOLESCENT SPRAGUE DAWLEY RAT MANDIBLES

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Student number: 2144943

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy in Anatomy

Johannesburg, (July) 2025

DECLARATION

I, Akaashni Nareschandra Bhika declare that this dissertation entitled “The effect of binge-alcohol consumption on the development of adolescent Sprague Dawley rat mandibles” is my own work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Akaashni Nareschandra Bhika

July 2025

CONFERENCE PRESENTATIONS

1. Bhika, A., Pillay, D., Ihunwo, A.O. A preliminary study on the effect of binge-alcohol consumption on the adolescent *Sprague Dawley* rat mandible development. 49th Anatomical Society of Southern Africa (ASSA) conference. 25-28 October 2022. Poster presentation.
2. Bhika, A., Ihunwo, A.O., Pillay, D. Impact of binge alcohol consumption on the cytoarchitecture and remodeling of the mandible in adolescent Sprague Dawley (SD) rats. Faculty of Health Sciences, University of the Witwatersrand, Biennial Research Day. 5 September 2024. Poster presentation.

PUBLISHED AND ACCEPTED ARTICLES

1. Bhika, A., Pillay, D., Ihunwo, A.O. (2024). Effect of a Dose-Dependent Administration of Binge Alcohol on the Mandible in the Adolescent Sprague Dawley Rat. *International Journal of Morphology,* 1(2):607-613
2. Bhika, A., Pillay, D., Ihunwo, A.O. (2024). Examining the Impact of Acute Binge Alcohol Consumption on Trabecular Morphometry and Tensile Strength in Adolescent Sprague Dawley Rat Mandibles. *International Journal of Morphology,* 42(4):905-910
3. Bhika, A., Ihunwo, A.O., Pillay, D. (2025). The effects of acute and chronic binge drinking followed by alcohol abstinence on mandibular morphometry in female adolescent Sprague Dawley rats has been accepted for publication in *International Journal of Morphology*, 43(1):41-46

DEDICATION

"To my parents, who taught me the value of hard work and education, and to my husband, whose patience, assistance, and love sustained me throughout this journey."

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This has been an immense time of learning for me, both academically and personally. Completing this PhD journey has been as much about personal growth as it has been about academic achievement, and it could not have been done without the help and support of numerous people.

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ABSTRACT

Underage binge drinking has become a major public concern as it can have a negative impact on the growth and development of the adolescent skeleton, affecting many people worldwide, including in South Africa. Peak bone mass is attained during adolescence and any disturbances during this stage can lead to long term consequences on the skeleton. Little is known about the effects of binge drinking on the craniofacial structures such as the mandible, which is essential for mastication and is also involved in processes such as speech, facial expression, and dentition. Improving our understanding of the effects of binge drinking on the adolescent mandible can help us identify potential intervention measures and raise public awareness about the dangers of alcohol use. The present study was conducted to investigate the impact of acute and chronic binge drinking on mandibular parameters in adolescent Sprague Dawley (SD) rats and to evaluate if recovery of the mandible is possible once alcohol is removed from the diet.

The study involved seventy-two (36 male and 36 female) Sprague Dawley rats, aged 7 weeks, divided into either an alcohol-exposed group or a pair-fed control group for acute binge, chronic binge, or alcohol abstinence models for both males and females. The alcohol-exposed group received 3 g/kg of 20 % alcohol 3 days a week for 7 or 28 days, followed by a 30-day abstinence period in the abstinence model. The pair-fed control groups received a caloric equivalent dose of maltose dextrin. The animals were euthanized on day 7, 28, or 54 using pentobarbital injections. Food and water intake, body mass gain, mandibular osteometry, trabecular morphometry (using 3D-MicroCT analysis), biomechanical tensile testing, cytoarchitecture and immunohistochemistry using the Ki-67 and ALP proteins were analysed.

Results indicated that alcohol-exposed female rats ate less during acute binge drinking but increased their food and water intake after abstinence. In males, alcohol reduced water consumption during the abstinence phase. Body mass changes mirrored food intake: female rats lost weight during acute binge drinking but gained after abstinence, while males exhibited decreased body mass after abstinence. Mandibular measurements indicated increased lower mandibular length (M3-Id) in alcohol-exposed males during acute binge drinking and greater ramus height (Cd-Go) in females. In the chronic model, females had increased coronoid height (Cr-Ag), while abstinent animals, especially females, exhibited increases in anterior mandibular dimensions (MF-Al^{I} and MF-M1). Trabecular parameters were disrupted across all binge models, with females most affected, especially after chronic exposure. These changes, involving bone volume to total volume ratio (BV/TV), trabecular thickness (TbTh), trabecular number (TbN), and trabecular spacing (TbSp), persisted even after abstinence, indicating lasting effects. Tensile strength was unchanged in the acute model but decreased in alcohol-exposed males after chronic binge drinking, with no female changes. After abstinence, males displayed no differences, while females had increased maximum and break forces. Across all three groups, alcohol exposure increased trabecular spacing and resulted in less mature bone with disorganized, wavy collagen in both male and female animals compared to the pair-fed controls. Acute binge drinking did not alter cell proliferation, while chronic exposure reduced it in both sexes. After abstinence, proliferation remained decreased in females but was unchanged in males. Binge alcohol exposure reduced osteoblast numbers, affecting only females in the acute model and both sexes in the chronic and abstinent models, with a marked decline in males after abstinence.

Differences between male and female animals was evident throughout the parameters analysed in this study, where alcohol had a more pronounced effect on females when compared to males, highlighting sexual dimorphism.

We concluded that binge alcohol exposure negatively impacts the adolescent skeleton across all models, with more severe and lasting effects after prolonged exposure. The female mandible was particularly vulnerable, showing persistent damage even after alcohol withdrawal.

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LIST OF ABBREVIATIONS

%:	Percent
°C:	Degree celsius
µm:	micrometer
dL:	deciliter
g:	gram
kg:	kilogram
kcal:	kilocalories
kV:	kilovolt
L:	litre
ml:	millilitre
mg:	milligram
mm:	millimeter
min	minute
nm:	nanometer
µA:	microAmpere
µCT:	Microfocus x-ray computed tomography
µm:	micrometer
3D:	Three-dimensional

Ag:	Ante-gonion
ALP:	Alkaline phosphatase
APES:	3-aminopropyltriexothysilane
AREC:	Animal Research Ethics Committee
AS:	angular synchondrosis
BAC:	Blood alcohol concentration
BMD:	bone mineral density
BV/TV:	Bone volume to total volume
C:	Canine
Cd:	Condylar head
Cr:	Coronoid
CSC:	condylar secondary cartilage
DAB:	3-3’ di-amino benzidine
DALYs:	Disability-Adjusted Life Years
E:	Embryonic
EDTA:	Ethylenedeaminetetraacetic acid
FAS:	Fetal Alcohol Syndrome
Fig:	Figure
GH	Growth hormone

Go:	Gonion
H_2O_2:	Hydrogen peroxide
H&E:	Haematoxylin and eosin
IHC:	Immunohistochemistry
I:	Incisor
Id^I:	Infradentale
M:	Molar
M1:	Molar 1
M3:	Molar 3
Me:	menton
MF:	Mental foramen
MS:	mandibular midline synchondrosis
MVA:	Motor vehicle accident
NECSA:	Nuclear Energy Association of South Africa
OPG	Osteoprotegerin
P:	Premolar
PBS:	Phosphate buffer solution
PFA:	Paraformaldehyde
PTH	Parathyroid hormone

ROI:	region of interest
SA:	South Africa
SD:	Sprague Dawley
SPSS:	Statistical Package for Social Service
TbTh:	Trabecular thickness
TbN:	Trabecular number
TbSp:	Trabecular spacing
WRAF:	Wits Research Animal Facility

CHAPTER 1: *Introduction, Aims and Objectives, and Thesis Layout*

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1 INTRODUCTION

1.1 Background to the study

The prevalence of excessive alcohol consumption constitutes a significant problem in South Africa (SA) and worldwide (Morojele and Ramsoomar, 2016). Adolescent alcohol consumption in SA is also a significant concern. The drinking pattern of this group is commonly binge or heavy episodic (Morojele and Ramsoomar, 2016). Binge drinking in adolescents is a risk factor for long-term progression to excessive chronic alcohol consumption in adulthood. Binge drinking is a large risk factor for alcohol-related problems such as motor vehicle accidents (MVAs) and deaths, interpersonal violence, crime, sexual risk which leads to sexually transmitted diseases, and maternal drinking which leads to fetal alcohol syndrome (Kuntsche *et al.*, 2017; Morojele and Ramsoomar, 2016). The above alcohol-related problems all have a negative impact on the economy (Morojele and Ramsoomar, 2016). Alcohol consumption in adolescents may contribute to decreased bone density and osteoporosis later in life, resulting in a higher likelihood of bone fractures. Clinically, periodontal disease that results in alveolar bone loss can also occur. This affects both bone quality and quantity, causing adverse effects on implant placement and stability (Bannach *et al.*, 2015). Binge drinking is likely to negatively affect the growth of the mandible in adolescents, which could play a role in facial aesthetics, airway problems, and complications with lack of space in the dentition. Binge drinking also lowers inhibitions and can lead to interpersonal violence and MVAs which increase the risk of maxillofacial fractures and their severity and healing time (Lee and Snape, 2008; O'Meara *et al.*, 2012).

Adolescence is a period of rapid skeletal growth and development between the ages of 10 and 19 years. Adolescence is divided into early adolescence (10-15 years), late adolescence (15-19 years), and young adulthood (20-24 years) (Das *et al.*,2017). Approximately 90 % of skeletal development and peak bone mass is achieved during adolescence. Insufficient peak bone mass greatly increases the risk of developing osteoporosis later in life (Das *et al.*, 2017; Monge, 2018; Weaver, 2002). Peak bone mass is primarily determined by genetics but can be affected by nutritional, environmental hormonal, and lifestyle factors, including diet, alcohol, and tobacco consumption (Sampson, 1998). Adolescents are thus recognized as a nutritionally at-risk group, as numerous factors can influence their growth and development.

The main mode of drinking in adolescents is binge drinking. Binge drinking, which is also referred to as heavy episodic drinking or risky single-occasion drinking, involves approximately five drinks for males or four drinks for females in a single drinking session (Morojele and Ramsoomar, 2016), or consuming ≥ 60 g of pure alcohol at least once per month (WHO, 2018). Binge drinking is a hazardous practice and can lead to risky behavior, intentional and unintentional injuries, and alcohol poisoning. Binge drinking amongst adolescents is more common than chronic drinking and commonly occurs on weekends, with little to no drinking during weekdays. This type of drinking is a hazardous practice that is associated with acute consequences with long-lasting effects such as irreversible disabilities due to injuries caused by risky behavior, or even death (Kuntsche *et al.*, 2017; Zhang *et al.*, 2018). These poor behavioral choices can also be carried over to everyday life and then affect social, educational, and occupational functioning, contributing to the emergence of long-term drinking problems, and even the development

of psychiatric illness (Friedlander *et al.*, 2003; Morojele and Ramsoomar, 2016). While alcohol misuse is widely recognized as a major general health issue, its specific impact on skeletal growth and development, particularly during adolescence, warrants closer attention. Peak bone mass occurs during adolescence and alcohol consumption may cause a disturbance in bone growth during this period (Sampson, 1998).

Alcohol consumption during adolescence can affect bone growth and development. Bone is a living tissue that changes throughout life. This involves the development and growth of bones, including deposition and removal during childhood and adolescence, as well as bone remodeling in adulthood, even after reaching full height. Alcohol consumption can affect growth and remodeling, leading to reduced bone density and an increased risk of fracture. Studies reporting on the possible effect of alcohol consumption during adolescence on long bones have been conducted in animal models. The results showed a significant decrease in bone growth, volume, density, and strength (Hogan *et al.*, 1997; LaBrie *et al.*, 2018; Sampson *et al.*, 1996, 1999; Sampson, 1997). The effects of alcohol on flat and craniofacial bones have been studied less extensively than on long bones. A study by Nackaerts and colleagues (2009) investigated the effects of alcohol consumption on bone quantity and quality in a European female population. The results revealed that moderate alcohol consumption caused reduced bone mineral density and cortical width in the mandible (Nackaerts *et al.*, 2009). A study by Maia et al (2021) looked at the effect of binge-like exposure in adolescents on alveolar bone. Their results indicated a decrease in trabecular thickness and volume ratio, with an increase in trabecular separation, which indicated that binge drinking during adolescence results in alveolar bone damage which can persist in adulthood (Maia *et al*., 2021).

Alcohol consumption has also been linked to harmful effects in the oral cavity. Excessive alcohol consumption has also been reported to cause periodontal disease and alveolar bone loss and is aggravated by bacterial plaque accumulation due to poor oral hygiene. In a controlled animal study, alcohol alone, without any other periodontal disease-inducing factors, was reported to induce periodontal disease and alveolar bone loss (Bannach *et al.*, 2015). Clinically, periodontal disease and bone loss lead to a loss of implant stability, which relies on good bone quality and quantity. Alcohol consumption is also known to reduce bone density and impact bone repair and formation, leading to bone loss (Sampson, 1998).

The effect of fetal alcohol syndrome on long bones has been studied for decades (Miralles-Flores and Delgado-Baeza, 1992; Pillay and Ndou., 2022; Rai *et al.*, 2008; Rosa *et al.*, 2019). The effects of binge drinking on the adolescent skeleton are, however, limited in the literature, even more so relating to the craniofacial skeleton. Given alcohol's broad public health impact, understanding its specific effects on the mandible, a structure vital for numerous functions including mastication, speech, and facial function is particularly important. However, research in this area remains limited, and further studies in this niche area are warranted.

1.2 AIM

The aim of this study is to investigate the impact of excessive alcohol consumption on the growth and functional integrity of mandibular bone morphology in adolescent rats, as well as the subsequent recovery of the mandible following alcohol abstinence. The investigation focuses on changes in baseline measurements, osteometric parameters, trabecular structure, bone strength, cytoarchitecture, cellular proliferation, and alkaline phosphatase activity, while also examining potential sex-specific differences in response to alcohol exposure.

1.3 OBJECTIVES

1. To determine the effect of alcohol on parameters such as, body mass, food and water consumed in the acute binge, chronic binge, and chronic binge alcohol-abstinent models.
2. To determine how binge alcohol consumption affects mandibular dimensions, growth, and osteometric parameters in the acute binge, chronic binge, and chronic binge alcohol-abstinent models. These measurements were taken using a digital vernier caliper.
3. To determine the effects of binge alcohol consumption in adolescents on the mandibular trabecular parameters relating to trabecular volume, thickness, number and spaces, using three-dimensional Micro-Focus X-ray Computed Tomography (3D-µCT), in the acute binge, chronic binge, and chronic binge alcohol-abstinent models.
4. To assess how alcohol consumption induces weakening in the developing mandibles in the acute binge, chronic binge, and chronic binge alcohol-abstinent

models. This was tested by subjecting mandibles to 3-point bending tests using a universal tensile tester.

5. To assess how binge alcohol consumption affects mandibular cytoarchitecture by using Hematoxylin and Eosin (H & E) staining in the acute binge, chronic binge, and chronic binge alcohol-abstinent models.
6. To determine how binge alcohol consumption affects proliferation and remodeling by immunolocalization of proliferating cells and osteoblasts using the anti-Ki-67 and anti-alkaline phosphatase (ALP) antibodies respectively, in the acute binge, chronic binge, and chronic binge alcohol-abstinent models.
7. To determine the differences in the effect that binge alcohol consumption has on sex by comparing the results of the above tests between adolescent male and female Sprague Dawley rats, in the acute binge, chronic binge, and chronic binge alcohol-abstinent models.

1.4 HYPOTHESIS

Binge alcohol consumption negatively affects the growth and development of the adolescent Sprague Dawley rat mandible. The expected outcome of the study is that alcohol would adversely affect the baseline parameters, osteometric parameters, trabecular structure, bone strength, cytoarchitecture, cellular proliferation, and alkaline phosphatase activity, with differences between males and females in response to alcohol exposure.

1.5 RATIONALE FOR THE STUDY

Binge drinking in adolescents constitutes a significant public health concern in South Africa. While chronic alcohol exposure in adults is a well-established risk factor for

osteoporosis, leading to decreased bone density and a higher risk of fractures, there is a significant gap in our understanding of how binge drinking affects skeletal health in adolescents (Sampson *et al.*, 1996, Sampson *et al.*,1999; Sampson, 1997). Even less is known about the effect of binge alcohol consumption on the adolescent mandible. The current literature focuses on the effect of alcohol on long bones. For example, Hogan et al. (1997) investigated the effect of alcohol consumption on young growing rats, which demonstrated detrimental effects on cortical bone (Hogan *et al.*, 1997). Föger-Samwald et al. (2018) studied the effects of binge alcohol consumption on bone using prepubescent pigs as a model. The results indicated a reduction in serum calcium and phosphate levels, as well as decreased bone density and trabecular number in the femur (Föger-Samwald *et al.*, 2018). There is a notable gap in the literature in understanding how adolescent binge drinking specifically affects craniofacial structures such as the mandible.

Addressing this gap, the present study investigates the impact of acute and chronic binge drinking, as well as recovery following abstinence, on the adolescent mandible. This study should be useful in providing clinicians with valuable information regarding the effect of binge drinking on the growth and structure of the mandible at puberty, including information on the effect on mandibular size, trabecular effects, bone strength, along with the effects on cytoarchitecture and specific proteins important for bone formation and mineralisation. The information gained from this study should also improve the understanding of the effect of adolescent binge drinking on orthodontic treatment, as alcohol is known to affect bone growth and remodeling which may impair the response of alveolar bone to orthodontic forces. Alcohol may also affect trabecular structures which could possibly compromise anchorage, tooth stability, and thus treatment outcomes.

Adolescent binge drinking could possibly compromise dental implant stability later on in life by impacting peak bone mass and by altering bone microarchitecture during this critical period. These changes could lead to lower bone density, poorer trabecular quality, and could reduce overall bone quality and quantity, which could negatively impact implant osseointegration and stability. This could increase the risk of preimplant bone loss and implant failure. It should also provide an understanding of the prevalence of craniofacial fractures in this age group and the healing patterns of these fractures, as alcohol consumption impairs mental state, leading to a greater incidence of interpersonal violence and accidents which can lead to fractures. Alcohol can also affect bone quality, which could lead to weaker bones, which fracture easier. Alcohol can also affect bone healing with altered osteoblast function and inflammatory responses, which can lead to delayed fracture and poorer-quality fracture healing.

1.6 LAYOUT OF THE THESIS

The thesis consists of 7 chapters as follows:

Chapter 1

Provides an overview of the general introduction, aims, objectives, and rationale for the study.

Chapter 2

This chapter provides a literature review on the epidemiology of binge alcohol consumption among adolescents in South Africa and its impact on the growth and development of the adolescent skeleton. It also covers the growth and development of

the human and rat mandible, the effect of alcohol on bone development and remodeling, and clinical implications related to binge drinking on the adolescent skeleton. Additionally, it discusses the concept of biomechanics on bone, as well as the concepts of immunohistochemistry involving the protein Ki-67, the enzyme Alkaline Phosphatase (ALP), and sexual dimorphism on the adolescent skeleton.

Chapter 3

The methodology of the project is outlined in this chapter. It includes details of how the animals were housed, how alcohol was administered to them, how the animals were terminated and their mandibles harvested, as well as the methods used to achieve the study objectives (baseline data analysis, osteometric measurements, 3D micro-CT analysis, tensile strength testing, histology and immunohistochemistry respectively).

Chapter 4

This chapter discusses the impact of an acute binge alcohol model on the growth and development of the mandible in adolescent Sprague Dawley rats.

Chapter 5

This chapter discusses the impact of an chronic binge alcohol model on the growth and development of the mandible in adolescent Sprague Dawley rats.

Chapter 6

This chapter entails the impact of prolonged binge drinking followed by a period of alcohol abstinence on the development of the mandible in adolescent Sprague Dawley rats.

Chapter 7

This chapter provides a general discussion and conclusions, along with limitations of the study.

CHAPTER 2: *Literature Review*

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2. LITERATURE REVIEW

Prevalence and history of alcohol consumption

Globally, alcohol consumption negatively affects health, contributing to approximately 3.8 % of all deaths and 4.6 % of disability-adjusted life years (DALYs). DALYs is a measure that quantifies the overall burden of disease by combining the effects of premature mortality with years lived with disability or illness. One DALY represents one lost year of healthy life. In South Africa, alcohol use was responsible for 7.1 % of all deaths and 7.0 % of total DALYs in 2000 (Peltzer *et al*., 2011). Regarding alcohol-attributable disabilities, alcohol use disorders were the leading cause (44.6 %), followed by interpersonal violence (23.2 %), and Fetal Alcohol Syndrome (FAS) (18.1 %) (Norman *et al*., 2007; Peltzer *et al*., 2011). The pattern of drinking within a population is also of importance. Alcohol consumption needs to be understood in the context of drinking patterns, which vary significantly across different societies. The drinking patterns prevalent among South Africans are a significant public health concern. In addition to being recognized globally as one of the countries with the riskiest alcohol drinking behaviors, a national review highlighted the elevated rates of binge and heavy episodic drinking (Morojele and Ramsoomar, 2016). In many developing regions like South Africa, the typical pattern involves infrequent but heavy drinking, especially among males. This is indicated by factors such as the frequency of heavy drinking episodes, the large typical amount of alcohol consumed, drinking in public settings, and drinking at community events (Peltzer *et al*., 2011). Many of these developing subregions are marked by hazardous drinking behaviors. Hazardous drinking describes a pattern or level of alcohol consumption that increases the risk of negative health outcomes for individuals. In contrast, harmful

drinking is characterized by alcohol use that leads to negative effects, such as physical or psychological harm (Reid *et al*., 1999). The trend of rising alcohol consumption among South African adolescents is concerning, with the age of initiation being less than 13 years, which is alarming. Alcohol consumption in South African adolescents is more dominant in males than in females and is usually consumed by binge or heavy episodic drinking (Ramsoomar and Morojele, 2012; Morojele and Ramsoomar, 2016).

Binge drinking decreases bone density and increases the risk of fractures

Binge drinking is a common practice in adolescents and young adults (Föger-Samwald *et al.*, 2018). However, there is limited knowledge regarding its effects on the skeletal system, even less so on the impact on the craniofacial structures. It is known that bone mass naturally decreases over the course of life as part of the aging process and that failure to obtain optimum peak bone mass during adolescence increases the risk of developing osteoporosis later in life (Chakkalakal, 2005). Alcohol consumption during adolescence may disrupt the attainment of peak bone mass resulting in poor skeletal health. The impact of binge drinking on bone growth remains unclear, indicating a need for further research. Lauing et al. (2008) reported on the consequences of binge alcohol treatment on adolescent rats followed by alcohol abstinence and its association with site-specific differences in bone loss and incomplete recovery of bone mass and strength. The results from their study indicated a significant decrease in cancellous bone mass in the tibia after acute binge exposure, but bone mass did recover after the abstinence period. A decrease in bone mass was noted in the vertebra after chronic binge exposure and was resistant to recovery after the abstinence period. Compressive bone strength was also affected. The amount of bone deposition and resorption after binge alcohol exposure and

abstinence is likely to be dependent on the proliferation rates and rates of turnover in the bone being examined e.g. the tibia has higher rates of turnover than the vertebrae and thus has more initial resorption, but also recovers quicker (Lauing *et al.*, 2008). Therefore, a decrease in bone mass and strength, with an increased risk of interpersonal violence, is likely to increase the risk of all fractures, but more specifically craniofacial fractures such as fractures of the mandible. These fractures are seen to be more severe where alcohol is involved, increasing the need for surgery, and have post-operative complications such as poor healing and increased healing time (Lee and Snape, 2008).

Bone structure

Bone is classified into two types: cortical and trabecular. Cortical bone, or compact bone, is thick and dense, making up the outer layer of the bone. Trabecular bone, or cancellous bone consists of a thin and porous meshwork (thin trabecular plates). The complex and hierarchical structure of bone can be categorized at various levels: macroscale (trabecular/cancellous bone and cortical bone), microscale (Haversian canals and osteons), sub-microscale (consists of a single layer of lamella containing collagen fibres), nanoscale (collagen fibrils), and sub-nanoscale (minerals and collagen molecules) (Fig. 2.1). Spongy trabecular bone is found inside the bone and consists of interwoven bone trabeculae. Cortical bone is highly dense, giving it significant strength against compression and distortion, and is found on the outer surface of bone. Running parallel to the bone shaft we find osteons, which are cylindrical structures containing osteocytes. Each osteon is made up of concentric lamellae that encircle a central Haversian canal, which contains blood vessels and fibre arrays. The subunits of these osteons consist of mineralised collagen fibrils, which form the organic component, and crystallized

hydroxyapatite, which makes up the inorganic component and is located between the collagen fibrils (Fan *et al*., 2022) (Fig. 2.1). Alcohol affects both types of bone, with trabecular bone being the most affected (Sampson, 1998). Alcohol exposure has been seen to alter the balance between bone deposition and bone resorption during the process of bone remodeling. This results in a reduction in trabecular bone volume, a decrease in the numbers of osteoblasts, and diminished bone formation, ultimately resulting in impaired bone formation and mineralisation (Sampson, 1998).

Bone development

The development of bone is either by endochondral or intramembranous ossification. Endochondral ossification is the process whereby bones undergo ossification within a cartilage template and in the surrounding fibroblastic perichondral sheath resulting in the formation of a bone collar. In contrast, intramembranous ossification occurs through the direct differentiation of osteoblasts within the mesenchyme (Galea *et al*., 2021) (Fig. 2.2).

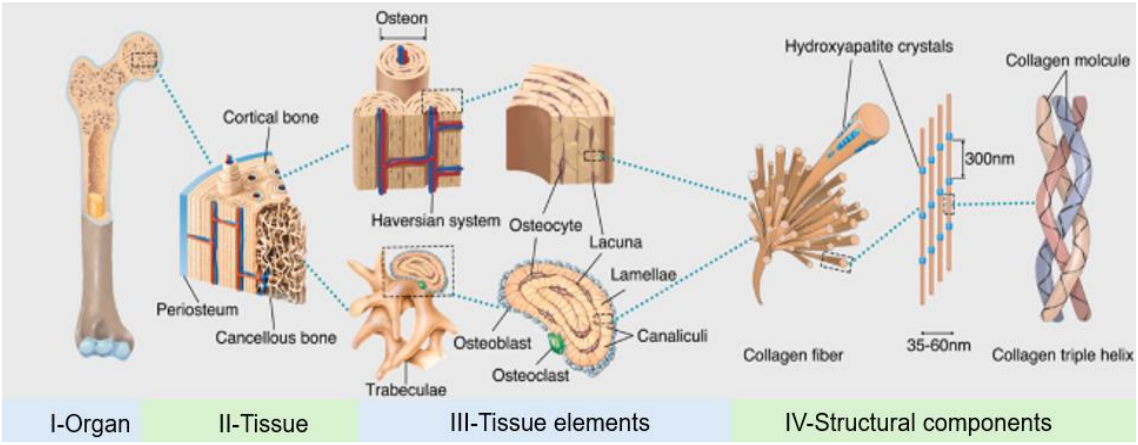

Figure 2.1: The hierarchal structure of bone. Bone consists of different levels: Levels III, IV, and V form the mechanical support structure for bone tissues, while Levels I and II create the microenvironment for bone tissue cells (Adapted from Zhu *et al*., 2021).

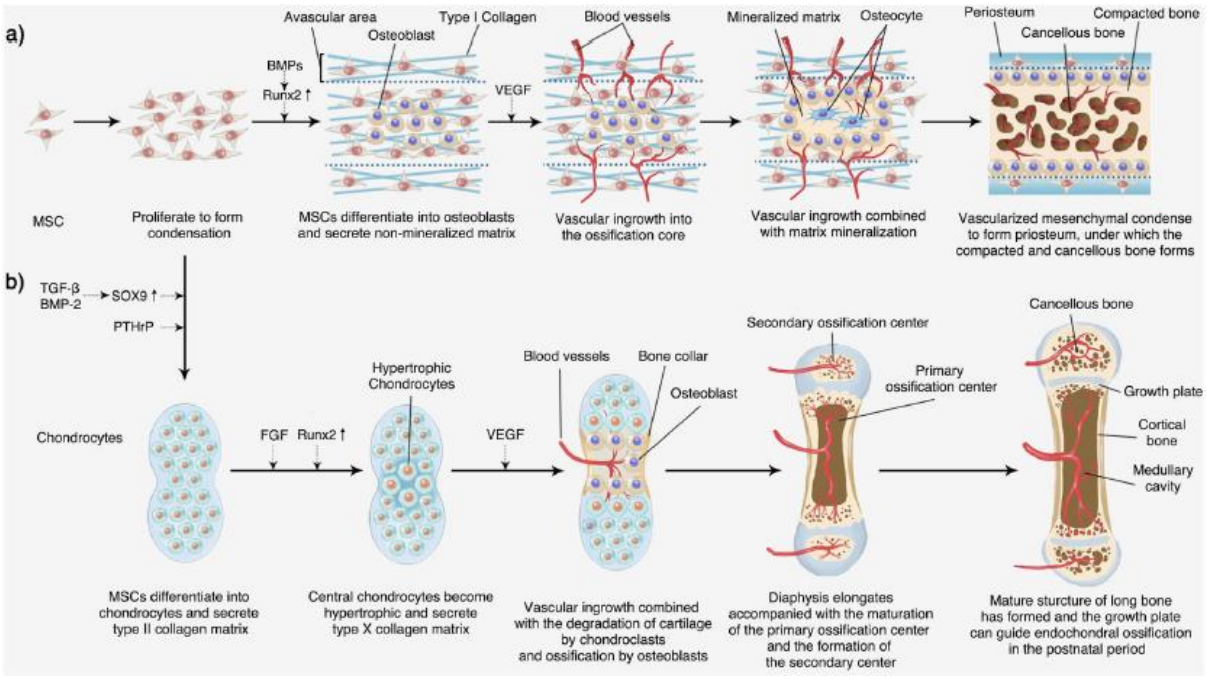

Figure 2.2: Different types of embryonic bone development. (a) Intramembranous bone formation; (b) endochondral bone formation. (Adapted from Zhu *et al*., 2021).

Bone remodeling influence on bone quantity and structural integrity

Bone undergoes continuous changes in development and growth during childhood and adolescence, and remodeling in adulthood, involving deposition and removal (bone remodeling). Bone is a dynamic structure that constantly undergoes changes and adapts throughout one's life. Bone contains three main cell types that are responsible for bone remodeling. Osteocytes which sense external stimuli, osteoclasts whose function is to resorb bone, and osteoblasts which are responsible for the formation of new bone. A schematic diagram of the changes that occur during remodeling is displayed in Figure 2.3. These phases include activation, resorption, reversal, formation, and mineralisation. During activation, bone lining cells move away from the bone, forming a raised covering above the surface remodeled. Osteoclast progenitor cells are also recruited during this

phase. The mononuclear osteoclast progenitor cells fuse and differentiate into multinucleated osteoclasts. The next phase is resorption. During this phase, old or damaged bone is resorbed. The subsequent phase is the reversal phase, which consists of mononuclear macrophage-like cells smoothing out the bone surface and preparing it for matrix deposition. This is the phase that links resorption to formation. The next phase is the formation phase, which is controlled by osteoblasts that secrete osteoid. Some of these osteoblasts become trapped in the matrix and differentiate into osteocytes. During the final stage, the remaining osteoblasts can undergo two processes. They can either undergo apoptosis or they can become bone-lining cells as the osteoid mineralises into new bone (Truesdell and Saunders, 2019).

Bone remodeling is required to maintain structural integrity. Bone loss induced by alcohol occurs due to an interruption in bone remodeling, where the formation of new bone (controlled by osteoblasts) is suppressed, and bone resorption (controlled by osteoclasts) either remains the same or increases. Alcohol interferes with osteoblast function by inhibiting their proliferation, differentiation, and activity. It disrupts key signaling pathways and reduces the expression of bone formation markers like alkaline phosphatase, osteocalcin, and collagen type I. This leads to decreased synthesis of the organic bone matrix and impaired mineralization. Alcohol also induces oxidative stress and promotes apoptosis in osteoblasts, further reducing bone formation capacity. On the other hand, alcohol stimulates osteoclast differentiation and activity by increasing the expression of RANKL while decreasing osteoprotegerin (OPG), tipping the balance towards enhanced bone resorption. This accelerates the breakdown of bone tissue, contributing to net bone loss (Chakkalakal, 2005; Sampson, 1998). This leads to decreased bone formation,

diminished bone mass, and an increased risk for bone fracture with delayed healing after fracture. The same principle applies to alcohol consumption where and the effects of alcohol on bone remodeling are likely to have an impact on the growth and development of the mandible by altering the balance between bone formation and resorption. (Chakkalakal, 2005; Sampson, 1998). Bone density and structure are essential in dentistry as they give structure and protection to teeth along with giving them anchorage (Bannach *et al.*, 2015).

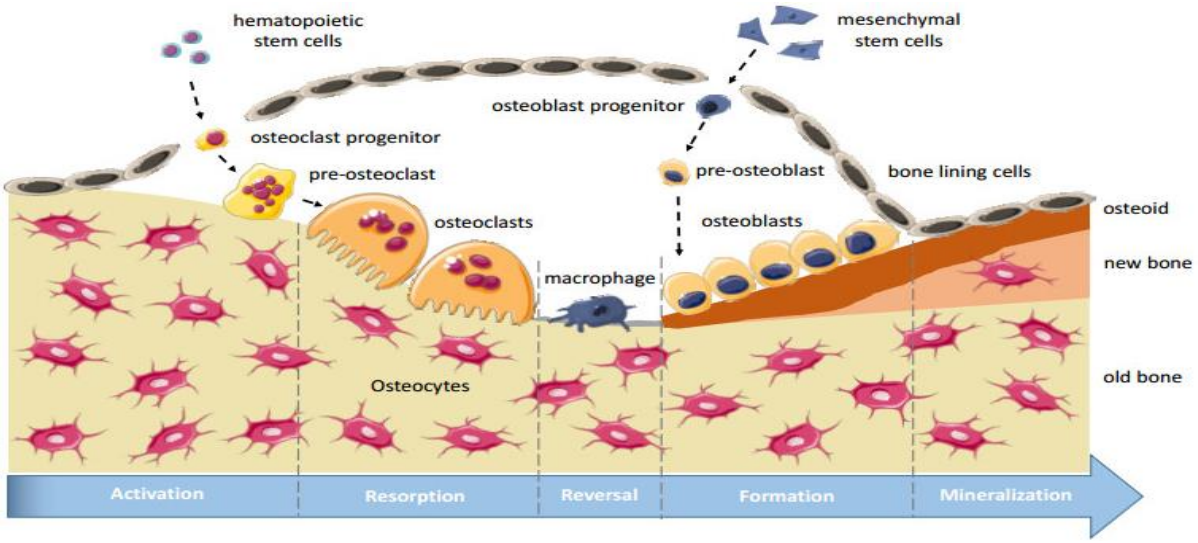

Figure 2.3: Phases of bone remodeling. A schematic diagram representing the different phases of bone remodeling: activation, resorption, reversal, formation, and mineralisation (Adapted from Truesdell and Saunders, 2019).

Anatomy of the developing human mandible

The mandible is derived from the first pharyngeal arch during the 4th week of embryonic development. This stage involves Meckel's cartilage which is the initial non-ossifying template for early mandibular growth, which then develops ossification centers that allow

for further growth and ossification of the mandible (Smartt *et al*., 2005). Meckel's cartilage is eventually replaced by intramembranous bone. Secondary cartilages develop that will give rise to the coronoid process, the condylar head, and the mental protuberance and contribute to endochondral components at later stages of development. Close to the final stages of fetal development, the secondary cartilage of the condyle is almost totally replaced by bone, with the exception of the upper end, which continues into adulthood. This cartilage acts as both an articular cartilage and a growth center. At birth, the mandible consists of two separate bones connected by a cartilaginous region that has not yet undergone ossification. As the child grows, the body of the mandible develops in structure, size, and function and houses both primary and permanent dentition. At ± 6 years of age, the ramus reaches its maximum height and increases its length in an anteroposterior direction. The vertical growth mimics that of the maxilla, to accommodate the eruption of permanent dentition. Lengthening of the dental arches also takes place. The mandible then undergoes remodeling which results in the correct positioning of both maxilla and mandible for proper occlusion. The overall size of the mandible will increase significantly between the ages of 11 and 17 (puberty), with a significant increase in condylar height during this adolescent period (Smartt *et al.,* 2005).

The anatomy of the rat mandible

Sprague Dawley (SD) rats are widely used in research and were used in this study. The anatomy of the SD rat mandible is thus important to know and understand. The rat mandible consists of paired bones namely the right and the left hemi-mandibles. These two hemi-mandibles are joined by fibrous connective tissue in the midline, called the mandibular symphysis. The mandible is made up of the body and the ramus. The

mandibular body consists of alveolar bone which houses the dentition. The rat dentition is as follows (per hemi-mandible): 1 incisor (I) tooth, no canine (C) teeth, no premolar (PM) teeth, and 3 molar (M) teeth. (I 1-1, C 0-0, PM 0-0, M 3-3) = 8 teeth (Kim *et al.*, 2018). The rat mandible consists of the alveolar process, mandibular angle (angular process), and mandibular body which form part of the body of the mandible. The body of the mandible contains the mental foramen (MF), which transmits the mental nerve and vessels. The ramus of the mandible consists of the condylar (Cr) and coronoid (Cd) processes. (Fig. 2.4). The lowest, most posteroinferior point on the angle of the mandible is called the gonion (Go). The ante-gonion (Ag) is the highest point of the notch or concavity of the lowest border of the ramus of the mandible where it joins the body of the mandible, and the most inferior point of the mandibular symphysis is called the menton (Me) (Fig. 2.4) (Maki *et al.*, 2002).

The mandible is a unique bone that undergoes both endochondral and intramembranous ossification. The condylar process of the mandible is the only part of this osseous tissue that develops through endochondral ossification (Mizoguchi *et al*., 2013). The rest of the mandible develops from intramembranous ossification. Intramembranous ossification typically occurs earlier in prenatal development. During the initial stages of mandibular development in the rat embryo, mesenchymal cells condense and differentiate directly into osteoblasts. This process starts around embryonic days 13-14 (E13-E14). These osteoblasts then lay down bone matrix directly, forming the mandibular bone proper or mandibular body (Chen *et al*., 2015; Robinson *et al*., 2015).

Endochondral ossification occurs later in prenatal development and is mainly associated with the development of the condylar process of the mandible. This process begins with

the formation of a cartilage template within the condylar region. The condylar cartilage begins to form from mesenchymal cells around embryonic day 16-17 (E16-E17) in rats (Chen *et al*., 2015; Robinson *et al*., 2015). Chondrocytes within this cartilage model undergo proliferation and hypertrophy, leading to the formation of primary growth centers in the condylar secondary cartilage (CSC), angular synchondrosis (AS), and the mandibular midline synchondrosis (MS). The AS and MS fuse and cause the mandibular condyle to revert to a secondary growth site. Blood vessels and osteoblasts invade the calcified cartilage, replacing it with bone tissue. This process continues postnatally and throughout early life as the condylar process undergoes growth and remodeling (Chen *et al*., 2015; Robinson *et al*., 2015). The development of the mandibular condyle not only contributes to an increase in the size of the mandible but also results in the anteroinferior displacement (transposition) of the mandible.

It is established that alcohol perturbs bones formed by endochondral ossification, where it disrupts chondrocyte proliferation and maturation in the growth plate, impairing the replacement of cartilage with mineralized bone. It also alters the expression of growth factors such as IGF-1 and reduces vascular invasion needed for ossification. These effects slow longitudinal bone growth, reduce bone length, and compromise the quality and strength of newly formed bone during this critical developmental period (Pillay and Ndou, 2022; Mizoguchi *et al*., 2013). However, there is still a gap in the literature about how alcohol affects the mandible which is formed through both endochondral and intramembranous ossification. However, from our knowledge, alcohol impairs the differentiation and function of osteoblasts derived from mesenchymal stem cells, reducing new bone matrix production and mineralization (Mizoguchi *et al*., 2013). It also interferes

with growth factors and signaling pathways critical for osteoblast proliferation and activity, which is likely how it will affect intramembranous ossification in the mandible.

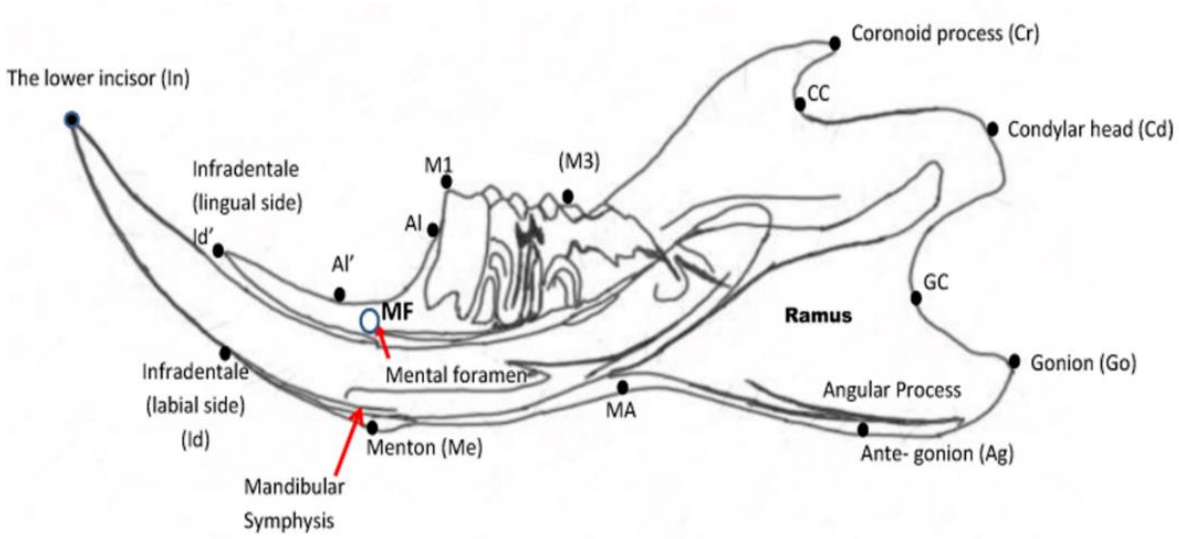

Figure 2.4: Diagram of a rat mandible. CC; the upper mandibular notch, **GC;** lower mandibular notch, **MA;** the deepest point of the outer margin of bone that connects Me and Ag, **Al;** the highest point of the mesial alveolar bone at the lower first molar; **Al^{l};** the deepest point of the outer margin of the bone (Al^{l}) connecting Al and Id^{l}; **M1**; the highest point of the mesiobuccal cusp of the lower incisor**, M3;** the highest point of the central cusps of the lower 3rd molar (Adapted from Maki *et al*., 2002).

The role of bone quality in dental implant stability

The stability of an implant is strongly linked to bone density and is a crucial factor in ensuring successful osseointegration (Merheb *et al*., 2018). Therefore, it is important to consider bone density and quality during implant placement to maximize the chances of success. Research has indicated that both current and past alcohol consumption may hinder the success of implant surgery and osseointegration by delaying bone growth and mineralisation around the implants (Torricelli *et al*., 2008). Binge drinking during adolescence could compromise dental implant stability later in life by interfering with peak

bone mass acquisition and modifying bone microarchitecture during this critical developmental window. Such changes may result in reduced bone density and poorer trabecular quality, diminishing the overall quantity and quality of bone necessary for successful implant osseointegration. This increases the risk of peri-implant bone loss and implant failure (Bannach *et al.*, 2015).

Effect of alcohol on orthodontic conditions and treatment such as tooth crowding, dento-alveolar, and jaw relationships

Orthodontic treatment predominantly takes place during adolescence. With binge drinking being an area of concern in adolescents, the effect of alcohol on orthodontic tooth movement should also be an area of concern. Orthodontic tooth movement involves bone resorption and deposition (remodeling), which is affected by alcohol consumption, which leads to more resorption than deposition. Thus, orthodontists treating patients who consume alcohol should take precautions to avoid excessive force application which could lead to tooth mobility (Krishnan *et al.*, 2012). Akhoundi et al. (2016) reported an increase in orthodontic tooth movement in an alcohol-treated group of Wistar rats, which they attributed to alcohol-induced osteopenia and a decrease in bone density (Ahmad Akhoundi *et al.*, 2016). Alterations in trabecular bone structure caused by alcohol could undermine anchorage and tooth stability, ultimately affecting treatment success. The effect of alcohol consumption on the growth of the adolescent mandible and maxilla is unknown, thus the effect of alcohol consumption on dental crowding is also unknown but may be affected if alcohol has a negative effect on maxillary or mandibular growth.

Effect of alcohol on facial fractures

Alcohol consumption impairs cognitive function and judgment, contributing to a higher likelihood of accidents and interpersonal violence that cause facial injuries. Coupled with alcohol's negative effects on bone strength and healing, through altered osteoblast activity and inflammatory responses, these factors can lead to more frequent fractures and delayed or compromised fracture repair (Lee and Snape, 2008; O'Meara *et al.*, 2012).

Bone biomechanics

Bone is a crucial load-bearing tissue that offers mechanical support to the body, with the bone matrix being a fundamental constituent of bone. The bone matrix has long been the focus of extensive research due to its vital role in the body and its distinctive mechanical properties (Ma *et al*., 2022). Studying the impact of alcohol on bone requires understanding its effect on bone biomechanics. Bone matrix has unique biomechanical properties in response to its surrounding mechanical environment. Bone possesses both strength and toughness, attributed to its macro and microstructure. Mechanical stimulation is an important biomechanical factor that impacts bone. Compressive and tensile stress are common mechanical stimuli on bone (Ma *et al*., 2022). Biomechanical properties of bone can be tested using 3-point bending tests which analyse numerous properties of bone including maximum force, maximum displacement, maximum time, break force, and energy to fracture (Pillay and Ndou., 2022).

Ki-67 protein

Ki-67 is a protein linked to cellular proliferation, found in the nucleus during the active phases of the cell cycle (G1, S, G2, and M phases) but is reduced in resting cells (G0

phase) (Sun and Kaufman, 2018). The Ki-67 antibody specifically binds to the Ki-67 protein and is widely used in immunohistochemistry (IHC) to detect and quantify specific proteins in tissue samples to assess cell proliferation. Therefore, utilizing the Ki-67 antibody enabled us to analyse how binge alcohol consumption affected proliferation rates in the adolescent mandible. Short-term alcohol exposure often results in suppressed cellular proliferation in sensitive tissues, including bone and gastrointestinal epithelium. Studies have reported a decrease in Ki-67 expression following acute or binge alcohol exposure, likely due to oxidative stress, inflammation, and disrupted signaling pathways that regulate the cell cycle. Long-term or chronic alcohol use typically exacerbates these effects. Prolonged alcohol consumption leads to sustained suppression of Ki-67 expression across multiple organ systems. Abstinence following prolonged alcohol use shows variable outcomes depending on the tissue type and duration of abstinence. Some studies demonstrate partial or full restoration of Ki-67 expression after a period of abstinence, suggesting that proliferative capacity can recover over time if alcohol is withdrawn. However, this recovery may be incomplete or delayed in tissues with significant alcohol-induced damage or in cases of long-term dependency (Eby *et al*., 2022; Golub *et al*.,2015; Nixon *et al*., 2008).

Alkaline phosphatase (ALP) enzyme

Alkaline phosphatase (ALP) is an enzyme that plays a role in bone mineralisation. Osteoblasts produce ALP which acts as an early indicator of osteoblast differentiation. Increased expression of ALP is seen when there is an increase in the differentiation of osteoblasts. ALP is also implicated in the process of osteoid mineralisation. ALP levels increase during bone formation and are a clinical marker for bone activity. Anti-ALP anti-

body is used to detect osteoblast expression. (Bassi *et al.*, 2011). Wezeman et al. (2007) found that acute binge alcohol exposure caused a decrease in the expression of bone formation markers osteocalcin and ALP (Wezeman *et al.*, 2007). Wang et al. (2003) investigated the effect of alcohol-induced adipogenesis in bone and marrow. The findings of their study also indicated that alcohol-exposed cells had a decreased activity of osteocalcin and ALP in marrow stromal cells which differentiate into osteoblasts (Wang *et al.*, 2003).

Sexual dimorphism and its effect on the skeletal system

Dimorphism refers to the existence of two distinct forms. "Sexual dimorphism" specifically means that the two sexes of a species differ in external appearance or other characteristics (Ralls and Mesnick., 2009).

The differences in bone mass between males and females at various ages are influenced by multiple factors, including the effects of sex steroids, genetics, age, environment, and behavior. Sexual dimorphism in skeletal development and the incidence of skeletal diseases is well-established. Generally, females have a lower bone mass than males at any given age. Females are more prone to osteoporosis, while males are more likely to develop Paget's disease. The functional skeleton, which provides structural integrity, is shaped by the actions of osteoclasts (responsible for bone resorption), osteoblasts (involved in bone formation), and osteocytes (which coordinate these activities) (Lorenzo *et al.*, 2020).

The differences in bone mass and disease incidence between sexes arise from several factors, including the influence of sex steroids (oestrogens and androgens), genetics, and

inflammation. These factors influence the development, breakdown, and death of osteoclasts, often leading to varying outcomes between males and females (Lorenzo *et al*., 2020).

Studies have reported a decrease in testosterone levels with alcohol consumption (Maurel *et al*., 2012a; Tentler and Kelley., 1997). In female alcoholics and alcohol-treated female rats, serum oestradiol levels have been shown to decrease. Low oestradiol is an important factor in alcohol-induced bone loss. However, small amounts of alcohol have been linked with an increase in oestrogen levels in females and elevated oestradiol levels in males, which may help explain the higher bone mineral density (BMD) observed in light drinkers (Maurel *et al*., 2012a). Thus, several factors contribute to the sexual dimorphism of the skeleton and the activity of cells in bone.

CHAPTER 3: *Methodology*

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A pilot study using a dosage of 1.5 g/kg was conducted prior to the completion of the final study. This was to determine in the dosage in question was safe to use without causing harm to the animals while still obtaining significant results as recommended by the Animal Research Ethics Committee, University of the Witwatersrand. This dosage proved to be too low to cause significant changes; therefore, a higher dose of 3 g/kg was proposed. This dosage has been safely used in previous studies without causing harm to the animals.

3.1 Animal housing

This study received ethical approval from the Animal Research Ethics Committee, University of the Witwatersrand (AREC 2020/11/02C). Seventy-two adolescent (male and female) Sprague Dawley rats aged 7 weeks (49 days) weighing approximately 150 g - 350 g were used in this study. The chosen sample size allows for a sufficient number of animals to detect meaningful differences, while accounting for biological variability. There was a balanced number of males and females to study sexual dimorphism. This sample size was a reasonable compromise between statistical power and logistical limits. It also respected the 3R principle (replacement, reduction, refinement) in animal research by minimizing animal use while still achieving scientifically valid results. Rats reach puberty at approximately 6 weeks (42 days), and adolescence occurs from approximately day 35 to day 63. The equivalence of 7-week (49-day) old rats in human years is 14.5 human years (Sengupta, 2013). All study animals were bred and kept at the Wits Research Animal Facility (WRAF), University of the Witwatersrand, Parktown Campus. These animals were maintained under controlled conditions which were free of most pathogens, in a temperature-controlled environment (26-28°C) and a 12-hour light/dark cycle. The

rats were housed in plastic cages 43 mm long, 220 mm wide, and 200 mm high with free movement of the rats within the cages. They were housed in pairs based on sex (e.g. 2 male rats were housed together). All the animals in the study were fed the standard rodent diet and water was provided *ad libitum*. Bias was prevented by randomly assigning animals to pair-fed control and alcohol exposed groups; by keeping feeding, handling and housing conditions consistent; by having equal numbers of male and female animals; by using validated, calibrated equipment to carry out measurements.

Food consumption was recorded daily using a calibrated digital scale. At the start of the study, approximately 650 g of food was provided by filling the food compartment of the cage, and the amount was precisely measured in grams. Each subsequent day, the remaining food was weighed, and the amount consumed over the previous 24 hours was calculated by subtracting this from the initial quantity. Food was replenished daily, with the exact amount provided measured each time.

Water intake was recorded daily. On the first day of the study, water bottles were filled to 400 ml. Each following day, the remaining volume was measured, and the amount consumed was calculated by subtracting this from the initial 400 ml. Fresh water was provided daily by refilling the bottles to 400 ml.

3.2 Group allocation and exposure

Before the commencement of the experiments, the animals were randomly divided into 6 groups, each consisting of 12 rats (6 male and 6 female rats). The study was conducted in 3 phases to minimize the number of animals handled at any given time. The groups are listed below:

Group A1 (1-week alcohol-exposed rats): These animals were exposed to alcohol for 7 days, which is equivalent to 1 week. They were referred to as acute binge alcohol-exposed rats. The alcohol was administered as a single daily dose through oral gavage of a 20 % (vol/vol) alcohol solution at a dose of 3 g/kg. This dosage was chosen to achieve peak blood alcohol concentrations (BACs) of around 100 mg/dL (Jeanblanc *et al*., 2019; Walker and Ehlers, 2009). An hour after exposure, BACs were measured by drawing blood using the tail prick method. The blood was stored in heparinized microcapillary tubes (Marienfeld). BACs were measured using an Alcohol Colorimetric Assay Kit (Sigma-Aldrich), and the readings were taken using an Absorbance Reader at a wavelength of 630 nm. The alcohol was administered 3 days per week (every alternate day), with no alcohol given during the remaining 4 days each week. Exposure for 3 days a week mimics a binge model of alcohol exposure, as opposed to a chronic model of exposure (Lauing *et al.*, 2008).

Group C1 (1-week pair-fed control rats): A pair-fed rat was individually matched to an alcohol-exposed rat based on initial body mass for precise caloric control. The following information pertains to the energy content of alcohol and maltose dextrin. One gram of alcohol yields 7 kilocalories (kcal), while 1 gram of maltose dextrin yields 3.89 kcal. Consequently, if 1 gram of alcohol equals 7 kcal/g, then a 20 % alcohol solution at a dose of 1.5 g/kg provides 1.4 kcal/g. The pair-fed group was matched with an isocaloric amount of maltose dextrin, which was also administered via oral gavage for 1 week, following the same schedule as Group A1 (Bertola *et al*., 2013; Hogan *et al*., 1999; Lauing *et al*., 2008).

Group A4 (4-week alcohol-exposed rats): The rats were exposed to alcohol for 28 days (4 weeks). They were known as the chronic binge alcohol-exposed rats. Alcohol was administered as per Group A1 (Lauing *et al.*, 2008).

Group C4 (4-week pair-fed control rats): This group was administered maltose dextrin for 28 days as per the pair-fed control Group C1.

Group A4-Abs (Chronic binge alcohol-abstinent rats): To assess the impact of binge alcohol exposure on bone recovery in rats, another group of rats was subjected to alcohol for 28 days (4 weeks) using the same method as Groups A1 and A4. Subsequently, they were kept alcohol-free for an additional 30 days before being euthanized (Bertola *et al.*, 2013; Hogan *et al*., 1999; Lauing *et al.*, 2008).

Group C4-Abs (Chronic binge alcohol-abstinent pair-fed control rats): These animals received maltose dextrin for 28 days according to pair-fed control Group C4. They were then kept for an additional 30 days without maltose dextrin before euthanasia.

Parameters such as, body mass, food and water consumed were recorded in all the groups to track the health of the animals throughout the study and to determine if alcohol has an impact on these parameters.

3.3 Determination of blood alcohol levels

Blood alcohol concentrations (BACs) were determined by drawing whole blood using the tail prick method one hour after alcohol and maltose dextrin administration. The blood was collected and stored in heparinized microcapillary tubes. After that, the blood was transferred from the microcapillary tubes into sterile 15 ml conical culture tubes and

centrifuged in a Hettich Rotofix 32A centrifuge (Germany) at 4000 rpm for 10 minutes. The plasma was collected, and its alcohol concentrations were tested using an Alcohol Colorimetric Assay Kit from Sigma-Aldrich, following the manufacturer's instructions. The readings were taken using an iMark Bio-Rad Microplate Absorbance Reader from Bio-Rad Laboratories Inc, USA, at a wavelength of 630 nm. All reactions and readings were conducted in an alcohol-free environment.

3.4 Skeletal harvesting

At the end of 7 days (groups A1 and C1), 28 days (groups A4 and C4), and 58 days (groups A4-Abs and C4-Abs), the animals were deeply anaesthetized using pentobarbitone. Animals were perfused with 4 % paraformaldehyde (PFA). The animals were then terminated by pentobarbital intraperitoneal injection. Once terminated, the heads were removed and placed in 10 % buffered formalin, and the rat's carcass was immersed in a 10 % buffered formalin solution after a midline incision was made to permit penetration of the fixative and stored in separate containers for storage and further fixation. The mandibles were then carefully dissected out and placed in 10 % buffered formalin for further fixation and processing.

3.5 Osteometric measurements

Binge alcohol consumption in adolescents is likely to cause a decrease in bone growth and bone mineral density. Thus, the width, height, and length along with general mandibular morphology and size may be affected by adolescent alcohol consumption. (Lauing *et al.*, 2008; Rehman *et al.*, 2019). Several osteometric measurements were taken for (i) estimation of general mandibular size, (ii), assessment of the linear mandibular length, and (iii) assessment of the linear mandibular height. Osteometric

measurements were taken suing using a Guanglu ISO9001:2000 digital vernier caliper (China).

When studying the growth of the mandible, the mental foramen is a reliable reference point for morphological measurement as it remains relatively stable during mandibular growth. General mandibular size estimates were based on measurements taken at the mental foramen (MF) as shown in the accompanying table (Table 3.1) and figure (Fig. 3.1). Measurements were made using a digital vernier caliper accurate to the nearest 0.05 mm, following the method described by Maki et al. (2002). Each measurement was taken three times, and then the average was used to assess the general mandibular size.

3.5.1. Estimation of general mandibular size

Measurements in Table 3.1 were used to assess the linear mandibular length of the adolescent rat mandible (Fig. 3.1).

Table 3.1: Measurements for estimation of general mandibular size (mm) (Adapted from Maki *et al.,* 2002).

Measurement	Description
MF-Cd	Distance between the mental foramen (MF) and most posterior point of the condylar head (Cd)
MF-Go	Distance between the mental foramen (MF) and the gonion (Go)
MF-Ag	Distance between the mental foramen (MF) and the ante-gonion (Ag)
MF-Me	Distance between the mental foramen (MF) and the menton (Me)
MF-Al^{l}	Distance between the mental foramen (MF) and the deepest point of the outer margin of the bone (Al^{l}) connecting Al and Id^{l}
MF-M1	Distance between the mental foramen (MF) and the highest point of the mesiobuccal cusp of the lower incisor (M1)
MF-M3	Distance between mental the foramen (MF) and the highest point of central cusps of the lower 3rd molar (M3)

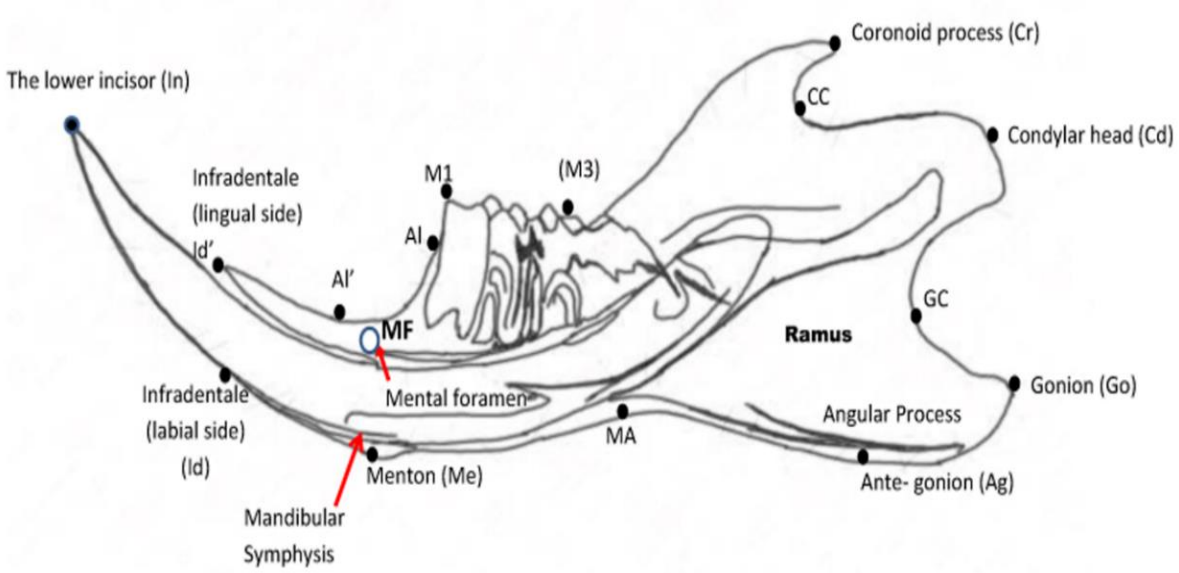


Figure 3.1: Reference points for the estimation of the general size of the rat mandible. CC; the upper mandibular notch, **GC**; lower mandibular notch, **MA**; the deepest point of the outer margin of bone that connects **Me** and **Ag**, **AI**; the highest point of the mesial alveolar bone at the lower first molar, **AI^{l}**; the deepest point of the outer margin of the bone **(AI^{l})** connecting **AI** and **Id^{l}**, **M1**; the highest point of the mesiobuccal cusp of the lower 1st molar, **M3**; the highest point of the central cusps of the lower 3rd molar (Adapted from Maki *et al*., 2002).

3.5.2. Assessments of linear mandibular length (mm)

Measurements in Table 3.2 were used to assess the linear mandibular length of the adolescent rat mandible (Fig 3.2).

Table 3.2: Measurement for the assessment of the linear mandibular length (mm) (Adapted from Maki *et al.,* 2002).

Measurement	Description
Cd-Id	Length of the base of the mandible which is the distance between the condyle to the infradentale (labial side)
Go-Id	Mandibular body length which is the distance between the gonion (Go) and the infradentale (Id) on the labial side
Al-Id^{l}	Length of the lingual side of the alveolar bone which is the distance between the highest point of the mesial alveolar bone at the lower first molar and the infradentale (Id^{l}) on the lingual side
M3-Id^{l}	Length of the lower dental arch which is the distance between the highest point of the central cusps of the lower 3rd molar (M3) and the infradentale (Id^{l}) on the lingual side

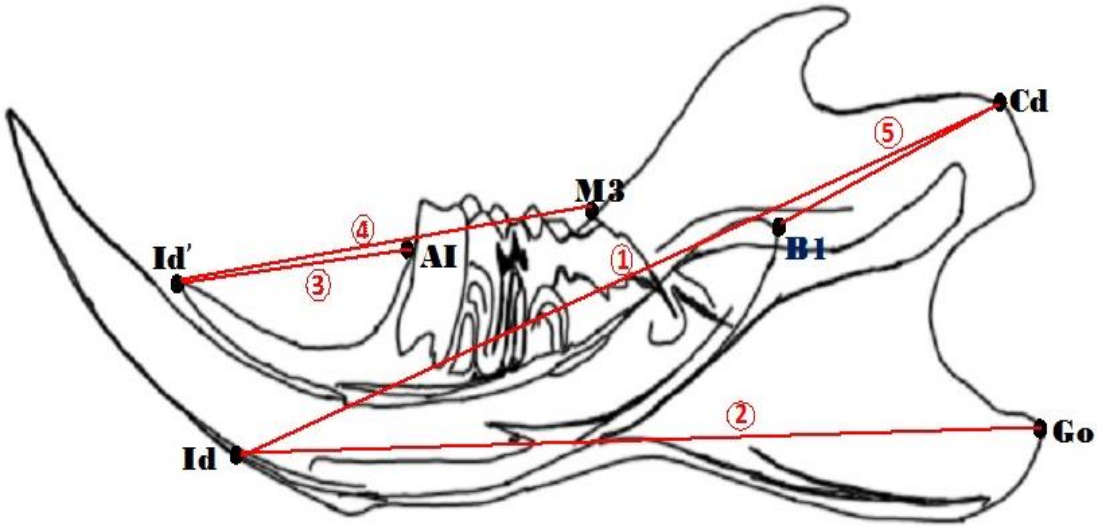


Figure 3.2: The medial aspect of the mandible. Reference points for linear measurements of the mandibular length of the rat mandible. **B1** indicates the alveolar base of the lower incisor and the Infradentale **(Id^{l})** on the lingual side, **Cd**; Coronoid process, **Go**; Gonion, **Id**; Infradentale (lingual side); **Id^{l}**; Infradentale (labial side), **AI**; length of lingual side of the alveolar bone, **M3**; the highest point of the central cusps of the lower 3rd molar (Adapted from Maki *et al*., 2002).

3.5.3. Assessment of linear mandibular height (mm)

Measurements in Table 3.3 were conducted to assess linear mandibular height in the adolescent rat mandible (Fig. 3.3).

Table 3.3: Measurements for the assessment of the linear mandibular height (mm) (Adapted from Maki *et al.,* 2002).

Measurement	Description
Cd-Go	Indicates the mandibular ramus height which is the distance between the condyle (Cd) and the gonion (Go)
Cd-Ag	Height of condylar head which is the distance between the condyle (Cd) and the ante-gonion (Ag)
Cr- Ag	Height of coronoid which is the distance between the coronoid (Cr) and ante-gonion (Ag)
Al^{I}-Me	Height of the central portion of the alveolar bone which is the distance between the deepest point of the outer margin of bone (Al^{I}) that connects menton (Me) and the ante-gonion (Ag)

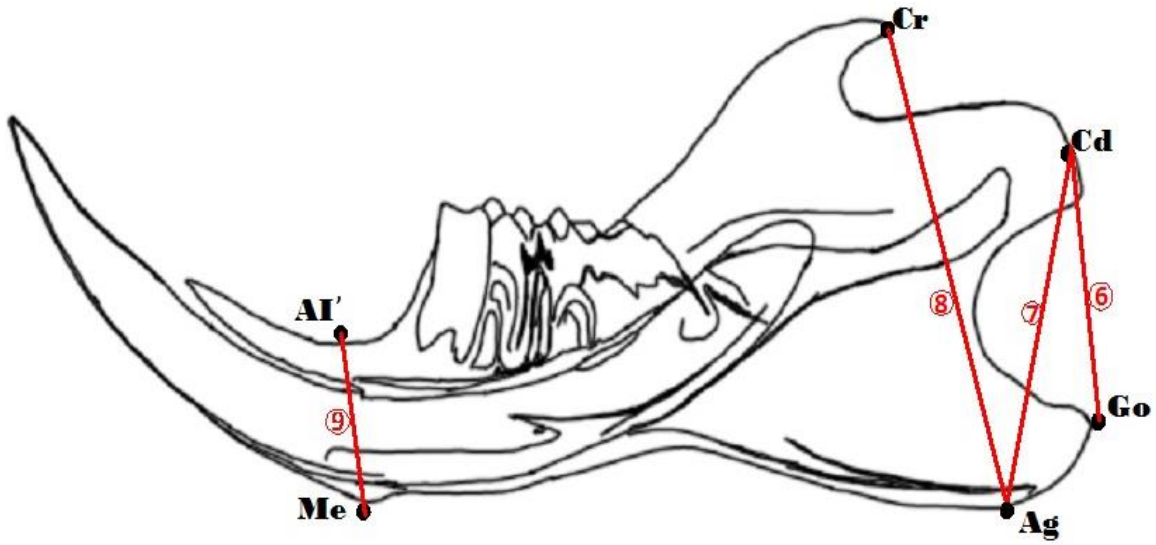


Figure 3.3: Reference points for linear measurements of the mandibular height of the rat mandible. Cr; Coronoid process, **Go**; Gonion, **Ag**; Ante-gonion, **Me**; Mention; **Al**^{I}; the deepest point of the outer margin of the bone (Adapted from Maki *et al*., 2002).

3.6 Three-dimensional Micro-Focus X-ray Computed Tomography (3D-µCT)

A Nikon XTH 225/320 LC X-ray microtomograph was used to scan the mandibles for 3D-µCT examination at the Nuclear Energy Association of South Africa (NECSA). To keep the samples steady during scanning, the bones were placed in low-density Styrofoam and positioned on the 360º rotating sample manipulator in the scanning chamber during scanning. The scanning parameters used are displayed in Table 3.4.

Table 3.4: 3D-µCT scanning parameters

Parameter	Value
X-ray source voltage	70 kV
X-ray source current	400 µA
Filter	1 mm aluminium
Scanning resolution	15 µm
Tomographic rotation	360 degrees
Rotation step	1 degree
Frame averaging	4
Scan duration	8 minutes

3.6.1. Region of interest and trabecular parameters analysed

After reconstruction of the mandibles, Volume Graphics® Max Studio (3.2) software was used for data analysis of the samples. The trabecular morphometric parameters were analysed in the chosen region of interest (ROI), in the interradicular trabecular bone between the first and second molar teeth. The trabecular morphometry assessed were: bone volume to total volume (BV/TV), trabecular thickness (TbTh), trabecular number (TbN), and, trabecular spacing (TbSp) (Table 3.5).

Table 3.5: Trabecular parameters assessed from the 3D-µCT scans (Adapted from Bouxsein *et al.,* 2010).

Parameter	Parameter description	Unit of measurement
Bone volume fraction (BV/TV)	A ratio that represents the bone volume to the total volume of the region of interest	%
Trabecular thickness (TbTh)	The mean thickness of the bone trabeculae in calculated as follows: TbTh = (2/BS)/BV (BS/BV is the bone surface-to-bone volume ratio)	mm
Trabecular number (TbN)	The measure of the mean number of trabeculae per unit length is calculated as follows: TbN = (BV/TV)/ TbTh	mm^{-1}
Trabecular spacing (TbSp)	The mean distance between trabeculae in calculated as follows: TbSp = (1/TbN) - TbTh	mm

3.7 Tensile strength testing

A Shimadzu universal testing machine was used for 3-point bending tests. Specimens (left hemi-mandibles) were placed on two rounded bars set 15 mm apart and the load was applied to the area halfway between the two support bars (Fig.3.4). Load-displacement curves (Fig 3.5) were recorded at a constant speed of 3 mm/min until failure

occurred. Tensile strength was determined from the parameters derived from the load-displacement curves (Table 3.6).

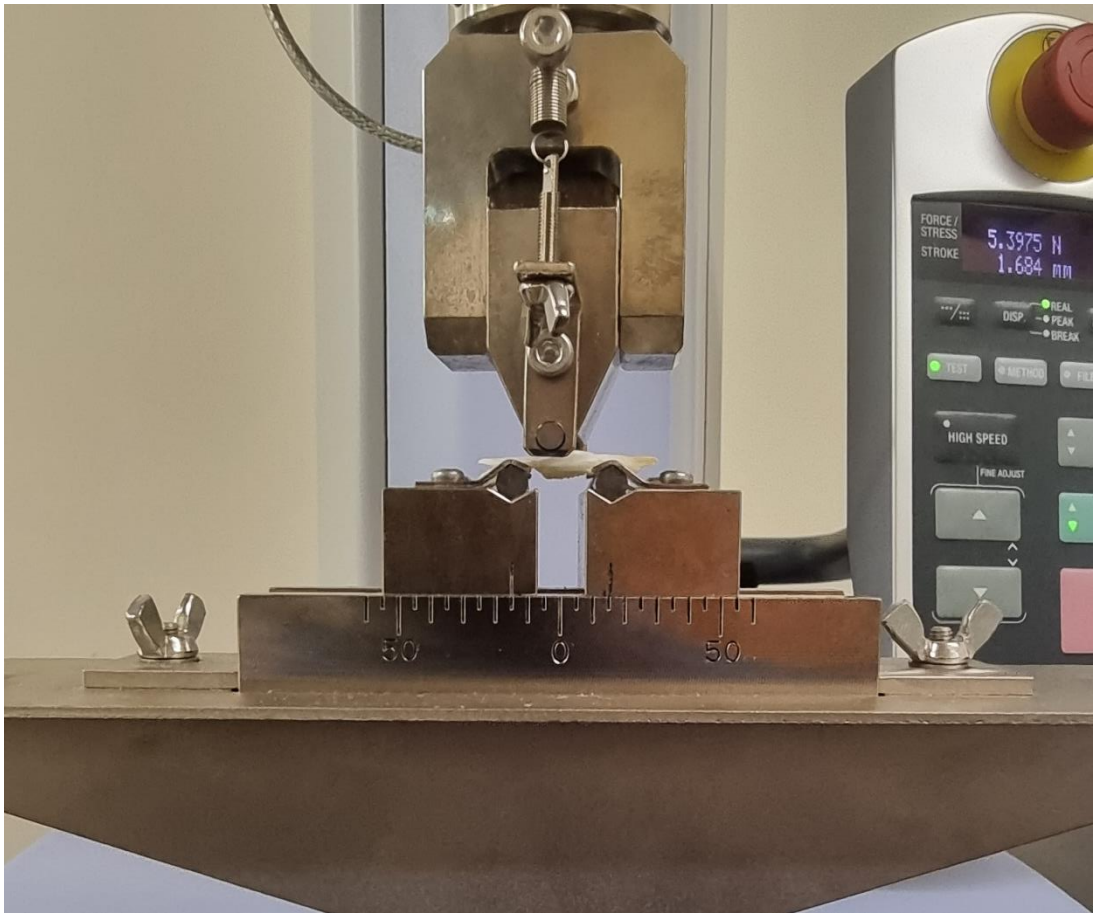


Figure 3.4: Three-point bend testing. The load was applied at the midpoint of the mandible. (Illustration provided by Bhika A, 2023).

Table 3.6: Tensile strength parameters derived from load displace curves (Adapted from Pillay and Ndou, 2022).

Tensile strength parameter	Parameter description
Maximum force (N)	The amount of force required for deformation of the bone to occur
Maximum displacement (mm)	Vertical deviation from the horizontal plane is required for the bone to fracture.
Maximum Time (sec)	The duration for which the bone resists the force until a fracture occurs
Break force (N)	The amount of force required for a bone fracture to occur

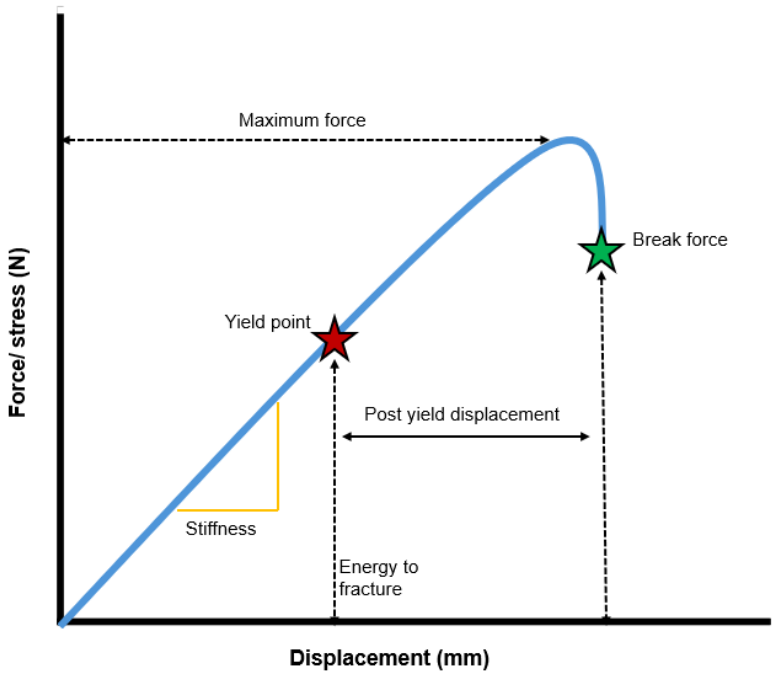


Figure 3.5: Load-displacement curve. Illustration of a load-displacement curve which indicates bone strength parameters of compact bone (Illustration provided by Bhika A, 2024)."

3.8 Tissue processing for histology and immunohistochemistry

The right hemi-mandible was used for Haematoxylin and Eosin (H & E) staining and immunohistochemistry. Formalin-fixed samples were decalcified in 14 % w/v ethylenediaminetetraacetic acid (EDTA, pH 7.4) in an incubator with continuous shaking at 45°C for 7-14 days (depending on the group, as larger bones took longer to decalcify). Decalcification was determined radiographically (Aribex NOMAD examiner portable x-ray system). Calcium in bone produces a white (radiopaque) appearance on X-ray images. In contrast, decalcified bone loses this radiopacity and appears radiolucent. Thus, the radiolucent appearance of the mandibles confirmed complete decalcification. The time

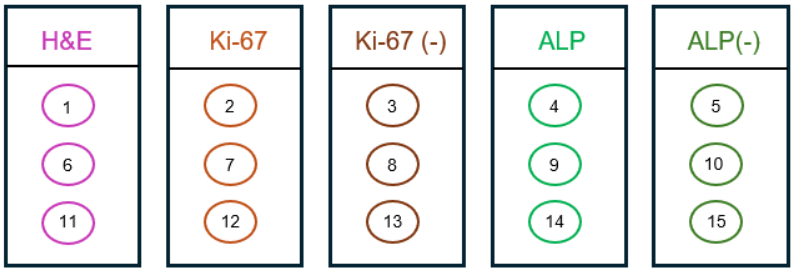
required for bone decalcification varied by bone size, with G1 taking 7 days, G4 10 days, and G4 -Abs 14 days. Although EDTA is generally gentler than acid-based decalcifiers and better preserves cellular details, prolonged decalcification can still cause artifacts. Uneven decalcification may also occur if the bone is very dense. To minimize these risks, it is essential to monitor decalcification progress regularly and use appropriate agitation and temperature control.

Subsequently, the bones were rinsed, and the region of interest was isolated by cutting through the mandible anteriorly, anterior to the 1st molar tooth at the area of the menton, and posteriorly, posterior to the third molar tooth. These bone samples were then placed in 10 % buffered formalin for 24 hours prior to processing. The samples were then processed through ascending grades of alcohol overnight using an automatic tissue processor (Citadel 2000, Thermo Fisher Scientific, USA), before embedding in paraffin. During embedding, special care was taken to orient the bone samples longitudinally, with the buccal aspect of the bone facing the cutting surface of the paraffin block to minimize variations in the sectioning angle. Serial sections were cut with a microtome (Leica RM2125 RTS; Leica Biosystems) at 5 µm thickness in a horizontal plane and placed on Epredia Superfrost^{TM} plus adhesion microscope slides (Netherlands).

The first sections were stained with H & E to assess normal cytoarchitecture. The second and third sections were immunolabeled with Ki-67 antibody (NB 500-170) and its negative control, respectively. The fourth and fifth sections were immunolabeled with alkaline phosphatase (ALP) antibody (Ab95462) and its negative control, respectively (Fig. 3.6). The process was repeated after discarding the initial 20 sections. Consequently, each experiment was conducted in triplicate.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
H&E	Ki-67	Ki-67 (-)	ALP	ALP (-)	H&E	Ki-67	Ki-67 (-)	ALP	ALP (-)	H&E	Ki-67	Ki-67 (-)	ALP	ALP (-)

(a)



(b)

Figure 3.6: Serial sections of mandibular bone samples. (a) illustrate how serial sections were acquired and (b) illustrate the process of labeling slides and selecting sections.

3.8.1 Haematoxylin and Eosin staining

For normal cytoarchitecture analysis, H & E staining was conducted. Firstly, slides containing tissue sections were dewaxed in xylene for 10 minutes X 2, hydrated in a graded series of alcohol (from 100-70 %), and rinsed in running tap water for 5 minutes. Sections were then stained with Meyer's Haematoxylin for 20 minutes, rinsed in running tap water for 5 minutes and differentiated in 1 % acid alcohol. Tissue sections were again rinsed in running tap water for 5 minutes. Tissue sections were then immersed in Scott's tap water, which acts as a bluing agent, for 5 minutes and was rinsed again in running tap water for 5 minutes. Thereafter, sections were stained with Eosin for 2 minutes, rinsed

briefly in running water, then dehydrated in a graded series of alcohols (from 70-100 %), cleared in xylene, mounted in Entellen, and covered with a coverslip (See appendix J)."

3.8.2 Immunohistochemistry of proliferating cells and osteoblasts

Immunolabeling of proliferating cells expressing the Ki-67 protein and osteoblasts expressing the alkaline phosphatase (ALP) protein was achieved using the Ki-67 antibody (NB 500-170) (Whitehead Scientific) and the Abcam ALP antibody (Ab95462) respectively. Tissue sections were deparaffinized for 20 minutes in xylene, rehydrated in a graded series of alcohol (from 100-70 %), and rinsed in running tap water for 5 minutes. After which, tissue sections were placed in a citrate buffer solution (pH 6) in a water bath at 60 °C for 2 hours to allow for antigen retrieval. Subsequently, the sections were cooled at room temperature for 20 minutes and washed in phosphate buffer solution (PBS) (pH 7.4) for 5 minutes. To block endogenous peroxidase, the sections were treated with a mixture containing 1 % hydrogen peroxide in methanol for 30 minutes and washed in PBS for 5 minutes, repeated three times. The tissue sections were then incubated with a protein block (Ab156024) for 20 minutes and washed in PBS for 5 minutes, repeated three times. Following this, the sections were incubated with the primary antibody (Ki-67 using a 1:200 dilution factor) or (ALP using a 1:1000 dilution factor) overnight at 4°C. After the overnight incubation, the sections were brought to room temperature for approximately 1 hour and then washed in PBS for 5 minutes, repeated four times, before being incubated with a biotinylated secondary antibody (Biotinylated Goat Anti-Rabbit IgG (H+L) (Ab64256) for 10 minutes. Subsequently, the sections were washed with PBS for 5 minutes, repeated four times, and incubated with Streptavidin HRP (Ab64269) for 10 minutes to reduce non-specific background staining and enhance antibody binding.

Following this, the sections were again washed in PBS for 5 minutes, repeated four times, before being incubated with 3-3’ diaminobenzidine (Ab64238 - DAB substrate kit) for 10 minutes to aid in the visualization of stained cells under a light microscope. The sections were then rinsed in running tap water for 5 minutes. Finally, the tissue sections were dehydrated through graded alcohol (from 70-100 %), cleared in xylene, mounted with Entellan, and covered with a coverslip (See appendix K).

3.9 Photography and quantification of cells

Photomicrographs of slides stained with H & E, and Ki-67 and ALP immunolabeled slides were captured using a light microscope fitted with an axiocam HRC digital camera (Zeiss Axioscope 2 plus). Slides were analysed for normal cytoarchitecture (H & E). Images of the immunolabeled slides were imported into Fiji Image J software for quantification of cells, where the manual counting method was utilized. Cell counts were conducted in duplicate. The counting was conducted on the entirety of the region of interest between the roots of the first and second molar teeth.

3.10 Data analysis

The data was managed in Microsoft Excel 2023 (Microsoft Corporation) and analysed using the Statistical Package for Social Service (SPSS) version 28 (IBM®) software. The Shapiro-Wilk test was used to assess the normality of the data. The Independent Samples t-test was used to analyse normally distributed data, comparing means between the pair-fed control and alcohol-exposed groups within the sexes, as well as comparing the same groups between sexes. Non-parametric data was analysed using the Mann-Whitney U test. The threshold for statistical significance was set at $p < 0.05$.

For clarity and ease of interpretation, the results and discussions relating to the three different study groups are presented separately across three consecutive chapters. This structure allows for a more detailed and systematic analysis of each group, facilitating clearer comparison and understanding of the findings.

CHAPTER 4: *The effect of acute binge alcohol consumption on the development of the mandible in adolescent Sprague Dawley rats*

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4.1 INTRODUCTION

Adolescence is a critical period marked by profound physical, cognitive, and emotional development. During this phase, individuals are particularly vulnerable to external influences, and experimentation with alcohol is common among this age group (Morojele and Ramsoomar, 2016). Binge drinking is characterized by the consumption of a large amount of alcohol in a brief period, posing significant risks to the health and well-being of adolescents. Binge drinking, also referred to as heavy episodic drinking, is defined as the intake of five or more drinks for males and four or more drinks for females during a single occasion or drinking session, or as the consumption of at least 60 grams of pure alcohol once a month (Morojele and Ramsoomar, 2016). Chronic alcohol exposure in adults is known to elevate the risk of osteoporosis, leading to decreased bone density and an increased likelihood of fractures. However, there is a lack of literature concerning the effects of acute binge drinking on the adolescent skeleton (Sampson *et al.*, 1996; Sampson, 1997). There is even less understanding of the impact of binge alcohol consumption on the adolescent mandible.

Research using animal models has examined the effects of alcohol consumption on long bones during adolescence, revealing a significant reduction in bone growth, volume, density, and strength. Additionally, Föger-Samwald et al. (2018) investigated the effects of binge alcohol consumption using prepubescent pigs as a model. Their results indicated a reduction in serum calcium and phosphate levels, along with decreased density and fewer trabeculae in the femur (Föger-Samwald *et al.*, 2018). The effects of binge drinking on trabecular parameters and the tensile strength of adolescent bone remain limited, particularly concerning craniofacial bones, indicating a need for further research.

The mandible is an unpaired bone of the craniofacial skeleton. This bone is essential for mastication, speech, facial expression, and dentition (Hatcher., 2022). The growth and development of the mandible requires control of cell proliferation, differentiation, and the ossification processes. Alcohol consumption is known to disturb these processes (Thobane *et al*., 2024). Therefore, this study investigated the effects of acute binge alcohol consumption on adolescent Sprague Dawley rat mandibles. The acute binge alcohol model in this study was a 7-day model where animals were exposed to a binge model of alcohol exposure for three out of the 7 days (every alternate day). The study used microtomography to examine the impact of alcohol on trabecular bone structure, 3-point bending tests to measure the tensile strength of bone tissue, Hematoxylin and Eosin (H & E) staining to analyse the effects of alcohol on bone cell structure, and immunohistochemistry with anti-ki-67 and anti-alkaline-phosphatase antibodies to determine the consequences of acute binge alcohol on cell proliferation and the number of osteoblasts in the adolescent mandible.

4.2 RESULTS

4.2.1 Baseline Data

4.2.1.1. Blood Alcohol Concentration

The mean blood alcohol concentration (BAC) was 105.00 mg/dL (± 0.37) in the male alcohol group; and 111.04 mg/dL (± 22.15) in the female alcohol-exposed group.

4.2.1.2 Food Consumption

Males and females exhibited differences in the pattern of food consumption. Male pair-fed control (mean = 48.06 g ± 2.69) and alcohol-exposed rats (mean = 48.69 g ± 2.54) showed no significant differences in the amount of food consumed ($p = 0.390$). Female

pair-fed control rats (mean = 30.94 g ± 0.84) consumed significantly more food than the alcohol-exposed rats (mean = 29.25 g ± 0.44) ($p = 0.018$).

The amount of food consumed by the male pair-fed control group was significantly greater than that consumed by the female pair-fed control group ($p < 0.001$). The same was observed in the alcohol-exposed groups where the male alcohol-exposed group consumed more food than the female alcohol-exposed group) ($p < 0.001$) (Fig. 4.1).

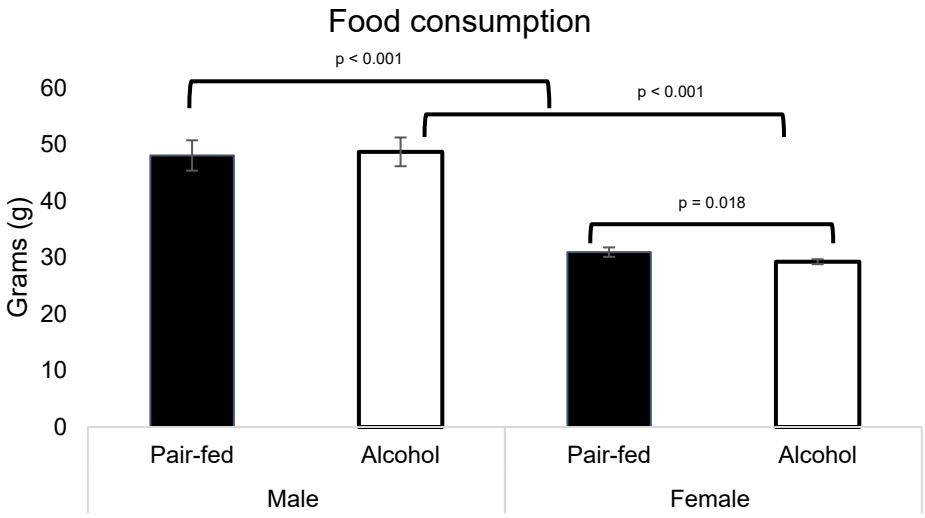


Figure 4.1: Food consumption in the acute binge alcohol model. The daily food consumption is shown as an average for the week for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

4.2.1.3 Water Intake

Males and females exhibited differences in water intake. Male pair-fed control rats (mean = 120.00 ml ± 5.46) consumed more water than the male alcohol-exposed rats (mean = 111.94 ml ± 5.42) ($p = 0.072$). The difference, however, was not significant. Again, no

significant differences were noted in the water intake between female pair-fed control (mean = 83.61 ml ± 10.72) and female alcohol-exposed rats (mean = 82.50 ml ± 10.83) (p = 0.453).

Water intake in the male pair-fed control group was significantly greater than that of the female pair-fed control group (p = 0.003). The same was exhibited in the alcohol-exposed groups where the male alcohol-exposed group consumed significantly more water than the female alcohol-exposed group) (p = 0.007) (Fig. 4.2).

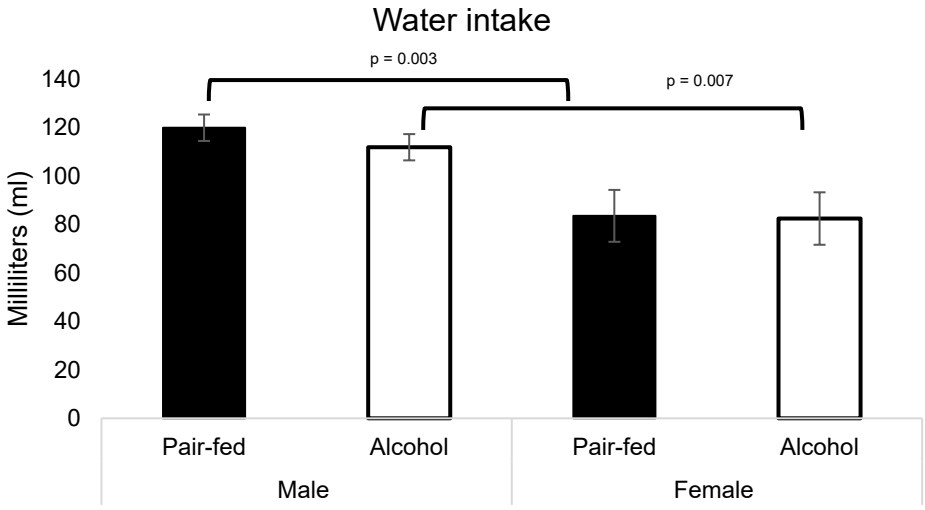


Figure 4.2: Water intake in the acute binge alcohol model. The daily water intake is shown as an average for the week for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

4.2.1.4 Percentage Body Mass Gained

Regarding the percentage (%) of body mass gained, male and female groups showed differences. Male pair-fed control rats (mean = 12.29 % ± 1.45) showed no significant

differences when compared to the male alcohol-exposed rats (mean = 12.16 % ± 1.55) ($p = 0.445$). However, in the female rats, the pair-fed controls (mean = 7.00 % ±1.14) had a significantly greater gain in body mass percentage than the female alcohol-exposed rats (mean = 4.97 % ± 1.45) ($p = 0.017$).

When comparing males and females, the percentage of body mass gained in the male pair-fed control group was significantly greater than that of the female pair-fed control group ($p < 0.001$). The same was noted in the alcohol-exposed groups where the male alcohol-exposed group had gained a significantly greater percentage of body mass than the female alcohol-exposed group) ($p < 0.001$) (Fig.4.3).

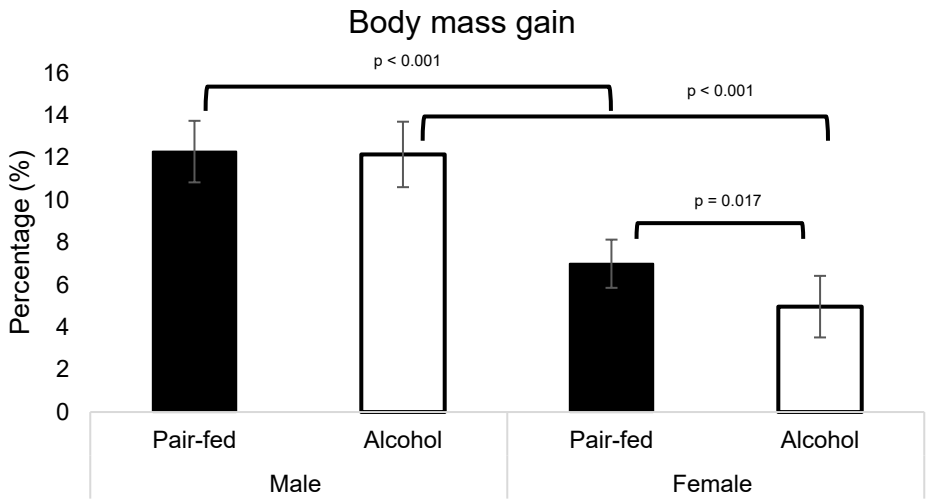


Figure 4.3: Percentage (%) body mass gain in the acute binge alcohol model. The percentage of body mass gained is shown for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

4.2.2 Osteometric Measurements

4.2.2.1 Mandibular Weight

Males and females exhibited differences in bone weight. Mandibular bone weight in males lower in the alcohol-exposed group (mean = 0.93 g ± 0.03) than the pair-fed control (mean = 0.98 g ± 0.06) ($p = 0.050$). This, however, was not statistically significant. In the female rats, no significant difference was detected in the weight of the mandible between the alcohol-exposed (mean = 0.84 g ± 0.04) and the pair-fed control groups (mean = 0.86 g ± 0.03) ($p = 0.215$).

The weight of the mandible was significantly greater in the male pair-fed control than in the female pair-fed control ($p = 0.001$). Similarly, mandibular weight was significantly greater in the male alcohol-exposed group than in the female alcohol-exposed group ($p = 0.002$) (Fig. 4.4).

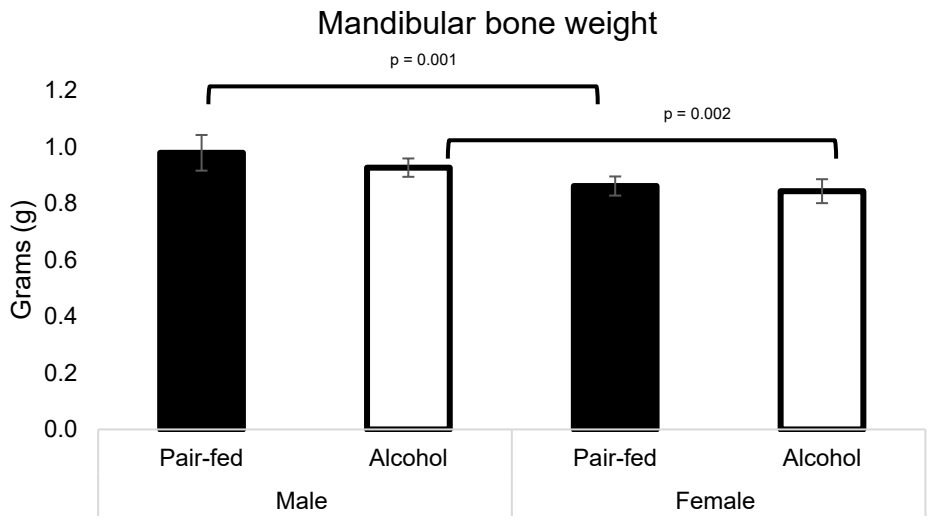


Figure 4.4: Mandibular weight in the acute binge alcohol model. The mean weights are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

4.2.2.2 General Mandibular Size

Both males and females showed similar general size of the mandible. There were no significant differences in measurements between male pair-fed control and alcohol-exposed rats (Table 4.1), and similarly, no significant differences were found between female pair-fed control and alcohol-exposed rats (Table 4.2).

When comparing the two sexes, the male pair-fed control rats had significantly greater measurements in the distance between the mental foramen (MF) and most posterior point of the condylar head (Cd) (MF-Cd), the distance between the mental foramen and the gonion (Go) (MF-Go), the distance between the mental foramen and the ante-gonion (Ag) (MF-Ag), the distance between the mental foramen and the menton (Me) (MF-Me), the distance between the mental foramen and the highest point of mesiobuccal cusp of the

lower 1st molar (M1) (MF-M1), and the distance between the mental foramen and the highest point of central cusps of the lower 3rd molar (M3) (MF-M3) than the female pair-fed controls ($p < 0.001$, $p = 0.003$, $p = 0.002$, $p = 0.033$, and $p < 0.001$, respectively). The distance between the mental foramen (MF) and the deepest point of the outer margin of the bone (Al^{l}) connecting Al and Id^{l} (MF-Al^{l}) showed no significant differences ($p = 0.107$). In the alcohol-exposed groups, the male rats also had significantly greater MF-Cd, MF-Go, MF-Ag, MF-Me, MF-Al^{l}, and MF-M3 measurements than the female alcohol-exposed group ($p < 0.001$, $p = 0.007$, $p = 0.005$, $p = 0.022$, $p = 0.001$, $p = 0.022$, respectively). MF-M1 was similar between the two groups ($p = 0.111$).

Table 4.1: General mandibular size in male rats in the acute binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
MF-Cd	Pair-fed control	6	19.41	0.41	0.291
	Alcohol	6	19.31	0.13	
MF-Go	Pair-fed control	6	18.61	0.39	0.115
	Alcohol	6	18.38	0.17	
MF-Ag	Pair-fed control	6	14.34	0.40	0.141
	Alcohol	6	14.10	0.32	
MF-Me	Pair-fed control	6	4.03	0.18	0.275
	Alcohol	6	3.98	0.09	
MF-Al^{I}	Pair-fed control	6	2.01	0.29	0.485
	Alcohol	6	2.11	0.09	
MF-M1	Pair-fed control	6	4.06	0.19	0.283
	Alcohol	6	4.00	0.13	
MF-M3	Pair-fed control	6	7.86	0.11	0.471
	Alcohol	6	7.85	0.15	

Table 4.2: General mandibular size in female rats in the acute binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
MF-Cd	Pair-fed control	6	18.60	0.22	0.436
	Alcohol	6	18.63	0.35	
MF-Go	Pair-fed control	6	17.91	0.28	0.252
	Alcohol	6	17.75	0.49	
MF-Ag	Pair-fed control	6	13.56	0.31	0.263
	Alcohol	6	13.42	0.42	
MF-Me	Pair-fed control	6	3.82	0.18	0.452
	Alcohol	6	3.83	0.13	
MF-AI^{I}	Pair-fed control	6	1.84	0.07	0.345
	Alcohol	6	1.87	0.12	
MF-M1	Pair-fed control	6	3.87	0.11	0.420
	Alcohol	6	3.89	0.16	
MF-M3	Pair-fed control	6	7.51	0.11	0.198
	Alcohol	6	7.60	0.22	

4.2.2.3 Linear Mandibular Length

Similarities were depicted in the length of the mandible in the male and female study groups. The only significant difference identified between the male groups was the

distance between the infradentale on the lingual side and the highest point of the central cusp of the lower third molar (M3-Id^{l}), which was significantly greater in the alcohol-exposed male group (mean = 14.576 mm ± 0.171) than in the pair-fed control (mean = 14.194 mm ± 0.280) ($p = 0.010$) (Table 4.3). No significant differences in measurements were displayed between the female pair-fed control and the alcohol-exposed groups (Table 4.4).

The length of the mandible was significantly greater with regards to the length of the base of the mandible which is the distance between the condyle to the infradentale on the labial side (Cd-Id) and the mandibular body length which is the distance between the gonion (Go) and the Infradentale (Id) on the labial side (Go-Id) in the male pair-fed control group when compared to the female pair-fed control group ($p = 0.001$ and $p = 0.011$, respectively). The length of lingual side of the alveolar bone which is the distance between the highest point of the mesial alveolar (AI) bone at the lower first molar and the Infradentale (Id^{l}) on the lingual side (AI-Id^{l}) and the length of the lower dental arch which is the distance between the highest point of the central cusps of the lower 3rd molar (M3) and the infradentale (Id^{l}) on the lingual side (M3-Id^{l}) showed no differences between the groups ($p = 0.194$ and $p = 0.144$, respectively). The length of the mandible was also significantly greater in the male alcohol-exposed group than the female alcohol-exposed group regarding all the measurements taken; Cd-Id ($p < 0.001$), Go-Id ($p = 0.001$), AI-Id^{l} ($p = 0.008$), and M3- Id^{l} ($p = 0.002$).

Table 4.3: Mandibular length in male rats in the acute binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Id	Pair-fed control	6	26.13	0.60	0.339
	Alcohol	6	26.02	0.22	
Go-Id	Pair-fed control	6	25.59	0.72	0.438
	Alcohol	6	25.64	0.24	
AI-Id[I]	Pair-fed control	6	8.41	0.39	0.146
	Alcohol	6	8.59	0.15	
M3-Id[I]	Pair-fed control	6	14.19	0.28	0.010*
	Alcohol	6	14.57	0.17	

Table 4.4: Mandibular length in female rats in the acute binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Id	Pair-fed control	6	25.04	0.31	0.181
	Alcohol	6	25.24	0.41	
Go-Id	Pair-fed control	6	24.73	0.29	0.302
	Alcohol	6	24.84	0.42	
Al-Idl	Pair-fed control	6	8.24	0.23	0.390
	Alcohol	6	8.19	0.23	
M3-Idl	Pair-fed control	6	14.06	0.12	0.449
	Alcohol	6	14.07	0.29	

4.2.2.4 Linear Mandibular Height

A similar pattern was identified between pair-fed control and alcohol-exposed groups in male and female rats. No significant differences were identified between male pair-fed control and alcohol-exposed groups (Table 4.5). Female rats showed a difference in the mandibular ramus height which is the distance between the condyle (Cd) and the gonion (Go) (Cd-Go) measurement where the alcohol-exposed group had a significantly greater Cd-Go (mean = 7.49 mm ± 0.15) than the pair-fed control group (mean = 7.23 mm ± 0.17) ($p = 0.011$). All other height measurements showed no significant differences between the female groups (Table 4.6).

Male pair-fed control rats had significantly greater mandibular height measurements than the female pair-fed control rats for the following measurements: Cd-Go: $p < 0.001$; the height of the condylar head which is the distance between the condyle (Cd) and the ante-gonion (Ag) (Cd-Ag) $p = 0.006$; the height of the coronoid which is the distance between the coronoid (Cr) and ante-gonion (Ag) (Cr-Ag): $p = 0.006$; and the height of the central portion of alveolar bone which is the distance between the deepest point of the outer margin of bone (Al^{I}) that connects to the menton (Me) and the ante-gonion (Ag) to the menton (Me) (Al^{I}-Me): $p = 0.014$. The mandibular height was also significantly larger in male alcohol-exposed rats than the female alcohol-exposed rats (Cd-Go: $p < 0.001$; Cd-Ag: $p = 0.017$; Cr-Ag: $p = 0.006$; Al^{I}-Me: $p < 0.001$).

Table 4.5: Mandibular height in male rats in the acute binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Go	Pair-fed control	6	7.84	0.26	0.232
	Alcohol	6	7.93	0.13	
Cd-Ag	Pair-fed control	6	11.84	0.50	0.357
	Alcohol	6	11.76	0.28	
Cr-Ag	Pair-fed control	6	13.42	0.31	0.267
	Alcohol	6	13.33	0.18	
All-Me	Pair-fed control	6	4.41	0.25	0.463
	Alcohol	6	4.39	0.12	

Table 4.6: Mandibular height in female rats in the acute binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Go	Pair-fed control	6	7.23	0.17	0.011*
	Alcohol	6	7.49	0.15	
Cd-Ag	Pair-fed control	6	11.11	0.32	0.106
	Alcohol	6	11.34	0.30	
Cr-Ag	Pair-fed control	6	12.87	0.31	0.255
	Alcohol	6	12.71	0.46	
All-Me	Pair-fed control	6	4.09	4.05	0.295
	Alcohol	6	0.15	0.12	

4.2.3 Three-dimensional Micro-focus X-ray Computed Tomography (3D- µCT)

4.2.3.1 Mandibular Bone to Total Volume Ratio (BV/TV)

Males and females exhibited differences in the bone to total volume ratio (BV/TV). The BV/TV in males was marginally lower in the pair-fed control group (mean = 58.40 % ± 3.65) than the alcohol-exposed group, however this difference was not significant (mean = 60.88 % ± 6.53) ($p = 0.223$). However, in the female rats, the pair-fed control group (mean = 68.27 % ± 4.85) had a significantly higher BV/TV than the alcohol-exposed group (mean = 61.20 % ± 3.45) ($p = 0.011$).

The BV/TV between the male and female pair-fed controls was statistically different, with the female controls BV/TV being greater than the BV/TV in males ($p = 0.020$). The BV/TV

between male and female alcohol-exposed groups showed no differences (p = 0.458) (Fig. 4.5).

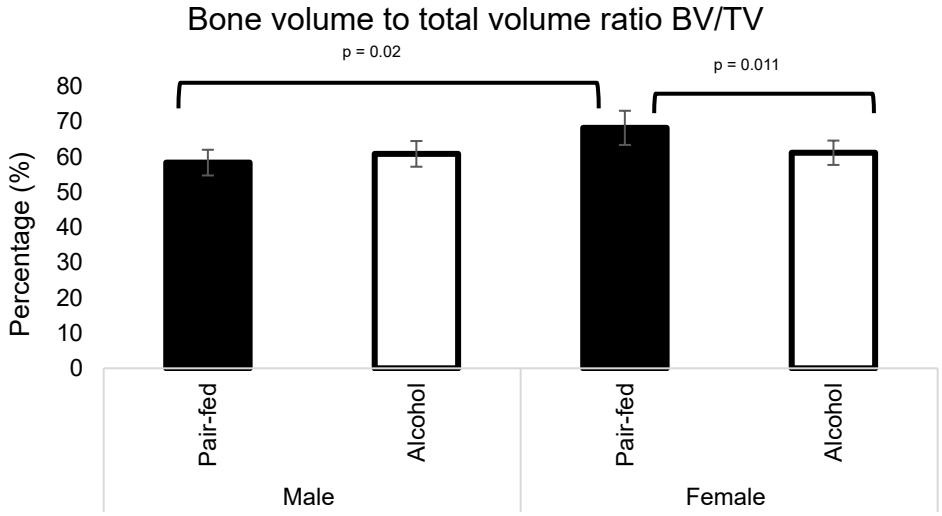


Figure 4.5: Mandibular total bone to volume ratio (BV/TV) in the acute binge alcohol model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

4.2.3.2 Mandibular Trabecular Thickness (TbTh)

With regards to trabecular thickness similar patterns were identified in males and females. The pair-fed control groups had thicker trabeculae in both males and females. Trabeculae were thinner in the male alcohol-exposed group (mean = 0.14 mm ± 0.03) than the pair-fed control group (mean = 0.16 mm ± 0.03) however, no significance was detected (p = 0.140). Thicker trabeculae were displayed in the female pair-fed control group (mean = 0.18 mm ± 0.03) than the alcohol-exposed group (mean = 0.16 mm ± 0.01), again this difference was not significant (p = 0.180).

Similarities were exhibited between the male and female pair-fed and the alcohol groups ($p = 0.124$ and $p = 0.134$, respectively) (Fig. 4.6).

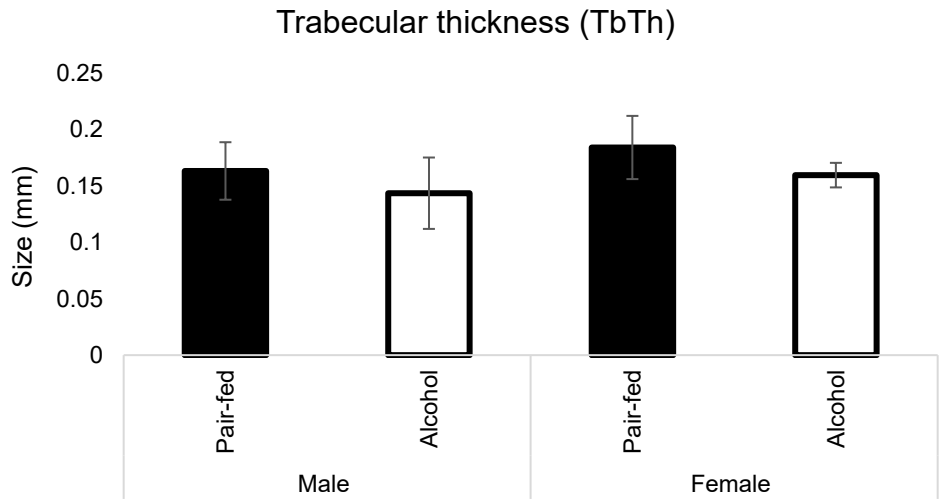


Figure 4.6: Mandibular trabecular thickness (TbTh) in the acute binge alcohol model. The mean values are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

4.2.3.3 Mandibular Trabecular Number (TbN)

The trabecular number was similar between the study groups in the mandible for both the male and female rats. The trabecular number (TbN) in the male pair-fed control and alcohol-exposed groups were similar (mean = 3.63 mm^{-1} ± 0.38 and mean = 3.86 mm^{-1} ± 0.42, respectively) ($p = 0.186$). Again, in the female rats the pair-fed control and the alcohol-exposed groups exhibited similarities (mean = 3.76 mm^{-1} ± 0.37 and mean = 3.84 mm^{-1} ± 0.18, respectively) ($p = 0.329$).

Also, trabecular distribution showed no differences between male and female pair-fed control groups (p = 0.302) as well as between male and female alcohol-exposed groups (p = 0.462) (Fig. 4.7).

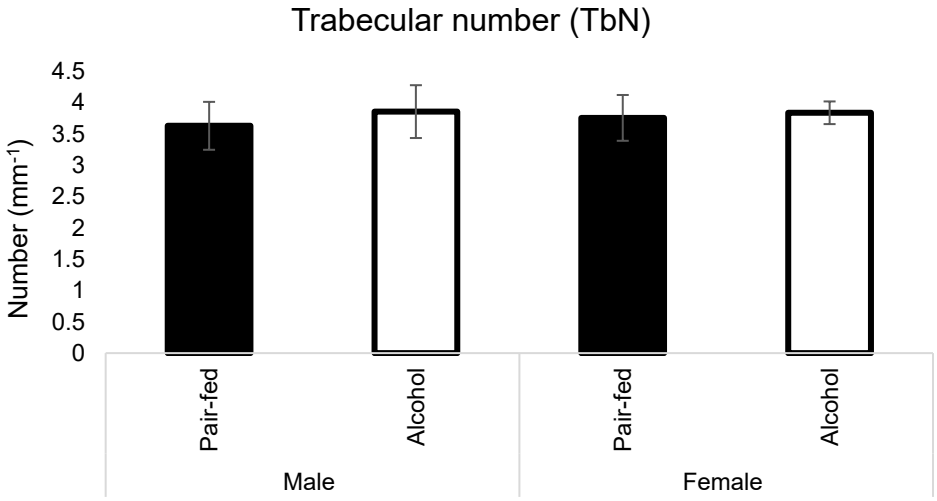


Figure 4.7: Mandibular trabecular number (TbN) in the acute binge alcohol model. The mean values are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

4.2.3.4 Mandibular Trabecular Spacing (TbSp)

Trabecular spacing stratified by study group showed a varied pattern in the mandible. In the male rats, the trabecular spacing was not statistically different for the pair-fed control than the alcohol-exposed rats (mean = 0.12 mm ± 0.01 and mean = 0.12 mm ± 0.05, respectively) (p = 0.240). However, the female rats, exhibited significantly wider trabeculae in the alcohol-exposed group than the pair-fed control group in females (mean = 0.1 mm ± 0.01 and mean = 0.08 mm ± 0.01) (p = 0.014).

A significant difference was exhibited in trabecular spacing (TbSp) between the male and female pair-fed control groups ($p < 0.001$) where male rats had significantly larger trabecular spaces. However, no significant differences in TbSp were observed between male and female alcohol-exposed groups (Fig. 4.8 and Fig. 4.9).

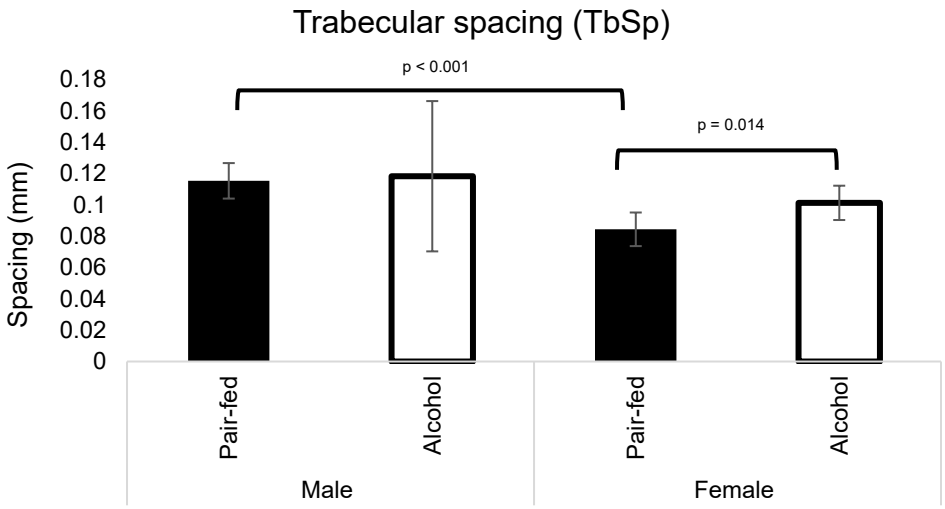


Figure 4.8: Mandibular trabecular spacing (TbSp) in the acute binge alcohol model. The mean values are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

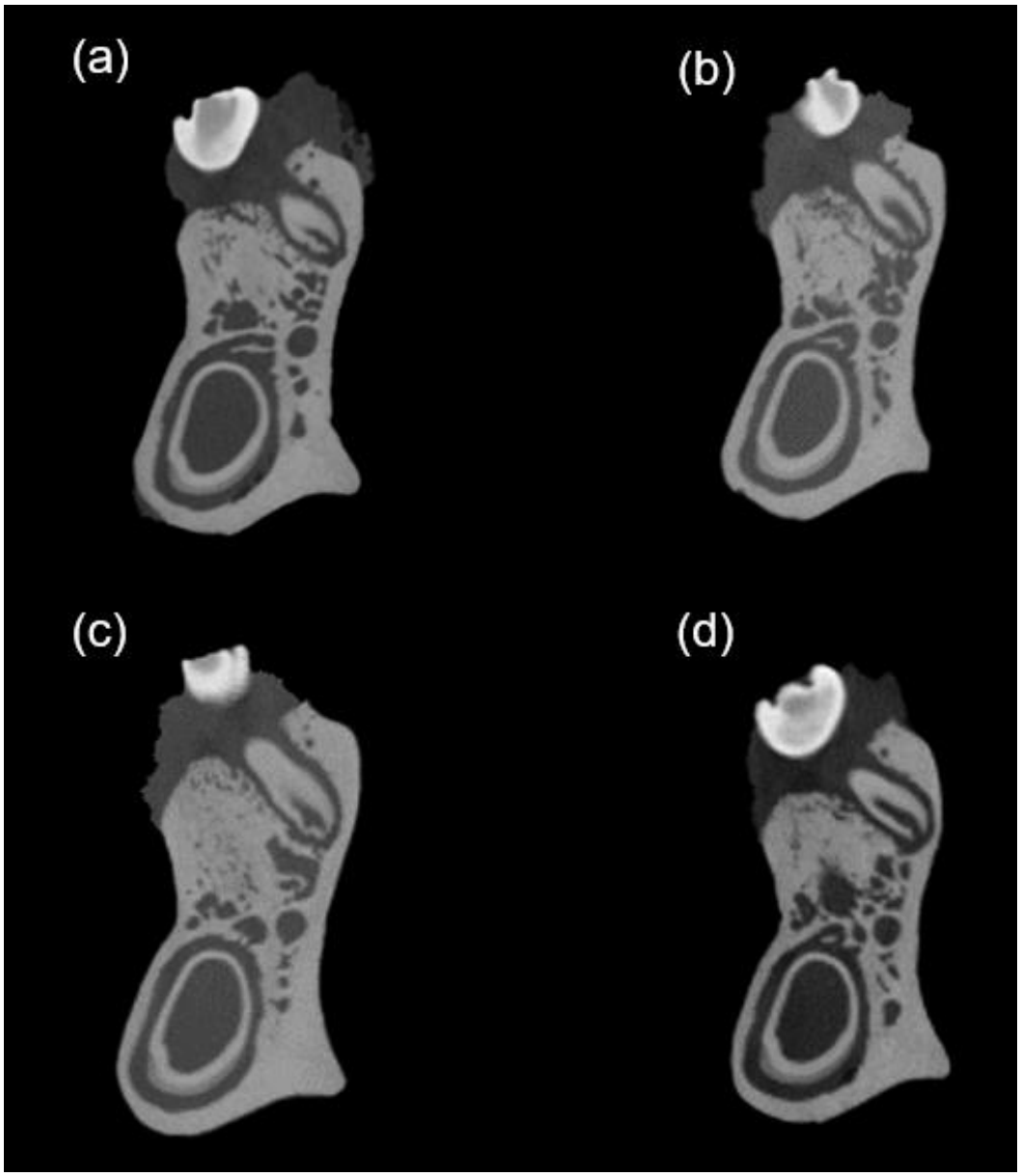


Figure 4.9: Trabecular morphology in the mandible in the acute binge alcohol model. Representative slices of (a) male pair-fed control group showing the similar number of trabeculae and spacing to the male alcohol-exposed group; (b) male alcohol-exposed group; (c) female pair-fed control group showing the similar number of trabeculae to the alcohol-exposed group with less spacing; (d) female alcohol-exposed group showing wider trabecular spaces than the control. Slices were taken between the 1st and 2nd mandibular molar teeth.

4.2.4 Tensile Strength of the Mandible using 3-point Bending Tests

Differences in tensile strength parameters were displayed between male and female rats. In male rats, the maximum force and break force were greater in the alcohol-exposed group, however, no significance was detected ($p = 0.102$ and $p = 0.132$, respectively). The time required to fracture the bones when applying force (maximum time), was greater in the pair-fed control group, again, this difference was not significant ($p = 0.132$). The maximum displacement between the pair-fed control and alcohol-exposed groups in male rats was similar ($p = 0.132$) (Table 4.7). With regards to the female rats, the maximum force and break force were greater in the pair-fed control group than the alcohol-exposed group, no significance was detected ($p = 0.065$ and $p = 0.057$, respectively). The maximum displacement and maximum time were similar between the pair-fed control and alcohol-exposed groups in female rats ($p = 0.294$ and $p = 0.294$, respectively) (Table 4.8).

No significant differences were shown in the maximum force ($p = 0.093$), break force ($p = 0.077$), maximum displacement ($p = 0.195$), and maximum time ($p = 0.195$) between male and female pair-fed control animals. The maximum force ($p = 0.002$), and the break force ($p = 0.003$) were significantly higher in the male alcohol-exposed rats than the female alcohol-exposed rats. A comparison between the male and female alcohol-exposed rats, displayed no significant differences in the maximum displacement and maximum time ($p = 1.0$ for both comparisons, respectively).

Table 4.7: Tensile strength of the mandible in male rats in the acute binge alcohol model

Measurement	Group	N	Mean	SD	P-value
Maximum force (N)	Pair-fed control	6	57.27	2.62	0.102
	Alcohol	6	66.50	16.44	
Maximum displacement (mm)	Pair-fed control	6	6.23	0.22	0.132
	Alcohol	6	5.22	2.14	
Maximum time (sec)	Pair-fed control	6	124.68	4.38	0.132
	Alcohol	6	121.05	5.53	
Break force (N)	Pair-fed control	6	56.01	2.73	0.114
	Alcohol	6	64.98	16.92	

Table 4.8: Tensile strength of the mandible in female rats in the acute binge alcohol model

Measurement	Group	N	Mean	SD	P-value
Maximum force (N)	Pair-fed control	6	49.30	13.49	0.065
	Alcohol	6	37.96	9.99	
Maximum displacement (mm)	Pair-fed control	6	6.11	0.27	0.294
	Alcohol	6	6.00	0.39	
Maximum time (sec)	Pair-fed control	6	122.14	5.38	0.294
	Alcohol	6	119.97	7.81	
Break force (N)	Pair-fed control	6	47.24	13.70	0.057
	Alcohol	6	31.95	16.72	

4.2.5 Mandibular Cytoarchitecture (Hematoxylin and Eosin staining)

Male and female groups showed slight differences regarding the cytoarchitecture identified with Hematoxylin and Eosin (H & E) staining. Male pair-fed control rats had abundant osteocytes scattered evenly throughout the tissue. Abundant osteoblasts were also detected on the inner surface of trabeculae. Numerous small trabecular spaces were exhibited throughout the tissue and were filled with erythrocytes. Widespread bone matrix was identified. Bone matrix, collagen fibres, and periodontal ligament fibres were evident surrounding the roots of the teeth. Bone still appeared less mineralised in some areas with wavy somewhat irregular appearance of collagen fibres in the bone matrix, with a large cell number (osteocytes and osteoblasts). Widespread small blood vessels were

evident throughout the tissue (Fig. 4.11a). The cytoarchitecture of the male alcohol-exposed group showed the same pattern as the male pair-fed control group except for a few differences. Trabecular spaces in this group were slightly larger than those exhibited in the pair-fed control group. Bone in this group was also less mineralised when compared to the pair-fed control group as the collagen fibres were less organised (Fig. 4.11b).

Female pair-fed control animals showed the same pattern as the male pair-fed control group except for a few differences. Numerous large trabecular spaces were identified in this group throughout the tissue and were filled with erythrocytes. Large trabecular spaces with small islands/spicules of osteoid were found in between the erythrocytes. Widespread larger blood vessels were noted. Bone in this group appeared less mineralised than the bone displayed in the male pair-fed control group (Fig. 4.11c). Female alcohol-exposed rats showed the same patterns observed in the female pair-fed control rats however the bone in this group was even less mineralised than the bone in the pair-fed control group with less organisation of the collagen fibres (Fig. 4.11d).

Male pair-fed control and alcohol-exposed groups had smaller trabecular spaces than the female-pair-fed control and alcohol-exposed groups and lacked the small islands/spicules of osteoid found in between the erythrocytes in these trabecular spaces. The male pair-fed control and alcohol-exposed groups contained smaller blood vessels, and slightly more organised collagen fibres in the bone matrix compared to the female groups.

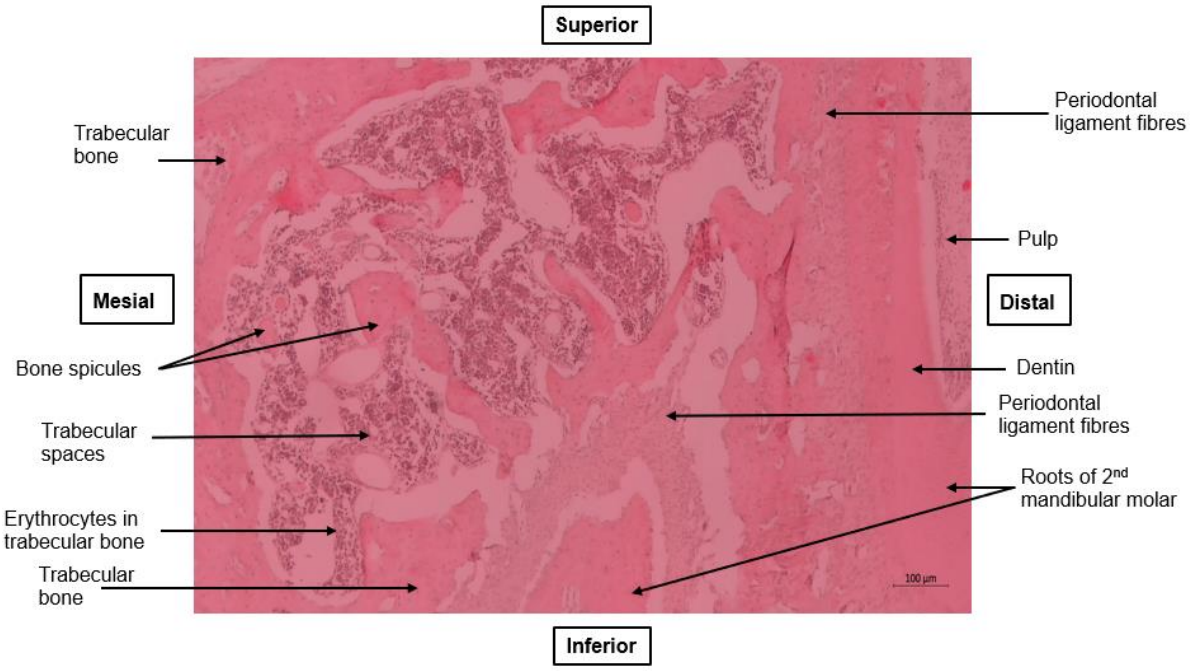
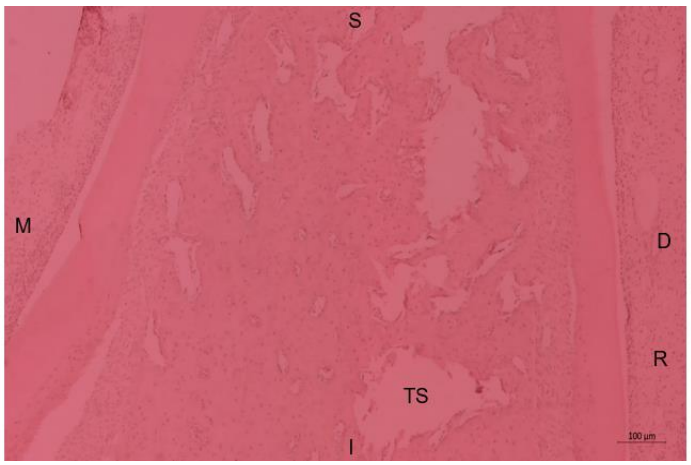
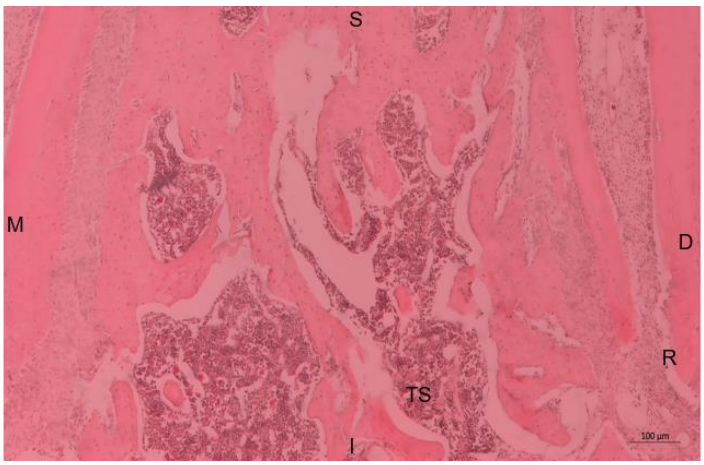


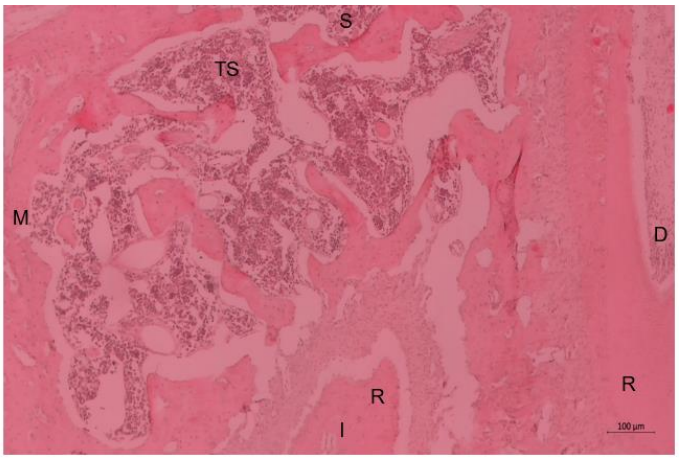
Figure 4.10: A photomicrograph showing a coronal section of the area between the roots of the first and second molar teeth of the mandible (taken from the buccal aspect of the bone), in the acute binge alcohol model. Scale bar = 100 microns at x 5 magnification. For orientation purposes.



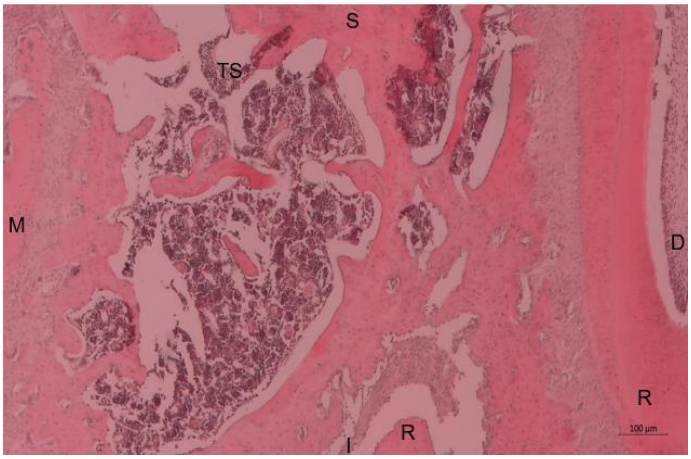
(a)



(b)



(c)



(d)

Figure 4.11: Representative sections of the mandible between the roots of the first and second molar teeth of the mandible in the acute binge alcohol model. Representative sections of (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. H & E staining. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space.

4.2.6 Immunohistochemistry

4.2.6.1 Mandibular Proliferative Cells, Ki-67 Immuno-Positive Cells

A similar pattern was displayed regarding the number of proliferating cells in male and female rats in the region between the 1st and 2nd molar teeth. There were no significant differences shown between the male pair-fed control (mean = 156.00 ± 8.46) and the alcohol-exposed group (mean = 160.00 ± 21.87) ($p = 0.409$). A slight decrease in proliferating cells was exhibited between the female pair-fed control group (mean = 129.00 ± 10.40) and the female alcohol-exposed group (mean = 141.00 ± 17.62). This difference was not statistically significant ($p = 0.165$).

A significant difference in the number of proliferating cells was exhibited between the male and female pair-fed control groups ($p = 0.011$) where the male pair-fed control rats had more proliferating cells than the female pair-fed control rats. The same pattern was detected between the male and female alcohol-exposed rats; however, this difference was not significant ($p = 0.160$) (Fig. 4.12 and Fig. 4.13).

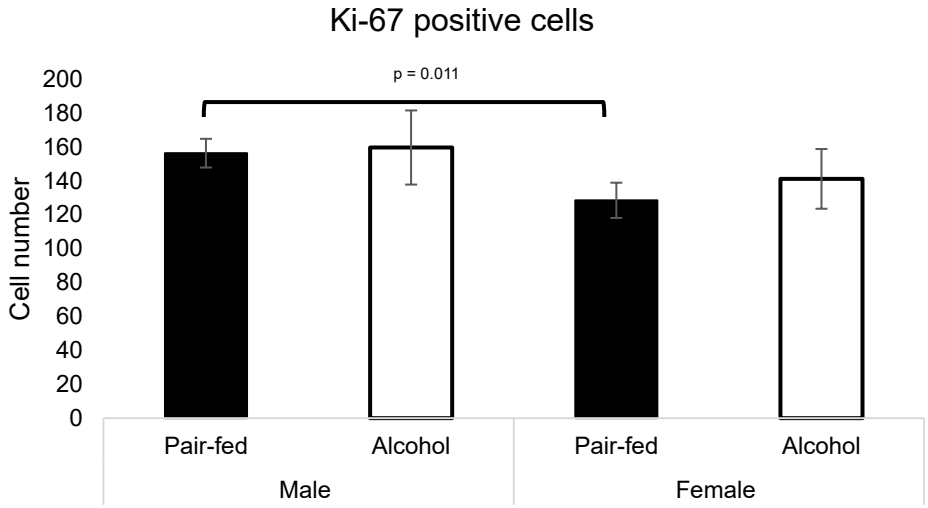


Figure 4.12: Ki-67 immuno-positive proliferative cells of the mandible in the acute binge alcohol model. The mean number of Ki-67 immuno-positive proliferative cells are shown for the region between the 1st and 2nd molar teeth in the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

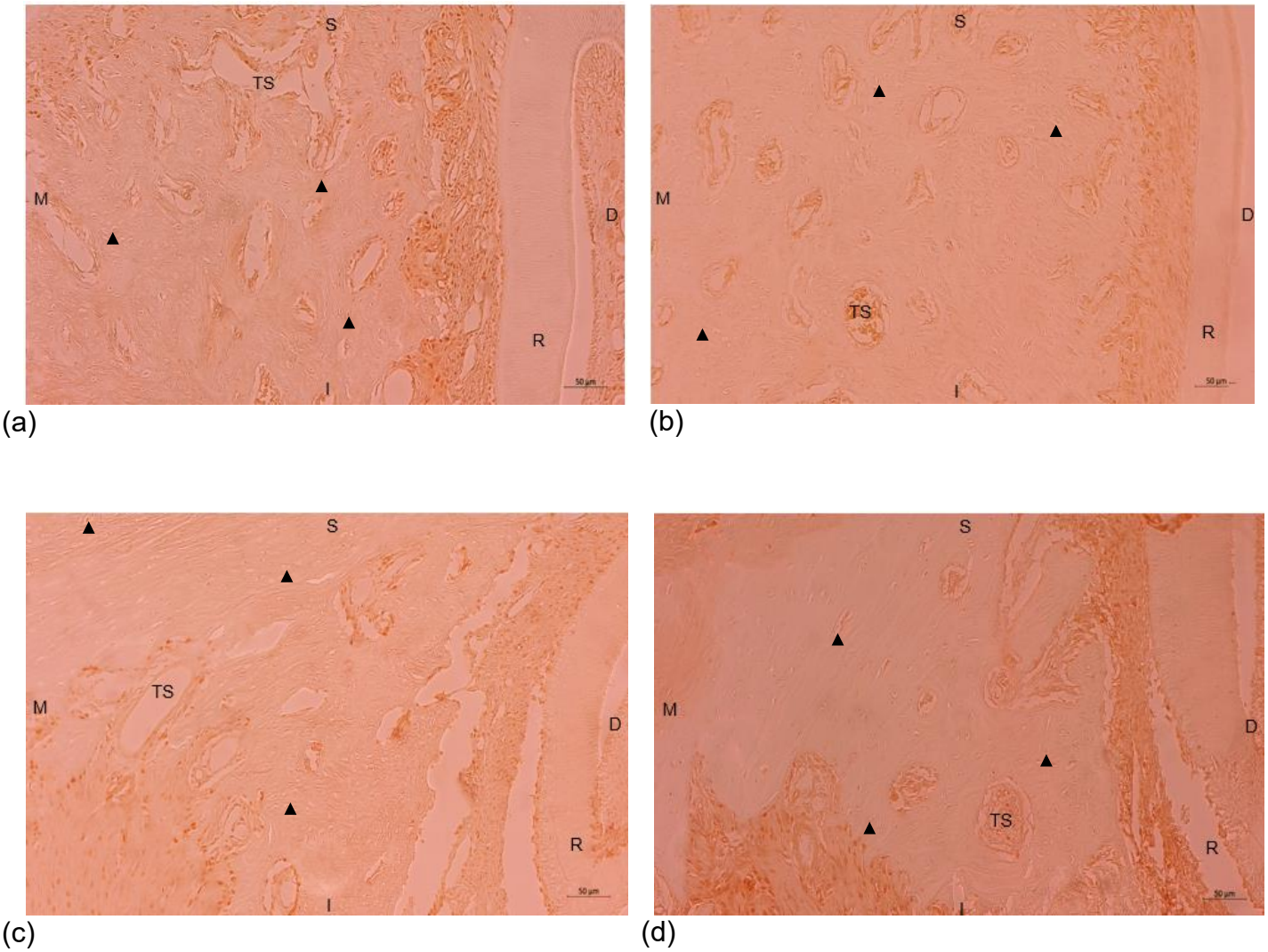


Figure 4.13: Representative photomicrographs of Ki-67 immunolabeled sections of the mandible between the roots of the first and second molar teeth of the mandible in the acute binge alcohol model. (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. Scale bar = 50 microns at x 10 magnification. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space. Black arrowheads indicate proliferating cells.

4.2.6.2 Mandibular Osteoblasts, Alkaline Phosphatase (ALP) Immuno-Positive Cells

Differences were identified in patterns in the number of osteoblasts between male and female groups in the region between the 1st and 2nd molar teeth. The number of osteoblasts in the male pair-fed control group (mean = 153.00 ± 13.04) did not differ from that exhibited in the male alcohol-exposed group (mean = 152.00 ± 17.54) ($p = 0.461$). Female pair-fed control animals (mean = 136.00 ± 11.81) had a significantly greater number of osteoblasts than the female alcohol-exposed group (mean = 106.00 ± 3.97) ($p = 0.008$).

No significant differences were noted in the number of osteoblasts in the male pair-fed control vs female pair-fed control groups ($p = 0.085$). The male alcohol-exposed rats had significantly greater amounts of osteoblasts than the female alcohol-exposed rats ($p = 0.006$) (Fig. 4.14 and Fig. 4.15).

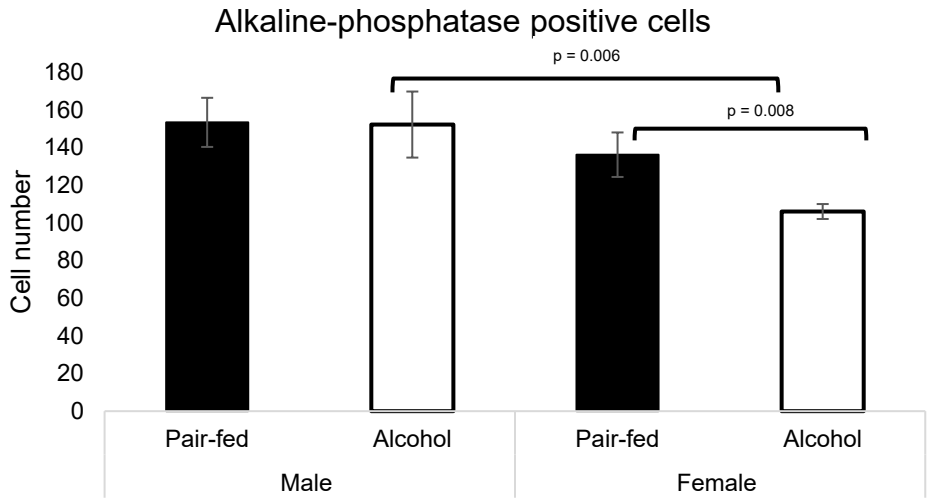


Figure 4.14: Alkaline phosphatase (ALP) immuno-positive osteogenic cells of the mandible in the acute binge alcohol model. The mean number of osteoblasts are shown for the region between the 1st and 2nd molar teeth in the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

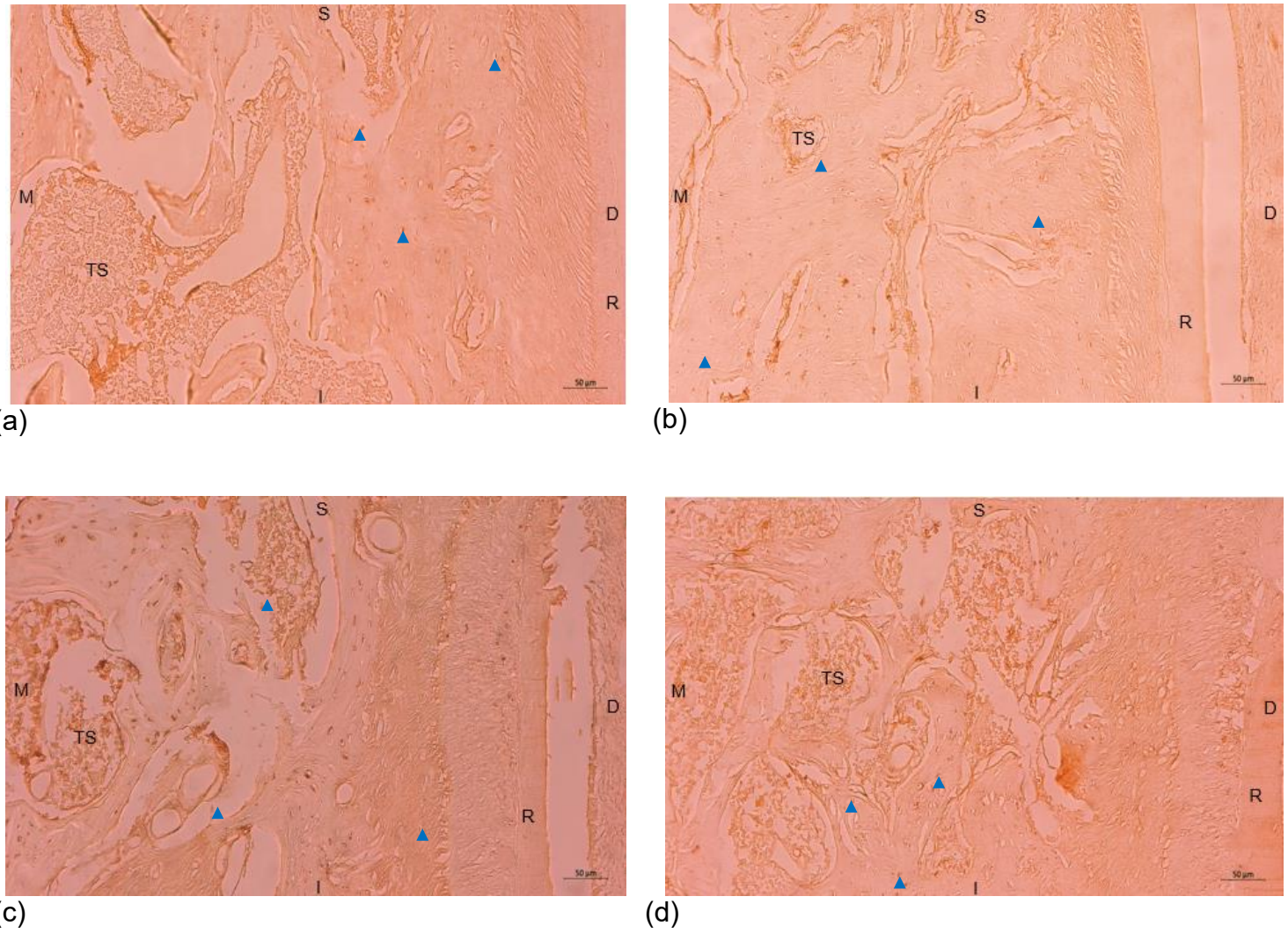


Figure 4.15: Representative photomicrographs of ALP immunolabeled sections of the mandible between the roots of the first and second molar teeth of the mandible in the acute binge alcohol model. (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. Scale bar = 50 microns at x 10 magnification. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space. Blue arrowheads indicate osteoblasts (on the surface of the bone) and osteogenic cells.

4.3 DISCUSSION

The current study investigated the effects of alcohol exposure on the growth and development of the mandible using an acute binge drinking model in adolescent Sprague Dawley rats. The baseline data (regarding food consumption, water intake, and body mass gain), and the size of the bone using osteometric measurements were analysed. Trabecular morphometry using 3D-μCT was evaluated. The tensile strength of the mandible was accessed using a 3-point bending test. The cytoarchitecture of the cells were evaluated with Hematoxylin and Eosin (H & E) staining, immunohistochemistry (IHC) was performed to quantify the effect of alcohol on proliferating and osteoblast cells. There were some differences in the baseline data between female pair-fed control and alcohol-exposed animals, along with differences between the sexes. Osteometric measurements showed differences in the length and height of the pair-fed control and alcohol-exposed animals in both male and female groups. Three-dimensional micro-focus X-ray computed tomography (3D-μCT) showed altered trabecular parameters in the alcohol-exposed groups in the acute binge model. Tensile strength testing showed no differences in bone strength between the male and female pair-fed control and alcohol-exposed groups. Some differences in cytoarchitecture were shown between male and female pair-fed control and alcohol-exposed groups. Differences were detected in all the parameters tested and analysed between the male and female groups.

Baseline Data

Few differences in food consumption were displayed with female alcohol-exposed rats consuming less food. The only differences noted with regards to water intake were between male and female groups. Female rats exposed to alcohol gained a lower

percentage body mass over the 7-day period compared to the pair-fed control group. Differences between male and female groups were identified in all parameters measured with males exhibiting greater overall values.

Female rats exposed to alcohol showed a significant reduction in food intake compared to pair-fed controls. This aligns with Nelson et al. (2016), who found that intraperitoneal alcohol delivery suppressed appetite, possibly due to alcohol-induced food aversion and disruption of gut-brain axis signaling. While some studies report alcohol increases appetite (Caton *et al*., 2004; Fong *et al*., 2021), others, like Nelson et al. (2016), observed the opposite. Ghrelin, an appetite-stimulating peptide, was found to increase in male but not female rats after alcohol exposure (Healey *et al*., 2020), which may explain the reduced food intake in females in our study. The non-voluntary mode of alcohol delivery and absence of a ghrelin response in females likely contributed to the observed decrease in appetite.

The hypothalamic-pituitary-gonadal axis also plays a role in food consumption between the sexes. Androgens in males have been shown to function as an appetite stimulant and oestrogen as an appetite suppressor (Asarian and Geary., 2013). This is another reason as to why male rats in our study may have consumed more food than the female rats. The increase in food consumption in male rats also explains why they had a greater percentage body mass gain than the female rats in our study. Alcohol may have affected the percentage body mass gain in the female alcohol-exposed rats in our study which may also be correlated to the decrease in food consumption.

Water intake remained unaffected by alcohol between pair-fed control and alcohol-exposed groups. Male groups in general consumed significantly more water than female groups. This is due to their larger size and increased food consumption. The above reasons can all be associated with sexual dimorphism and the fact that androgens act as an appetite stimulant in males leading to a greater food intake and thus a greater body mass and greater size (Asarian and Geary., 2013).

Osteometric Measurements

Alterations in general mandibular size, length and height were also noted when comparing osteometric measurements which were taken using a digital vernier caliper. Altered measurements included a greater M3-Id^{i} (linear mandibular length) measurement in the male alcohol-exposed group; and a greater Cd-Go (linear mandibular height) measurement in the female alcohol-exposed group.

In the present study, differences in bone weight were detected between the male pair-fed control and alcohol-exposed groups, where the alcohol-exposed group bones weighed less than the pair-fed control group. Other studies have found a decrease in bone mass in alcohol-exposed animals (Broulík *et al.,* 2010; Chakkalakal., 2005). The decrease in bone mass in alcohol-exposed animals could be due to altered bone remodeling where bone formation is suppressed and an increase in bone resorption is exhibited, which could lead to a low bone mass (Broulík *et al.,* 2010).

Chen et al. (2022) found that alcohol delays the senescence of endothelial cells which are responsible for maintaining bone mass, which may be another mode in which alcohol influences bone mass. Alcohol-induced bone loss is usually characterized by damage in

bone structure, a decrease in bone mass and an increase in the risk of bone fractures (Chen *et al*., 2022). No adverse effects on bone mass were observed in the female rats. Eby et al. (2020) suggested that oestrogen may have a protective role against the potential negative effects that alcohol consumption could have on maintaining the equilibrium of bone structure and function (Eby *et al*., 2020). The duration of exposure used in the acute group in our study and the possible antagonistic effect that oestrogen may have had a protective effect against alcohol may suggest why no differences in bone mass was observed in the female groups.

A difference in bone mass was detected when comparing male and female rats in both pair-fed and alcohol-exposed groups. This is due to sexual dimorphism, because in general, females, at any age, have a lower bone mass than males (Lorenzo., 2020).

With regards to the effect of acute binge drinking on the size of the adolescent mandible, our study found that acute binge drinking had no significant effect on the general size of the mandible in adolescent rats, however individual length and height parameters were affected. The length of the lower dental arch (M3-Id) which is one of the measurements that was used to depict length in the mandible was larger in male rats exposed to alcohol. The mandibular ramus height (Cd-Go) was greater in the female alcohol-exposed rats. The result of the present study contradicts previous reports which exhibited a decrease in bone length in alcohol-exposed animals (Hogan *et al*., 1997; Sampson *et al*., 1996; Sampson *et al*., 1997, Wezeman *et al*., 2000). This contradiction in results may be due to the other studies reporting on the effect of chronic drinking and not binge drinking as in our study. A study by Sampson et al. (1999) used a binge alcohol model which reported alcohol to have detrimental effects when given for a longer period. The duration of

exposure in the acute binge drinking model (7 days) in the current study may have been too short to elicit negative effects on the mandible. This was a case-controlled study, eliminating selection bias or chance from the study. The following were also taken into consideration to minimise any influence on the results. Animals were randomly assigned to experimental groups to reduce selection bias, and all procedures were standardized to ensure consistency across groups.

Furthermore, previous studies mentioned above reported on the effects alcohol may have on long bones and not the mandible. The mandible is a flat bone that undergoes both endochondral and intramembranous ossification, whereas most long bones only undergo endochondral ossification. Alcohol intake in the current study had less of an impact on the mandible. This may be partially explained by differences in the ways that bones form. The precise biological processes and timing of mandibular development and growth might make it less susceptible to alcohol's harmful effects. Intramembranous ossification is a process that may be less sensitive to disruptions in chondrocyte activity, a known alcohol target. Moreover, the mandible is subjected to early and continuous mechanical loading from mastication and muscle attachments, which stimulates bone remodeling and may provide a protective effect against alcohol-induced bone loss. Therefore, a new question regarding whether alcohol consumption influences intramembranous ossification differently from endochondral ossification is raised by the study's findings.

The development of the mandibular condyle not only leads to an increase in the size of the mandible but also results in the anteroinferior displacement (transposition) of the mandible. The length of the body of the mandible increases in a rectilinear direction, whereas the condylar process exhibits growth in various directions from anterosuperior

to posterior (Mizoguchi *et al*., 2013). This diverse growth pattern enables a wide range of growth and shapes of the mandible. The growth of the condyle is closely related to transposition of the mandible, which will in turn influence the angle of the mandible, thus the maxillary-mandibular relationship. In individuals with low angles (short face, brachyfacial pattern, and deep bites), the mandibular growth is characterized by anterosuperior growth of the condyle. In individuals with high angles (long face, dolichofacial pattern, and skeletal open bite), posterosuperior growth of the condyle is noted (Mizoguchi *et al*., 2013). Disturbances in growth can thus have a varying range of effects on the length and height of the mandible. In a case of juvenile rheumatoid arthritis, the posterior margin of the ramus had bone apposition, and grew in a posterior direction (Mizoguchi *et al*., 2013). This could indicate that the exposure to alcohol in the acute binge model in our study showed a similar growth pattern as was observed in the case above, with more bone apposition in the posterosuperior direction, which could lead to a mandible with a high angle, with complications such as malocclusion and an open bite. Additional investigation is still needed in this area.

Males showed an overall greater general size, length and height in mandibular size when compared to the females. This is attributed to sexual dimorphism as males are usually larger in size than females (Lorenzo., 2020). Several factors may be responsible for sexual dimorphism such as age, genetics, actions of sex steroids, environment, and behavior (Lorenzo., 2020).

Three-Dimensional Micro-focus X-ray Computed Tomography (3D- µCT)

Three-dimensional micro-focus X-ray computed tomography (3D-µCT) was used to access trabecular volume, thickness, number, and spacing in the mandible (between the

first and second molar teeth). Altered trabecular parameters were identified in the alcohol-exposed groups in the acute binge, chronic binge, and chronic binge alcohol-abstinent models.

In the present study, numerous trabecular parameters were noted including the bone-to-total volume ratio (BV/TV) which was lower in the alcohol-exposed group in female rats. When comparing the two sexes, the BV/TV was greater in the female pair-fed control rats than in the male pair-fed control rats. No differences in trabecular thickness (TbTh) and trabecular number (TbN) were shown in the acute binge alcohol model in our study. This may be attributed to the short duration of the acute binge alcohol model, or even mandibular bone resilience. Wider trabeculae were depicted in the female alcohol-exposed group than in the pair-fed control. Differences were also noted where wider trabeculae were evident in the male pair-fed controls than the female pair-fed controls.

The effect of alcohol on trabecular parameters including bone to total volume ratio (BV/TV) and trabecular spacing (TbSP) in female rats in our study corroborate with numerous studies conducted. A decrease in BV/TV and an increase in TbSp were exhibited in alcohol-exposed rats in a study conducted by Sampson et al. (1998) where the effect of alcohol consumption on adult and aged bone in a rat model was studied (Sampson *et al*., 1998). Maia et al. (2021) also detected a decrease in BV/TV and an increase in TbSp in the alveolar bone of female adolescent rats after 30 and 60 days of alcohol exposure, and Föger-Samwald et al. (2018) identified a decrease in BV/TV in the femur of a prepubescent pig model exposed to a binge drinking model (Föger-Samwald *et al*., 2018; Maia *et al*., 2021). The narrower trabecular spaces exhibited in the female pair-fed controls may also be due to female rats having high serum oestrogen levels

during adolescence which may have had a positive impact on bone development (Schiessl *et al*., 1998). Bone development also occurs at a faster rate in female rats, which may lead to earlier maturation of bone (Jiao *et al*., 2010).

The findings of the present study regarding the effect of alcohol on the male rats is similar to the study conducted by Callaci et al. (2004) who found that binge drinking had no effect on male rats in the first 2 weeks of exposure but caused a decrease in BV/TV after 3 weeks (Callaci *et al*., 2004). Our study suggests that binge alcohol-exposure for a period as short as 1 week may influence the trabecular parameters of female rats while having no apparent significant impact on males. Callaci et al. (2006) observed a decrease in BV/TV in female rats exposed to a binge alcohol model for a time of 2 and 4 weeks. A more prominent decrease was noted in the 4-week exposed group (Callaci *et al*., 2006). The duration of exposure may be the reason for no noticeable changes in the male rats. The differences detected between male and female rats, where the female pair-fed control group in our study had a greater BV/TV than the male pair-fed control group may be attributed to the fact that female rats have high serum oestrogen levels during adolescence which may have a positive impact on bone development (Schiessl *et al*., 1998). Bone development also occurs at a faster rate in female rats, which may lead to earlier maturation of bone (Jiao *et al*., 2010).

Maia et al. (2021) identified thinner trabeculae in alveolar bone of adolescent female rats exposed to a binge alcohol model (Maia *et al*., 2021). Sampson et al. (1998) whose study model also consisted of female Sprague Dawley rats, exhibited no differences in TbTh between pair-fed control and alcohol-exposed rats. There were differences in study designs between our study and the two studies mentioned above. Maia et al. (2021)

exposed the animals to 3 g/kg of a 20 % vol/vol of alcohol for 4 weeks (3 days per week). We used the same dosage and volume of alcohol as Maia et al. (2021); however our acute group was only exposed to alcohol for 7 days (3 days per week). Sampson et al. (1998) exposed their animals to an 8.1 % vol/vol of alcohol for 3, 6, 9, 12, 15, 18 and 21 months. The low dose of alcohol used by Sampson et al. (1998) could have contributed to their lack of changes in trabecular thickness (TbTh). The duration of exposure may be the reason for no noticeable changes exhibited in our study. Sampson et al. (1998) and Föger-Samwald et al. (2018) identified a decrease in TbN in animals exposed to alcohol. Maia et al. (2021) however noticed no differences in TbN. These contradictory results could again likely be due to differences in study design, and the duration of exposure may be the reason for no noticeable changes in trabecular number (TbN) in our study. It is also possible that certain parameters were not significantly affected by acute alcohol exposure due to the pattern and timing of mandibular bone development. The mandible undergoes intramembranous ossification and is subjected to early mechanical stimulation through muscle function and mastication, which may promote bone remodeling and stability even during short-term stress. This, combined with the slower or more gradual developmental changes in mandibular bone compared to other skeletal sites, could render it less responsive to the immediate effects of acute alcohol exposure.

Tensile Strength

In the present study, no differences in bone strength were observed between the male pair-fed control and alcohol-exposed groups. No differences were evident between the female pair-fed control and alcohol-exposed groups either. No comparable studies examining the tensile strength of the mandible were identified. The only sex differences

noted were the increase in the maximum force, and the break force which were significantly higher in the male alcohol-exposed rats than in the female alcohol-exposed rats. In a study conducted by Callaci et al. (2004), they detected no significant effects on vertebral compressive bone strength in male Sprague Dawley rats exposed to binge alcohol exposure after 1 and 2 weeks of exposure. A decrease in vertebral compressive strength, however, was exhibited after a 3-week exposure period of binge alcohol (Callaci *et al*., 2004). A decrease in vertebral compressive bone strength in female Sprague Dawley rats exposed to binge alcohol exposure after 2 and 4 weeks of exposure was detected in a study conducted by Callaci et al. (2006). Hogan et al. (1997) studied the effects of alcohol consumption on young actively growing rats. A three-point bending test was used to report the mechanical properties of the femur. Animals were exposed to alcohol for 2, 4 6, 8 and 10 weeks and parameters tested and analysed at each interval. Mechanical properties (bone stiffness and whole bone strength) showed significantly lower values in the alcohol-exposed rats compared to the control rats at all time intervals (Hogan *et al*., 1997).

Lauing et al. (2008) investigated the effects of binge alcohol consumption in adolescent rats where animals were exposed to a binge alcohol model for 1 week (acute group), 4 weeks (control group) and an additional group was exposed of 4 weeks and held for an additional 30 days without treatment (chronic binge alcohol-abstinent group). Vertebral compressive strength in the lumbar vertebra was not significantly reduced in the acute group but was significantly reduced by 31 % in the chronic group (Lauing *et al*.,2008).

The literature shows that a decrease in tensile bone strength was displayed, however after a duration of exposure of 2 or more weeks, thus the duration of exposure of the

acute group of our study (7 days) may be the reason for no noticeable changes in tensile strength results except for the increase in the maximum force, and the break force being significantly higher in the male alcohol-exposed rats than in the female alcohol-exposed rats. This may be because bone health is affected by numerous factors, sex being one of them, as females have a lower bone mass than males. This could be attributed to sexual dimorphism, as males typically have larger, and stronger bones compared to females (Gordon and Gordon., 2020). During puberty in males, periosteal apposition of bone increases the width of bone and endosteal resorption enlarges the medullary cavity. This leads to an increase in cortical thickness as the periosteal apposition is greater than the endosteal resorption. In females, periosteal apposition decreases at an earlier stage with no changes in medullary size, which leads to bone with a smaller total size and medullary size to males. This leads to a similar cortical thickness (Wang and Seeman., 2008). Even though cortical thickness is similar between sexes, the mass of cortical bone is greater in males due to the greater perimeter of the larger bone. This is one reason for males having stronger bones when compared to females (Seeman., 2001). Thus, sexual dimorphism is the reason for the differences identified between male and female animals in our study.

Mandibular Cytoarchitecture

Only qualitative analysis of the cytoarchitecture was assessed, with consistent findings observed across all samples within each group. Samples from all study animals were included in the quantitative analysis. Some differences were identified between male pair-fed control and alcohol-exposed animals, between female pair-fed control and alcohol-exposed animals, and lastly between male and female pair-fed control and alcohol-exposed groups.

The cytoarchitecture of male alcohol-exposed animals showed larger trabecular spaces when compared to the pair-fed control animals. Smaller trabecular spaces are detected in more mature bone, possibly indicating that alcohol played a role in delaying bone formation in the alcohol-exposed male rats (Galea *et al*., 2021). No information was available in the literature to confirm this suggestion. The bone matrix in the alcohol-exposed group displayed disorganised collagen fibres when compared to the pair-fed control group which likely indicates less mineralization. Alcohol can affect the skeleton by altering mineral homeostasis and directly affect the bone cells. Alcohol can also decrease the mineralisation rate and the rate of synthesis of the bone matrix (Turner *et al*.,1987), which is likely what occurred in the present study in both male and female alcohol-exposed animals.

Differences were also displayed between male and female pair-fed control and alcohol-exposed animals, where the male animals appeared to have more mature, more mineralised bone as opposed to the female groups, as the female groups had much larger trabecular spaces and still contained bone spicules which are indicative of earlier bone development (Nanci., 2018). This is contrary to the principles of growth in general where females develop at a faster rate than males. Changes related to interradicular bone may also be a result of increased mechanical forces, which would lead to greater bone volumes (Nenda *et al*., 2016). This could likely also lead to accelerated bone development which may be the reason for the differences identified in our study, as male rats had a greater food intake than the female rats, thus a greater mechanical force applied to the mandible and the alveolar bone.

Cell proliferation and osteoblast activity

The only significant differences in proliferating cells identified were between male and female animals. Acute binge alcohol consumption however did have an impact on the number of osteoblasts in female rats, and differences were also exhibited between the male and female groups.

The alcohol-exposed groups did not show any differences in cell number when compared to the pair-fed control rats in both male and female groups. This is contrary to what Miralles-Flores and Delgado-Baeza. (1992) reported in their study, which reported on the effects of chronic prenatal alcohol-exposure. These authors found that alcohol caused a decrease in the number of proliferating cells in the growth plate of the hypertrophic zone in rats after prenatal alcohol exposure. No studies were found in the literature which reported on Ki-67 immuno-positive proliferative cells in the mandible following alcohol exposure. Proliferating cells in an adolescent study indicate active growth. A lack of differences between the alcohol-exposed and pair-fed animals could be due to the duration of the acute phase. Alkaline phosphatase, which more specifically expresses for osteoblasts, was also used in the present study to determine if acute binge alcohol consumption influenced bone forming cells.

A decrease in the number of osteoblasts was displayed in the female rats exposed in an acute model of binge alcohol exposure. This corroborates with previous studies which suggest that alcohol consumption can be associated with osteopenia which is likely due to a decrease in osteoblast activity (Chakkalakal., 2005; Eby *et al*., 2020; Turner, 2000). Dyer et al. (1998) also identified a decrease in the histology of bone formation in three-month-old female Sprague Dawley rats after 6 weeks of alcohol exposure which they

attributed to the suppression of both osteoblast number and function by alcohol (Dyer *et al.,*1998). Lower levels of osteocalcin have also been displayed in humans and animals exposed to alcohol. Osteocalcin serves the role of binding and concentrating calcium within bone tissue. It is exclusively produced by osteoblasts and is recognized as a biomarker indicative of active bone formation (Eby *et al*., 2020), thus a decrease in osteocalcin levels also supports a decrease in osteoblast number and activity. Bone biomarker levels can be sensitive to sex hormones (Maurel *et al*., 2012a). A reduction in serum oestradiol levels has been documented in female alcoholics and in female rats exposed to alcohol. Diminished serum oestradiol has been identified as a significant contributor to alcohol-induced bone loss (Maurel *et al*., 2012a). Bone biomarkers and oestradiol which are both affected by alcohol consumption may be the reason for the decrease in the number of osteoblasts detected in our female rats and not in the males.

The number of proliferating cells along with the number of osteoblasts were both greater in the male groups than the female groups. This is due to sexual dimorphism, as males have larger, more robust bones (Gordon and Gordon., 2020), thus it is plausible that they would also have more proliferating cells and osteoblasts.

4.4 CONCLUSION

During a 7-day period, exposing adolescent Sprague Dawley rats to alcohol for only 3 days through acute binge drinking had an impact on the growth and development of their mandibles, affecting the following parameters: mandibular length and height, trabecular parameters, and the expression of ALP. The effect was more significant and extensive in females compared to males. Acute binge drinking also affected the appetite of female

rats, leading to a decrease in food consumption and subsequent reduction in percentage body mass gain.

After acute binge drinking, male alcohol-exposed rats showed a decrease in the weight of the mandibular bone. This was likely an increase in the size of the trabecular spaces observed in the Hematoxylin and Eosin (H & E) stained sections when analyzing the effect of acute alcohol exposure on the bone's structure. Additionally, certain parameters related to the length (in males) and height (in females) of the mandible increased in the alcohol-exposed group. This increase may be due to the short duration of exposure in the acute binge group (7 days), which might not have been long enough to cause detrimental effects on the length and height of the mandible.

When analyzing the effect of binge alcohol exposure on the microarchitecture of the bone, it had a negative effect on the bone volume to total volume ratio (BV/TV) in female alcohol-exposed rats and lead to larger trabecular spaces, thus weaker bones in these animals. The study confirmed that the bone structure in female rats exposed to alcohol had larger trabecular spaces and lower mineralisation compared to the control group. Additionally, the alcohol-exposed rats showed larger amounts of wavy and unorganised collagen fibres.

Female rats exposed to acute binge drinking showed reduced osteoblast numbers, negatively impacting bone growth. In contrast, male rats were less affected, possibly due to the short 7-day study duration. Differences in development and size between male and female rats were also noted, reflecting sexual dimorphism.

CHAPTER 5: *The impact of chronic binge alcohol consumption on the development of the mandible in adolescent Sprague Dawley rats*

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5.1 INTRODUCTION

Alcohol consumption among adolescents in South Africa (SA) is a major concern, with many engaging in binge drinking (Morojele and Ramsoomar, 2016). Binge drinking is defined as consuming five or more drinks for males and four or more drinks for females in a single session or ingesting 60 grams or more of pure alcohol at least once a month (Morojele and Ramsoomar, 2016). In rodent models of binge drinking, it is necessary to achieve pharmacologically relevant blood alcohol concentrations (BACs) of approximately 1 g/L (100 mg/dL), as rodents metabolize alcohol much faster than humans. A dose of 1.5 g/kg in rodents typically results in a BAC of around 100 mg/dL one hour after administration (Jeanblanc *et al*., 2019; Walker and Ehlers, 2009). Binge drinking is generally associated with a BAC of 80 mg/dL or higher (Föger-Samwald *et al*., 2018).

Adolescence, which occurs between the ages of 10 and 19, is a crucial period for rapid skeletal growth and development, during which about 90 % of skeletal development and peak bone mass are attained. A deficiency in peak bone mass poses a significant risk for osteoporosis later in life (Weaver, 2002). The impact of binge drinking on bone growth is still not fully understood, requiring further research. Lauing et al. (2008) investigated the effects of binge drinking on adolescent rats and discovered a marked decrease in cancellous bone mass in the tibia and vertebrae after acute binge exposure. Chronic binge exposure also resulted in reduced bone mass in the vertebrae and compromised compressive bone strength (Lauing *et al*., 2008). Furthermore, a study by Föger-Samwald and colleagues (2018) using a prepubescent pig model revealed fewer trabeculae in the femur (Föger-Samwald *et al*., 2018). There is limited information on the effects of binge

drinking on the adolescent skeleton, particularly regarding craniofacial structures. Research on the impact of alcohol on the adolescent mandible is especially scarce. Given the mandible's importance for facial aesthetics, implant stability, and dental spacing, studies in this area are highly warranted.

The present study utilized three-dimensional microtomography (3D-μCT) to examine the effects of alcohol on trabecular morphology, 3-point bending tests to assess the tensile strength of bone tissue, and Hematoxylin and Eosin (H & E) staining to analyse the impact of alcohol on bone cytoarchitecture. Additionally, immunohistochemistry with anti-Ki-67 and anti-alkaline phosphatase antibodies was used to determine whether an acute binge alcohol model influenced the number of proliferating cells and osteoblasts in the adolescent mandible, respectively, utilizing a chronic binge alcohol consumption model.

5.2 RESULTS

5.2.1 Baseline Data

5.2.1.1. Blood Alcohol Concentration

The mean blood alcohol concentration (BAC) was 141.01 mg/dL (± 22.53) in the male alcohol group; and 272.12 mg/dL (± 34.02) in the female alcohol-exposed group.

5.2.1.2 Food Consumption

Males and females exhibited similarities in the pattern of food consumption where food consumed throughout the 4 weeks was consistent. Male pair-fed control (week 1: mean = 48.00 g ±2.58; week 2: mean = 49.98 g ±2.43; week 3: mean = 51.43 g ±1.78; week 4: 51.19 g ±2.59) and alcohol-exposed rats (week 1: mean = 47.60 g ±2.14; week 2: mean = 49.57 g ±2.10; week 3: mean = 51.55 g ±2.35; week 4: 50.31 g ±1.62) showed no

significant differences in the amount of food consumed in weeks 1 to 4 ($p = 0.423$; $p = 0.400$; $p = 0.479$; $p = 0.322$, respectively).

A similar pattern continued in the female pair-fed control (week 1: mean = 30.93 g ±0.31; week 2: mean = 32.10 g ±0.62; week 3: mean = 33.53 g ±1.07; week 4: 34.45 g ±1.05) and alcohol-exposed rats (week 1: mean = 32.14 g ±1.57; week 2: mean = 32.55 g ±1.61; week 3: mean = 35.58 g ±1.56; week 4: 35.69 g ±3.77) where no significant differences in the amount of food consumed in weeks 1 to 4 was exhibited ($p = 0.130$; $p = 0.338$; $p = 0.067$; $p = 0.306$, respectively).

The amount of food consumed by the male pair-fed control group was significantly greater than that consumed by the female pair-fed control group in all 4 weeks ($p < 0.001$ for all 4 weeks). The same was displayed in the alcohol-exposed groups where the male alcohol-exposed group consumed more food than the female alcohol-exposed group for all 4 weeks (week 1: $p < 0.001$; week 2: $p < 0.001$; week 3: $p < 0.001$; week 4: $p = 0.002$) (Fig. 5.1).

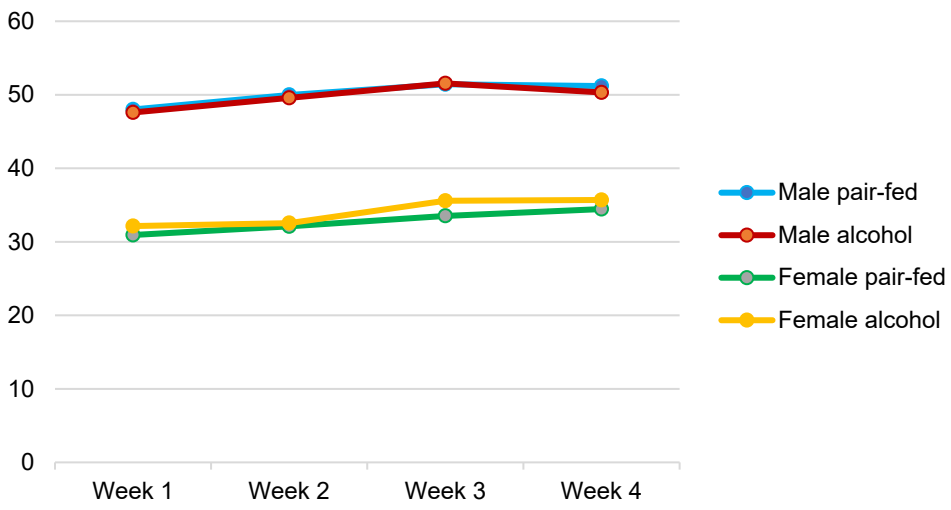

Figure 5.1: Food consumption in the chronic binge alcohol model. The daily food consumption is shown as an average per week for the pair-fed control and alcohol-exposed groups in both male and female rats.

5.2.1.3 Water Intake

Males and females exhibited similarities in water intake, where water intake throughout the 4 weeks was relatively consistent, with the greatest water intake being in week 3 for all groups. No significant differences were identified in the water intake between male pair-fed control (week 1: mean = 77.86 ml ±1.89; week 2: mean = 82.86 ml ±0.71; week 3: mean = 88.33 ml ±4.12; week 4: 84.29 ml ±4.34) and male alcohol-exposed rats (week 1: mean = 77.86 ml ±23.11; week 2: mean = 82.38 ml ±4.06; week 3: mean = 87.38 ml ±2.30; week 4: 82.86 ml ±1.89) ($p = 0.500$; $p = 0.425$; $p = 0.372$; $p = 0.314$, respectively).

This pattern continued in the female rats where no significant differences were observed in the water intake between female pair-fed control (week 1: mean = 57.14 ml ±4.34; week

2: mean = 66.67 ml ±4.54; week 3: mean = 75.00 ml ±1.89; week 4: 67.86 ml ±3.57) and alcohol-exposed rats (week 1: mean = 62.86 ml ±5.85; week 2: mean = 63.57 ml ±6.89; week 3: mean = 73.33 ml ±5.27; week 4: 68.33 ml ±3.60) ($p = 0.123$; $p = 0.126$; $p = 0.317$; $p = 0.440$, respectively).

Water intake in the male pair-fed control group was significantly greater than that of the female pair-fed control group observed weekly (week 1: $p < 0.001$; week 2: $p = 0.002$; week 3: $p = 0.004$; week 4: $p = 0.004$). The same was observed in the alcohol-exposed groups where the male alcohol-exposed group consumed more water than the female alcohol-exposed group weekly (week 1: $p = 0.009$; week 2: $p = 0.008$; week 3: $p = 0.007$; week 4: $p = 0.002$). (Fig. 5.2).

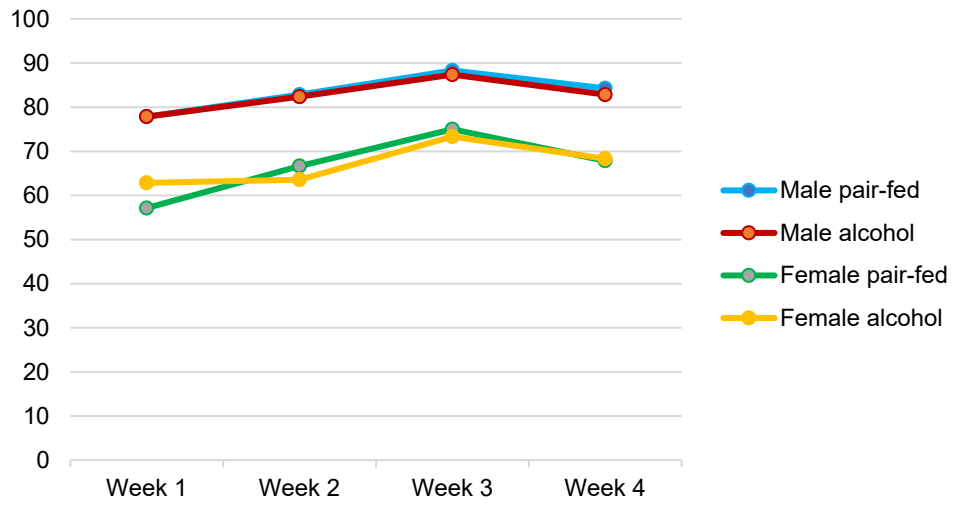

Figure 5.2: Water intake in the chronic binge alcohol model. The daily water intake is shown as an average per week for the pair-fed control and alcohol-exposed groups in both male and female rats.

5.2.1.4.4 Percentage Body Mass Gained

In terms of the percentage (%) of body mass gained the male and female groups showed differences. A two-way ANOVA displayed that in the male rats, the only differences exhibited related to the weeks ($P < 0.001$), with week 3 specifically showing a significant decrease in body mass gain. No differences were observed between the pair-fed control and alcohol-exposed groups ($p = 0.310$).

Female rats exhibited no differences between pair-fed control and alcohol-exposed groups ($p = 0.476$) and between weeks ($p = 0.794$).

When comparing male and female pair-fed groups, the percentage body mass gained in the male pair-fed control group was significantly greater compared to the female pair-fed

control group ($p < 0.001$). A significant difference in body mass gain was also noted between the weeks ($p = 0.005$), with a significant decrease in week 3 specifically. When comparing the male and female alcohol-exposed groups, there was a significant difference between the groups ($p < 0.001$). A difference between weeks was also observed ($p = 0.003$) with week 3 showing a statistically lower percentage of body mass gain than the other weeks (Fig. 5.3).

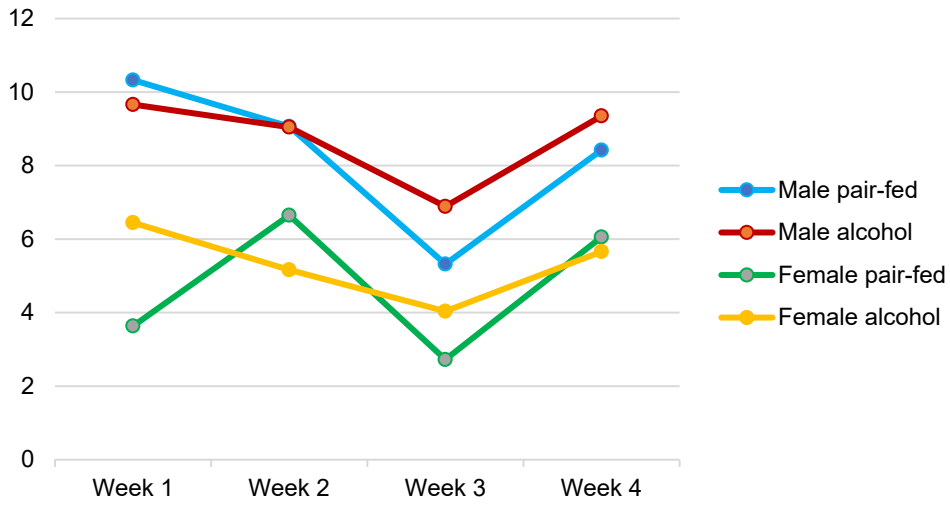

Figure 5.3: Percentage (%) body mass gained in the chronic binge alcohol model. The % body mass gained is shown for the pair-fed control and alcohol-exposed groups in both male and female rats per week.

5.2.2 Osteometric Measurements

5.2.2.1 Mandibular Weight

Males and females exhibited similarities in mandibular weight. The bone weight in males was similar in the pair-fed control (mean = 1.08 g ±0.06) and the alcohol-exposed group

(mean = 1.09 g ±0.07) (p = 0.937). Female rats also showed no differences in the mandibular weight between the pair-fed control (mean = 0.94 g ±0.04) and the alcohol-exposed groups (mean = 0.90 g ±0.08) (p = 0.182). The weight of the mandible was significantly greater in male rats in both the pair-fed control and alcohol-exposed groups when compared to the female pair-fed control and alcohol-exposed groups (p < 0.001 for both groups) (Fig. 5.4).

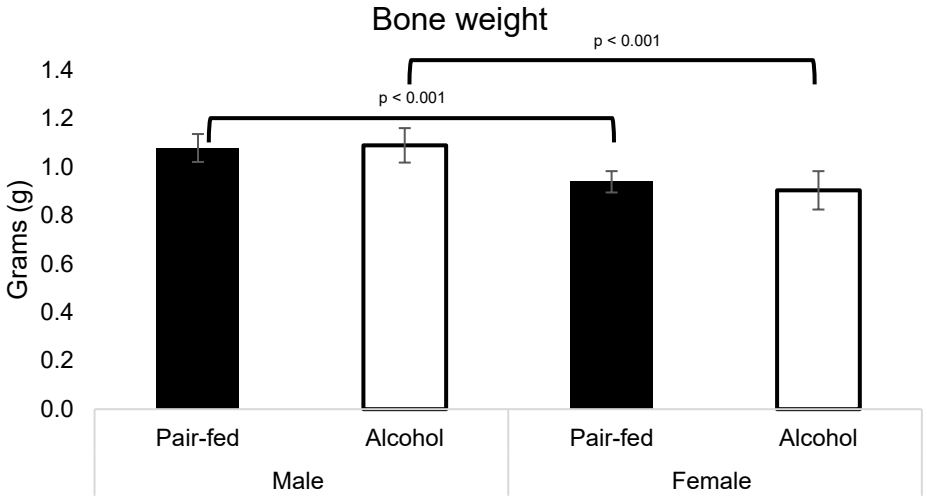

Figure 5.4: Mandibular weight in the chronic binge alcohol model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

5.2.2.2 General Mandibular Size

Males and females exhibited similarities concerning the general size of the mandible. No differences in measurements were displayed between the alcohol-exposed and pair-fed control groups in both male (Table 5.1) and female animals (Table 5.2).

When comparing males and females, the male pair-fed control rats had significantly greater measurements relating to: the distance between the mental foramen (MF) and the most posterior point of the condylar head (Cd) (MF-Cd), the distance between the mental foramen (MF) and the gonion (Go) (MF-Go), the distance between the the mental foramen (MF) and the ante-gonion (Ag) (MF-Ag), the distance between the mental foramen (MF) and the menton (Me) (MF-Me), the distance between the mental foramen (MF) and the deepest point of outer margin of the bone (Al^{l}) connecting Al and Id^{l} (MF-Al^{l}), the distance between the mental foramen (MF) and the highest point of mesiobuccal cusp of the lower 1st molar (M1) (MF-M1), and the length of the lingual side of the alveolar bone which is the distance between the highest point of the mesial alveolar bone at the lower 3rd molar and the infradentale (Id^{l}) on the lingual side (MF-M3) than the female pair-fed control rats ($p < 0.001$, $p = 0.001$, $p < 0.001$, $p = 0.001$, $p = 0.001$ and, $p < 0.001$, $p = 0.005$, respectively). In the alcohol-exposed groups, the male rats also displayed significantly greater MF-Cd, MF-Go, MF-Ag, MF-Me, MF-Al^{l}, MF-M1, and, MF-M3 measurements than the female alcohol-exposed group ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p = 0.003$, $p = 0.002$, respectively).

Table 5.1: General mandibular size in male rats in the chronic binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
MF-Cd	Pair-fed control	6	20.11	0.53	0.452
	Alcohol	6	20.15	0.56	
MF-Go	Pair-fed control	6	19.22	0.46	0.247
	Alcohol	6	19.38	0.35	
MF-Ag	Pair-fed control	6	14.4	0.42	0.319
	Alcohol	6	14.344	0.38	
MF-Me	Pair-fed control	6	4.13	0.15	0.240
	Alcohol	6	4.19	0.12	
MF-Al^{l}	Pair-fed control	6	2.50	0.13	0.375
	Alcohol	6	2.47	0.17	
MF-M1	Pair-fed control	6	4.28	0.14	0.363
	Alcohol	6	4.32	0.18	
MF-M3	Pair-fed control	6	7.92	0.19	0.353
	Alcohol	6	7.96	0.16	

Table 5.2: General mandibular size in female rats in the chronic binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
MF-Cd	Pair-fed control	6	18.91	0.19	0.307
	Alcohol	6	18.81	0.40	
MF-Go	Pair-fed control	6	19.30	0.34	0.172
	Alcohol	6	18.12	0.35	
MF-Ag	Pair-fed control	6	13.64	0.18	0.274
	Alcohol	6	13.56	0.25	
MF-Me	Pair-fed control	6	3.85	0.10	0.246
	Alcohol	6	3.82	0.06	
MF-AII	Pair-fed control	6	2.04	0.07	0.222
	Alcohol	6	2.02	0.0	
MF-M1	Pair-fed control	6	3.98	0.07	0.275
	Alcohol	6	4.01	0.111	
MF-M3	Pair-fed control	6	7.64	0.10	0.589
	Alcohol	6	7.62	0.14	

5.2.2.3 Linear Mandibular Length

Similarities were displayed in the length of the mandible in male and female study groups.

No differences in measurements were identified between the alcohol-exposed and pair-fed control groups in both male (Table 5.3) and female animals (Table 5.4).

The length of the mandible was significantly greater with regards to the length of the base of the mandible which is the distance between the condyle to the infradentale on the labial side (Cd-Id), the mandibular body length which is the distance between the gonion (Go) and the infradentale (Id) on the labial side (Go-Id), the length of lingual side of the alveolar bone which is the distance between the highest point of the mesial alveolar bone at the lower first molar and the infradentale (Id^{I}) on the lingual side (AI-Id^{I}), and the length of the lower dental arch which is the distance between the highest point of the central cusps of the lower 3rd molar (M3) and the infradentale (Id^{I}) on the lingual side (M3- Id^{I}) in the male pair-fed control group than the female pair-fed control group ($p < 0.001$; $p < 0.001$; $p = 0.002$; $p = 0.010$, respectively). The length of the mandible was also significantly greater in the male alcohol-exposed group than the female alcohol-exposed group regarding Cd-Id ($p = 0.001$); Go-Id ($p = 0.001$); AI-Id^{I} ($p < 0.001$); and M3- Id^{I} ($p = 0.002$).

Table 5.3: Mandibular length in male rats in the chronic binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Id	Pair-fed control	6	26.81	0.49	0.406
	Alcohol	6	26.88	0.61	
Go-Id	Pair-fed control	6	26.33	0.49	0.313
	Alcohol	6	26.47	0.50	
Al-Idl	Pair-fed control	6	9.04	0.34	0.440
	Alcohol	6	9.02	0.21	
M3-Idl	Pair-fed control	6	14.84	0.36	0.206
	Alcohol	6	15.01	0.34	

Table 5.4: Mandibular length in female rats in the chronic binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Id	Pair-fed control	6	25.42	0.27	0.305
	Alcohol	6	25.24	0.78	
Go-Id	Pair-fed control	6	25.04	0.27	0.362
	Alcohol	6	24.92	0.78	
Al-Idl	Pair-fed control	6	8.46	0.14	0.070
	Alcohol	6	8.28	0.23	
M3-Idl	Pair-fed control	6	14.39	0.16	0.232
	Alcohol	6	14.27	0.36	

5.2.2.4 Linear Mandibular Height

A similar pattern was identified between pair-fed control and alcohol-exposed groups in the male and female rats.

No differences in measurements were exhibited between the alcohol-exposed and pair-fed control groups in male rats (Table 5.5). Female rats showed a difference in the height of the coronoid which is the distance between the coronoid (Cr) and ante-gonion (Ag) (Cr-Ag) measurement where the alcohol-exposed group had a scientifically greater Cr-Ag (mean = 12.967 mm ±0.266) than the pair-fed control group (mean = 12.711 mm ±0.111) ($p = 0.027$) (Table 5.6).

Male pair-fed control rats exhibited significantly greater mandibular height measurements than the female pair-fed control group in the mandibular ramus height which is the distance between the condyle (Cd) and the gonion (Go) (Cd-Go): $p < 0.001$; in the height of condylar head which is the distance between the condyle (Cd) and the ante-gonion (Ag) (Cd-Ag): $p < 0.001$; in the Cr-Ag: $p = 0.002$; and in height of the central portion of the alveolar bone which is the distance between the deepest point of the outer margin of bone (Al^{l}) that connects to the menton (Me) and the ante-gonion (Ag) to the menton (Me) (Al^{l}-Me): $p < 0.001$). The mandibular height was also significantly larger in male alcohol-exposed rats than the female alcohol-exposed rats (Cd-Go: $p < 0.001$; Cd-Ag: $p = 0.004$; Cr-Ag: $p = 0.005$; Al^{l}-Me: $p = 0.001$).

Table 5.5: Mandibular height in male rats in the chronic binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Go	Pair-fed control	6	8.17	0.29	0.295
	Alcohol	6	8.26	0.27	
Cd-Ag	Pair-fed control	6	12.21	0.41	0.310
	Alcohol	6	12.34	0.48	
Cr-Ag	Pair-fed control	6	13.91	0.44	0.467
	Alcohol	6	13.88	0.67	
All-Me	Pair-fed control	6	4.43	0.11	0.422
	Alcohol	6	4.42	0.17	

Table 5.6: Mandibular height in female rats in the chronic binge alcohol model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Go	Pair-fed control	6	7.62	0.11	0.310
	Alcohol	6	7.49	0.23	
Cd-Ag	Pair-fed control	6	11.34	0.15	0.208
	Alcohol	6	11.47	0.32	
Cr-Ag	Pair-fed control	6	12.71	0.11	0.027*
	Alcohol	6	12.97	0.27	
All-Me	Pair-fed control	6	4.11	0.10	0.460
	Alcohol	6	4.10	0.09	

*Indicates =a p-value of ≤ 0.05

5.2.3 Three-dimensional Micro-focus X-ray Computed Tomography (3D- µCT)

5.2.3.1 Mandibular Bone to Total Volume Ratio (BV/TV)

The male and female rats showed varied findings in the bone-to-total volume ratio (BV/TV) in the mandible. The BV/TV in males exhibited no significant differences in the alcohol-exposed group (mean = 59.71 % ±4.04) compared to the pair-fed control group (mean = 63.45 % ±4.23 (p= 0.083). However, the female rats exhibited a significant decrease in the BV/TV in the alcohol-exposed group (mean = 55.68 % ±5.62) than the pair-fed control group (mean = 68.76 % ±5.54) (p= 0.002). No significant differences were noted in BV/TV between male and female pair-fed control (p = 0.06) and the alcohol-exposed groups (p= 0.092) (Fig. 5.5).

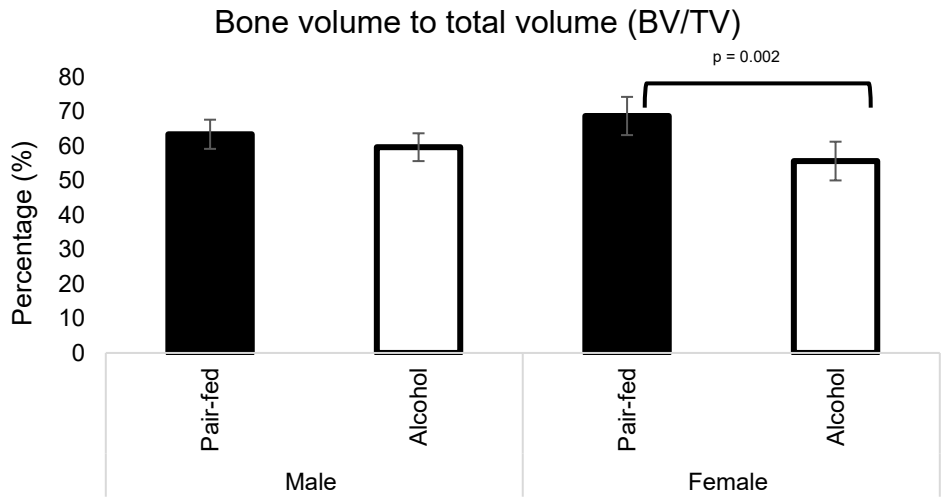

Figure 5.5: Mandibular total bone to volume ratio (BV/TV) in the chronic binge alcohol model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

5.2.3.2 Mandibular Trabecular Thickness (TbTh)

No differences were seen in TbTh in the alcohol-exposed group (mean = 0.15 mm ± 0.03) compared to the pair-fed control group (mean = 0.18 mm ± 0.03) ($p = 0.485$). However, we found significantly thinner trabeculae in the female alcohol-exposed group (mean = 0.15 mm ± 0.04) compared to the pair-fed control group (mean = 0.21 mm ± 0.05) ($p = 0.019$). There were no significant differences between the male and female pair-fed control groups ($p = 0.130$) or the alcohol-exposed groups (male vs female) ($p = 0.498$). (Fig. 5.6).

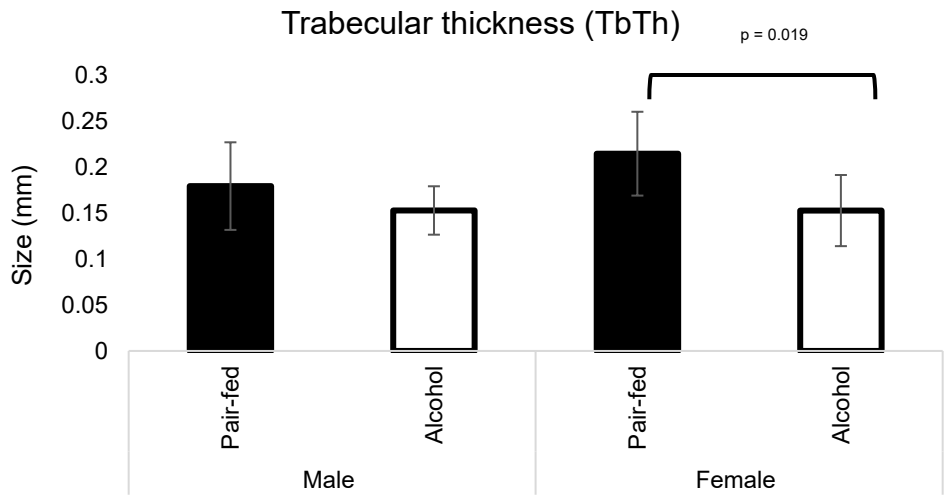

Figure 5.6: Mandibular trabecular thickness (TbTh) in the chronic binge alcohol model. The mean values are given for the pair-fed control and alcohol exposure in both male and female rats. Error bars indicate standard deviation.

5.2.3.3 Mandibular Trabecular Number (TbN)

Trabecular distribution showed a varied pattern in the study groups when stratified between males and females. The trabecular number (TbN) in the male pair-fed control (mean = 3.36 mm^{-1} ±0.45) was marginally fewer than in the alcohol-exposed group (mean = 3.78 mm^{-1} ±0.41), however, no significant differences were detected (p = 0.186). In the female rats, statistically fewer trabeculae were observed in the pair-fed control (mean = 3.29 mm^{-1} ±0.42) than in the alcohol-exposed groups (mean = 3.82 mm^{-1} ±0.45) (p= 0.035). Similarities were exhibited between the male and female pair-fed and the alcohol-exposed groups (p = 0.408 and p = 0.436, respectively) (Fig. 5.7).

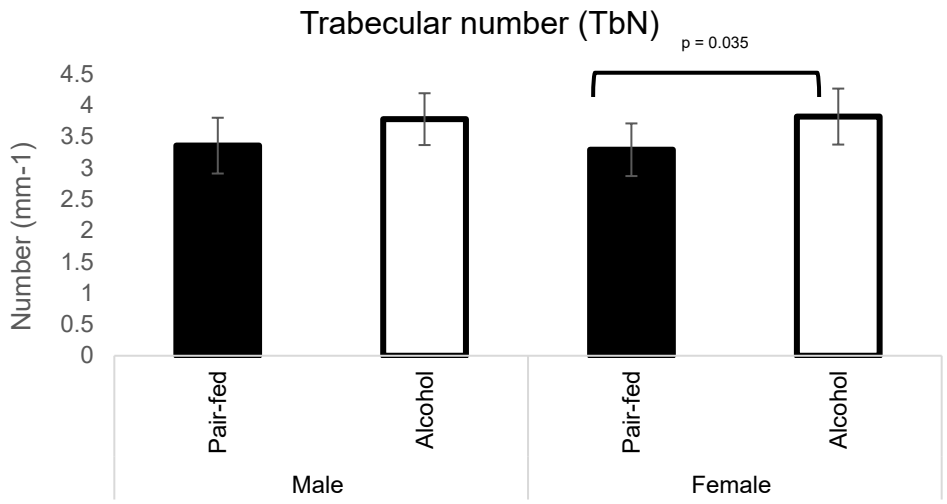

Figure 5.7: Mandibular trabecular number (TbN) in the chronic binge alcohol model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

5.2.3.4 Mandibular Trabecular Spacing (TbSp)

The trabecular spacing (TbSp) was similar in the pair-fed control (mean = 0.12 mm ±0.03) and the alcohol-exposed (mean = 0.11 mm ±0.03) groups in male rats (p = 0.292). However, in the female rats, significantly wider trabeculae (TbSp) were displayed in the alcohol-exposed group (mean = 0.11 mm ±0.01) than in the pair-fed control group (mean = 0.09 mm ±0.01) (p= 0.019). No significant differences were found in TbSp between male and female pair-fed control (p = 0.065) and alcohol-exposed groups (p = 0.428) (Fig. 5.8 and Fig. 5.9).

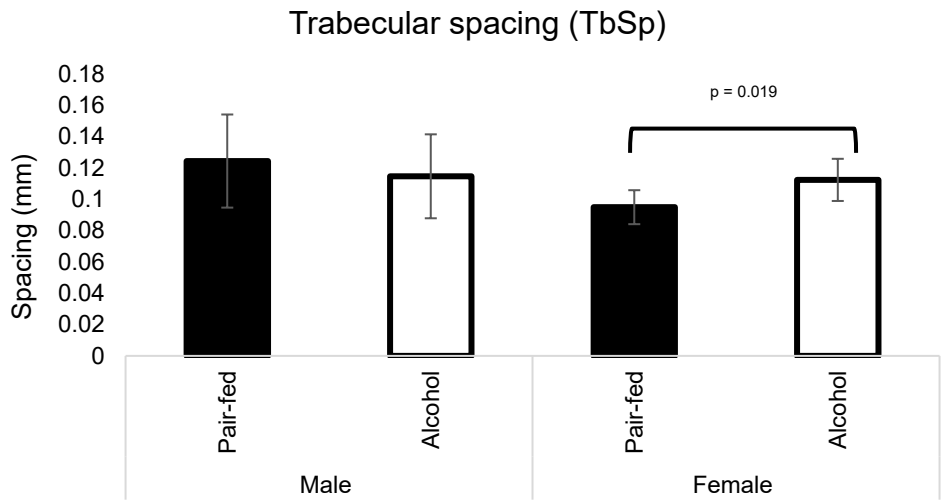

Figure 5.8: Mandibular trabecular spacing (TbSp) in the chronic binge alcohol model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

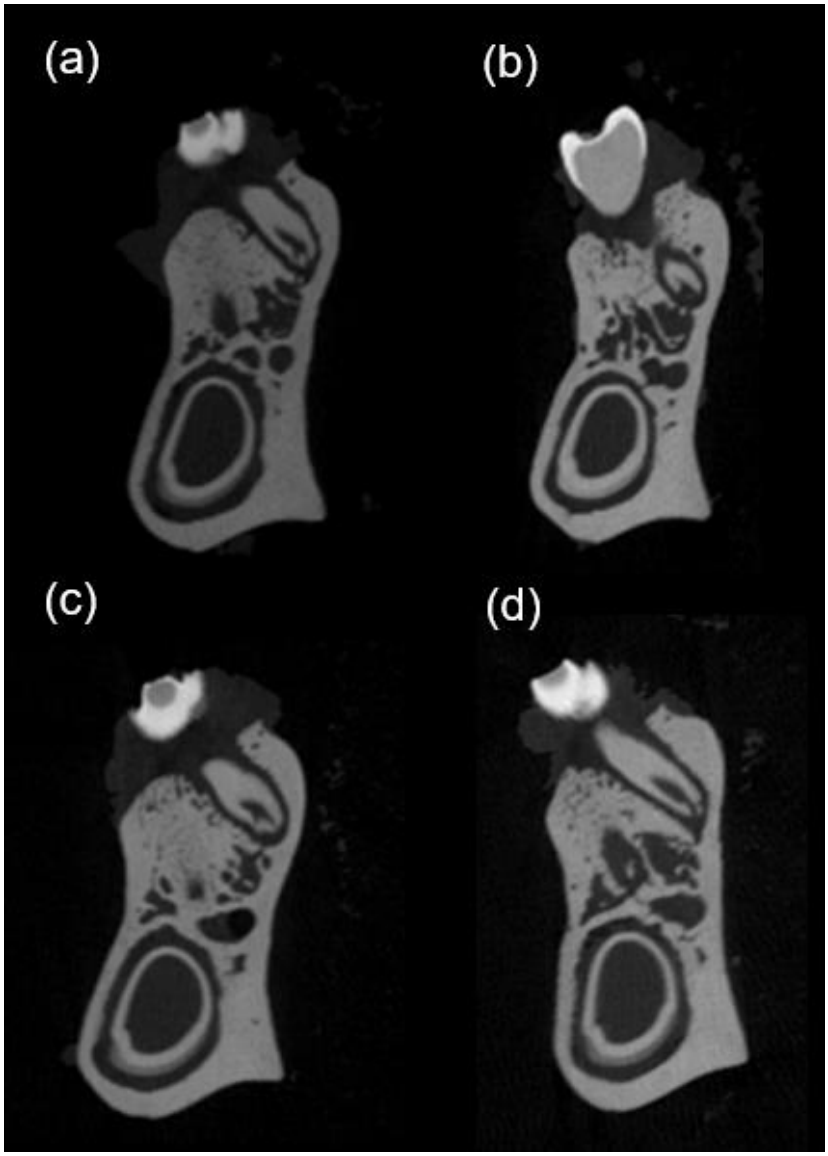

Figure 5.9: Trabecular morphology in the mandible in the chronic binge alcohol model. Representative slices of (a) male pair-fed control group showing slightly less trabeculae and similar spacing to the male alcohol-exposed group; (b) male alcohol-exposed group; (c) female pair-fed control group showing less trabeculae when compared to the alcohol-exposed group, with less spacing; (d) female alcohol-exposed group showing a greater trabecular number with wider trabecular spaces than the control. Slices were taken between the 1st and 2nd mandibular molar teeth.

5.2.4 Tensile Strength of the Mandible Using 3-point Bending Tests

Differences in tensile strength parameters were observed between male and female rats. In male rats, the pair-fed control group showed significantly greater maximum force, maximum displacement, maximum time, and break force ($p = 0.04$, $p = 0.015$, $p = 0.015$, and $p = 0.04$, respectively) (Table 5.7). Among female rats, the maximum force was slightly greater in the alcohol-exposed group, but this difference was not significant ($p = 0.217$). In female rats, the maximum displacement, maximum time, and break force were similar between the alcohol-exposed and pair-fed control groups ($p = 0.246$, $p = 0.246$, and $p = 0.192$, respectively) (Table 5.8). Furthermore, all four tensile strength parameters (maximum force, maximum displacement, maximum time, and break force) were significantly higher in the male pair-fed control group than in the female pair-fed control group ($p = 0.005$, $p = 0.019$, $p = 0.019$, and $p = 0.007$, respectively). However, when comparing the tensile strength parameters between male and female alcohol-exposed groups, similarities were found (maximum force, $p = 0.239$; maximum displacement, $p = 0.161$; maximum time, $p = 0.161$; break force, $p = 0.230$).

Table 5.7: Tensile strength of the mandible in male rats in the chronic binge alcohol model

Measurement	Group	N	Mean	SD	P-value
Maximum force (N)	Pair-fed control	6	68.13	8.15	0.004*
	Alcohol	6	53.76	7.17	
Maximum displacement (mm)	Pair-fed control	6	5.95	0.71	0.015*
	Alcohol	6	5.16	0.33	
Maximum time (sec)	Pair-fed control	6	119.08	14.29	0.015*
	Alcohol	6	103.22	6.64	
Break force (N)	Pair-fed control	6	67.99	8.00	0.004*
	Alcohol	6	53.58	7.24	

Table 5.8: Tensile strength of the mandible in female rats in the chronic binge alcohol model

Measurement	Group	N	Mean	SD	P-value
Maximum force (N)	Pair-fed control	6	53.12	8.42	0.217
	Alcohol	6	57.14	8.65	
Maximum displacement (mm)	Pair-fed control	6	5.13	0.45	0.246
	Alcohol	6	4.98	0.27	
Maximum time (sec)	Pair-fed control	6	102.65	8.96	0.246
	Alcohol	6	99.61	5.32	
Break force (N)	Pair-fed control	6	52.18	10.07	0.192
	Alcohol	6	57.12	8.64	

5.2.5 Mandibular Cytoarchitecture (Hematoxylin and Eosin staining)

Male and female groups showed slight differences regarding the cytoarchitecture identified with Hematoxylin and Eosin (H & E) staining. Male pair-fed control rats exhibited abundant osteocytes scattered evenly throughout the tissue. Small trabecular spaces were exhibited throughout the tissue and were filled with erythrocytes. A widespread bone matrix was identified. Bone matrix, collagen fibres, and periodontal ligament fibres were exhibited surrounding the roots of the teeth. Bone appeared more mineralised than the bone exhibited in the acute binge alcohol group. Widespread small blood vessels were exhibited throughout the tissue (Fig. 5.11a). The cytoarchitecture of the male alcohol-exposed group displayed similar patterns to the male pair-fed control group except for a

few differences. Trabecular spaces in this group were slightly larger and more abundant than those exhibited in the pair-fed control group. Bone in this group appeared slightly less mineralised when compared to the pair-fed control group as the collagen fibres were more evident and less organised (Fig. 5.11b).

Female pair-fed control animals showed the same pattern as the male pair-fed control group except for a few differences. A greater number of small trabecular spaces were identified in this group throughout the tissue and were filled with erythrocytes. Bone in this group appeared less mineralised than the bone displayed in the male pair-fed control group (an increase in wavy collagen fibres was present) (Fig. 5.11c). Female alcohol-exposed rats showed similar patterns to the female pair-fed control rats however the bone in this group was even less mineralised than the bone in the pair-fed control group with less organisation of the collagen fibres. Larger trabecular spaces were also identified in this group (Fig. 5.11d).

Male pair-fed control and alcohol-exposed groups exhibited fewer trabecular spaces than the female-pair-fed control group and alcohol-exposed groups. Male groups also displayed bone that appeared slightly more mineralised. The male pair-fed control and alcohol-exposed groups contained smaller trabecular spaces, and slightly more organised collagen fibres in the bone matrix compared to the female groups.

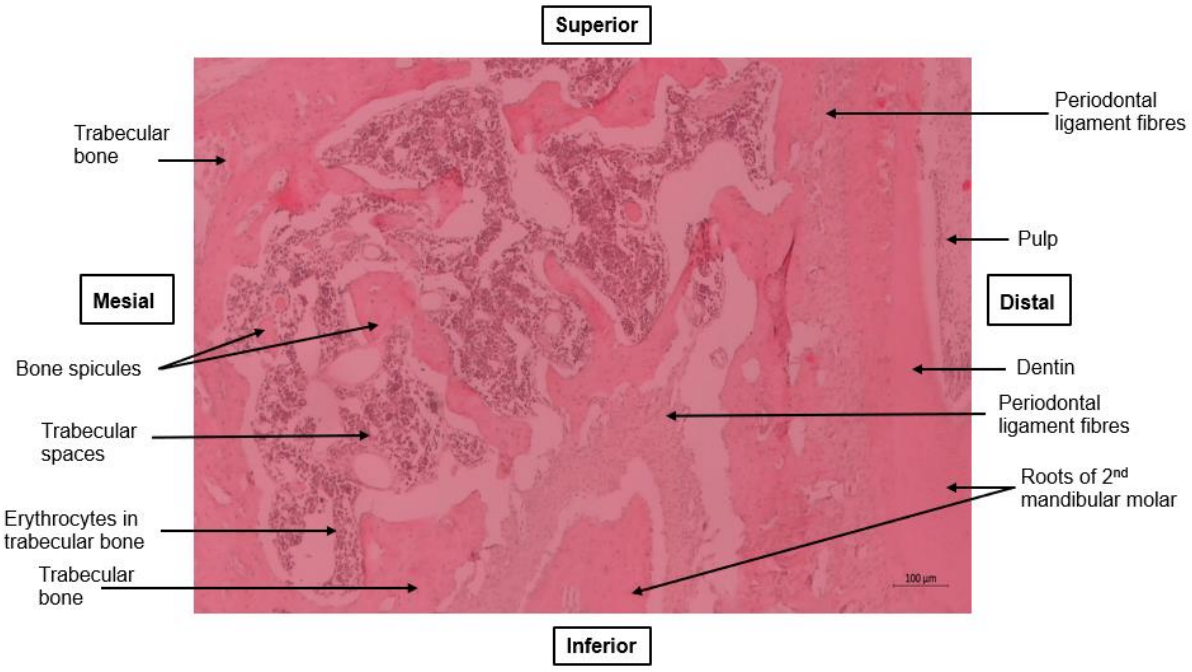

Figure 5.10: A photomicrograph showing a coronal section of the area between the roots of the first and second molar teeth of the mandible (taken from the buccal aspect of the bone), in the chronic binge alcohol model. Scale bar = 100 microns at x 5 magnification. For orientation purposes.

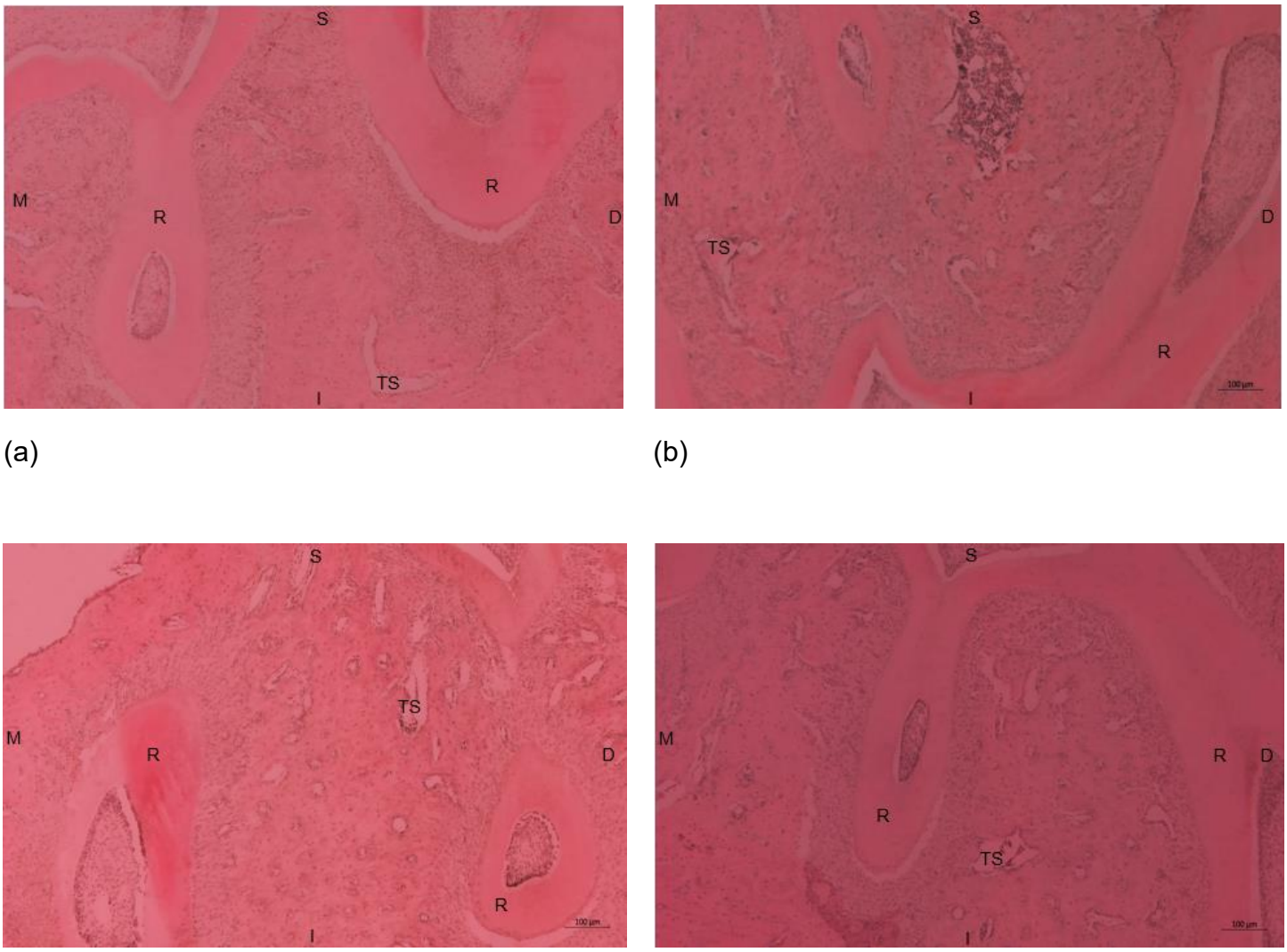

Figure 5.11: Representative photomicrograph of the mandible between the roots of the first and second molar teeth of the mandible in the chronic binge alcohol model. (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. H & E staining. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space.

5.2.6 Immunohistochemistry

5.2.6.1 Mandibular Proliferative Cells, Ki-67 Immuno-Positive Cells

Similarities were found regarding the number of proliferating cells in male and female rats in the region between the 1st and 2nd molar teeth. Male pair-fed control animals had a significantly greater number of proliferative cells (mean = 283 ±.11.25) than the alcohol-exposed group (mean = 192 ± 9.66) ($p < 0.001$).

The female pair-fed control group displayed a significantly higher number of proliferating cells (mean = 171 ± 9.02) compared to the female alcohol-exposed group (mean = 124 ± 2.52) ($p < 0.001$)

A significant difference in the number of proliferating cells was observed between the male and female pair-fed control groups ($p < 0.001$). The male pair-fed control rats had more proliferating cells than the female pair-fed control rats. This difference was also observed between the male and female alcohol-exposed rats ($p < 0.001$).) (Fig. 5.12 and Fig. 5.13).

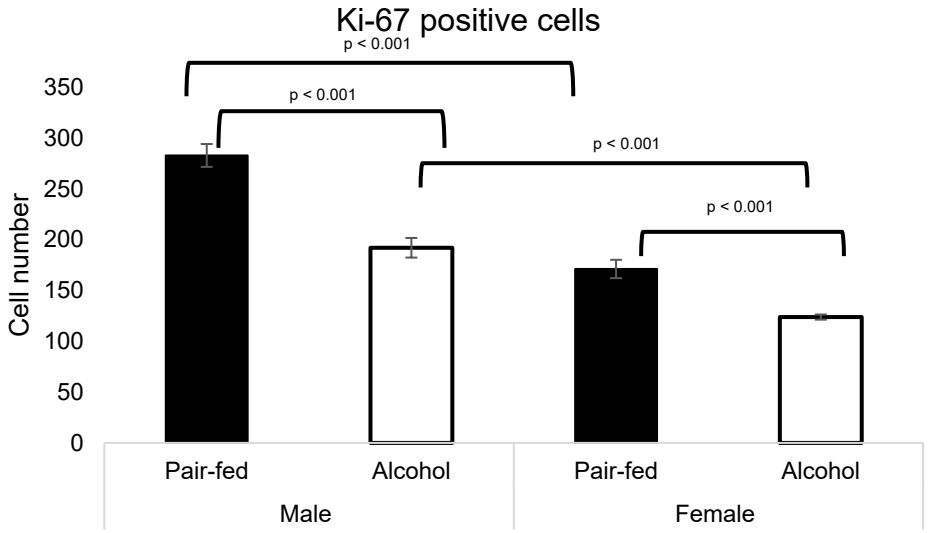

Figure 5.12: Ki-67 immuno-positive proliferative cells of the mandible in the chronic binge alcohol model. The mean number of Ki-67 immuno-positive proliferative cells is shown for the region between the 1st and 2nd molar teeth in the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

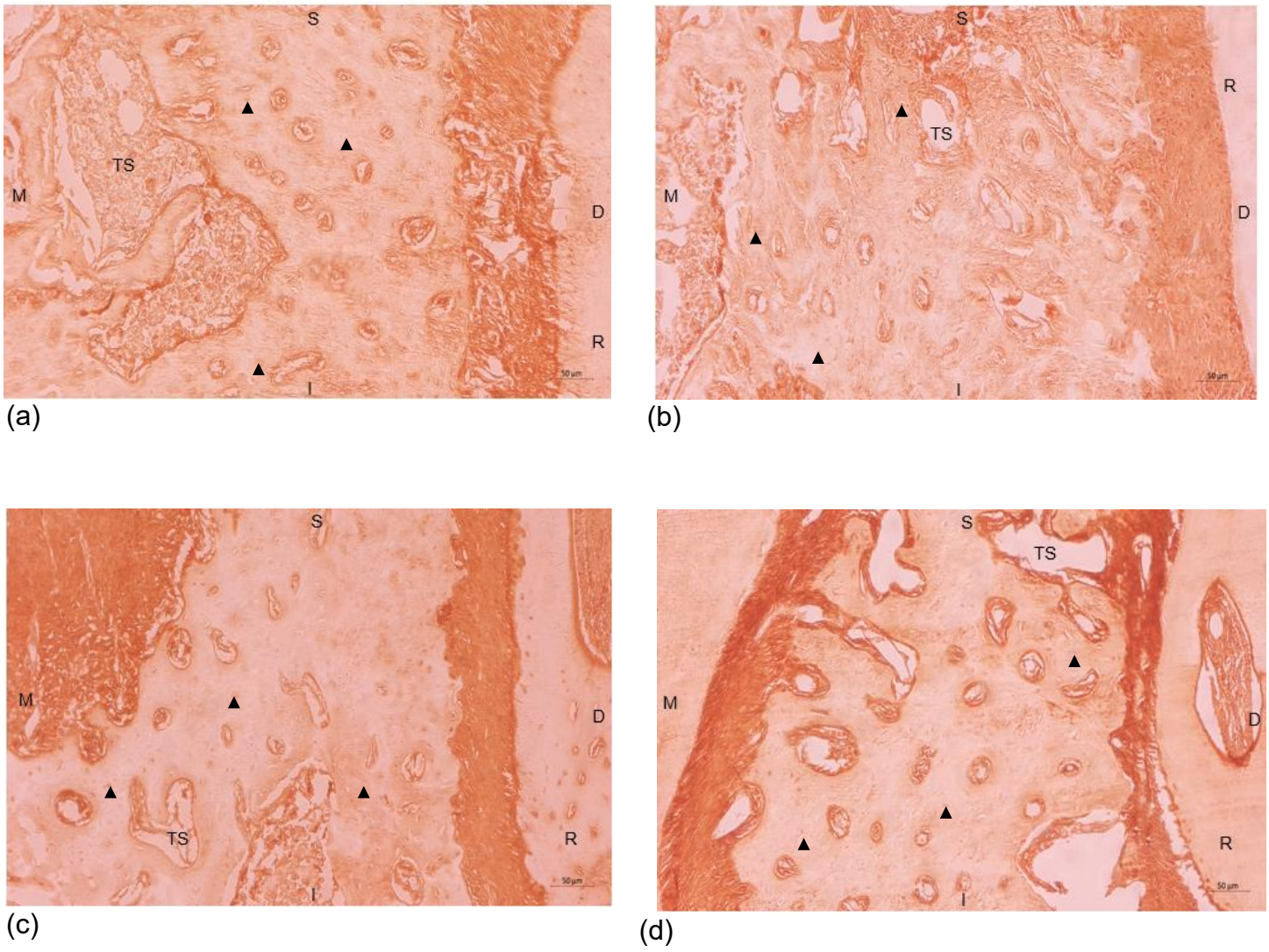

Figure 5.13: Representative photomicrographs of Ki-67 immunolabeled sections of the mandible between the roots of the first and second molar teeth of the mandible in the chronic binge alcohol model. (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. Scale bar = 50 microns at x 10 magnification. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space. Black arrowheads indicate proliferating cells.

5.2.6.2 Mandibular Osteoblasts, Alkaline Phosphatase (ALP) Immuno-Positive Cells

Similar patterns in the number of osteoblasts between male and female groups in the region between the 1st and 2nd molar teeth were found. The number of osteoblasts in the male pair-fed control group (mean = 222 ± 9.25) was significantly higher than the number of osteoblasts in the male alcohol-exposed group (mean = 159 ± 5.07) ($p < 0.001$). Similarly, female pair-fed control animals (mean = 173 ± 11.25) also had a significantly greater number of osteoblasts than the female alcohol-exposed group (mean = 128 ± 4.77) ($p = 0.002$).

Significant differences were observed in the number of osteoblasts in the male pair-fed control vs female pair-fed control groups ($p = 0.002$) where the male rats had significantly greater amounts of osteoblasts than the female rats. Male alcohol-exposed rats also had significantly greater amounts of osteoblasts when compared to the female alcohol-exposed rats ($p < 0.001$) (Fig. 5.14 and Fig. 5.15).

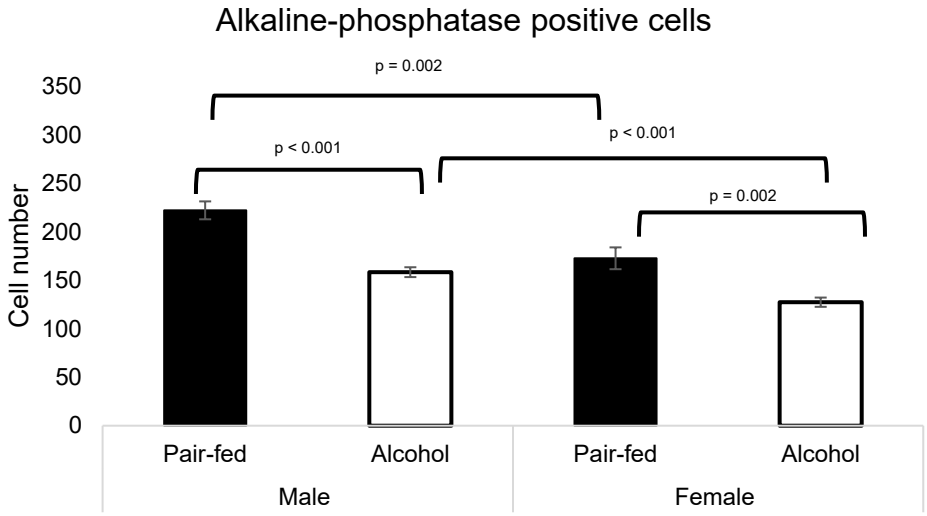

Figure 5.14: Alkaline phosphatase (ALP) immuno-positive osteogenic cells of the mandible in the chronic binge alcohol model. The mean number of osteoblasts are shown for the region between the 1st and 2nd molar teeth in the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

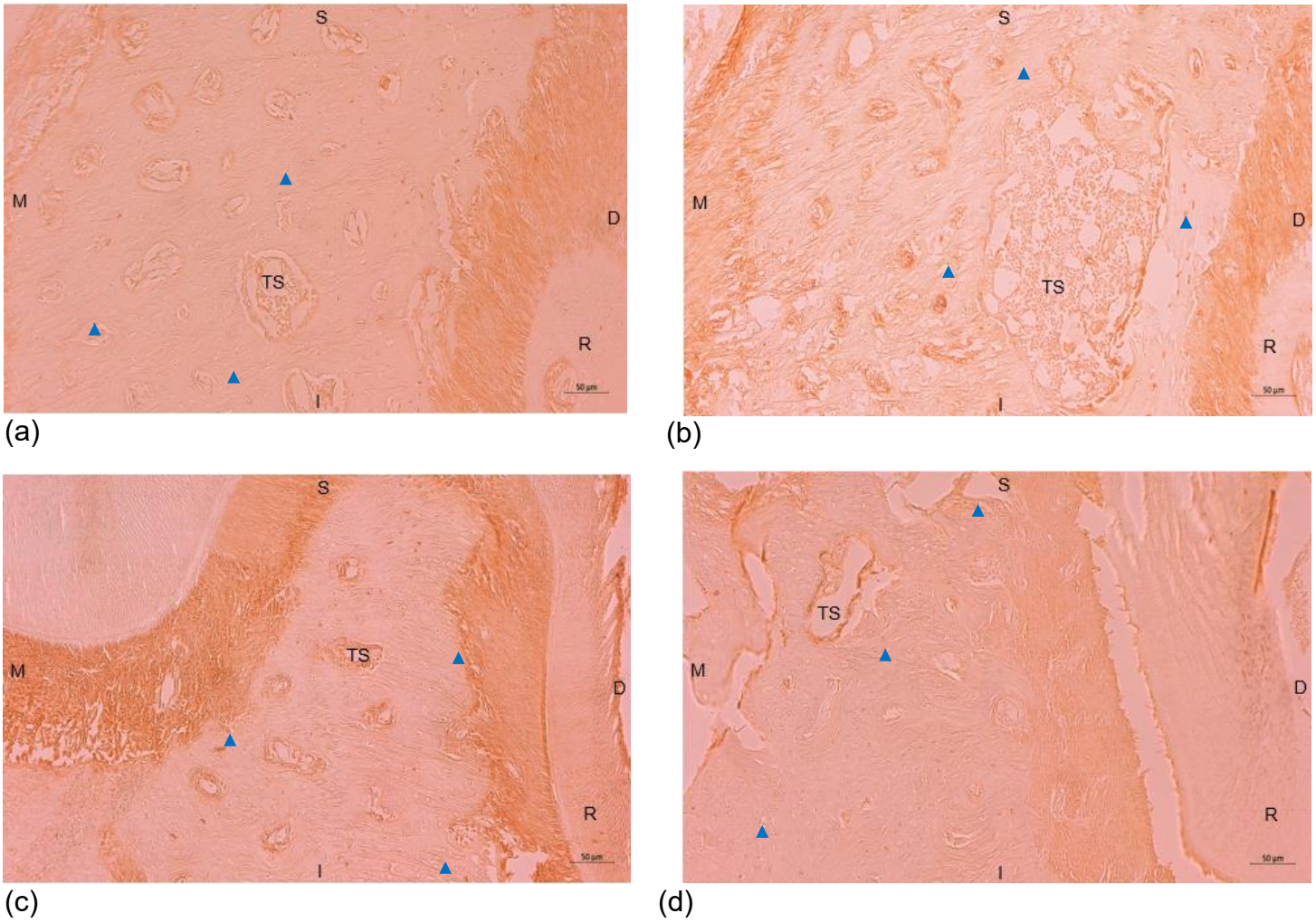

Figure 5.15: Representative photomicrographs of ALP immunolabeled sections of the mandible between the roots of the first and second molar teeth of the mandible in the chronic binge alcohol model. (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. Scale bar = 50 microns at x 10 magnification. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space. Blue arrowheads indicate osteoblasts (on the surface of the bone) and osteogenic cells.

5.3 DISCUSSION

The study investigated the impact of alcohol exposure on the mandible development in adolescent rats using a chronic binge drinking model. It analysed bone size, trabecular morphometry, mandible tensile strength, cell cytoarchitecture, and immunohistochemistry. The present study revealed differences in the baseline data, particularly between pair-fed control and alcohol-exposed animals. Osteometric measurements indicated variations in the height of pair-fed and alcohol-exposed female animals. Three-dimensional micro-focus X-ray computed tomography (3D-μCT) illustrated altered trabecular parameters in the alcohol-exposed groups in the chronic binge model. Tensile strength testing showed significant differences in all 4 parameters between male pair-fed control and alcohol-exposed animals, with no significant differences observed between female groups. Differences in cytoarchitecture were observed between male and female pair-fed control and alcohol-exposed groups, specifically regarding trabecular size and the degree of bone mineralisation. Immunohistochemistry demonstrated differences in the number of proliferating cells and osteoblasts present in the pair-fed and alcohol-exposed rats in both male and female groups. Differences were observed across all the parameters tested and analysed between male and female groups.

Baseline Data

The study found no significant differences in food consumption between male pair-fed control and alcohol-exposed animals, and between female pair-fed control and alcohol-exposed animals per week. However, male pair-fed control and alcohol-exposed rats consumed more food than the female pair-fed control and alcohol-exposed rats. Reports

have shown conflicting arguments where some studies indicate that alcohol causes an increase in appetite (Caton *et al*., 2004; Fong *et al*., 2021) and some show that alcohol acts as an appetite suppressant (Nelson *et al*., 2016). Some studies also indicate that alcohol had no impact on food consumption (Nelson *et al*., 2016), where animals were exposed to alcohol voluntarily. The mode of delivery of alcohol played a role in appetite suppression, where animals exposed to alcohol via intraperitoneal injection presented with signs of appetite suppression and animals allowed to consume alcohol voluntarily displayed no effects on food consumption (Larue-Achagiotis *et al*.,1990; Nelson *et al*., 2016).

In the present study alcohol did not affect appetite when administered through oral gavage. This finding is similar to the study of Nelson et al. (2016) where a similar outcome was observed, however the study by Nelson et al. (2016) involved voluntary alcohol consumption by animals. The above findings indicate that the effect on appetite is likely related to the study design, dosage of alcohol, and duration of alcohol exposure. In the present study, differences were observed, with male rats generally consuming more food than female rats. This difference can be attributed to sexual dimorphism. It has been shown that androgens in males act as an appetite stimulant (Asarian and Geary, 2013), leading to increased food consumption, greater percentage body mass gain, and ultimately larger size in male animals.

Water intake remained unaffected in both male and female groups in the present study. However, a study by Larue-Achagiotis et al. (1990) reported that alcohol caused a decrease in water intake (Larue-Achagiotis *et al.,* 1990). Our study findings are similar to Rothwell and Stock (1984) which reported that alcohol consumption did not affect the

water intake of the animals (Rothwell and Stock., 1984). Male rats consumed significantly more water than female rats. This is likely due to the male rats in our study being significantly greater in size than the female rats and due to their greater food consumption. This can be attributed to sexual dimorphism (Asarian and Geary., 2013).

The data observed in the percentage body mass gained were intriguing. Both male and female pair-fed control and alcohol-exposed groups showed a significant decrease in percentage body mass gain in week 3, followed by an increase in week 4.

The percentage of body mass gain can be directly linked to appetite and food consumption. In our study, food consumption in the chronic binge alcohol model was not affected, and food intake remained consistent between pair-fed control and alcohol-exposed animals. It's important to note that the alcohol-exposed animals in this study were paired with a maltose-dextrin pair-fed caloric equivalent control animal, so caloric differences would not have influenced the observed weight changes.

Various studies have reported different effects of alcohol on body mass gain. For example, some studies have shown a decrease in body mass gain in male rats exposed to 20 % alcohol, while others have found no influence on body mass gain in adolescent male rats due to alcohol consumption (Kolota *et al*., 2019; Umamaheswari *et al*., 2012; Velvhizi *et al*., 2002). The decrease observed in body mass gain in week 3 correlates with the studies that observed a decrease in body mass in alcohol-exposed animals.

Differences were displayed with male rats gaining a greater percentage of body mass than females in both control and alcohol-exposed groups. This can be attributed to sexual dimorphism. Androgens in males have been shown to act as an appetite stimulant

(Asarian and Geary., 2013), which results in greater food consumption, a greater percentage of body mass gain, and thus a greater overall size of male animals.

Osteometric Measurements

No negative effects on bone mass were exhibited in the chronic binge alcohol model in both male and female animals in the current study. Previous studies have indicated a decrease in bone mass in alcohol-exposed animals (Broulík *et al.,* 2010; Chakkalakal., 2005). Light to moderate alcohol consumption is reported to have a beneficial effect on the skeleton, whereas heavy alcohol consumption decreases bone quality and quantity (Gaddini *et al*., 2016). The lack of significant differences observed in our study could be related to the study design, dosage, and duration of alcohol exposure. Sampson et al. (1999) investigated the effect of binge drinking on bone metabolism in a young actively growing rat model. They identified a decrease in the weight of the femur. However, no effect was observed on the tibia. The lack of changes in the bone weight could thus also be bone-specific which may explain why no differences were detected in our study.

When observing the differences in general mandibular size, linear mandibular length, and linear mandibular height, the only significant difference identified was the Cr-Ag measurement (height of the coronoid which is the distance between the coronoid (Cr) and ante-gonion (Ag)) which was significantly greater in the female alcohol-exposed group. This measurement represents the mandibular height in the posterior mandible. Changes in mandibular height were also noted in female alcohol-exposed rats in the acute binge alcohol model of our study.

The increase in the Cr-Ag mandibular height parameter in the female group in our study is likely due to one of two reasons, or a combination of both. The first reason is the study design (mode of alcohol administration and duration of exposure), and the second reason is the process of the development of the mandible. In the prenatal development of the rat mandible, both intramembranous and endochondral ossification processes occur at different stages.

Intramembranous ossification typically occurs earlier in prenatal development. During the initial stages of mandibular development in the rat embryo, mesenchymal cells condense and differentiate directly into osteoblasts. This process starts around embryonic days 13-14 (E13-E14) in rats, and these osteoblasts then lay down bone matrix directly, forming the mandibular bone proper (mandibular body) (Chen *et al*., 2015).

Endochondral ossification occurs later in prenatal development and is primarily associated with the development of the condylar process of the mandible. This process starts with the formation of a cartilage template within the condylar region. The condylar cartilage begins to develop from mesenchymal cells around embryonic day 16-17 (E16-E17) in rats (Chen *et al*., 2015). Chondrocytes within this cartilage model undergo proliferation and hypertrophy, leading to the formation of primary growth centers in the condylar secondary cartilage (CSC), angular synchondrosis (AS), and mandibular midline synchondrosis (MS). The AS and MS fuse and the mandibular condyle reverts to a secondary growth site. Blood vessels and osteoblasts invade the calcified cartilage, replacing it with bone tissue. This process continues postnatally and throughout early life as the condylar process undergoes growth and remodeling (Chen *et al*., 2015).

The development of the mandibular condyle not only leads to an increase in the size of the mandible but also results in the anteroinferior displacement (transposition) of the mandible. The length of the body of the mandible increases in a rectilinear direction, whereas the condylar process exhibits growth in various directions from anterosuperior to posterior (Mizoguchi *et al*., 2013). This diverse growth pattern enables a wide range of growth and shapes of the mandible. The growth of the condyle is closely related to the transposition of the mandible, which will in turn influence the angle of the mandible, thus the maxillary mandibular relationship. In individuals with low angles (short face, brachyfacial pattern, and deep bites), the mandibular growth is characterized by anterosuperior growth of the condyle. In individuals with high angles (long face, dolichofacial pattern, and skeletal open bite), posterosuperior growth of the condyle is exhibited (Mizoguchi *et al*., 2013).

Disturbances in growth can thus have a varying range of effects on the length and height of the mandible. In the case of juvenile rheumatoid arthritis, the posterior margin of the ramus had bone apposition and grew in a posterior direction (Mizoguchi *et al*., 2013). This could indicate that the exposure to alcohol in both the acute binge and chronic binge alcohol models in our study showed a similar growth pattern as was displayed in the case above, with more bone apposition in the posterosuperior direction, which could lead to a mandible with a high angle, with complications such as malocclusion and an open bite.

Males showed an overall greater general size, length, and height in mandibular size when compared to females in the chronic binge alcohol model. This is likely due to sexual dimorphism. Males are generally larger than females (Lorenzo., 2020). Several factors are responsible for sexual dimorphism some of them being age, genetics, actions of sex

steroids, environment, and behavior (Lorenzo., 2020) which can all lead to the differences identified between the male and female animals in our study.

Three-Dimensional Micro-focus X-ray Computed Tomography (3D- μCT)

Three-dimensional micro-focus X-ray computed tomography (3D-μCT) was used to access trabecular volume, thickness, number, and spacing in the mandible (between the first and second molar teeth). Altered trabecular parameters were observed in the alcohol-exposed groups in the chronic binge alcohol model.

In the present study, the bone-to-total volume ratio (BV/TV) was significantly lower in the alcohol-exposed group in the female rats. Thinner trabeculae were observed in the alcohol-exposed groups in both male and female rats, with the differences between female rats exhibiting statistical significance. The number of trabeculae (TbN) was significantly lower in the female pair-fed control group in the chronic binge alcohol model. Trabeculae were significantly wider in the alcohol-exposed female rats in the present study.

In the present study, we observed that chronic binge alcohol exposure for 4 weeks affected the trabecular parameters of female rats but had no significant impact on alcohol-exposed males. The findings of this study regarding the female rats are consistent with the literature, where alcohol had an impact on the trabecular parameters of alcohol-exposed male and female rats. Bone to total volume ratio (BV/TV) is a ratio representing the bone volume to the total volume ratio of the region of interest and can be affected by both internal and external factors. Alcohol can have an impact on bone volume by affecting bone remodeling by either decreasing the number and function of osteoblasts,

increasing the number and function of osteoclasts, or by both mechanisms (Sampson., 1998). Sampson. (1998) reported a decrease in BV/TV in the tibia of alcohol-exposed female rats. Maia et al. (2021) observed a decrease in BV/TV in the alveolar bone of female adolescent rats after 30 and 60 days of alcohol exposure, and Föger-Samwald et al. (2018) noted a decrease in BV/TV in the femur of a prepubescent pig model exposed to a binge drinking model (Föger-Samwald *et al*., 2018; Maia *et al*., 2021). Callaci et al. (2004) found that binge drinking had no effect on male rats in the first 2 weeks of exposure but caused a decrease in BV/TV after 3 weeks (Callaci *et al*., 2004). Callaci et al. (2006) depicted a decrease in BV/TV in female rats exposed to a binge alcohol model for a time frame of 2 and 4 weeks, with a more prominent decrease detected in the 4-week alcohol-exposed group (Callaci *et al*., 2006). Thinner trabeculae were noted in the alveolar bone of adolescent female rats exposed to a binge alcohol model (Maia *et al*., 2021). Trabecular thickness is related to alveolar bone quality, thus a decrease in thickness leads to a decrease in bone quality which indicates that alcohol influences the microarchitecture of the mandible. The mechanism behind the deleterious effects on bone integrity of the animals exposed to a chronic binge alcohol model is likely because of the effect that alcohol has on the cells involved (osteoblasts and osteoclasts), and the interruption of normal bone modeling and remodeling (Callaci *et al*., 2006; Maia *et al*., 2021). A decrease in trabecular number is expected to be detected in the alcohol-exposed animals, however, the opposite was observed in our study. Föger-Samwald et al. (2018) indicated a decrease in TbN in the femur and an increase in TbN in the humerus of binge alcohol-exposed prepubescent pigs (Föger-Samwald *et al*.,2018). Differences in trabecular number at different bone sites indicate that variations in bone structure, mechanical

properties, and bone metabolism across different body sites, known as skeletal heterogeneity, may manifest in how alcohol impacts bone health.

Wider trabecular spaces indicate disturbances and modification in the microarchitecture of the mandibular bone by a chronic binge alcohol model. The consumption of alcohol directly disrupts the equilibrium between osteoclasts and osteoblasts, leading to disturbances in bone remodeling and homeostasis (Maia *et al*., 2021).

The observed differences, with females being more affected than males, are likely due to variations in hormonal profiles between male and female rats. In adolescent females, alcohol disrupts normal oestrogen levels and signals in the hypothalamus–pituitary–gonadal axis, impairing growth hormone release. This hormonal disruption can blunt the pubertal surge needed for bone growth more in females than males and can lead to the increase in negative effects of alcohol on the skeleton in female rats (Corona *et al*., 2022). The actions of testosterone in male rats might also explain the lack of effect on the BV/TV observed in our study. Testosterone, which is found in males, plays a crucial role in maintaining bone health at all life stages. It does this by interacting with the androgen receptor present on osteoblasts, osteoclasts, osteocytes, and marrow stromal cells. These cells also express the oestrogen receptor, which responds to oestradiol produced from the aromatization of testosterone (Corona *et al*., 2022).

During puberty, both testosterone and oestradiol promote periosteal apposition and trabecular bone growth, contribute to pubertal growth spurts, and facilitate the attainment of peak bone mass. Testosterone leads to the development of broader bones with a thicker cortex and a higher peak bone mass than females (Corona *et al*., 2022).

Testosterone also helps maintain a balance between bone resorption and bone remodeling (Corona *et al*., 2022).

The actions of testosterone found in male rats may explain why no adverse effect were observed in BV/TV in the male animals. The increased negative effects observed in the female rats in our study are also likely due to alcohol's impact on oestrogen levels. Further studies focusing on specific hormones would be necessary to confirm this. Mngoma et al. (2024) discovered that BV/TV decreased in male alcohol-exposed rats in the distal epiphysis of the femur in a chronic binge alcohol model (Mngoma *et al*., 2024).

The femur is a long bone that develops through endochondral ossification. The present study examined the effect of alcohol on the mandible, which is a flat bone that develops through both intramembranous and endochondral ossification. This suggests that the effect on trabecular parameters may be both bone and site-specific.

Tensile Strength

Significant differences in all parameters regarding tensile strength were detected in the male animals, with alcohol-exposed groups exhibiting a decrease in all parameters. No significant differences were observed in the female group.

No significant differences were identified in the *3D-μCT* analysis of the trabecular parameters in the male group, and we thus expected no differences in the tensile strength to be detected. However, we observed the opposite with alterations in all parameters regarding bone strength. Lauing et al. (2008) observed a 31 % decrease in compressive strength in the L4 vertebra of rats exposed to binge alcohol exposure for 28 days (Lauing *et al*., 2008). Both Hogan et al. (1999) and Chakkalakal (2005) identified a decrease in

cortical bone strength in rats exposed to alcohol (Chakkalakal 2005; Hogan *et al*., 1999). The results exhibited in our study thus corroborate with the results observed in previous studies (Chakkalakal 2005; Hogan *et al*., 1999; Lauing *et al*., 2008). A decrease in cortical strength may be attributed to a decrease in mineral content, which was not analysed in this study. Further investigations would be needed to access this. Maurel et al. (2012b) found cortical bone to be more sensitive to dose effects of alcohol than trabecular bone in male Wistar rats and that the damage caused to the cortical bone also had a negative impact on bone strength (Maurel *et al*., 2012b). The results in our study correlate with the results displayed by Maurel et al. (2012b), where alcohol had a greater impact on cortical bone and bone strength in our male animals than it did on the trabecular parameters.

The opposite effect was noted on the tensile strength in the female rats in our study where alcohol had no significant effect on the bone strength. This was an interesting observation as the trabecular parameters in the female rats were impacted greatly by binge alcohol exposure. Chronic binge alcohol consumption may have induced a compensatory increase in cortical bone formation in the female animals in response to its overall negative effects on trabecular bone. This could be due to increased mechanical loading or a biological response to preserve structural integrity. Literature regarding this possible compensatory mechanism is not available. This represents a gap in the current literature, and further research is needed to clarify this observation.

Regarding differences between the tensile strength in male and female rats, all four parameters tested were significantly greater in the male pair-fed control group than in the female pair-fed control group, with no differences observed between male and female alcohol-exposed groups. The differences in the pair-fed control groups are likely attributed

to sexual dimorphism as males typically have larger, and stronger bones compared to females (Gordon and Gordon., 2020). The similarities identified between the male and female alcohol-exposed groups could be attributed to the fact that alcohol caused a decrease in strength in the male alcohol-exposed group, thus evening out the differences in strength between the groups.

Mandibular Cytoarchitecture

Only qualitative analysis of the cytoarchitecture was assessed, with consistent findings observed across all samples within each group. Samples from all study animals were included in the quantitative analysis. Some differences were noted between male pair-fed control and alcohol-exposed animals, between female pair-fed control and alcohol-exposed animals, and lastly between male and female pair-fed control and alcohol-exposed groups.

The trabecular spaces in the male pair-fed control group appeared small, and the bone appeared mature and mineralised. However, in the male alcohol-exposed groups, the trabecular spaces appeared slightly larger and more abundant compared to the pair-fed control group. The bone in this group appeared less mineralised, as the collagen fibres were more evident and less organised. Smaller trabecular spaces are typically detected in more mature bone, which possibly indicates that alcohol played a role in delaying bone maturation in the alcohol-exposed male group (Galea *et al*., 2021).

Similarly, in the female alcohol-exposed group, the bone appeared less mineralised compared to the pair-fed control group, with visually less organised collagen fibres and larger trabecular spaces present. The presence of larger trabecular spaces with more

abundant wavy collagen fibres which may indicate that alcohol delayed the process of bone maturation (Galea *et al.,* 2021).

Male pair-fed control and alcohol-exposed groups exhibited bone cytoarchitecture that appeared more mature and more mineralised than that observed in the female pair-fed control and alcohol-exposed groups. This finding contradicts the general principle that females develop at a faster rate than males (Nanci., 2018). The changes related to interradicular bone may also result from increased mechanical forces, leading to greater bone volumes (Nenda *et al*., 2016). This could likely accelerate bone development and may explain the differences identified in our study as male rats had a greater food intake than female rats, resulting in greater mechanical force applied to the mandible and alveolar bone.

Cell proliferation and osteoblast activity

In this objective, our aim was two-fold: first, to investigate the impact of chronic binge alcohol consumption on proliferating cells in the adolescent mandible, and second, to evaluate its effects on osteoblasts in the same population.

A significant decrease in the number of proliferating cells was observed in both male and female alcohol-exposed rats when compared to their pair-fed controls, which indicates that chronic binge alcohol consumption had an adverse effect on the proliferation of cells. The results exhibited in our study correlate with a study by Miralles-Flores and Delgado-Baeza. (1992), which reported a decrease in proliferating cells in the growth plate in prenatal rats exposed to alcohol. Prolonged alcohol consumption has been associated with a persistent reduction in Ki-67 expression (Eby *et al*., 2022). Pillay et al. (2024)

looked at the effect of alcohol on prenatal alcohol exposure. They looked at the effect of alcohol on both TGFβ-1 and Ki-67. Their findings showed a reduction in both markers in following alcohol-exposure. They proposed that alcohol may disrupt the TGFβ-1 signaling pathway involved in cell proliferation, leading to a subsequent decrease in proliferating cells, as indicated by the reduced Ki-67 expression (Pillay *et al*.,2024). There were no studies in the literature that reported on Ki-67 immuno-positive proliferative cells in the mandible following alcohol exposure in adolescent rats.

In this study, we investigated the impact of chronic binge alcohol consumption on bone-forming cells using alkaline phosphatase, which is expressed in osteoblasts. The findings revealed a significant decrease in the number of osteoblasts in male and female rats exposed to alcohol compared to their pair-fed controls. This sugests that chronic binge alcohol consumption has a negative influence on osteoblasts, which are crucial for bone formation. Our results align with existing literature indicating that alcohol can detrimentally affect osteogenic cells, leading to a decrease in osteoblast number and function, as well as reduced bone formation, potentially resulting in osteopenia (Chakkalakal, Dyer *et al*., 1998; Eby *et al*., 2020; Turner, 2000). The authors Wezeman et al. (2007) and Wang et al. (2003) also reported that alcohol exposure reduces the activity of bone formation markers, osteocalcin and ALP. While Wezeman focused on acute binge exposure, Wang's study showed similar effects in marrow stromal cells undergoing osteogenic differentiation, suggesting alcohol impairs bone formation (Wang *et al*., 2003; Wezeman *et al*., 2007). ALP is produced by osteoblasts and plays a role in bone mineralisation. A decrease in ALP expression would then likely indicate a decrease osteoblast

differentiation, which may lead to a decrease in osteoid formation and mineralisation (Bassi *et al.*, 2011).

The number of proliferating cells and osteoblasts was higher in the male groups compared to the female groups. This difference is due to sexual dimorphism, as males typically have larger, more robust bones (Gordon and Gordon, 2020). This makes it plausible for males to have more proliferating cells and osteoblasts.

5.4 CONCLUSION

The study examined the effects of chronic binge drinking on the mandible growth in adolescent Sprague Dawley rats. The results indicate that binge drinking had a more significant impact on females compared to males, leading to increased body mass gain in both male and female rats. Additionally, chronic binge alcohol exposure weakened bones in female rats and may have delayed bone mineralisation in male rats. The study also observed a decrease in bone growth cells in both males and females, with more pronounced effects in females. The differences were attributed to various factors such as study design, maturation times, and hormonal differences.

CHAPTER 6: *Investigating the impact of long-term binge drinking followed by a period of abstinence on the development of the adolescent Sprague Dawley rat mandible*

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6.1 INTRODUCTION

Binge drinking is a widespread phenomenon among adolescents in South Africa and across the globe, presenting considerable risks to their skeletal development and growth (Morojele & Ramsoomar, 2016). Furthermore, it heightens the probability of excessive chronic alcohol intake in later life and is associated with alcohol-related complications, including motor vehicle collisions, fatalities, interpersonal violence, and criminal behavior (Kuntsche *et al*., 2017; Morojele & Ramsoomar, 2016). Binge drinking is characterized as the consumption of five or more alcoholic beverages for males and four or more for females within a single occasion or the intake of at least 60 grams of pure alcohol once per month (Fillmore & Jude, 2011; Morojele & Ramsoomar, 2016).

Adolescence, which encompasses the age range of 10 to 19 years, represents a pivotal phase characterized by significant skeletal growth and development, during which approximately 90 % of skeletal maturation and the attainment of peak bone mass occurs. Throughout this developmental stage, adolescents are deemed to be nutritionally susceptible due to a myriad of factors that can adversely influence their growth and overall development (Das *et al*., 2017; Monge, 2018; Weaver, 2002). Alcohol is recognized as a teratogenic substance that exerts detrimental effects on skeletal integrity (Callaci *et al*., 2006; Sampson *et al*., 1999) and heightens the likelihood of fractures alongside prolonged healing processes (Chakkalakal, 2005; Hogan *et al*., 1997; LaBrie *et al*., 2018; Sampson *et al*., 1996; Sampson, 1997).

However, because adolescents are still developing, their skeletal systems may be able to recover from alcohol-induced damage if the teratogen is removed from their diet. Lauing et al. (2008) reported that the tibia could recuperate from bone mass deficiencies following binge alcohol consumption, but the vertebrae could not (Lauing *et al*., 2008).

Research indicates that alcohol has different effects on different bones. In young rats, chronic alcohol exposure led to a reduction in tibial length (Rosa *et al*., 2019). After subjecting rats to an alcohol-liquid diet for various durations (2, 4, 6, 8, and 10 weeks), Sampson et al. (1996) observed a decrease in femur length. However, Hogan et al. (1999) found that alcohol did not impact bone length over different time intervals in a chronic alcohol consumption model using a liquid alcohol diet. In a binge drinking model, alcohol administered via oral gavage increased the length of the femur and tibia (Sampson *et al*., 1999). These differences are likely due to variations in study design, models, and alcohol exposure durations. Although many studies have concentrated on the effects of alcohol on long bones (Hogan *et al*., 1999; Sampson *et al*., 1996; Sampson *et al*., 1999), there is limited research on the impact of alcohol on craniofacial bones, such as the mandible.

The mandible is a unique bone that undergoes both endochondral and intramembranous ossification. The condylar process of the mandible develops through endochondral ossification, while the rest of the mandible forms via intramembranous ossification (Mizoguchi *et al*., 2013). The mandible's overall size increases significantly between ages 11 and 17, with notable growth in condylar height during adolescence (Smartt *et al*., 2005).

Intramembranous ossification occurs earlier in prenatal development. In the early stages of mandibular development in the rat embryo, mesenchymal cells condense and directly differentiate into osteoblasts, starting around embryonic days 13-14. These osteoblasts then form the mandibular bone proper (mandibular body) by directly laying down the bone matrix (Chen *et al*., 2015).

Endochondral ossification, primarily associated with developing the condylar process of the mandible, occurs later in prenatal development. In rats, this process begins around embryonic day 16-17 when a cartilage template forms within the condylar region. Chondrocytes within this cartilage model proliferate and undergo hypertrophy, creating primary growth centers in the condylar secondary cartilage (CSC), the angular synchondrosis (AS), and the mandibular midline synchondrosis (MS). The AS and MS eventually fuse, and the mandibular condyle becomes a secondary growth site. Blood vessels and osteoblasts invade the calcified cartilage, replacing it with bone tissue. This process continues postnatally and throughout early life, as the condylar process undergoes growth and remodeling (Chen *et al*., 2015). The development of the mandibular condyle increases the mandible's size and results in its anteroinferior displacement (transposition).

It is well-established that alcohol disrupts bones formed by endochondral ossification (Mizoguchi *et al*., 2013). However, there is still a gap in the literature regarding the effect of chronic alcohol consumption on the mandible.

This study, therefore, investigated the effects of chronic binge drinking followed by a period of alcohol abstinence. This was implemented to determine if the removal of alcohol

from the diet would result in recovery of the adolescent Sprague Dawley rat mandible by assessing the baseline parameters, osteometric morphometry, trabecular morphometry, tensile strength, cytoarchitecture, and different cells using Ki-67 and ALP immunohistochemistry (IHC), with the implementation of a chronic binge alcohol consumption model, followed by a period of alcohol abstinence.

6.2 RESULTS

6.2.1 Baseline Data

6.2.1.1. Blood Alcohol Concentration

The mean blood alcohol concentration (BAC) was 164.05 mg/dL (± 24.70) in the male alcohol-exposed group; and 270.97 mg/dL (± 20.63) in the female alcohol-exposed group.

6.2.1.2 Food Consumption

Male and female study groups displayed similarities in the pattern of food consumption where food consumed throughout the 8 weeks was relatively consistent, except for week 5 where a non-significant decrease in food consumption was observed in all groups. Male pair-fed control (week 1: mean = 46.43 g ±4.71; week 2: mean = 48.17 g ±4.31; week 3: mean = 50.66 g ±2.64; week 4: mean = 49.79 g ±2.94; week 5: mean= 46.31 g ±2.00; week 6: mean = 52.92 g ±3.98; week 7: mean = 53.17 g ±4.14; week 8:mean = 52.71 g ±2.68) and alcohol-exposed rats week 1: mean = 45.88 g ±3.50; week 2: mean = 47.21 g ±2.26; week 3: mean = 47.02 g ±2.85; week 4: mean = 48.62 g ±3.36; week 5: mean =45.00 g ±1.60; week 6: mean = 50.64 g ±2.14; week 7: mean = 50.19 g ± 1.96; week 8: mean = 48.45 g ±2.32) showed no significant differences in the amount of food consumed throughout weeks 1 to 8 ($p = 0.440$; $p = 0.377$; $p = 0.107$; $p = 0.337$; $p = 0.213$; $p = 0.216$; $p = 0.162$; $p = 0.053$, respectively).

A similar pattern was observed in female pair-fed control (week 1: mean = 31.81 g ±1.38; week 2: mean = 32.60 g ±1.76; week 3: mean = 32.90 g ±2.87; week 5: mean= 31.95 g ±0.48; week 6: mean = 35.64 g ±2.40; week 7: mean = 31.89 g ±2.00; week 8: mean = 31.40 g ±2.39) and alcohol-exposed groups (week 1: mean = 32.67 g ±3.08; week 2: mean = 32.33 g ±1.36; week 3: mean = 33.45 g ±1.20; week 5: mean= 28.62 g ±6.71; week 6: mean = 35.21 g ±2.04; week 7: mean = 34.14 g ±2.41; week 8: mean = 33.72 g ±1.21), where no significant differences in the amount of food consumed was exhibited in weeks 1,2,3,5,6,7and 8 ($p = 0.341$; $p = 0.424$; $p = 0.388$; $p = 0.700$; $p = 0.412$; $p = 0.141$; $p = 0.105$, respectively). A significant increase in food consumption was identified in week 4 in the alcohol-exposed group (mean = 34.88 g ±0.85) when compared to the pair-fed control group (mean = 32.24 g ±1.41) ($p=0.025$).

The amount of food consumed by the male pair-fed control group was significantly greater than the amount consumed by the female pair-fed control group in all 8 weeks ($p = 0.003$; $p = 0.002$; $p < 0.001$; $p < 0.001$; $p = 0.050$; $p = 0.002$; $p < 0.001$; $p < 0.001$, respectively). The same pattern was exhibited in the alcohol-exposed groups where the male alcohol-exposed group consumed significantly more food than the female alcohol-exposed group for all 8 weeks ($p = 0.004$; $p < 0.001$; $p < 0.001$; $p = 0.001$; $p = 0.007$; $p < 0.001$; $p < 0.001$; $p < 0.001$, respectively) (Fig. 6.1).

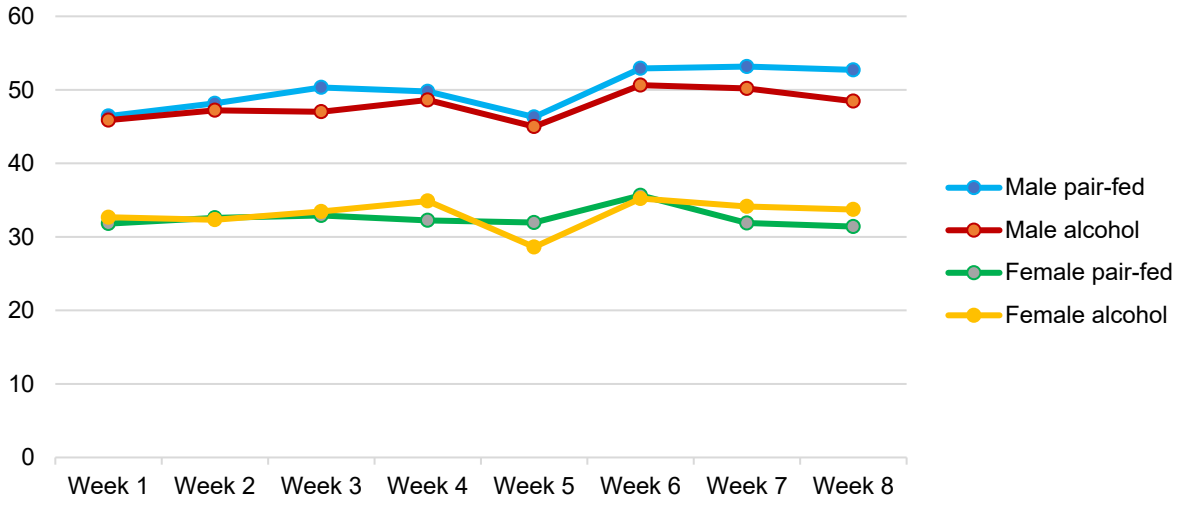


Figure 6.1: Food consumption in the chronic binge alcohol-abstinent model. The daily food consumption is shown as an average per week for the pair-fed control and alcohol-exposed groups in both male and female rats.

6.2.1.3 Water Intake

Males and females displayed similarities in water intake, where water intake from weeks 1 to 4 was relatively consistent, with an increase in water intake in week 5, and a decline to pre-spike levels in weeks 6 7, and 8. No significant differences were identified in the water intake between male pair-fed control animals for weeks 1-6 (week 1: mean = 83.57 ml ±5.58 ; week 2: mean = 87.62 ml ±3.67; week 3: mean = 95.71 ml ±3.98; week 4: mean = 92.86 ml ±3.11; week 5: mean = 127.14 ml ±8.92; week 6: mean = 102.62 ml ±3.27) and male alcohol-exposed rats (week 1: mean = 81.43 ml ±8.24; week 2: mean = 84.52 ml ±7.64; week 3: mean = 84.76 ml ±12.82; week 4: mean = 84.52 ml ±8.64; week 5: mean = 113.81 ml ±11.61; week 6: mean = 92.38 ml ±12.86) ($p = 0.364$; $p = 0.281$; $p = 0.115$; $p = 0.096$; $p = 0.095$; $p = 0.144$, respectively). Even though no significant

differences were identified, a decrease was exhibited in the water intake in the male alcohol-exposed animals. A significant decrease in water intake was identified in the alcohol-exposed group in weeks 7 and 8 (week 7: mean = 84.52 ml ±7.84; week 8: mean = 84.29 ml ±6.23) when compared to the pair-fed control group (week 7: mean = 97.14 ml ±3.27; week 8: mean = 97.76 ml ±3.30) ($p = 0.031$ and $p = 0.031$, respectively).

A similar pattern was displayed in female rats where no significant differences were observed in the water intake between female pair-fed control (week 1: mean = 69.76 ml ±2.51; week 2: mean = 69.29 ml ±3.98; week 3: mean = 71.19 ml ±3.93; week 4: mean = 70.00 ml ±4.46; week 5: mean = 87.14 ml ±5.67; week 6: mean = 69.76 ml ±1.09; week 7: mean = 60.00 ml ±0.71; week 8: mean = 61.90 ml ±2.06) and alcohol-exposed rats in all 8 weeks (week 1: mean = 73.10 ml ±8.64; week 2: mean = 72.38 ml ±6.64; week 3: mean = 76.67 ml ±9.18; week 4: mean = 77.86 ml ±8.24; week 5: mean = 106.90 ml ±19.33; week 6: mean = 78.33 ml ±10.01; week 7: mean = 69.76 ml ±10.26; week 8: mean = 72.38 ml ±11.64) ($p = 0.278$; $p = 0.263$; $p = 0.198$; $p = 0.110$; $p = 0.082$; $p = 0.400$; $p = 0.100$; $p = 0.100$, respectively). Even though no significant differences were identified, an increase was exhibited in the water intake in the female alcohol-exposed animals.

Water intake in the male pair-fed control group was significantly greater than that of the female pair-fed control group in each week (week 1: $p = 0.009$; week 2: $p = 0.002$; week 3: $p < 0.001$; week 4: $p < 0.001$; week 5: $p = 0.001$; week 6: $p < 0.001$; week 7: $p < 0.001$; week 8: $p < 0.001$). The opposite was observed in the alcohol-exposed groups where the male alcohol-exposed group consumed similar amounts of water to the female alcohol-exposed group in each of the 8 weeks (week 1: $p = 0.147$; week 2: $p = 0.053$; week 3: p

= 0.212; week 4: p = 0.194; week 5: p = 0.312; week 6: p = 0.105; week 7: p = 0.059; week 8: p = 0.097) (Fig. 6.2).

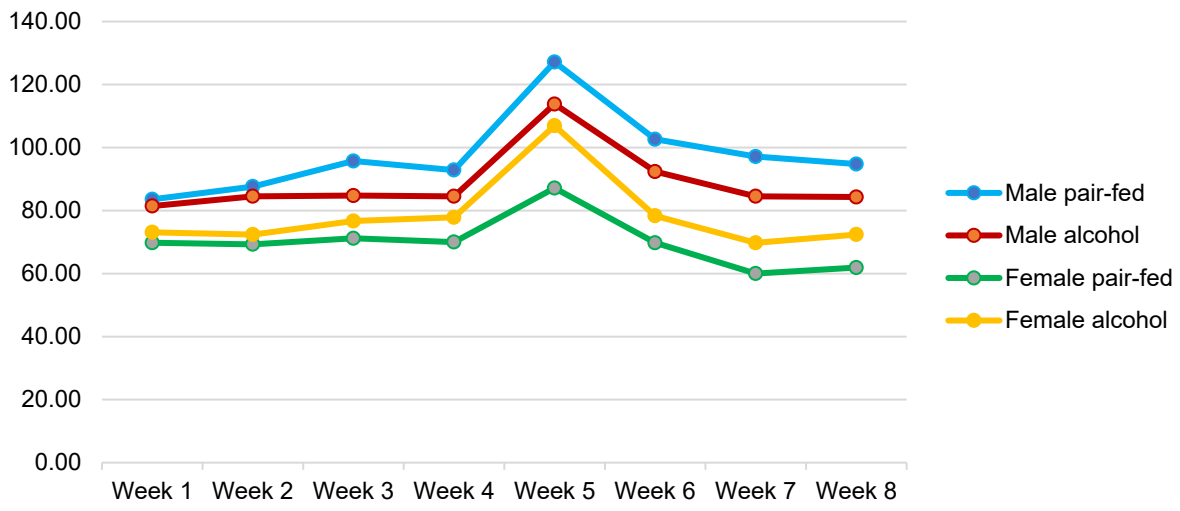


Figure 6.2: Water intake in the chronic binge alcohol-abstinent model. The daily water intake is shown as an average per week for the pair-fed control and alcohol-exposed groups in both male and female rats.

6.2.1.4 Percentage Body Mass Gained

The percentage body mass gained showed some variations between the groups. A two-way ANOVA in the male rats revealed no differences between the pair-fed control and alcohol exposed groups ($p = 0.051$) and a significant main effect of week on the dependent variable ($p < 0.001$), indicating values differed across time points. However, post hoc comparisons using Tukey's HSD did not identify any significant pairwise differences between weeks.

The same pattern was observed in the female rats where no differences were noted between the pair-fed control and alcohol-exposed groups ($p = 0.699$). A significant main effect of week on the dependent variable ($p = 0.002$) was observed, indicating values differing across time points. However, post hoc comparisons using Tukey's HSD did not identify any significant pairwise differences between weeks.

When comparing male and female pair-fed groups, the percentage body mass gained in the male pair-fed control group was significantly greater than that of the female pair-fed control group ($p < 0.001$). A significant main effect of week on the dependent variable ($p < 0.001$) was observed indicating values differing across time points. However, post hoc comparisons using Tukey's HSD did not identify any significant pairwise differences between weeks. When comparing the male and female alcohol-exposed groups, there was a significant difference between the groups ($p < 0.001$) where males had a greater percentage body mass gain than females. A significant main effect of week on the dependent variable ($p < 0.001$) was observed indicating values differing across time points. However, post hoc comparisons using Tukey's HSD did not identify any significant pairwise differences between weeks (Fig. 6.3).

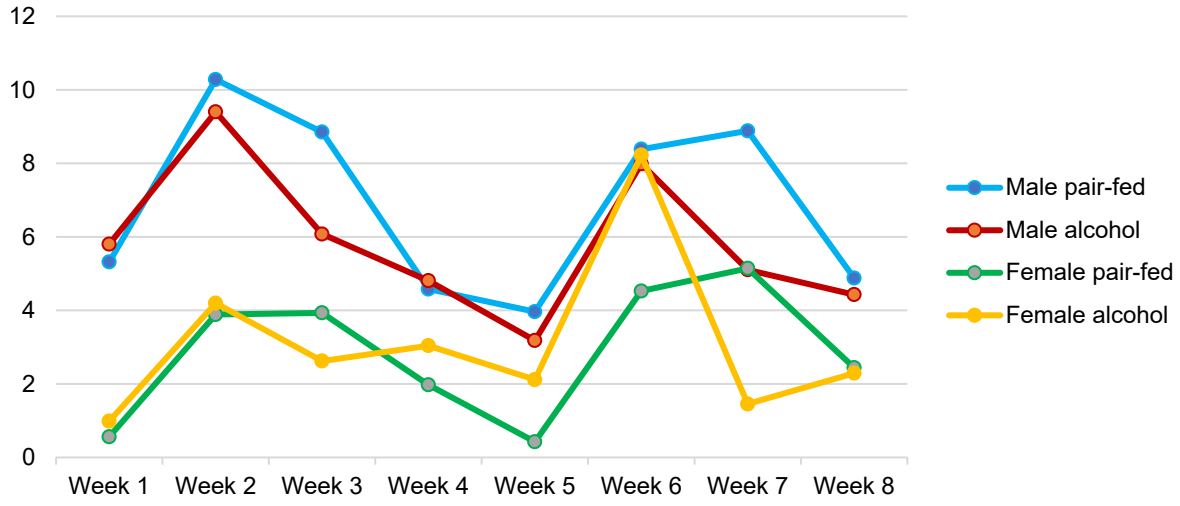


Figure 6.3: Percentage (%) body mass gain in the chronic binge alcohol-abstinent model. The percentage of body mass gained is shown for the pair-fed control and alcohol-exposed groups in both male and female rats per week.

6.2.2 Osteometric Measurements

6.2.2.1 Mandibular Weight

Similarities in mandibular weight patterns were exhibited in male and female animals. The bone weight in males was similar in the pair-fed control (mean = 1.31 g ±0.08) and the alcohol-exposed group (mean = 1.32 g ±0.08) ($p = 0.424$). Female rats also showed no differences in the mandibular weight between the pair-fed control (mean = 1.16 g ±0.03) and the alcohol-exposed groups (mean = 1.15 g ±0.1) ($p = 0.383$). The weight of the mandible was significantly greater in male rats in both the pair-fed control and alcohol-exposed groups when compared to the female pair-fed control and alcohol-exposed groups ($p < 0.001$ and $p = 0.004$, respectively) (Fig. 6.4).

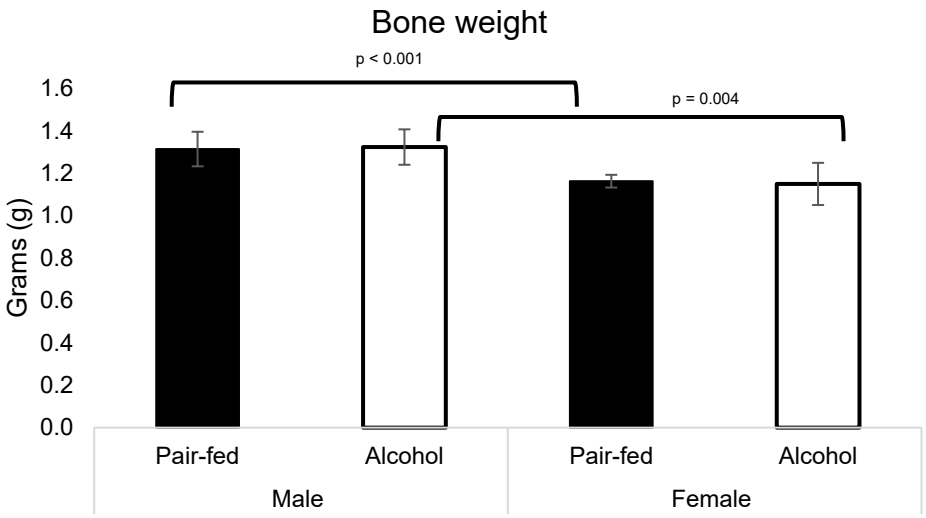


Figure 6.4: Mandibular weight in the chronic binge alcohol-abstinent model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

6.2.2.2 General Mandibular Size

Males and females exhibited a few differences with regard to the general size of the mandible. No differences in measurements were displayed between the alcohol-exposed and pair-fed control groups in the male groups (Table 6.1). Significant differences were observed in the female animals in the distance between the mental foramen (MF) and the deepest point of outer margin of the bone (AI^{l}) (MF- AI^{l}) where the alcohol-exposed group (mean = 2.88 mm ±0.03) displayed a greater distance than the pair-fed control group (mean = 2.76 mm ±0.08) ($p = 0.004$). The distance between the mental foramen (MF) and the highest point of mesiobuccal cusp of the lower 1^{st} molar (M1) (MF-M1) was also significantly larger in the alcohol-exposed female group (mean = 4.49 mm ±0.09) than it was in the pair-fed control group (mean = 4.31 mm ±0.12) ($p = 0.006$) (Table 6.2).

When comparing males and females, the male pair-fed control rats had significantly greater measurements relating to all seven of the measurements conducted (MF-Cd: $p = 0.014$; MF-Go: $p = 0.013$; MF-Me: $p = 0.002$; MF AI^{l}: $p = 0.004$; MF-M1: $p = 0.003$; MF-M3: $p < 0.001$; MF-Ag: $p = 0.006$). In the alcohol-exposed groups, male rats displayed significantly greater distances relating to: the distance between mental foramen (MF) and the most posterior point of condylar head (Cd) (MF-Cd), the distance between mental foramen (MF) and the gonion (Go) (MF-Go), the distance between the mental foramen (MF) and the ante-gonion (Ag) (MF-Ag), the distance between the mental foramen (MF) and the menton (Me) (MF-Me), and the length of the lingual side of the alveolar bone which is the distance between the highest point of the mesial alveolar bone at the lower 3rd molar and the infradentale (Id^{l}) on the lingual side (MF-M3).

Table 6.1: General mandibular size in male rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean (mm)	SD	P-value
MF-Cd	Pair-fed control	6	20.82	0.82	0.290
	Alcohol	6	21.04	0.48	
MF-Go	Pair-fed control	6	20.22	0.87	0.357
	Alcohol	6	20.37	0.35	
MF-Ag	Pair-fed control	6	15.23	0.61	0.112
	Alcohol	6	14.87	0.28	
MF-Me	Pair-fed control	6	4.45	0.21	0.419
	Alcohol	6	4.43	0.15	
MF-Al^{l}	Pair-fed control	6	2.94	0.11	0.250
	Alcohol	6	2.99	0.11	
MF-M1	Pair-fed control	6	4.75	0.28	0.174
	Alcohol	6	4.61	0.19	
MF-M3	Pair-fed control	6	8.31	0.12	0.466
	Alcohol	6	8.31	0.10	

Table 6.2: General mandibular size in female rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean (mm)	SD	P-value
MF-Cd	Pair-fed control	6	19.78	0.57	0.401
	Alcohol	6	19.86	0.47	
MF-Go	Pair-fed control	6	19.16	0.47	0.301
	Alcohol	6	19.29	0.34	
MF-Ag	Pair-fed control	6	14.36	0.33	0.699
	Alcohol	6	14.51	0.88	
MF-Me	Pair-fed control	6	40.8	0.10	0.330
	Alcohol	6	4.11	0.11	
MF-Al^{l}	Pair-fed control	6	2.76	0.08	0.004*
	Alcohol	6	2.88	0.03	
MF-M1	Pair-fed control	6	4.31	0.12	0.006*
	Alcohol	6	4.49	0.09	
MF-M3	Pair-fed control	6	8.02	0.10	0.193
	Alcohol	6	8.10	0.19	

* Indicates $P \leq 0.05$

6.2.2.3 Linear Mandibular Length

Similarities in measurement patterns were displayed in the mandibular length in male and female study groups.

No differences in measurements were identified between the alcohol-exposed and pair-fed control groups in both male (Table 6.3) and female animals (Table 6.4).

The mandible was significantly larger in all four measurements taken in the male pair-fed control group compared to the female pair-fed control group. The measurements included the length of the base of the mandible (Cd-Id), the mandibular body length (Go-Id), the length of the lingual side of the alveolar bone (AI-IdI), and the length of the lower dental arch (M3-IdI) ($p = 0.009$; $p = 0.014$; $p = 0.011$; $p = 0.003$, respectively). Additionally, the length of the mandible was significantly greater in the male alcohol-exposed group than the female alcohol-exposed group for Cd-Id ($p = 0.049$), Go-Id ($p < 0.001$), AI-IdI ($p < 0.001$), and M3-IdI ($p < 0.001$).

Table 6.3: Mandibular length in male rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Id	Pair-fed control	6	27.7	0.99	0.342
	Alcohol	6	27.41	1.41	
Go-Id	Pair-fed control	6	27.47	1.06	0.310
	Alcohol	6	27.72	0.55	
Al-Idl	Pair-fed control	6	9.43	0.38	0.127
	Alcohol	6	9.5	0.21	
M3-Idl	Pair-fed control	6	15.68	0.37	0.119
	Alcohol	6	15.91	0.24	

Table 6.4: Mandibular length in female rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Id	Pair-fed control	6	26.39	0.56	0.366
	Alcohol	6	26.28	0.53	
Go-Id	Pair-fed control	6	26.19	0.57	0.273
	Alcohol	6	26.01	0.47	
AI-IdI	Pair-fed control	6	8.92	0.26	0.415
	Alcohol	6	8.96	0.35	
M3-IdI	Pair-fed control	6	14.98	0.30	0.097
	Alcohol	6	14.73	0.31	

6.2.2.4 Linear Mandibular Height

A similar pattern was found between the pair-fed control and alcohol-exposed groups in both male and female rats. No differences in measurements were observed between the alcohol-exposed and pair-fed control groups in male rats (Table 6.5). The same pattern was observed in female rats, with no significant differences between the groups (Table 6.6).

Male pair-fed control rats had significantly greater mandibular height measurements than the female pair-fed control group in several areas: mandibular ramus height (Cd-Go) ($p = 0.002$), height of condylar head (Cd-Ag) ($p = 0.010$), height of coronoid (Cr-Ag) ($p <$

0.001), and central portion of alveolar bone (AII-Me) (p = 0.041). The mandibular height was also significantly larger in male alcohol-exposed rats compared to female alcohol-exposed rats in the same areas (Cd-Go: p = 0.002; Cd-Ag: p = 0.004; Cr-Ag: p < 0.001; AII-Me: p < 0.001).

Table 6.5: Mandibular height in male rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Go	Pair-fed control	6	9.13	0.45	0.304
	Alcohol	6	2.26	0.38	
Cd-Ag	Pair-fed control	6	13.13	0.65	0.475
	Alcohol	6	13.16	0.52	
Cr-Ag	Pair-fed control	6	14.96	0.74	0.338
	Alcohol	6	14.80	0.49	
AII-Me	Pair-fed control	6	4.83	0.22	0.220
	Alcohol	6	4.91	0.13	

Table 6.6: Mandibular height in female rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean (mm)	SD	P-value
Cd-Go	Pair-fed control	6	8.37	0.21	0.172
	Alcohol	6	8.51	0.29	
Cd-Ag	Pair-fed control	6	12.21	0.49	0.358
	Alcohol	6	12.30	0.38	
Cr-Ag	Pair-fed control	6	13.63	0.25	0.135
	Alcohol	6	13.82	0.30	
All-Me	Pair-fed control	6	4.60	0.07	0.093
	Alcohol	6	4.52	0.11	

6.2.3 Three-dimensional Micro-focus X-ray Computed Tomography (3D- µCT)

6.2.3.1 Mandibular Bone to Total Volume Ratio (BV/TV)

The male and female rats showed different results in the bone to total volume ratio (BV/TV) in the mandible. In males, the BV/TV was slightly lower in the alcohol-exposed group (mean = 69.32 % ±4.80) compared to the pair-fed control group (mean = 71.57 % ±4.50), but this difference was not statistically significant ($p = 0.231$). On the other hand, the female group exhibited a significant decrease in BV/TV in the alcohol-exposed group (mean = 49.66 % ±6.77) compared to the pair-fed control group (mean = 79.94 % ±4.42) ($p < 0.001$). Additionally, pair-fed female rats had a significantly higher BV/TV than male

pair-fed control rats (p = 0.007), while male alcohol-exposed rats had a higher BV/TV than female alcohol-exposed rats (p < 0.001) (Fig. 6.5).

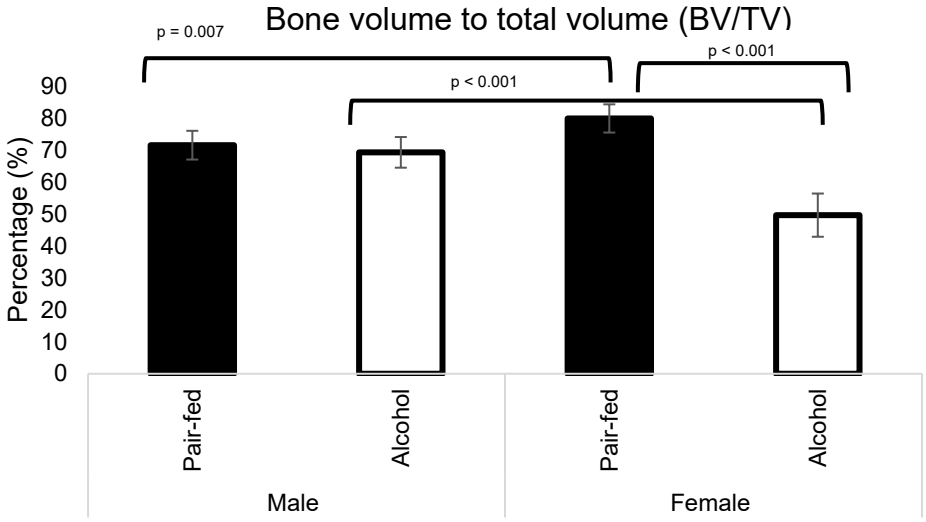


Figure 6.5: Mandibular total bone to volume ratio (BV/TV) in the chronic binge alcohol-abstinent model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

6.2.3.2 Mandibular Trabecular Thickness (TbTh)

Male rats exhibited similarities between the pair-fed control (mean = 0.27 mm ±0.05) and the alcohol-exposed groups (mean = 0.27 mm ±0.07) (p= 0.430). Significantly thinner trabeculae were observed in the female alcohol-exposed group (mean = 0.16 mm ±0.03) than the pair-fed control rats (mean = 0.34 mm ±0.09) (p < 0.001). No significant differences were noted between male and female pair-fed control groups (p = 0.130) however rats in the male alcohol-exposed group had significantly thicker trabeculae than the female alcohol-exposed rats (p = 0.007) (Fig. 6.6).

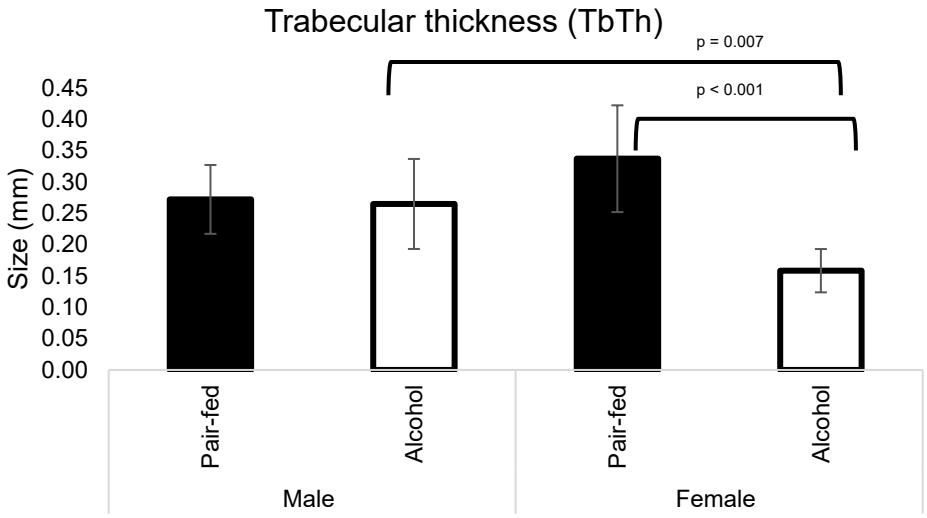


Figure 6.6: Mandibular trabecular thickness (TbTh) in the chronic binge alcohol-abstinent model. The mean values are given for the pair-fed control and alcohol-exposed in both male and female rats. Error bars indicate standard deviation.

6.2.3.3 Mandibular Trabecular Number (TbN)

Differences in trabecular distribution was observed between males and females. The trabecular number (TbN) in the male pair-fed control (mean = 2.78 mm^{-1} ±0.32) was similar to the alcohol-exposed group (mean = 2.79 mm^{-1} ±0.44) (p = 0.476). Statistically fewer trabeculae were observed in the female rat's pair-fed control group (mean = 2.71 mm^{-1} ±0.53) than the alcohol-exposed group (mean = 3.23 mm^{-1} ±0.19) (p= 0.033). Similarities were exhibited between the male and female pair-fed control animals (p = 0.407) Statistically fewer trabeculae were observed in the male alcohol-exposed group compared to the female alcohol-exposed group (p = 0.034) (Fig. 6.7).

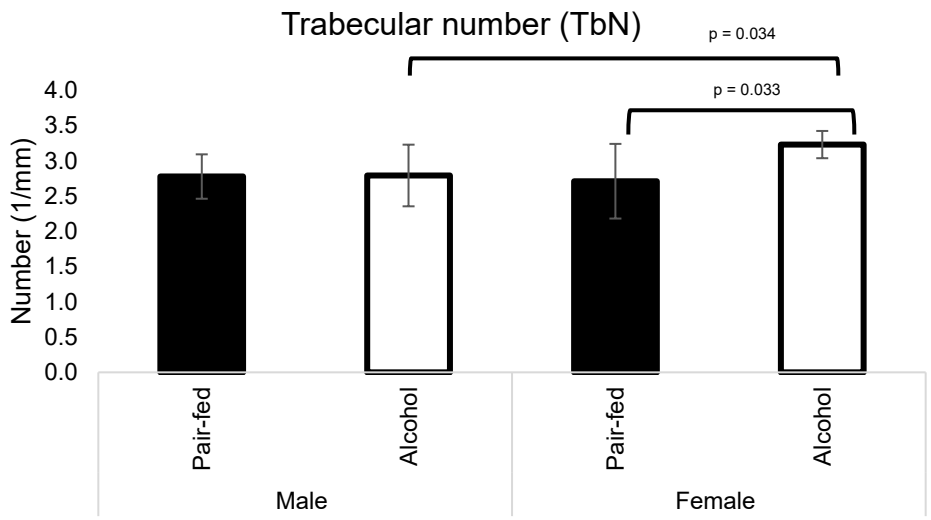


Figure 6.7: Mandibular trabecular number (TbN) in the chronic binge alcohol-abstinent model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

6.2.3.4 Mandibular Trabecular Spacing (TbSp)

The trabecular spacing (TbSp) was similar in the pair-fed control (mean = 0.11 mm ±0.01) and the alcohol-exposed (mean = 0.10 mm ±0.01) groups in male rats ($p = 0.409$). However, in the female rats, significantly wider trabeculae (TbSp) were displayed in the alcohol-exposed group (mean = 0.15 mm ±0.02) than in the pair-fed control group (mean = 0.13 mm ±0.02) ($p < 0.001$). No significant differences were found in TbSp between male and female pair-fed control animals ($p = 0.133$). Alcohol-exposed groups showed that trabecular spacing was wider in females than in males ($p < 0.001$) (Fig. 6.8 and Fig. 6.9).

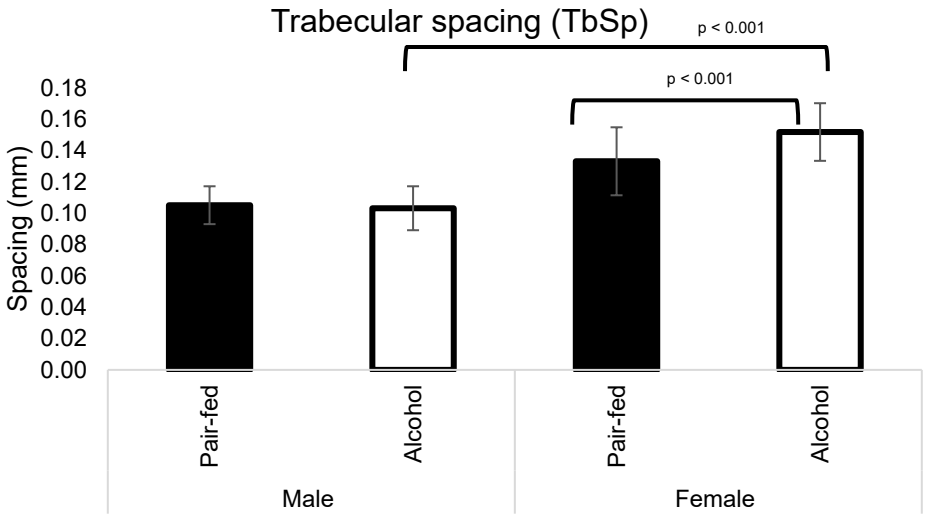


Figure 6.8: Mandibular trabecular spacing (TbSp) in the chronic binge alcohol-abstinent model. The mean percentages are given for the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

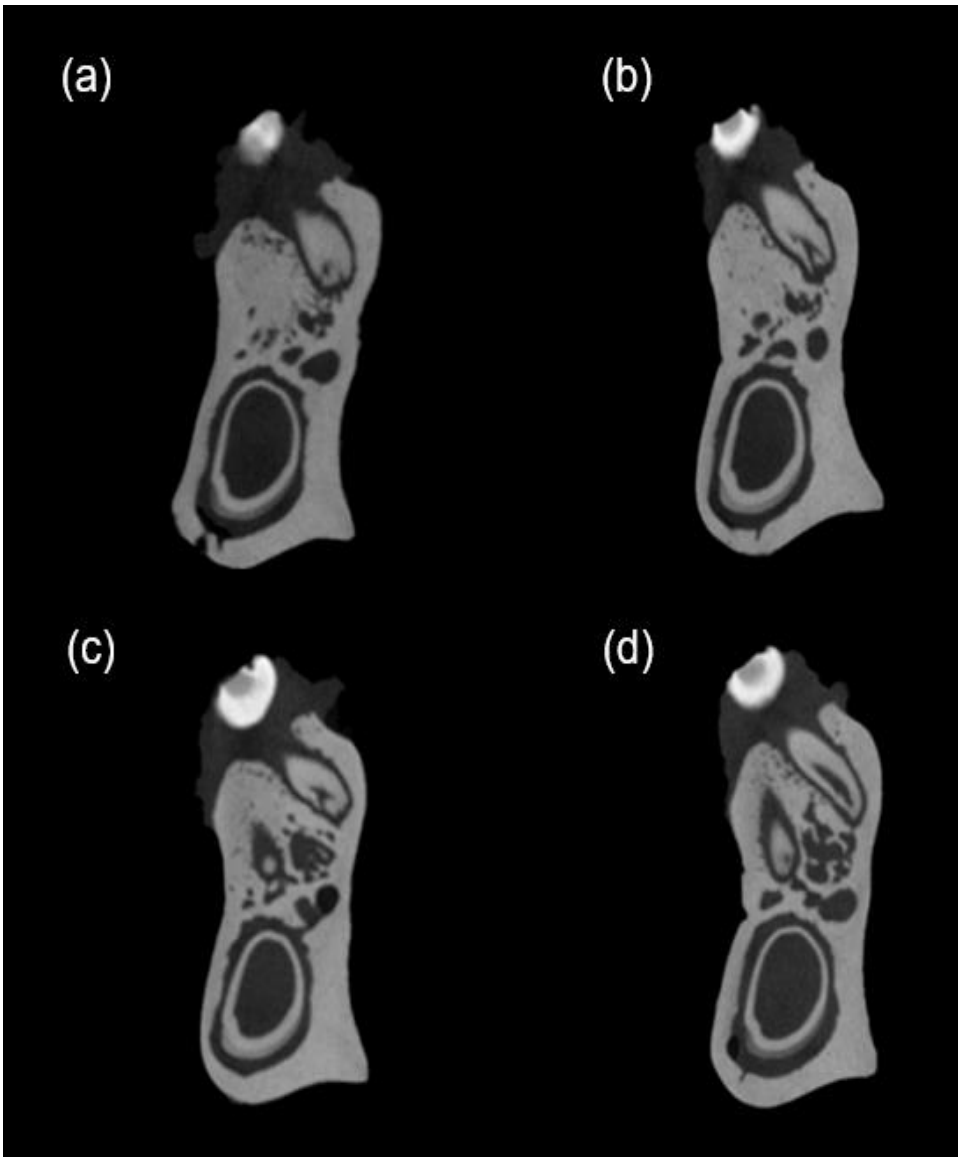


Figure 6.9: Trabecular morphology in the mandible in the chronic binge alcohol-abstinent model. Representative slices of (a) male pair-fed control group showing slightly less trabeculae and similar spacing to the male alcohol-exposed group; (b) male alcohol-exposed group; (c) female pair-fed control group showing less trabeculae when compared to the alcohol-exposed group, with less spacing; (d) female alcohol-exposed group showing a greater trabecular number with wider trabecular spaces than the control. Slices were taken between the 1st and 2nd mandibular molar teeth.

6.2.4 Tensile Strength of the Mandible Using 3-point Bending Tests

Differences were observed in the tensile strength parameters between male and female rats. In male rats, no significant variances were noted in any of the four tensile strength parameters tested in the pair-fed control and alcohol-exposed groups (maximum force, $p = 0.256$; maximum displacement, $p = 0.142$; maximum time, $p = 0.142$; break force, $p = 0.256$) (Table 6.7). However, in female rats, the alcohol-exposed group exhibited significantly greater maximum force and break force ($p = 0.001$ for both parameters), while the maximum displacement and maximum time were similar between the pair-fed control and alcohol-exposed female groups ($p = 0.240$ for both parameters) (Table 6.8).

Furthermore, when comparing the tensile strength parameters between male and female pair-fed control groups, all four parameters were higher in the male pair-fed control group (maximum force, $p < 0.001$; maximum displacement, $p = 0.015$; maximum time, $p = 0.015$; break force, $p < 0.001$). Additionally, the maximum force ($p = 0.012$) and break force ($p = 0.012$) were significantly greater in the male alcohol-exposed group, while the maximum displacement and maximum time were similar ($p = 0.450$ for both parameters).

Table 6.7: Tensile strength of the mandible in male rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean	SD	P-value
Maximum force (N)	Pair-fed control	6	86.27	11.64	0.256
	Alcohol	6	81.11	14.52	
Maximum displacement (mm)	Pair-fed control	6	4.92	0.15	0.284
	Alcohol	6	4.77	0.30	
Maximum time (sec)	Pair-fed control	6	98.35	2.91	0.142
	Alcohol	6	95.29	5.95	
Break force (N)	Pair-fed control	6	86.27	11.64	0.256
	Alcohol	6	81.11	14.52	

Table 6.8: Tensile strength of the mandible in female rats in the chronic binge alcohol-abstinent model

Measurement	Group	N	Mean	SD	P-value
Maximum force (N)	Pair-fed control	6	50.26	4.82	0.001
	Alcohol	6	63.86	6.64	
Maximum displacement (mm)	Pair-fed control	6	4.39	0.58	0.240
	Alcohol	6	4.78	0.24	
Maximum time (sec)	Pair-fed control	6	87.89	11.67	0.240
	Alcohol	6	95.69	4.72	
Break force (N)	Pair-fed control	6	50.19	4.70	0.001
	Alcohol	6	63.86	6.64	

6.2.5 Mandibular Cytoarchitecture (Hematoxylin and Eosin staining)

Male and female groups exhibited slight differences regarding the cytoarchitecture analysed with Hematoxylin and Eosin (H & E) staining. Male pair-fed control rats exhibited abundant osteocytes scattered evenly throughout the tissue. Numerous small trabecular spaces with blood vessels were identified throughout the tissue. A widespread mineralised bone matrix was displayed. The bone matrix, collagen fibres, and periodontal ligament fibres were observed surrounding the tooth roots. The bone in the chronic binge alcohol abstinent group appeared more mineralised compared to the two previously analysed groups in this study. (Fig. 6.11a). The cytoarchitecture of the male alcohol-exposed group showed patterns similar to those of the male pair-fed control group, with

a few exceptions. In the alcohol-exposed group, trabecular spaces were slightly larger and more numerous compared to the pair-fed control group. Bone in this alcohol-exposed group appeared slightly less mineralised when compared to the pair-fed control group as the collagen fibres were more evident and less organised (Fig. 6.11b).

Female pair-fed control animals showed a different pattern to the male pair-fed control groups. Larger trabecular spaces were identified in this group throughout the tissue and were filled with erythrocytes. Bone in this group also appeared less mineralised than the bone displayed in the male pair-fed control group (indicated by an increase in wavy collagen fibres) (Fig. 6.11c). Female alcohol-exposed rats showed similar patterns to the female pair-fed control rats, however the bone in this group was even less mineralised than the bone in the female pair-fed control group with less organisation of the collagen fibres, and larger trabecular spaces. The female alcohol-exposed groups contained the least mineralised bone, with the largest trabecular spaces from all the animals in the chronic binge alcohol-abstinent model (Fig. 6.11d).

The male pair-fed control and alcohol-exposed groups displayed fewer trabecular spaces than the female pair-fed control and alcohol-exposed groups. The male groups also displayed bone architecture that appeared more mineralised and more mature. The male pair-fed control and alcohol-exposed groups contained smaller trabecular spaces, and slightly more organised collagen fibres in the bone matrix compared to the female groups.

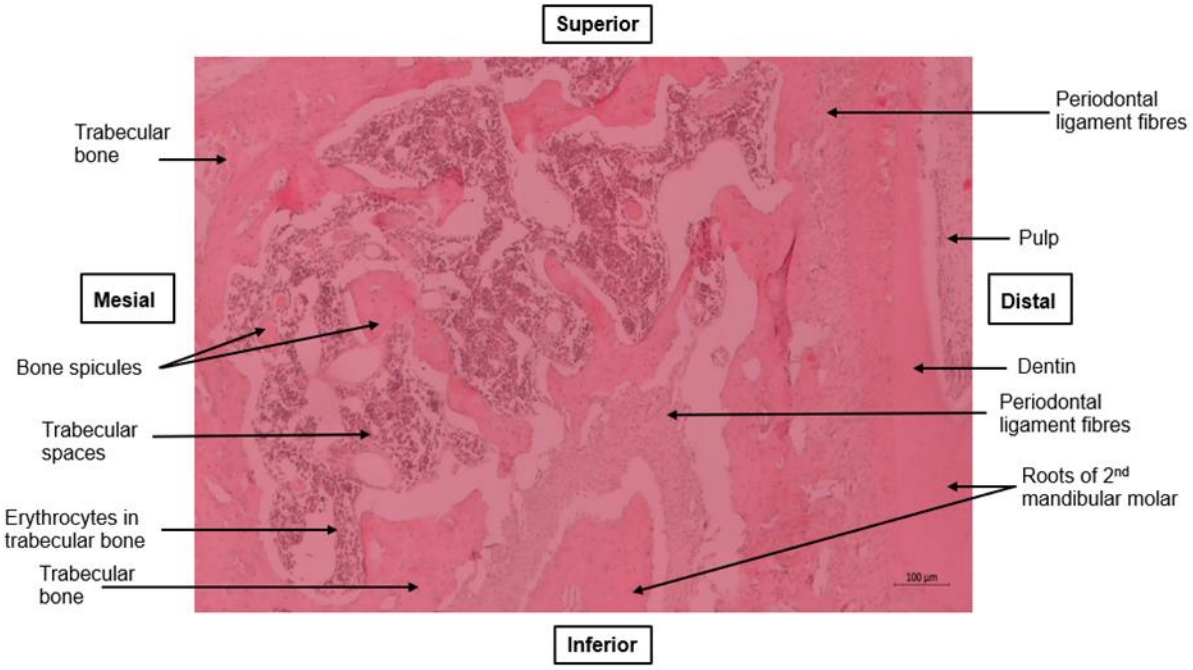


Figure 6.10: A photomicrograph showing a coronal section of the area between the roots of the first and second molar teeth of the mandible (taken from the buccal aspect of the bone), in the chronic binge alcohol-abstinent model. Scale bar = 100 microns at x 5 magnification. For orientation purposes.

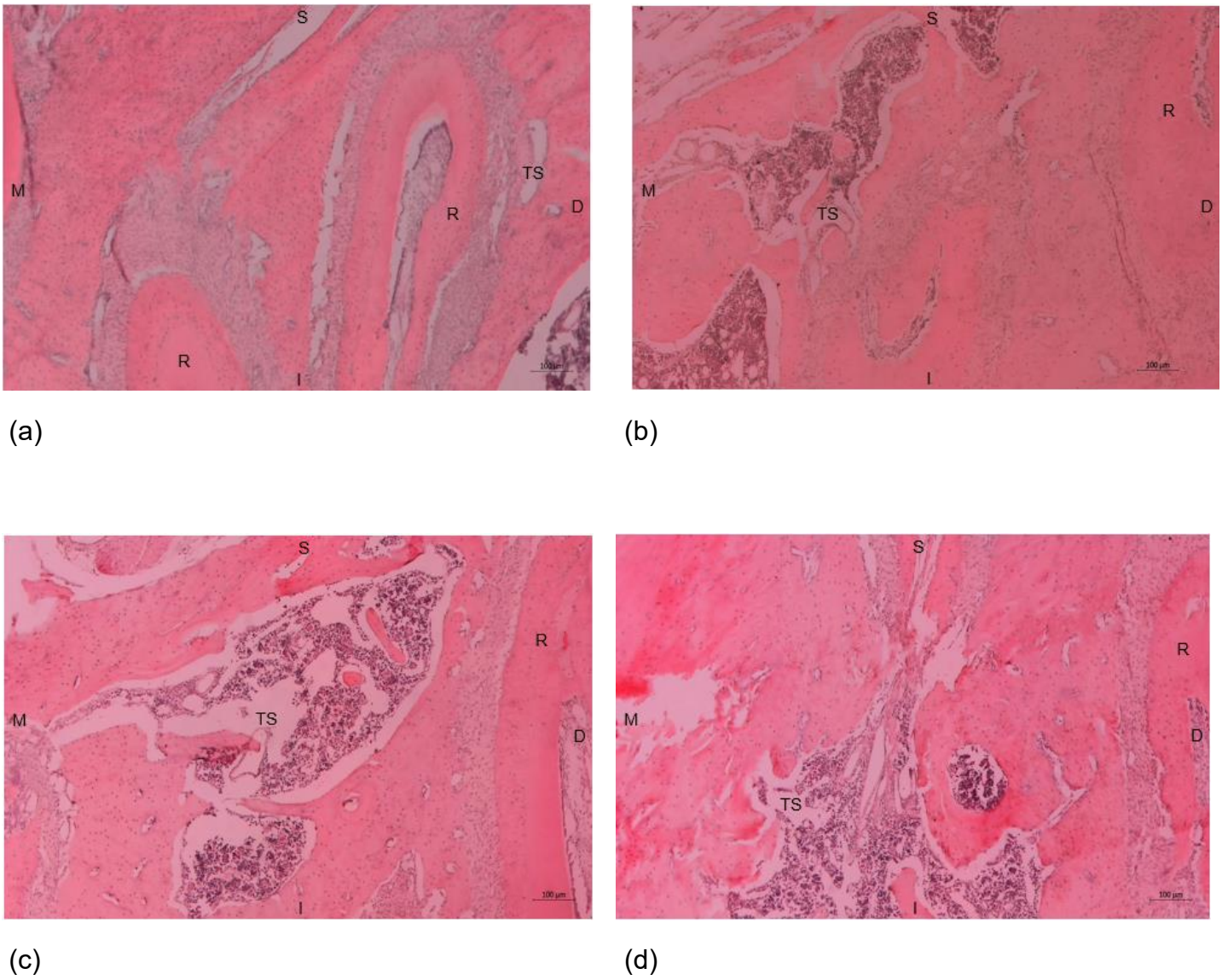


Figure 6.11: Representative sections of the mandible between the roots of the first and second molar teeth of the mandible in the chronic binge alcohol-abstinent model. Representative sections of (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. H & E staining. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space.

6.2.6 Immunohistochemistry

6.2.6.1 Mandibular Proliferative Cells, Ki-67 Immuno-Positive Cells

Varied patterns were observed regarding the number of proliferating cells in male and female rats in the region between the 1st and 2nd molar teeth. Male pair-fed control animals had a similar number of proliferative cells (mean = 142 ±.10.54) than the alcohol-exposed group (mean = 140 ± 3.04) ($p = 0.405$).

The female pair-fed control group exhibited a significantly greater number of proliferating cells (mean = 167 ± 0.76) when compared to the female alcohol-exposed group (mean = 125 ± 13.71) ($p = 0.003$).

A significant difference in the number of proliferating cells was exhibited between the male and female pair-fed control groups ($p = 0.008$) where the female pair-fed control rats displayed more proliferating cells than the male pair-fed control rats. However, in a comparison between the male and female alcohol-exposed rats ($p = 0.006$), the male rats had more proliferating cells than the female rats (Fig. 6.12 and Fig. 6.13).

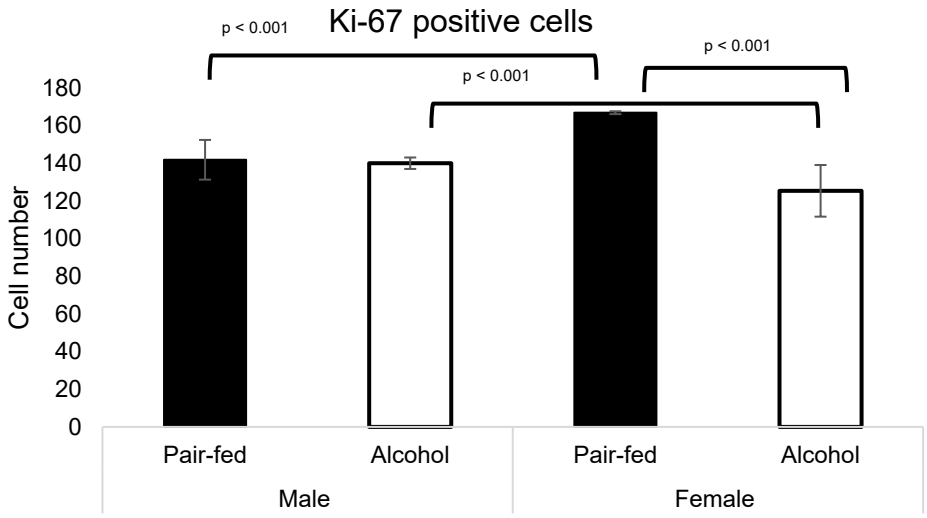


Figure 6.12: Ki-67 immuno-positive proliferative cells of the mandible in the chronic binge alcohol-abstinent model. The mean number of Ki-67 immuno-positive proliferative cells is shown for the region between the 1st and 2nd molar teeth in the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.

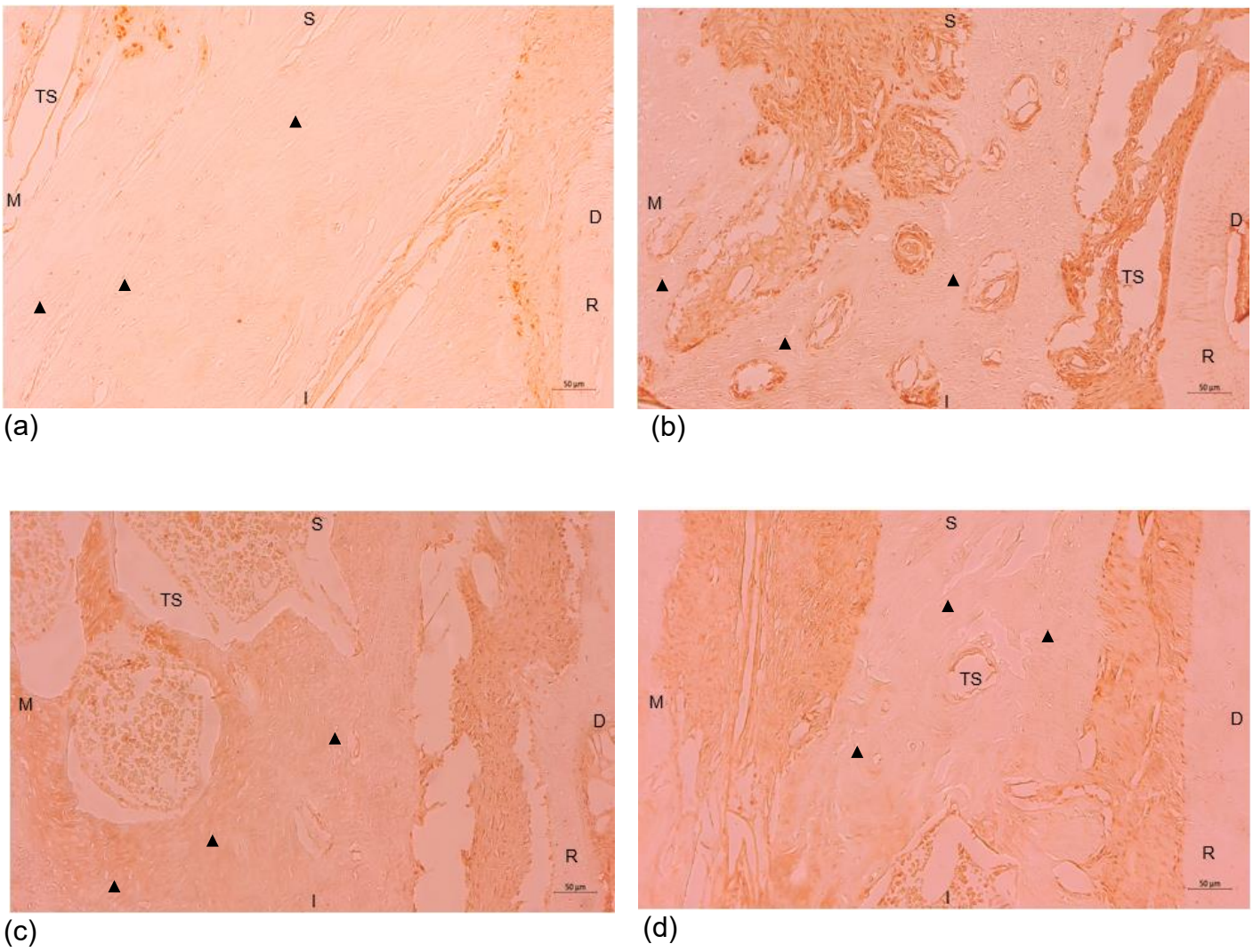


Figure 6.13: Representative photomicrographs of Ki-67 immunolabeled sections of the mandible between the roots of the first and second molar teeth of the mandible in the chronic binge alcohol-abstinent model. (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. Scale bar = 50 microns at x 10 magnification. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space.

6.2.6.2 Mandibular Osteoblasts, Alkaline Phosphatase (ALP) Immuno-Positive Cells

Differences were depicted in the pattern of the number of osteoblasts between male and female groups in the region between the 1st and 2nd molar teeth. The number of osteoblasts in the male pair-fed control group (mean = 243 ± 13.94) was significantly higher than the number of osteoblasts observed in the male alcohol-exposed group (mean = 182 ± 11.86) ($p = 0.002$). Female pair-fed control animals (mean = 224 ±12.29) displayed a greater number of osteoblasts than the female alcohol-exposed group (mean = 201 ± 15.41). This difference, however, was not statistically significant ($p = 0.056$).

No significant differences were found in the number of osteoblasts in the male pair-fed control versus female pair-fed control groups ($p = 0.076$). The same pattern continued between male alcohol-exposed and female alcohol-exposed rats ($p = 0.081$) (Fig. 6.14 and Fig. 6.15).

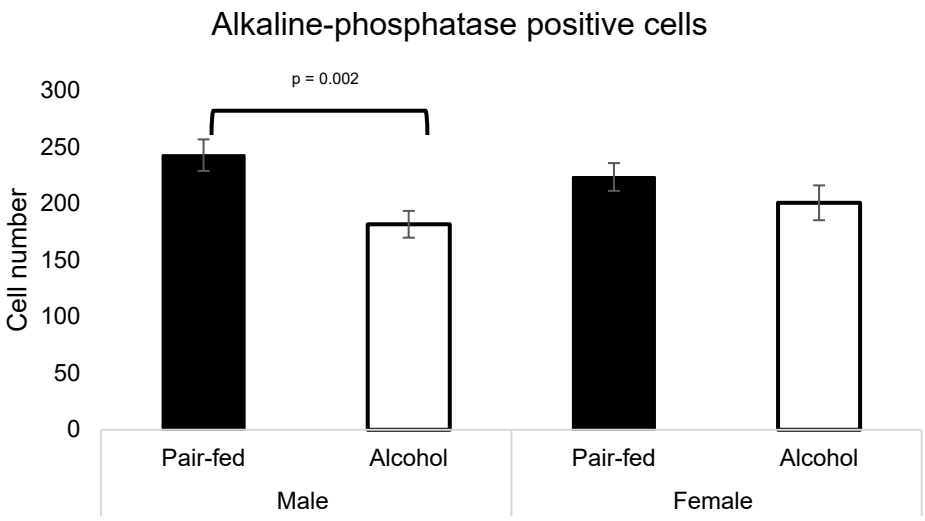
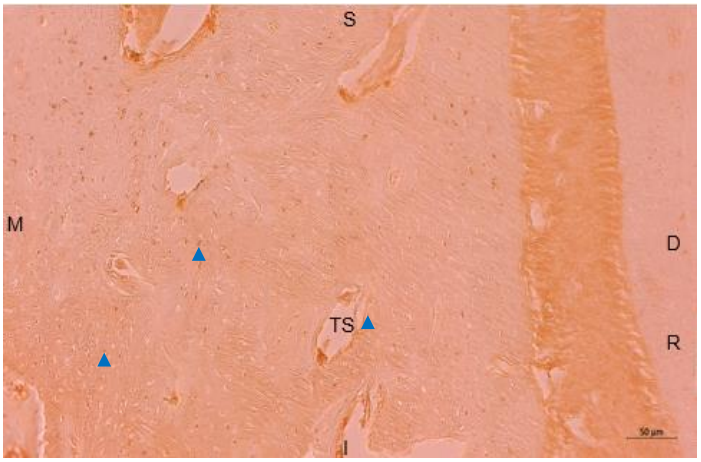
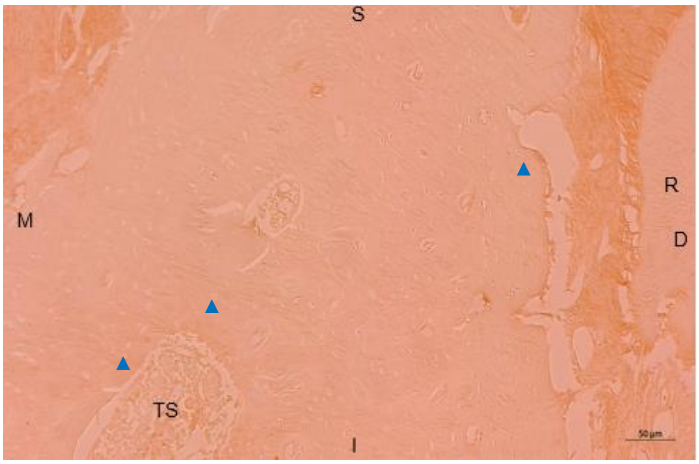


Figure 6.14: Alkaline phosphatase (ALP) immuno-positive osteogenic cells of the mandible in the chronic binge alcohol-abstinent model. The mean number of osteoblasts is shown for the region between the 1st and 2nd molar teeth in the pair-fed control and alcohol-exposed groups in both male and female rats. Error bars indicate standard deviation.



(a)



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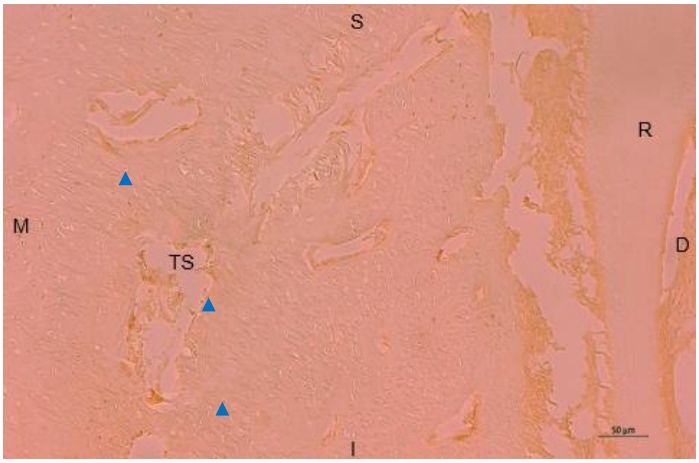
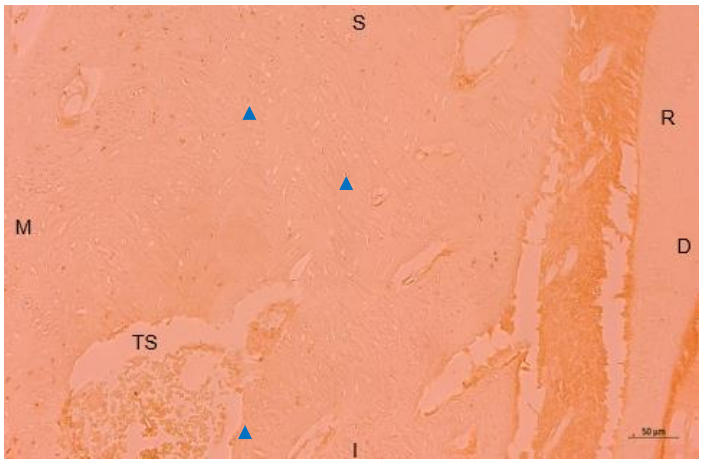


(c)



(d)

Figure 6.15: Representative photomicrographs of ALP immunolabeled sections of the mandible between the roots of the first and second molar teeth of the mandible in the chronic binge alcohol-abstinent model. (a) male pair-fed control; (b) male alcohol-exposed; (c) female pair-fed control; (d) female alcohol-exposed. Scale bar = 50 microns at x 10 magnification. M=mesial; D=distal; S=superior; I=inferior; R=root; TS=trabecular space. Blue arrowheads indicate osteoblasts (on the surface of the bone) and osteogenic cells.

6.3 DISCUSSION

This study investigated the effects of alcohol exposure on the growth and development of the mandible using a chronic binge drinking model followed by a period of alcohol abstinence to determine if the mandible can recover from the adverse effects of alcohol in an adolescent Sprague Dawley rat model. The size of the bone using osteometric measurements was analysed. Trabecular morphometry using microtomography was evaluated. The tensile strength of the mandible was accessed using 3-point bending tests. The cytoarchitecture of the cells was evaluated with Hematoxylin and Eosin (H & E) staining, and Ki-67 and ALP immunohistochemistry (IHC) was performed to quantify the effect of alcohol on proliferating and osteogenic cells.

The findings showed some differences in the baseline data between pair-fed control and alcohol-exposed animals. Osteometric measurements indicated differences in the general mandibular size of the pair-fed control and alcohol-exposed animals in the female group. Three-dimensional micro-focus X-ray computed tomography (3D-μCT) confirmed long-term alterations in trabecular parameters in the alcohol-exposed groups in female rats. Tensile strength testing exhibited significant differences in 2 of the 4 parameters between female pair-fed control and alcohol-exposed animals, with no significant differences observed between male groups. Some differences in cytoarchitecture were displayed between male and female pair-fed control and alcohol-exposed groups regarding trabecular size and the degree of mineralisation of the bone. Differences were exhibited in the number of proliferating cells and the number of osteogenic cells present in the pair-fed and alcohol-exposed rats in both male and female groups. Differences

between male and female animals were depicted across the board in all the parameters tested and analysed.

Baseline Data

Similarities in food consumed per week were observed between pair-fed control and alcohol-exposed groups for both male and female animals. A significant increase was however observed in week 4 in the female alcohol-exposed group when compared to the pair-fed control. Caton et al. (2004) reported that alcohol may cause short-term appetite stimulation which may be the reason for the increase in food consumption in the female alcohol-exposed animals during week 4 in this study. Fong et al. (2021) stated that alcohol is energy-dense, causes weak satiety responses, reduces dietary fat oxidation, and may also stimulate appetite (Fong *et al*., 2021). Differences in food intake between males and feamles were observed where male pair-fed control animals consumed significantly more food than the female pair-fed control animals. No significant differences in food consumption were however identified between male and female alcohol-exposed groups. When looking at the data, male alcohol-exposed animals consumed marginally less food than male pair-fed control animals, and female alcohol-exposed animals marginally more food than the pair-fed control animals throughout the 8 weeks, which has aided in closing this gap between the two groups. Previous research has shown conflicting arguments regarding the effect of alcohol on appetite and food consumption, where some studies indicate that alcohol causes an increase in appetite (Caton *et al*., 2004; Fong *et al*., 2021) and others indicate that alcohol acts as an appetite suppressant (Nelson *et al*., 2016). The former corroborates to the female animals whereas the latter to the males.

An increase or decrease in appetite may be due to any of these three mechanisms. Alcohol has the ability to disinhibit eating restraint and satiety hormones, which would increase food intake. Another mechanism relates to alcohol's pharmacological effects on various neurotransmitters in the brain that influence behavior for example, alcohol binds to $GABA_A$ receptors which is linked to the control of food intake. Alcohol has also been shown to stimulate the release of opioid peptides which also influences food intake (Kwok *et al*., 2019). The above mechanisms may have different effects on males and females. Females in this study were largely affected by alcohol exposure, more so than males, which may be the reason for alcohol having a greater effect on their appetite as well.

Water intake displayed similar patterns to food consumption. Overall male alcohol-exposed animals consumed less water than male pair-fed control animals with a significant decrease noted in weeks 7 and 8, whereas female alcohol-exposed animals consumed more water than the pair-fed controls (this increase was however non-significant). A spike in water consumption was identified in week 5 in all groups. The type of water bottle used during this week was different (due to reasons out of our control). The bottles in week 5 contained a longer nozzle and water flowed out of them more easily, when compared to the bottles used in the previous weeks. The bottles were then switched back to the original bottles thereafter. The change in bottles used in that week was likely the reason for the sudden increase in water intake and is likely a false interpretation of the amount of water consumed.

Increased diuresis can be caused by binge or acute alcohol consumption as alcohol causes the inhibition of vasopressin release in the neurohypophysis which causes an increase in urination (Madeira *et al*., 1993). If alcohol acts as a diuretic, the loss of water

caused by alcohol would need to be replenished, which would then lead to an increase in water intake. Females may be more prone to some long-term consequences of alcohol exposure, which likely indicates why they were affected more than males (Middaugh *et al*., 1992).

The percentage mass gain was relatively consistent between the groups following a similar pattern between male and female study groups. Throughout the 8 weeks, male pair-fed control animals maintained a marginally higher percentage of body mass gain than alcohol-exposed animals. However, female alcohol-exposed animals maintained a marginally higher percentage of body mass gained than the female pair-fed controls. This data correlates with the animal's food consumption patterns which indicate the same pattern. The percentage of body mass gain can be directly related to appetite and food consumption. The non-significant increase in appetite noted in the females is likely due to alcohol being energy dense, causing weak satiety responses, reducing dietary fat oxidation, and may act as an appetite stimulant (Fong *et al*., 2021), which caused these rats to eat more (marginally) resulting in a marginally greater percentage body mass gain.

Male rats showed the opposite with a decrease in food consumption in the alcohol-exposed animals, a decrease in water intake, and a decrease in the percentage body mass gain. This finding corroborates with Nelson et al. (2016) who found that alcohol can act as an appetite suppressant (Nelson *et al*., 2016). Differences between male and female animals are consistent with those of sexual dimorphism (Asarian and Geary., 2013).

Osteometric Measurements

As observed in the chronic binge alcohol group (Chapter 4), no negative effects on bone mass were exhibited in the chronic binge alcohol-abstinent model either in the male or female animals.

A decrease in bone mass in alcohol-exposed animals was reported in previous studies (Broulík *et al.,* 2010; Chakkalakal., 2005). Gaddini et al. (2016) reported that light to moderate alcohol consumption has a beneficial effect on the skeleton, whereas heavy alcohol consumption decreases bone quality and quantity (Gaddini *et al*., 2016). The lack of significant differences observed in our study may be related to the study design, dosage, and duration of alcohol exposure. Sampson et al. (1999) conducted a study where they investigated the effect of binge drinking on bone metabolism in a young actively growing rat model. They found a decrease in the weight of the femur; however, no effect was displayed on the tibia (Sampson *et al*., 1999). The lack of changes in bone weight may therefore be bone specific. This may also explain why no differences were detected in our study which investigated the effects of binge alcohol consumption on the mandible.

In examining the general mandibular size, linear mandibular length, and linear mandibular height, no significant differences were displayed in the male rats. The only parameters affected in the female group were the distance between the mental foramen (MF) and the deepest point of the outer margin of the bone (Al^{l}) (MF- Al^{l}), and the distance between the mental foramen (MF) and the highest point of mesiobuccal cusp of the lower 1^{st} molar (M1) (MF-M1), which indicate overall mandibular size, but more specifically the height of the anterior mandible, which were both significantly larger in the alcohol-exposed female

group. As described in the discussion of the chronic binge alcohol model (Chapter 4), the increase in mandibular height in the female group in our study is likely due to one of two reasons, or a combination of both. The first is the study design (mode of alcohol administration and duration of exposure), and the second is the development of the mandible. The mandible grows through both intramembranous and endochondral ossification. The length of the body of the mandible increases in a rectilinear direction, whereas the condylar process exhibits growth in various directions from anterosuperior to posterior (Mizoguchi *et al*., 2013). This diverse growth pattern enables a wide range of growth and shapes of the mandible. The growth of the condyle is closely related to the transposition of the mandible, which will in turn influence the angle of the mandible, thus the maxillary mandibular relationship. In individuals with low angles (short face, brachyfacial pattern, and deep bites), the mandibular growth is characterized by anterosuperior growth of the condyle. In individuals with high angles (long face, dolichofacial pattern, and skeletal open bite), posterosuperior growth of the condyle is exhibited (Mizoguchi *et al*., 2013). Disturbances in growth can thus have a varying range of effects on the length and height of the mandible. In the case of juvenile rheumatoid arthritis, the posterior margin of the ramus had bone apposition in a posterior direction (Mizoguchi *et al*., 2013). The developmental processes of the mandible, including mandibular transposition, in the context of acute and chronic binge alcohol exposure, reveal a consistent growth pattern. This pattern suggests increased bone apposition in the posterosuperior direction, potentially leading to the manifestation of a mandible with a high angle. This, in turn, may result in complications such as malocclusion and an open bite. In cases of binge alcohol abstinence, posterior mandibular height normalization

occurs, while an increase in anterior mandibular height is observed. This is characterized by an anterosuperior growth pattern. This pattern may stem from initial growth disturbances in the condyle due to alcohol exposure or due to compensatory adjustments following the initial posterosuperior growth.

The mandibular condyle in rats' fuses at approximately 14 weeks of age (Zoetis *et al*., 2003). The rats in our study were younger than this at termination which means that the condyle was not yet fused, thus allowing alcohol to have an adverse effect on both the endochondral and intramembranous growth of the mandible. No literature is available to validate these findings.

Male animals depicted an overall greater general size, length, and height of the mandible when compared to females in the chronic binge alcohol-abstinent model. These differences could be due to sexual dimorphism. Male animals are normally larger in size than female animals (Lorenzo., 2020). Numerous factors are responsible for sexual dimorphism including genetics, actions of sex steroids, environment, and behavior (Lorenzo., 2020) which can all impact the differences identified between the male and female animals in our study.

Three-Dimensional Micro-focus X-ray Computed Tomography (3D- µCT)

Three-dimensional micro-focus X-ray computed tomography (3D-µCT) was used to access trabecular volume, thickness, number, and spacing in the mandible (between the first and second molar teeth). Altered trabecular parameters were observed in the alcohol-exposed groups in the chronic binge alcohol-abstinent model.

In the current study, the bone to total volume ratio (BV/TV) was significantly lower in female alcohol-exposed rats. Male rats also exhibited a lower BV/TV in alcohol-exposed rats; however, this change was not significant. The results indicate that BV/TV in female rats remains lower in alcohol-exposed animals even after a 30-day abstinence from alcohol, which implies that chronic binge drinking has a long-term effect on this specific trabecular parameter in female animals yet has minimal to no effects on male animals. Previous studies have identified a decrease in BV/TV in alcohol-expose animals in numerous different bones including the tibia, femur, and mandible (Föger-Samwald *et al*., 2018; Maia *et al*., 2021; Sampson *et al*., 1998) which corroborates with the finding exhibited by the female animals in the present study. The duration of exposure and the study design may be the reason for no changes in the bone ratio of the male rats.

Testosterone found in males plays a vital role in maintaining bone health across all stages of life. Testosterone leads to the development of wider bones that have a thicker cortex and a greater peak bone mass than females (Corona *et al*., 2022). Testosterone also aids in maintaining a balance between bone resorption and bone remodeling (Corona *et al*., 2022). The reason for no significant differences in BV/TV observed in the male rats in this study may be due to the actions of testosterone. Mngoma et al. (2024) found that BV/TV was decreased in male alcohol-exposed rats in the distal epiphysis of the femur in a chronic binge alcohol model (Mngoma *et al*., 2024). The femur is a long bone which develops by endochondral ossification. The present study reported on the effect of alcohol on the mandible which is a flat bone developing from intramembranous and endochondral ossification. This indicates that the effect on BV/TV may be both bone and site specific.

Pair-fed control female rats displayed a greater BV/TV than male pair-fed control rats whereas female alcohol-exposed rats exhibited a smaller BV/TV than male alcohol-exposed rats. This may be attributed to the fact that female rats have high serum oestrogen levels during adolescence which may have a positive impact on bone development (Schiessl *et al*., 1998). Bone development also occurs at a faster rate in female rats, which may lead to earlier maturation of bone (Jiao *et al*., 2010). Once the animals were exposed to alcohol, the dynamic between males and females changed, indicating that alcohol had more of a long-term effect on the female rats decreasing their BV/TV, whereas alcohol had less of an effect on male rats, hence their BV/TV raised to levels higher than the females.

Significantly thinner trabeculae were identified in the female alcohol-exposed group, while no significant differences were displayed between the male groups. This indicates that binge alcohol consumption has a long-term deleterious effect on trabecular thickness even after alcohol is removed from the diet in female rats. The results of the present study correlate with a study conducted by Maia et al. (2021) who also reported thinner trabeculae in alveolar bone in female rats exposed to a binge drinking model (Maia *et al*., 2021). The lack of change identified in male rats is likely due to study design, dosage of alcohol, and method of ingestion and may also be due to the effect of sex hormones like testosterone that have a protective effect on the male skeletal system (Corona *et al*., 2022).

No differences were noted between the male and female pair-fed control groups, however, male alcohol-exposed animals had significantly thicker trabeculae than female

alcohol-exposed animals. This would be due to alcohol having a long-term negative effect on the trabecular thickness of female animals exposed to alcohol.

Non-significant differences were observed in the trabecular number in the male groups. A significant increase in trabecular number (TbN) was observed in the female alcohol-exposed group when compared to the female pair-fed control group. Föger-Samwald et al. (2018) exhibited a decrease in TbN in the femur and an increase in TbN in the humerus of binge alcohol-exposed prepubescent pigs (Föger-Samwald *et al*., 2018) which is the expected outcome on the trabecular number after alcohol exposure. We also expected to observe a decrease in trabecular number as all the other trabecular parameters were negatively affected in female alcohol-exposed animals, however we observed an increase in trabecular number. The mandible may have responded to the overall damage by increasing the number of trabeculae, to maintain bone strength and compensate for the negative effects of alcohol on BV/TV and TbTh. This compensatory mechanism would not fully offset the detrimental effects of alcohol on bone quality. Chronic alcohol exposure can disrupt the balance between bone formation and resorption, impairing the overall structural integrity of the bone despite changes in trabecular architecture, and can cause long-term effects on the bone even after alcohol withdrawal. Literature regarding this possible compensatory mechanism is not available. The lack of significant differences displayed in male rats is likely due to study design, dosage of alcohol, and method of ingestion and may also be due to the effect of sex hormones like testosterone that have a protective effect on the male skeletal system (Corona *et al*., 2022). The differences observed between male and female alcohol-exposed groups indicated that female

alcohol-exposed rats had significantly larger numbers of trabeculae than male alcohol-exposed rats. This again is likely due to a compensatory effect.

Trabecular spaces were significantly wider in female alcohol-exposed rats when compared to female pair-fed control rats. This correlates with Maia et al. (2021) who also noted an increase in trabecular spacing in alveolar bone in female rats exposed to alcohol. Wider trabecular spaces suggest disruptions and changes in the microarchitecture of the mandibular bone caused by chronic binge alcohol exposure. Alcohol consumption directly disrupts the balance between osteoclasts and osteoblasts, leading to disturbances in bone remodeling and homeostasis (Maia *et al*., 2021). Trabecular spaces in the female rats in our study remained wider in the alcohol-exposed group, even after alcohol removal from the diet. This indicates likely long-term disruptions to the bone that could not be rectified with the removal of alcohol. Alcohol-exposed female rats displayed wider trabeculae than alcohol-exposed male rats. This indicates that alcohol had lasting effects on female rats. This is likely due to sexual dimorphism (Corona *et al*., 2022), and the fact that alcohol seems to have a greater disruptive effect on the female adolescent skeleton as opposed to the male adolescent skeleton observed from previous parameters in the present study.

The results displayed some improvement compared to the chronic binge model, suggesting that alcohol may have long-term detrimental effects on the mandible. However, it could also indicate that bone recovery simply requires a longer period of time.

Tensile Strength

Tensile strength parameters differed between male and female study groups. No significant changes were observed between male pair-fed control and alcohol-exposed groups, however, a significant increase in maximum force and break force was observed in the female alcohol-exposed group when compared to the female pair-fed control.

Tensile strength parameters were not affected negatively in the chronic binge alcohol model in male animals, which indicates that removal of alcohol from the diet allowed for recovery of the mandible regarding this particular parameter in male animals exposed to alcohol. This aligns with Lauing et al. (2008), who found that after a 28-day binge period, alcohol removal led to an increase in tensile strength parameters in vertebrae following a 30-day abstinence period, despite the initial negative impact of chronic binge alcohol consumption (Lauing *et al*., 2008). Withdrawal of alcohol from the diet may have caused an increase in tensile strength parameters in female rats exposed to alcohol. The results from this study are contradictory to the literature, where alcohol exposure had caused a decrease in tensile strength parameters in alcohol-exposed animals (Hogan *et al*., 1997; Rosa *et al*., 2019). Lauing et al. (2008) establish that after alcohol exposure, tensile strength had increased to normal levels in the alcohol-exposed animals (Lauing *et al*.,2008). Turner and Sibonga (2001) reported that a positive correlation can be found between light to moderate alcohol consumption and a reduction in fracture risk in postmenopausal women (Turner and Sibonga,2001).

A possible explanation for the results of the current study could be that alcohol consumption may have had a compensatory protective mechanism on the cortical bone in female rats, in a way to protect the disruption caused to the internal structure of the

bone. No literature is available to compare these findings. Differences between male and female animals indicated that males had stronger mandibles that required greater tensile parameters to fracture than females in both pair-fed control and alcohol-exposed groups. This would be a result of sexual dimorphism, as male rats in our study also presented with larger bones (Corona *et al*., 2022),

Mandibular Cytoarchitecture

Only qualitative analysis of the cytoarchitecture was assessed, with consistent findings observed across all samples within each group. Samples from all study animals were included in the quantitative analysis. Several differences were observed between male pair-fed control and alcohol-exposed animals, between female pair-fed control and alcohol-exposed animals, and between the male and female pair-fed control and alcohol-exposed groups.

In the male pair-fed control group, the trabecular spaces were small, and the bone appeared mature and mineralised. In contrast, the male alcohol-exposed groups had larger and more numerous trabecular spaces compared to the pair-fed control group. Bone in this alcohol-exposed group appeared less mineralised when compared to the pair-fed control group as the collagen fibres were more evident and less organised. Smaller trabecular spaces are typically found in more mature bone, suggesting that alcohol may have contributed to a delay in bone maturation in the alcohol-exposed male group (Galea *et al*., 2021).

In the female alcohol-exposed group, the bone appeared less mineralised compared to the pair-fed control group, with visually less organised collagen fibres and larger

trabecular spaces. The occurrence of these larger trabecular spaces and the abundance of wavy collagen fibres suggest that alcohol may have delayed bone maturation (Galea *et al*., 2021).

The male pair-fed control and alcohol-exposed groups showed bone cytoarchitecture that appeared more mature and mineralised than the female pair-fed control and alcohol-exposed groups. This observation contradicts the general principle that females typically develop faster than males (Nanci, 2018). The differences in interradicular bone may be attributed to increased mechanical forces, leading to greater bone volume (Nenda *et al*., 2016). These forces might accelerate bone development, which could explain the differences observed in our study, as male rats had higher food intake than female rats, resulting in greater mechanical force on the mandible and alveolar bone. Alcohol demonstrated more significant negative effects in females in this study, which aligns with the observation that the cytoarchitecture in the female alcohol-exposed group was the most impacted.

Cell proliferation and osteoblast activity

This study used Ki-76 and alkaline phosphatase immunohistochemistry to examine the impact on proliferating cells followed by a more specific analysis of alcohol's effect on osteogenic cells.

A significant decrease was observed in the number of proliferating cells in the female alcohol-exposed animals than in the pair-fed control animals. The results of our study are consistent with those found by Miralles-Flores and Delgado-Baeza (1992). These authors reported a decrease in proliferating cells in the growth plate of prenatal rats exposed to

alcohol, using a chronic alcohol liquid diet. No significant difference was displayed in the male animals. When exposed to chronic binge alcohol, the proliferating cells in the male alcohol-exposed rats in the current study had decreased, thus removing alcohol from their diet, caused an increase in proliferating cells to an amount observed in the healthy pair-fed control rats indicating recovery after alcohol abstinence in this parameter in male rats.

A significant increase in proliferating cells was observed in female pair-fed control animals when compared to male pair-fed control animals. Ishida and Heersche. (1997) found that progesterone found in females promotes the proliferation and differentiation of osteoprogenitor cells (Ishida and Heersche.,1997). The results identified in our study are consistent with the findings of Ishida and Heersche (1997).

Male alcohol-exposed rats displayed a significant increase in proliferating cells when compared to female alcohol-exposed rats. This finding contrasts with the results of Ishida and Heersche. (1997). The difference is likely due to alcohol's negative impact on proliferating cells in the female mandible, reducing their numbers to levels below those observed in male rats whose cell proliferation was not impacted by alcohol exposure in this study.

Alkaline phosphatase, an enzyme specifically expressed in osteoblasts and osteogenic cells, was used to assess whether chronic binge alcohol consumption affected bone-forming cells and if these cells were able to recover once alcohol was removed.

Osteoblasts were negatively affected by alcohol exposure more so in males than in females, as the decrease in male alcohol-exposed rats was significant when compared

to the male pair-fed control animals. The decrease observed in female alcohol-exposed animals when compared to the pair-fed control animals was not significant. This decrease identified in alcohol-exposed animals is consistent with the literature where alcohol exposure has decreased the number of osteoblasts (Giuliani *et al*., 1999; Maurel *et al*., 2012).

6.4 CONCLUSION

The impact of chronic binge drinking, involving 3 days of alcohol exposure per week over 28 days followed by 30 days of alcohol abstinence, on the growth and development of the adolescent Sprague Dawley rat mandible was found to be more pronounced and widespread in female animals compared to males. This drinking pattern induced blood alcohol concentration (BAC) levels consistent with a binge drinking model. Notably, female animals exhibited an increase in appetite and water consumption during the 4th week, with subsequent normalization. Furthermore, throughout the 8 weeks, male pair-fed control animals displayed a slightly higher percentage of body mass gain than alcohol-exposed animals, whereas female alcohol-exposed animals maintained a marginally higher percentage of body mass gain relative to female pair-fed controls. These findings suggest differential effects of alcohol on food consumption, water intake, and percentage body mass gain between male and female rats. Surprisingly, no detrimental effects on bone mass were observed in the chronic binge alcohol abstinence model in both male and female animals. Nonetheless, certain parameters related to the anterior height of the mandible in female animals displayed an increase in the alcohol-exposed group. This increase may be attributed to the growth and development of the mandible, impacting on its length and height. In the chronic binge alcohol-abstinent model, the increased height

in the posterior mandible corrected itself after alcohol withdrawal, whereas the heightened anterior mandible may be associated with initial growth disturbances due to alcohol exposure. It is important to note that this alcohol-induced influence did not affect the general size, linear length, or linear height measurements in male study animals. When analyzing the effect of chronic binge alcohol exposure and recovery thereafter on the microarchitecture of the bone, alcohol displayed a significant negative effect on all the trabecular parameters analysed (BV/TV, TbTh, TbN, and TbSp) in the female animals, even after alcohol was removed from the diet. This indicates that alcohol elicited a significant and long-term negative effect on the trabecular morphometry of the mandible which was not able to recover once alcohol was removed from the diet. Alcohol did not affect the trabecular morphometry of the mandible in the male animals.

Tensile strength parameters indicated that the cortical bone and tensile strength in male animals exposed to alcohol showed signs of recovery once alcohol was removed from the diet. Withdrawal of alcohol from the diet caused an increase in tensile strength parameters in female rats exposed to alcohol which could indicate that alcohol consumption had a possible compensatory protective mechanism on the cortical bone in female rats, in a way to protect the disruption caused to the internal structure of the bone. Another possible reason for this could be a self-recovery mechanism (Lauing *et al.,* 2008).

An analysis of the cytoarchitecture revealed that alcohol may have induced a delay in bone mineralisation in male animals, persisting even after alcohol was eliminated from their diet. A similar effect was observed in female rats, with a more pronounced impact, suggesting that alcohol causes long-lasting alterations in the cytoarchitecture of the mandible.

The current study observed a significant decrease in the number of proliferating cells in the female alcohol-exposed rats, with a non-significant decrease in osteoblasts identified. This indicates that the removal of alcohol from the diet may have led to recovery of the damage caused to the osteoblasts in female rats, however, alcohol still had a negative impact on the rest of the proliferating cells in the long term. Male animals displayed a decrease in osteoblasts, indicating that alcohol had a long-term effect on these cells.

Chronic binge drinking had less pronounced and extensive effects on male animals compared to female animals, though several negative impacts were still observed. This indicates that sexual dimorphism played a role in the impact of chronic binge drinking on the mandible. Removing alcohol from the diet helped the mandible to recover in some of the analysed parameters, but in others, it did not, resulting in long-term effects.

The chronic binge abstinent model displayed noticeable improvement compared to the chronic binge model regarding some of the parameters analysed, suggesting that, with sufficient time, full recovery in some parameters may be possible.

CHAPTER 7: *General Discussion, Limitations, and Conclusions*

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7.1 GENERAL DISCUSSION

The study aimed to evaluate whether disruptions in bone growth, development, and structural integrity affect the adolescent mandible in acute and chronic binge alcohol models. Additionally, it investigated whether the mandible recovers following a period of alcohol abstinence.

Our study investigated the extent to which different models of binge alcohol exposure would affect bone growth and development in a rat adolescent model. The models used are appropriate in the South African context where binge drinking is common in the adolescent population (Morojele & Ramsoomar, 2016).

Animals were divided into an acute binge alcohol model, a chronic binge alcohol model, and a binge alcohol abstinence model. Animals were exposed to 3 g/ kg of a 20 % (vol/vol) alcohol solution (Bhika *et al.,* 2024a) for a period of 7 days, 18 days, and 28 days followed by a 30-day alcohol-abstinent period respectively.

To assess if these binge drinking models influence the developing adolescent skeleton, baseline data which included food and water consumption, along with percentage body mass gain in the animals was evaluated. Thereafter osteometric measurements of the mandible were captured, followed by three-dimensional micro-focus X-ray computed tomography (3D- µCT) to assess trabeculae thickness, volume, number, and spacing. Tensile strength was analysed using a 3-point bending test. We then analysed the cytoarchitecture of the bone with Haematoxylin and Eosin (H & E) staining. Finally, the effect of different binge alcohol consumption methods on the effect of proliferating cells and osteoblasts was assessed by immunolabeling of proliferating cells expressing the Ki-

67 protein and osteoblasts expressing the ALP enzyme using the Ki-67 antibody and the ALP antibody respectively.

Baseline data

In the acute binge alcohol model, female alcohol-exposed animals consumed less food than the female pair-fed control animals. No differences were noted in food consumption between the pair-fed control and alcohol-exposed animals in the chronic binge alcohol model. In the chronic binge alcohol-abstinent model, we observed an increase in food consumption in the alcohol-exposed female animals. No effect on food consumption was noted in the male animals.

In the acute and chronic binge alcohol models, water consumption was similar between the pair-fed control and alcohol-exposed groups. In the chronic binge alcohol-abstinent model, male alcohol-exposed animals consumed less water than the pair-fed control animals, and the female alcohol-exposed animals consumed more water than the female pair-fed control animals.

When looking at the effect of binge drinking on percentage body mass gain, female alcohol-exposed rats in the acute binge alcohol group had a decrease in body mass gain compared to the pair-fed control group. The chronic binge alcohol model indicated some differences with an increase in body mass gain in the female alcohol-exposed animals in week 1, and an increase in alcohol-exposed males in week 4. In the chronic binge alcohol-abstinent model, male alcohol-exposed animals showed a decrease in percentage body mass gain over the 4 weeks when compared to the pair-fed control animals, and the female animals showed the opposite results, with an increase in percentage body mass

gain in alcohol-exposed animals. Differences were observed between the male and female animals in all three groups in all the baseline data with male animals consuming more food and water than female animals and also exhibiting a greater percentage body mass gain than female animals.

In the acute binge alcohol model, alcohol may have decreased the appetite of female rats which may have led to a food aversion, leading to a decrease in food consumption. This negative effect of alcohol on appetite was also observed in previous literature (Caton *et al*., 2004; Fong *et al*., 2021; Nelson *et al*., 2016). The absence of observed differences as the study progressed may indicate that the rats developed a tolerance to alcohol. The female alcohol-exposed rats in the chronic binge alcohol-abstinent group consumed significantly more food than the pair-fed controls in week 4 which may indicate that after alcohol tolerance has occurred, alcohol may have caused a short-term appetite stimulation in the female rats. Alcohol is an energy-dense substance that causes weak satiety responses, reduces fat oxidation, and may also stimulate appetite (Caton *et al*., 2004; Fong *et al*., 2021). Alcohol plays a role in numerous pathways including satiety hormones, affecting neurotransmitters, and stimulating the release of opioid peptides which would all affect an increase or decrease in food consumption depending on which way the pathways are affected (Kwok *et al*., 2019). The differences noted in food consumption influenced the percentage of body mass gain where a decrease in food consumption led to a decrease in body mass gain and vice versa. The increase in water consumption in the chronic binge alcohol-abstinent model female rats may be because alcohol acts as a diuretic. This would lead to an increase in diuresis as alcohol inhibits vasopressin release in the neurohypophysis which leads to an increase in urination. The

loss of water would then need to be replenished, and this would be done by an increase in water intake (Maderia *et al.,* 1993).

Osteometric measurements

A decrease in bone mass in male alcohol-exposed animals was depicted in the acute binge alcohol model. The only other differences noted in bone mass in all three groups were differences between male and female animals, where males exhibited heavier mandibles. When assessing the general mandibular size, linear mandibular length, and linear mandibular height the following differences were noted; an increase in the lower mandibular length (M3-Id) in the alcohol-exposed male animals in the acute binge alcohol model and an increase in the ramus height (Cd-Go) in the alcohol-exposed female animals. The differences identified in the chronic binge alcohol model were in the female alcohol-exposed animals who had an increase in the height of the coronoid found in the posterior mandible (Cr-Ag). The chronic binge alcohol-abstinent animals showed an increase in the anterior mandible with an increase in parameters also noted in female alcohol-exposed animals (MF-Al^{l} and MF-M1) (Bhika *et al.,* 2025). Differences were identified between male and female animals in all three groups with male animals exhibiting greater measurements than female animals.

A decrease in bone length, mass, and size is the expected outcome in studies analyzing the effect of alcohol on the skeleton. A decrease in these parameters was noted in previous studies examining the effects of alcohol on long bones (Broulík *et al*., 2010, Chakkalakal., 2005; Hogan *et al*., 1997; Sampson *et al*., 1996; Sampson *et al*., 1997; Wezeman *et al*., 2000). The results identified in our study were the opposite, indicating that alcohol may have effects that are both bone and site-specific. Long bones develop

through endochondral ossification and most flat bones through intramembranous ossification. The mandible develops through both endochondral and intramembranous ossification (Smartt *et al*., 2005) which may likely have had an impact on the results of our study.

Growth disturbances can affect both the length and height of the mandible in various ways. In the case of juvenile rheumatoid arthritis, bone apposition on the posterior margin of the ramus resulted in the mandible growing in a posterosuperior direction (Mizoguchi *et al*., 2013). Mandibular development, including transposition, under acute and chronic binge alcohol exposure, reveals a consistent growth pattern, with increased bone apposition in the posterosuperior direction. During periods of alcohol abstinence, posterior mandibular height normalized, while anterior height increased, characterized by an anterosuperior growth pattern. This may be due to early growth disturbances in the condyle caused by alcohol exposure or compensatory adjustments following the initial posterosuperior growth. The mandibular condyle in rats' fuses at approximately 14 weeks of age (Zoetis *et al*., 2003). The rats in our study were younger than 14 weeks at the time of termination, meaning the condyle had not yet fused. This allowed alcohol to adversely affect both endochondral and intramembranous growth of the mandible. There is no available literature to compare these findings.

Three-Dimensional Micro-focus X-ray Computed Tomography (3D-μCT)

When analyzing the trabecular parameters in the acute binge alcohol model, only BV/TV and trabecular spacing were affected. BV/TV was decreased in the alcohol-exposed animals in the female group, which also exhibited an increase in trabecular spacing. BV/TV in this group was also greater in female groups when compared to male groups

(Bhika *et al*., 2024b). Trabecular parameters were greatly affected in the chronic binge alcohol model and in the binge alcohol abstinence model where a decrease in BV/TV and trabecular thickness were noted, with an increase in trabecular spacing and trabecular number in alcohol-exposed female rats. Some differences between males and females were identified between the groups with males exhibiting thicker trabeculae with narrower spaces.

Trabecular parameters were impacted across all three binge alcohol models, with varying degrees of severity between them. The female animals were most affected. The chronic binge alcohol model in females showed more pronounced effects than the acute binge alcohol model. The effects identified in the chronic binge alcohol model in females continued even after alcohol was removed in the binge alcohol abstinence model indicating that alcohol had lasting, long-term effects on trabecular parameters including bone to total volume ratio (BV/TV), trabecular thickness (TbTh), trabecular number (TbN) and trabecular spacing (TbSp).

The negative alterations in trabecular parameters in female animals are consistent with literature which has identified negative effects on trabecular parameters in several bones (Föger-Samwald *et al*., 2018; Maia *et al*., 2021; Sampson *et al*., 1998). The duration of exposure and the study design may be the reason for no changes in the bone ratio of the male rats. A significant increase in trabecular number (TbN) was observed in the female alcohol-exposed groups in both the chronic binge and binge alcohol abstinence models compared to the female pair-fed control groups. Föger-Samwald et al. (2018) found a decrease in TbN in the femur and an increase in TbN in the humerus of binge alcohol-exposed prepubescent pigs, which aligns with the expected effects of alcohol exposure

on trabecular number. However, the mandible may have responded to alcohol-induced damage by increasing the number of trabeculae to maintain bone strength and compensate for the negative effects on BV/TV, TbTh, and TbSp. This compensatory mechanism, however, may not fully mitigate the detrimental effects of alcohol on bone quality. Chronic alcohol exposure disrupts the balance between bone formation and resorption, weakening overall bone integrity despite changes in trabecular architecture, and can have persistent long-term effects on the bone, even after alcohol withdrawal. There is no available literature addressing this possible compensatory mechanism.

Biomechanics

No differences were identified in tensile strength between the pair-fed control and alcohol-exposed groups in male and female rats in the acute binge alcohol model. In the chronic binge alcohol model, male alcohol-exposed animals displayed a decrease in tensile strength in all parameters tested with no effect noted in the female groups. In the chronic binge alcohol-abstinent group, no differences were displayed in the male groups, however, differences were displayed in the female groups where female animals exposed to alcohol had a greater maximum force and break force. Differences were identified between male and female animals throughout the groups. Male animals displayed greater tensile strength than female animals, mainly in the pair-fed control groups with some increases observed in the alcohol-exposed groups.

No effects on tensile strength were identified in the acute binge alcohol model, indicating that a period of longer than 7 days is necessary for alcohol to illicit negative effects on the tensile strength of the mandible. The mode of alcohol administration may have influenced the outcomes of our study. Iwaniec and Turner. (2013) reported that intraperitoneal

alcohol injections induce dramatic bone-metabolism changes which are not seen with oral gavage (Iwaniec and Turner., 2013). The chronic binge alcohol model noted a negative impact on all tensile strength parameters in male alcohol-exposed rats. This was not the outcome that we had expected to observe as most other parameters in the study were not affected in male rats. A decrease in tensile strength parameters is identified in the literature and corroborates with our study (Callaci *et al*., 2004; Callaci *et al*., 2006; Chakkalakal., 2005; Hogan *et al*., 1997; Lauing *et al*., 2008; Maurel *et al*., 2012b). The decrease in cortical strength could be linked to reduced mineral content, which was not analysed in this study and requires further investigation. Maurel et al. (2012b) found that cortical bone in male Wistar rats was more sensitive to alcohol dose effects than trabecular bone (Maurel *et al*., 2012b). In the chronic binge alcohol-abstinent model, no negative impacts were observed in the male group, indicating that the negative impact that occurred in the chronic binge alcohol model was overcome once alcohol was removed. Lauing et al. documented the same effect on the vertebra of alcohol-exposed rats, where the vertebra had an increase in tensile strength parameters once alcohol was removed. An increase in maximum strength and break force was identified in the female animals in the chronic binge alcohol-abstinent model. This may be due to a self-compensatory protective mechanism on the cortical bone in female rats, in a way to protect the disruption caused to the internal structure of the bone. No literature is available to validate these findings.

Mandibular cytoarchitecture

The same pattern was observed throughout all three groups where male and female animals displayed visually larger trabecular spaces in the alcohol-exposed groups with

bone that appeared less mature which contained more disorganised wavy collagen than the pair-fed control groups. Differences were uniformly noted between male and female animals throughout the three groups where bone in the male animals contained larger trabecular spaces with visually less organised collagen and appeared more mature and mineralised than bone in the female animals in both alcohol-exposed and pair-fed control groups.

The effect of the different binge drinking models on mandibular cytoarchitecture remained uniform throughout the groups, where acute and chronic binge drinking caused wider and more numerous trabecular spaces, with bone with appeared less developed which contained visually more disorganised, wavy collagen fibres than the pair-fed control groups. Smaller trabecular spaces are typically found in more mature bone, suggesting that alcohol may have contributed to a delay in bone maturation in the alcohol-exposed groups. The occurrence of abundant wavy, disorganised collagen fibres also suggests that alcohol delayed bone maturation (Galea *et al*., 2021). Alcohol-related disturbances in bone remodeling, impacting osteoblast and osteoclast function, may be influenced by deficiencies or impairments in vitamin D, calcium, and parathyroid hormone (PTH). Serum deoxypyridinoline (a bone resorption marker) has also been observed to increase in animals exposed to alcohol. A reduction in the expression of bone resorption markers, such as ALP and osteocalcin, has also been observed in binge drinking models. Leptin, a protein produced by white adipose tissue that regulates appetite, food intake, and body weight, has also been implicated in the regulation of bone mass. It has been shown to reduce bone mass via central nervous system pathways and to influence bone directly by affecting the osteoblast cell lineage. Previous studies have observed that leptin

decreases osteoblastogenesis with an increase in adipogenesis. Growth hormone (GH) is a key regulator of bone growth. Alcohol has been reported to affect GH activity, altering the balance between osteoblast and adipocyte differentiation. Alcohol has been seen to impair the effects of GH on tibial growth and trabecular bone formation and has been shown to suppress osteoblastic cells within the bone marrow. By disrupting osteogenic differentiation of mesenchymal stem cells in the bone marrow, alcohol further reduces bone formation. Additionally, decreases in vitamin D and parathyroid hormone (PTH) can interfere with bone metabolism. Low serum levels of magnesium and phosphate, often observed in heavy drinkers, may also contribute to bone loss (Maurel *et al*.,2012a). The mechanisms described above, which impact bone growth and remodeling, may have contributed to the findings observed in this study. However, further investigations are necessary to determine precisely which pathways were involved and how they may have negatively affected the mandible.

These results were displayed in the binge abstinence model also, which indicates that alcohol had a lasting, long-term effect on the cytoarchitecture of the mandible.

Cell proliferation and osteoblast activity

Acute binge alcohol consumption had no impact on the number of proliferating cells between pair-fed control and alcohol-exposed groups. Chronic binge alcohol consumption negatively affected the number of proliferating cells in both male and female animals in the alcohol-exposed groups. In the chronic binge alcohol-abstinent model, female alcohol-exposed animals were still affected with a decrease in proliferating cells, however, male animals indicated no effects.

Binge alcohol exposure had an effect on the number of osteoblasts in all three groups with a decrease noted in the female alcohol-exposed group in the acute binge alcohol model, a decrease in both male and female alcohol-exposed groups in the chronic binge alcohol model, and a decrease in both male and female groups again in the chronic binge alcohol-abstinent model (with the decrease being significant in only the male animals).

Differences were identified between the male and female animals in both the number of proliferating cells and the number of osteoblasts where male animals displayed greater numbers of both cells when compared to female animals.

As identified in the other parameters, female animals were affected to a greater extent than male animals. An increase in alcohol exposure time led to an increase in negative effects on both the proliferating cells and on the number of osteoblasts in the mandible. Once alcohol was removed, some lasting negative effects were still noted. The results of our study corroborate with a study by Miralles-Flores and Delgado-Baeza. (1992), who reported a decrease in proliferating cells in the growth plate of prenatal rats exposed to alcohol. This suggests that chronic binge alcohol consumption negatively impacts osteoblasts, which are essential for bone formation. Our findings are consistent with existing literature showing that alcohol can detrimentally affect osteogenic cells, reducing both the number and function of osteoblasts and impairing bone formation, potentially leading to osteopenia (Chakkalakal, Dyer, *et al*., 1998; Eby *et al*., 2020; Turner, 2000).

Differences of sexual dimorphism on findings

Male animals in this study consumed more food and water than female animals and had a greater percentage of body mass gain. The differences between male and female

animals noted are consistent with those of sexual dimorphism (Asarian and Geary., 2013). Male animals are typically larger than females (Lorenzo, 2020). Several factors are responsible for sexual dimorphism, including genetics, sex steroid actions, environment, and behavior (Lorenzo, 2020), all of which may influence the differences observed between male and female animals in our study. These factors seem to primarily favour male growth and development, as male animals exhibited stronger bones and were less affected by the harmful effects of alcohol. The results also indicate that alcohol had a more pronounced effect on females when compared to males in all the parameters tested and analysed. Further studies are needed to clarify the specific reasons for these sex-based differences.

7.2 LIMITATIONS

1. A larger sample size per group and sex could provide more robust and reliable results by reducing variability and increasing statistical power. This would allow for more precise detection of subtle differences between groups, better representation of individual variations, and potentially reveal sex-specific effects or interactions that might be missed with smaller sample sizes.
2. The laboratory setting may not fully mimic the complex social and environmental factors that influence drinking behavior in humans.
3. Rat physiology and metabolism differ from humans, which may limit the generalizability of the findings to human alcohol consumption and its effects, therefore, the results must be applied with caution to humans. Some differences between rats and humans that may be a limitation include the following:

a. Rats have a much shorter growth period and faster skeletal maturation compared to humans, which may limit how well adolescent growth processes translate.
b. Rats have higher bone turnover and faster remodeling rates than humans, potentially exaggerating or masking alcohol-related effects.
c. Differences exist in mandibular shape, occlusion, masticatory muscle forces, and tooth eruption patterns, which may influence responses to alcohol-induced bone changes.
d. SD rats are an inbred strain with limited genetic variation, while human populations are genetically diverse, affecting generalizability.
e. Rats cannot replicate the complex behavioral, nutritional, and environmental factors linked to human adolescent binge drinking patterns.

4. The short exposure window in the acute binge alcohol model may have been too short to have a significant impact.

7.3 CONCLUSIONS

1. Underage alcohol exposure in adolescent females initially negatively impacts food consumption and body mass gain. However, after extended exposure, this effect appears to reverse, leading to increased appetite and body mass gain.
2. Binge drinking affects both intramembranous and endochondral ossification and causes disturbances in mandibular growth and development with long-lasting effects.
3. Our findings regarding trabecular morphometric parameters indicate that binge drinking impacts these parameters after just three exposure sessions in an acute

binge alcohol model. More significant effects are observed in a chronic binge model. Notably, these effects persist even after alcohol withdrawal.

4. In the male chronic binge alcohol model, tensile strength was significantly affected, but the mandible showed recovery after alcohol withdrawal. In females, chronic binge alcohol exposure may have triggered a compensatory response in the cortical bone.
5. Alcohol consumption in an acute binge and chronic binge model has negative lasting effects on the maturation and mineralisation of the bone in the adolescent mandible in both males and females.
6. Adolescent alcohol consumption reduces the number of proliferating cells in the mandible, with recovery observed in males after alcohol withdrawal. However, this effect persists in females.
7. Osteoblast number and likely activity are affected by binge drinking in male and female adolescents, with these effects persisting after alcohol withdrawal.
8. Adolescent exposure to binge alcohol consumption affects males and females differently, with females experiencing more pronounced effects on the adolescent mandible in an acute binge, chronic binge, and chronic binge alcohol-abstinent model.
9. The effects of acute and chronic binge drinking on the adolescent mandible partially support the idea that bone health can recover after alcohol withdrawal, as some parameters improve, while others show no signs of recovery.

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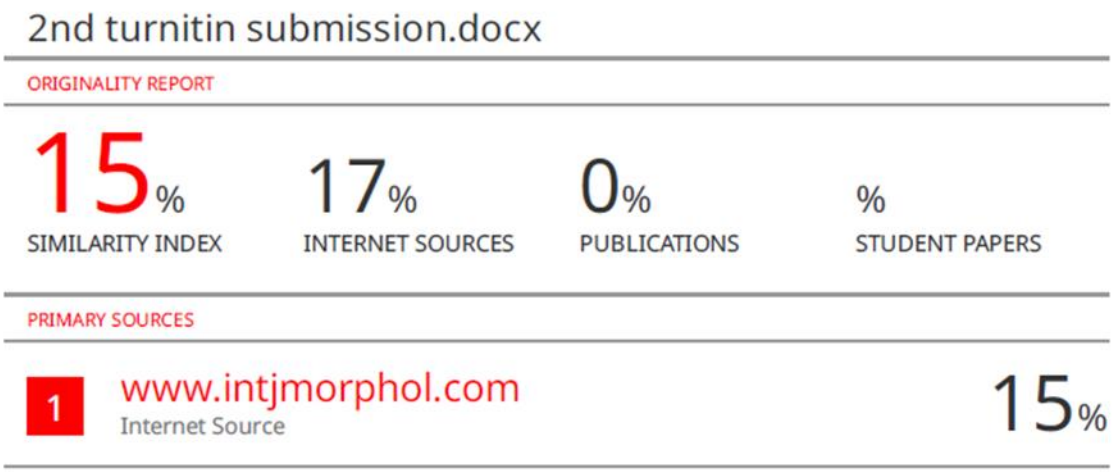
APPENDICES

Appendix A: Similarity index



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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG | FACULTY OF HEALTH SCIENCES | Anatomical Sciences

13 December 2024

TO WHOM IT MAY CONCERN

Student Akaashni N. Bhika's (student number: 2144943) PhD thesis, titled *"The Effect of Binge Alcohol Consumption on the Development of Adolescent Sprague Dawley Rat Mandibles,"* has a similarity index of 15%.

This is due to Turnitin identifying similarities between her dissertation and her two publications in the International Journal of Morphology.
This is the only source of similarity that has been noted.

We are satisfied with her similarity index, as there are minimal changes needed in this regard.

Kind regards,

Professor Amadi O. Ihunwo

Supervisor

Dr Diana Pillay

Co-Supervisor

Appendix B: Ethical Clearence Forms

AREC 2022 AM&E

Please note that only electronically completed applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
ANIMAL RESEARCH ETHICS COMMITTEE

registered as Animal Research Ethics Committee (AREC) with the South African National Health Research Ethics Council (NHREC) of the National Dept. of Health, reg. no. AREC -101210-002

APPLICATION FOR ADAPTATIONS OR MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Principal Investigator (PI) Name: Dr Akaashni Bhika

b. School / Department: School of Anatomical Sciences.

c. Email Address: akaashni.bhika@wits.ac.za

d. Experiment to be modified / extended

	AREC NO		
Original AREC Clearance Certificate Number	2020	11	02/C
Clearance Certificate Expiry date	4 Mar 2023		
Number of previous AM&Es			4

e. Project Title:

The effect of binge-alcohol consumption on the development of adolescent Sprague Dawley rat mandibles

		No.	Species
f.	Number and species of animals originally approved	72	*Sprague-Dawley rats*
g.	Number of additional animals previously approved on AM&Es	8	*Sprague-Dawley rats*
i.	Number of animals used and currently involved in the study to date	80	Animal study has been completed

j. Specific details of Adaptations/Modification/ Extension:

May I please apply for an extension to continue collecting data on the harvested bones, as my PhD has not yet been completed (All work at WARF has been completed and data is currently being collected).

k. Motivation for Adaptations/Modification/ Extension:

Data on the harvested bones is still being collected. This data is necessary for me to complete my PhD.

Date: 26/01/2023

PI Signature:

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

AREC 2022 AM&E

RECOMMENDATIONS:

Approval for the extension of ethics until 31 March 2025. Please note any further extension must be applied to prior to the revised expiry date.

Date:27 January 2023

Signature:

Deputy Chair, AREC

Appendix C: Reagents used

Acetic acid (glacial) (Saarchem 1021020LC., RSA)

Acetone (SAAR1022040LC, Merck Chemicals (Pty.) Ltd., RSA)

Alkaline phosphatase (ALP) antibody (Ab95462)

Citric Acid (19150 41, Riedel-de Haën AG, Germany)

3-3’ diaminobenzidine (Ab64238 - DAB substrate kit)

Disodium hydrogen orthophosphate anhydrate (CAS NO. 7558-79-4, Associated Chemicals Enterprise, RSA)

Entellen (HX 088232, Merck RGA, Germany)

Eosin yellowish (SAAR2186000DC, Merck Chemicals (Pty.) Ltd., RSA)

Formaldehyde (Associated Chemicals Enterprise., RSA)

Hydrochloric acid 1 % (HCl) (Associated Chemicals Enterprise., RSA)

Hydrogen peroxide (Catalog. 1037024, Merck (Pty.) Ltd., RSA)

Ki-67 antibody (NB 500-170)

Mayer’s solution (SAAR2422001LC, Merck Chemicals (Pty.) Ltd., RSA)

Paraffin Wax (Merck Chemicals (Pty.) Ltd., RSA)

Potassium Alum (CAS NO. 7784-24-9, Associated Chemical Enterprises)

Potassium chloride (CAS NO. 7447-40-7, Associated Chemicals Enterprise, RSA)

Potassium dihydrogen orthophosphate (CAS NO. 7778-77-0, Associated Chemicals Enterprise, RSA)

Rabbit Specific HPR/DAB (ABC) detection IHC kit (Ab64256 Abcam)

Sodium chloride (CAS NO. 7647-14-5, Associated Chemical Enterprise, RSA)

Sodium hydrogen carbonate (CAS NO. 582-28-20, Saarchem (Pty.) Ltd., RSA)

Sodium Iodate (The lab Depot, Inc)

Strept-Avidin HRP (Ab64269 Abcam)

Tween 20 (616-45-00KF, Saarchem, RSA)

Xylene (CAS NO. 108-38-3, Associated Chemicals Enterprise, RSA

Appendix D: Equipment

Alcohol Colorimetric Assay Kit (Sigma-Aldrich, USA)

Automatic tissue processor (Citadel 2000, Thermo Fisher Scientific, USA)

Axiocam HRC digital camera (Zeiss Axioscope 2 plus)

Cassettes (Merck Chemicals (Pty.) Ltd, RSA)

Digital Vernier caliper (Guanglu ISO9001:2000, China)

Embedding table (Miles Scientific, serial number 31602-554)

ESCO Laboratory fume hood (Model: EFA-4UDW-8. SN: 2011-60070)

iMark Bio-rad Microplate Absorbance Reader (Bio-rad Laboratories Inc, USA)

Incubator (Model-508-2U, Labcon shaker)

Microhaematocrit centrifuge (Haematokrit 210, SN 0031 386-04, Germany)

Microscope (axioscope) (Model Axiocom HRC, identification number 105- 041756)

Microscope Slides (Epredia Superfrost^{TM} plus adhesion microscope slides, Netherlands)

Microtome (Leica RM 2125RTS, Fabr. Nr. 07390329, Leica Biosystems)

Nikon XTH 255L 3D-microCT scanner (Nikon Metrology, Leuven, Belgium)

Pipettes (Finnpipette GLP F2 Kit 2 (0,2-1000ul))

Shimadzu universal tensile strength testing machine (China)

Water bath (Model number 416, SN: SE 9455)

Weighing scales: (Model- KERN EW600-2M, Germany) (Model- SARTORIUS GMBH, Germany)

Appendix E: Software packages

Fiji Image-J software

Statistical Package for Social Service (SPSS) version 28 (IBM®)

Microsoft Excel 2023 (Microsoft Corporation)

Volume Graphics® Max Studio (VGS) software Axiovision (AXIOVs) version 4.7.2.0

Appendix F: Calculation of alcohol (20 %) and maltose dextrin (isocaloric) dosages

20 % Alcohol:

Dose = 3 g/kg body weight

Alcohol density = 0.789 g/ml

Thus, to calculate the volume:

Volume = mass/density

=3g/0.789g/ml

= 3.8 ml

Thus, the required dosage = **3.8 ml/kg body weight**

Maltose Dextrin:

How to calculate the caloric equivalent of the alcohol dosage administered:

1 g of alcohol = 7 kCal

3 g of alcohol = 2 1kCal

20 % of alcohol was used, therefore: 3 g of 20 % alcohol = 20 % of 21 kCal

= **4.2 kCal** to match

1g maltose dextrin = **3.89kCal**

Thus, 4.2 kCal / 3.89kCal

= 1.08 g of maltose dextrin for caloric equivalence

Dissolve 1.08 g of maltose dextrin in 4 ml of distilled water to make a 27 % maltose dextrin solution.

1.08 g maltose dextrin in 4ml distilled water = 4.2 kCal/4ml

= 1.05 kCal/ml

How to calculate the maltose dextrin isocaloric dosage in **ml/kg** body weight:

= 4.2 kCal/kg /1.05 kCal/ml

= 4 ml/kg body weight

Appendix G: Solutions to be made

10 % Neutral Buffered Formalin (1000 ml):

Measure 100 ml of Formaldehyde (37 % - 40 %)

Add 900 ml of distilled water

Add 3.5 g of sodium dihydrogen phosphate (anhydrous)

Add 6.5 g of sodium hydrogen phosphate (anhydrous)

Add 8 g of sodium chloride

Store at room temperature

10mM Sodium Citrate Buffer pH 6 + 0.05% Tween 20 (1000 ml):

Tri- Sodium citrate (di-hydrate) 2.9g

Distilled water 1000ml

Mix to dissolve sodium citrate and adjust pH to 6 with 1M HCl

1M Phosphate Buffer pH 7.4 (2000 ml):

NaCl 16g

Na_2HPO_4 3.5g

KCl 0.4g

KH_2PO_4 0.4g

Distilled water 2000ml

14 % EDTA for Tissue Decalcification (100 ml):

Dissolve 14g of EDTA in 100 ml of distilled water

pH the ETDA solution to a pH of 7

Incubate the tissue in 14 % EDTA for 7 days at 45 ^{o}C, with continuous gentle shaking

Replace the decal solution with a fresh supply after 3-4 days

Continually monitor specimens to ensure that decalcification process is complete

Radiographs can be taken to confirm if the decalcification process is complete

Once decalcification is complete (tissue is pliable) wash specimens in running tap water for 10 mins

4 % Paraformaldehyde (PFA) (1000 ml):

750 ml distilled water

40 g PFA

250 ml 0.4 M PBS

Appendix H: Method of Tissue processing

Tissue was processed using an automatic tissue processor (Citadel 2000, Thermo Fisher Scientific, USA) overnight as follows:

10 % buffered formalin (4hrs)

70 % alcohol (1hr)

95 % alcohol (2hrs)

95 % alcohol (2hrs)

95 % alcohol (2hrs)

95 % alcohol (2hrs)

100 % alcohol (2hrs)

100 % alcohol (2hrs)

100 % alcohol (2hrs)

Chloroform (2hrs)

Chloroform (2hrs)

Paraffin wax (2hrs)

Paraffin wax (2hrs)

Appendix I: Paraffin wax embedding

Begin by gently heating both the paraffin wax and metal moulds, ensuring the temperature stays below 60°C. Have a pair of forceps on hand, warmed and ready for use

Once the paraffin wax has completely melted, carefully pour it into the metal mould

Using the warmed forceps, delicately place the specimens into the mould, ensuring that the anterior (lingual) surface of the bone is positioned facing the bottom of the mould

Allow the mixture to cool slightly before covering it with a plastic cassette

Add more melted paraffin wax to fill any gaps, and let it cool for approximately 20 minutes

Finally, carefully remove the metal mould from the plastic cassette

Store paraffin wax embedded samples at room temperature

Place paraffin wax embedded samples in a freezer (-20 °C) for 24 hours prior to sectioning

Remove paraffin wax embedded samples from the freezer 1 hour before sectioning, and place on ice

Appendix J: Mayer's Haematoxylin and Eosin Staining

Haematoxylin solution (To make up 1000ml):

Haematoxylin 1 g

Aluminium potassium sulphate (Potassium alum) 50 g

Sodium iodate 0.2 g

Citric acid 1 g

Chlorate hydrate 50 g

Allow haematoxylin, aluminium potassium sulphate and sodium iodate to dissolve overnight

Add chloral hydrate and citric acid

Boil solution for 5 minutes, thereafter, allow to cool

Eosin solution (To make up 1000ml):

Measure 1000mls of distilled water

Add 8g of stock eosin.

Add 2g of erythron.

Stir using a magnetic stirrer until completely dissolved

Scott’s tap water solution (To make up 1000ml):

Sodium hydrogen carbonate 2g

Magnesium sulphate 20g

Distilled water 1000ml

Dissolve the salts in the water and store the stock solutions at room temperature

Method for H&E Staining:

Hydration:

1. Dewax in xylene for 2 x 10 minutes

2. Immerse in 100 % alcohol (10 dips) X 2

3. Immerse in 95 % alcohol (10 dips)

4. Immerse in 70 % alcohol (10 dips)

5. Wash in distilled water for 5 minutes

Nuclear staining:

1. Stain with Mayer’s Haematoxylin for 20 minutes

2. Wash in running tap water for 5 minutes

3. Differentiate in 1 % acid alcohol (2 dips)

4. Wash in running tap water for 5 minutes

5. Blue in Scott's tap water for 5 minutes

6. Wash in running tap water for 5 minutes

Cytoplasm staining:

1. Stain in Eosin for 2 minutes

2. Wash briefly in running tap water

Dehydration and mounting:

1. Immerse in 70 % alcohol for 3 minutes

2. Immerse in 90 % alcohol for 10 minutes

3. Immerse in 100 % alcohol for 10 minutes

4. Clear in xylene (10 dips) X 2

5. Mount in Entellen and cover with a coverslip

Results:

Nuclei: Blue/purple

Cytoplasm and extracellular matrix: Shades of pink

Appendix K: Immunohistochemistry for Ki-67 and Alkaline Phosphatase (ALP) antibody (NB 500-170) and (Ab95462) respectively:

Method:

Day 1:

Deparaffinize sections in Xylene for 2 x10 mins

Rehydrate sections through a graded series of alcohol (from 100-70 %),

Wash slides in running tap water for 5 mins

Place slides in Citrate Buffer pH 6 overnight @ 60° C in a water bath for 2 hours (for antigen retrieval)

Remove slides from water bath and allow to cool at room temperature for 20 mins

Rinse in PBS (pH 7.4) for 5mins

Block endogenous peroxidase with 1 % H_2O_2 in methanol for 30 mins

Wash in PBS pH 7.4 for 3 x 5mins

Incubate with Protein Block for 20mins

Wash in PBS for 3 x 5mins

Incubate with primary antibody anti Ki-67 NB 500-170 (1: 200) or ALP Ab95462 (1:1000) overnight at 4°C

Day 2:

Remove sections from 4°C refrigerator and allow sections to reach room temperature

Wash in PBS pH 7.4 for 4 x 5mins

Incubate with biotinylated secondary antibody (Biotinylated Goat Anti-Rabbit IgG (H+L) (Ab64256) for 10mins

Wash in PBS pH 7.4 for 4 x 5mins

Incubate with Strept Avidin HRP (Ab64269) for 10mins

Wash in PBS pH 7.4 for 4 x 5mins

Incubate with Ab64238 - DAB substrate kit for 10mins

Rinse in running tap water for 5mins

Do not counterstain

Hydrate through graded alcohols, clear in xylene, mount with Entellen and cover sections with a cover slip