THE EFFECT OF CREATINE ON THE DEVELOPING RAT FOETUS

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DECLARATION

I, Frans Hendrik Badenhorst, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University

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FH Badenhorst

_____ day of _____ , 2005

To my father Frans, mother Annalie and brothers Mias, Francois, Darius and Andre, whose support and encouragement lifted me when times were hard.

And to Antonei, whose love carried me when I was tired and inspired me to be the best I can be.

ABSTRACT

Creatine is one of the most frequently or generally used ergogenic substances. It is used by professional and amateur athletes and the "man on the street". Creatine is involved in energy production and protein synthesis in muscle. Although studies have been carried out on the effect of creatine on adults, no study has yet determined whether creatine would have an influence on the developing rat foetus if taken by a female during pregnancy.

The aim of this study was thus to determine whether creatine had an effect on the developing foetus.

Dams were divided into two groups, which were injected between days 7-13 and on days 9 and 11 only of intra-uterine development respectively. Each group was subdivided into a control and two experimental groups. Experimental group one received a low dose of creatine (53.5mg/250g body weight); the other experimental group received a high dose of creatine (107mg/250g body weight). The control group received an equal volume (1ml) of the vehicle (saline) in which the creatine was constituted. Dams were sacrificed on day 20 of development. The foetuses were removed and their weight and length taken. Foetuses were examined for abnormalities. Two foetuses from each litter underwent skeletal staining. Tissue was excised from the remaining foetuses and processed for histology for histological investigation.

Creatine positively affected the growth of the foetuses of dams injected between days 7-13, while foetuses of dams injected only on days 9 and 11 in the B-group showed reduced growth. Creatine also had a slightly negative effect on the histological structure of the liver, but enhanced skeletal muscle growth, endocrine cell formation (pancreas) and skeletal formation.

From the results obtained it is hypothesized that creatine and insulin together may play a positive role from implantation to birth, while creatine given at certain stages of organogenesis delayed development of the foetus.

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1. INTRODUCTION

Following the discovery of phosphocreatine in 1927 and the metabolic reaction of creatine kinase (see section 2.4) in 1934 (Saks *et al.*, 1987; Conway and Clark, 1996), studies have focused on the biophysiological effect of creatine on muscle with specific focus on high-energy demands. Creatine (Greek *kreas*, flesh) metabolism was not studied intensively until recently (1980's) when a series of fascinating discoveries were made. It was demonstrated that creatine analogs have the potential to be potent anti-tumour agents (Miller *et al.*, 1993), while cyclocreatine exhibits the potential to reduce the damage to a cell that has undergone ischemic injury (Saks *et al.*, 1996).

Genetic endowment and proper training are the requirements for an athlete to excel in any given sport. Often a combination of physiology and biomechanical traits are combined with intense physical and mental training to ensure success in sports performance. Due to the increasing pressures and in the hope of breaking an elusive record, most athletes (Guzik *et al.*, 2000) resort to ergogenic aids (i.e. substances that improve sport performance beyond the physical effects of training)(Wyss and Kadurrah-Daouk, 2000; Persky and Brazeau, 2001), and which do not contain pharmacological agents such as nandrolone and anabolic steroids.

Creatine is intricately linked in the energy production and utilization reaction, which is why 95% of the total creatine content is stored in skeletal muscle (Williams *et al.*, 1999; Persky and Brazeau, 2001). Skeletal muscle needs large amounts of creatine since it requires huge amounts of ATP to generate energy while exercising.

Creatine is phosphorylated to form phosphocreatine (PCr). PCr is used in fast and slow twitch muscle fibres, although there are different mechanisms of ATP regeneration for each type of muscle. In fast twitch muscle fibres (skeletal muscle sprinting) there is a large pool of PCr in the fast twitch muscle. Due to the high creatine kinase activity in fast twitch muscle fibres, ADP to ATP regeneration (see section 2.4 for metabolic reaction) is kept at near equilibrium during short bursts of maximal exercise (Wyss and Kadurrah-Daouk, 2000). These high amounts of PCr thus buffer the cytosolic phosphorylation reaction potential that is crucial for the proper functioning of regeneration of ATP.

Slow twitch muscle contraction (i.e. the heart), is much more complex than fast twitch muscle fibres. Slow twitch myocytes need a continuous supply of phosphates to ATP production sites for optimal energy production and utilization (Wyss and Kadurrah-Daouk, 2000). Different isoenzymes of creatine kinase assist with this particular process. Mitochondrial creatine kinase (Mi-CK)(Brudnak, 2004) acts as a transport shuttle to allow creatine to be converted to PCr in the intermembranous space of the mitochondrion. ATP that has been

synthesized in the mitochondrial matrix diffuses into the intermembranous space. The γ-phosphate group of the ATP molecule is sliced from the ATP by Mi-CK and transferred to the creatine to form PCr. The PCr diffuses back into the cytosol to the sites of energy utilization (muscle). At the site of energy utilization, ATP is dephosphorylated to form ADP. Due to the large amount of PCr available in the cytosol, creatine kinase rephosphorilates the ADP back to ATP. This is a time saving process, since ADP does not have to diffuse back to the mitochondrial matrix to be rephosphorylated to ATP. However, the ATP that was dephosphorylated to ADP in the intermembranous space, when Mi-CK sliced off the γ-phosphate group, diffuses back into the mitochondrial matrix to be phosphorylated back to ATP (Wyss and Kadurrah-Daouk, 2000).

Because creatine is synthesized endogenously in the mammalian body, the International Olympic Committee does not classify it as a banned substance. Due to the fact that creatine is a nutritional supplement, it is exempted from all the rules and regulations set out by the Food and Drug Administration (Breithaupt, 2001). Athletes that wish to enhance sports performance use creatine as a non-steroid dietary supplement. Exogenous creatine supplementation can increase total creatine concentration in the mammalian body. There is, however, a threshold amount of creatine that can be stored in skeletal muscle (see section 2.4)(Hochachaka and Mossey, 1998). It is due to this attribute that sportsmen and sportswomen "load up" on exogenous creatine supplementation. Exogenous creatine supplementation improves muscle performance in three ways by:

1) increasing PCr, 2) increasing PCr resynthesis and 3) decreasing lactic acids and adenine nucleotides (Wyss and Kadurrah-Daouk, 2000). The higher the concentration of total creatine and phosphocreatine in the skeletal muscle, the longer and harder one can exercise.

2. SYNTHESIS AND METABOLIC FUNCTION OF CREATINE

Creatine is synthesized from several amino acids and since it is a natural dietary constituent of animal foods, it is considered as non-essential (Campbell, 1995).

2.1 Endogenous synthesis

A normal human being requires about 2 grams of creatine a day (Steenge *et al.*, 1998; Robinson *et al.*, 2000; Wyss and Kadurrah-Daouk, 2000; Persky and Brazeau, 2001; Brudnak, 2004). Fifty percent of creatine (1 gram) is obtained exogenously from fish and red meat (Walker, 1979), while Balsom *et al.* (1993) found trace amounts of creatine in plants. The other 50% of creatine is produced endogenously.

The amino acids arginine, glycine and methionine play an important role in the synthesis of creatine (Campbell, 1995; Persky and Brazeau, 2001). Glycine forms the backbone of the creatine molecule while arginine donates its only amidino group, and methionine its methyl group (Walker, 1979). The amidinotransferase enzyme (AGAT) mainly controls the creatine biosynthesis

reaction, while this reaction is completed by the methyltransferase (GAMT) reaction (Williams *et al*, 1999; Wyss and Kadurrah-Daouk, 2000).

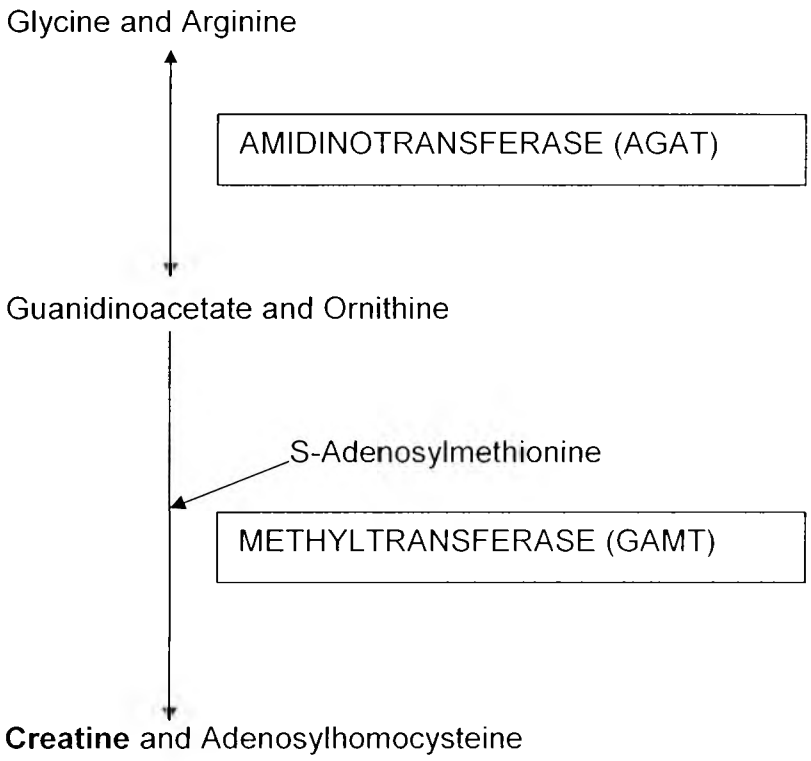


Figure 1. Biosynthesis of creatine (Williams *et al.*, 1999)

2.2 Main production sites and transportation of creatine in the body

Main production sites of endogenous creatine are found in the liver (Walker, 1979), pancreas and kidney (Horn *et al.*, 1998). Endogenous creatine is then released into the bloodstream to be taken up by various organs (heart, brain) and skeletal muscle. This uptake of creatine is mediated by a specific creatine transporter protein (Horn *et al.*, 1998). Horn *et al.* (1998) found that when adult

rats are supplemented with exogenous creatine, the creatine concentration in the liver and kidney is increased, but no significant increases were found in the skeletal muscle or brain tissue.

Although creatine is produced endogenously in the body, it needs to be transported to the areas with a high creatine kinase content. Creatine then needs to be transported through biological membranes (i.e. cell and mitochondrial membranes). Wyss and Kadurrah-Daouk (2000) stated that creatine uptake into skeletal muscle, kidney, brain or heart (creatine kinase-containing organs) is mediated by a "specific, saturable Na^+ -and Cl^- -dependent creatine transporter". These researchers also state that this transporter might be influenced by hormonal and/or dietary factors.

It has also been shown that an increase in creatine concentration tends to cause an increase in insulin-like growth factor (IGF) (Odoom *et al.*, 1996; Persky and Brazeau, 2001). Odoom *et al.* (1996) have shown that IGF has a stimulating effect on the sodium-potassium pump. The sodium-potassium pump transports creatine, against the concentration gradient, through the biological membranes and into the cells (Steenge *et al.*, 1998; Wyss and Kadurrah-Daouk, 2000; Persky and Brazeau, 2001).

Creatine is stored in two forms in the body, namely free creatine (FCr) (40%) and phosphorylated creatine, better known as phosphocreatine (PCr) (60%). Total

body creatine (both FCr and PCr) yields about 120 grams of creatine in a 70kg individual. Ninety-five percent of creatine is stored in skeletal muscle (Williams *et al*, 1999; Persky and Brazeau, 2001).

2.3 Placental crossing of creatine

As previously mentioned, the liver, pancreas and kidney are primary sites of production of creatine (Horn *et al.*, 1998). It was, however, recently discovered that high levels of AGAT were also present in the rat deciduoma (Wyss and Kadurrah-Daouk, 2000). In addition Sandell *et al.* (2003) observed that GAMT expression was highest in mouse embryonic and extraembryonic tissue from conceptuses between embryonic day E7.5 and E9.5. Sandell *et al.* (2003) compiled an expression profile of E10.5-E18.5 embryos (just embryonic tissue). GAMT expression was found to be very low in these tissues at E10.5 while the expression gradually increased in the embryo until day 17.5, at which stage the embryonic and placental GAMT expression became uniform (Sandell *et al.*, 2003).

The placenta is thus a production site of creatine (AGAT and GAMT expression)(Sandell *et al.*, 2003). In addition, creatine is said to readily cross the placenta into the foetal blood system (University of Michigan, Laboratory notes, unpublished data).

2.4 Metabolic functions

Creatine is well known as a supplement that increases muscle mass (see section 3), power, and enhances sport performance (Mujika and Padilla, 1997; Wyss and Kadurrah-Daouk, 2000; Persky and Brazeau, 2001; Brudnak, 2004). These effects may occur through creatine's involvement in several metabolic functions. The most common metabolic functions in which creatine is involved, are buffering, stabilization of the mitochondrial membranes, axonal elongation, insulin sensitivity, GLUT4 upregulation, as well as in the production of energy. During the production of energy, adenosinediphosphate (ADP) and PCr form adenosinetriphosphate (ATP) and Cr. Energy is set free as ATP loses an inorganic phosphate (Pi) through the enzyme ATPase, thus forming ADP and Pi (see section 1 for mechanism).

$$ADP + PCr \rightarrow ATP + Cr \xrightarrow{ATPase} ADP + Pi$$

Due to the degradation of PCr to Pi and creatine, the ATP pool has an effective turnover of several dozen times when ATP breaks up into ADP +Pi, releasing massive amounts of energy during high-intensity exercise (Campbell, 1995).

Daily degradation of creatine to creatinine is about 1.6% (Stöckler, 1997). This is a simple, irreversible reaction that occurs spontaneously inside the muscle

(Clark, 1997; Crim *et al.*, 1975, 1976). Creatinine enters the bloodstream and is filtered out through the kidneys and excreted in the urine (Greenhaff, 1997).

3. EFFECTS OF CREATINE SUPPLEMENTATION ON BODY MASS AND COMPOSITION

An increase in body mass is one of the effects of creatine supplementation (Guzik *et al.*, 2000; Wyss and Kadurrah-Daouk, 2000). Creatine is an osmotically active substance (Williams *et al.* 1999) such that an increase in the creatine concentration of the cell will result in an increase of water in the cell (Volek *et al.*, 1997a; 1997b; Ziegenfuss *et al.*, 1998; Wyss and Kadurrah-Daouk, 2000; Brudnak, 2004). This could explain the rapid increase in body weight following exogenous creatine supplementation. It has also been shown that an increase in intracellular water may stimulate protein synthesis or decrease protein degradation (Clark, 1997; Ingwall, 1996; Mujika and Padilla, 1997; Volek and Kraemer, 1996; Volek *et al.*, 1997a; 1997b).

Normal values for creatine in muscle are 115 – 120 mmol/kg dry muscle (determined by the snap-freezing technique) (Harris *et al.*, 1992). This concentration in muscle can increase to 160-mmol/kg dry muscle (Hochachaka and Mossey, 1998), which is the muscle threshold for creatine following exogenous creatine intake.

The human population can be divided into three groups: a sedentary group, a physically active group and a highly trained group of athletes. In experiments carried out to determine the effect of short-term creatine supplementation, subjects were chosen from these three population groups as well as matched control groups. The sedentary subjects (0.35g creatine/kg body weight for 5 days) showed a significant increase in body mass following exogenous creatine supplementation although they were not physically active (Ziegenfuss *et al.*, 1998). The physically active group (25g/day for 6 days, (0.35g creatine/kg bodyweight)) showed significant increases in body mass, while the control group had a decrease of body mass due to the rigorous training (Balsom *et al.*, 1993). The highly trained athletes also showed a significant increase in body mass. This gain in body mass was achieved with relatively small doses of creatine (20g/day for 5 days, 0.285g creatine/kg bodyweight) (Mujika *et al.*, 1996)

4. REPORTED SIDE EFFECTS

Anecdotal reports indicate that creatine may cause gastro-intestinal distress (stomach upset, diarrhoea), muscle cramping and muscle injury. However, Williams *et al.* (1999) reported that there is no scientific evidence to substantiate these findings. In a previous study carried out by Badenhorst and Vorster (2000, unpublished data), creatine caused growth retardation in the developing chick embryo. Abnormalities included degeneration of the retina and sclera and

massive subcutaneous haemorrhaging. The ventral thoracic wall failed to close, leaving the heart exposed.

5. MEDICAL USES OF CREATINE

Although creatine is mainly used for weight gain and muscle building, research has shown that creatine can be used as a supplement to aid in the recovery of certain disorders. Creatine is also said to have an effect on degenerative muscle diseases and loss of neural function during the aging process (Williams *et al*, 1999, Wyss and Kadurrah-Daouk, 2000).

5.1 Heart disease

Creatine has the ability to influence cardiac and neural functions. Research has been carried out to determine the effect of creatine on myocardial metabolism and degenerative diseases of the myocardium i.e. heart failure, ischemia and ventricular arrhythmias (Conway *et al.*, 1996; Saks *et al.*, 1996). Improved myocardial metabolism and the reduction of incidence of ventricular fibrillation in ischemic heart patients were found when PCr was intravenously administered (Constantin-Teodosiu *et al.*, 1995; Conway *et al.*, 1996; Andrews *et al.*, 1998). The explanation for this was that the viability of the ischemic cell membrane is improved by the administration of exogenous creatine, thus minimizing injury to the cell during ischemia (Saks *et al.*, 1996). All the abovementioned researchers

concluded that intravenous PCr injections offer an effective cardioprotective function in patients that suffer from ischemic heart disease.

5.2 Tumour growth inhibition

Miller *et al.* (1993) evaluated the effects of creatine and cyclocreatine on the growth rates of rat mammary tumours and sarcoma in male rats. In addition, Miller *et al.* (1993) evaluated the growth of tumour cells, which were injected subcutaneously in mice. They found that administration of cyclocreatine to the affected animals delayed the appearance and growth of the tumour cell populations subcutaneously in rats and mice, but concluded that additional research was necessary to explore its potential medical benefit.

Wyss and Kadurrah-Daouk (2000) found that cyclocreatine acts as a tumour repressor or anti-tumour agent (Persky and Brazeau, 2001). Cell lines with high creatine kinase concentrations were not inhibited following exposure to cyclocreatine (Wyss and Kadurrah-Daouk, 2000). It was speculated that in tumour cells, cyclocreatine did not cause excessive water influx into the cells; thus cyclocreatine acts as an efficient anti-tumour agent (Wyss and Kadurrah-Daouk, 2000). Wyss and Kadurrah-Daouk (2000) maintain that creatine can induce tumour growth; whereas creatine analogs can inhibit tumour growth.

5.3 Creatine synthesis deficiencies

Due to an inborn error in GAMT production (Wyss and Kadurrah-Daouk, 2000), some babies are unable to synthesize creatine endogenously (Arias-Mendoza *et al.*, 1998). These creatine-deficient babies displayed significantly decreased motor and neural functions (Wyss and Kadurrah-Daouk, 2000), which in turn caused abnormal mental, neuromuscular and physical maturation (Stöckler *et al.*, 1997). When oral creatine supplementation (for up to 25 month's duration) was given to these babies, they experienced normal physiological and mental development (Stöckler *et al.*, 1994; 1996a; 1996b; Stöckler and Hanefeld, 1997).

6. AIM OF STUDY

It has been shown that many female athletes take exogenous creatine supplementation to enhance their performance (Wyss and Kadurrah-Daouk, 2000; Persky and Brazeau, 2001). If the athlete was to become pregnant, and due to the ability of creatine to cross the placenta (University of Michigan, laboratory notes, unpublished data), as well as the placenta being a site for endogenous creatine synthesis, creatine may have an influence on the developing embryo/foetus. In a previous study carried out by Badenhorst and Vorster (2000, unpublished data), it was observed that creatine delayed development and growth in chick embryos. Creatine also induced abnormalities such as ectopia cordis and subcutaneous haemorrhaging.

The aim of the present study utilizing a mammalian model in order to mimic the human condition, was thus:

1. To investigate whether exogenous creatine (creatine supplementation) has an effect on the development of the rat embryo and/or foetus;
2. To determine whether abnormalities occur following either isolated intake or prolonged exposure to creatine;
3. To determine if abnormalities (should they occur) are dose-dependant.

7. MATERIAL AND METHODS

7.1 Rationale for the use of Sprague-Dawley rats

The rat has several advantages for embryological study, especially when a teratogen is involved. Some of the advantages of working with rats are that they are relatively disease resistant, have a short reproductive cycle (with well documented staging of the developing foetus), and the foetuses are large enough to work with conveniently. Sprague-Dawley rats were selected for this particular study, as they have a low spontaneous rate of malformation. In addition, Sprague-Dawley rats are freely available.

7.2 Rat breeding and maintenance

Four fertile male and forty-four female Sprague-Dawley rats were used for this study. The rats were obtained from the Central Animal Unit of the University of the Witwatersrand, (Animal ethics clearance number 2003/48/3)(Appendix A). Female rats were housed (four rats per cage) in the Central Animal Unit, with a controlled temperature of 21 ± 1°C and 50 – 60 % relative humidity. A 12-hour light: 12-hour dark cycle was maintained. Their diet consisted of water and rat cubes *ad libitum*.

7.3 Determination of pregnancy

Vaginal smears were carried out daily to determine the stage of the oestrus cycle of each animal. Swabs for vaginal smears were made of toothpicks that were rolled in cotton wool. The swabs were smaller than ear bud swabs and thus the risk of injury or discomfort to the animal was greatly decreased.

After the vaginal smear was taken, the sample was smeared on to a glass slide. The smear was then fixed with alcohol and immersed in Shorr's stain (Drury and Wallington, 1980)(Appendix B).

The smears were immersed in the staining solution for one minute and were then washed in 70% ethanol. The smears were dehydrated through a graded series of

alcohols (95%, 95% and 100%) and placed in Xylene. Coverslips were mounted with Entellan.

The smears were viewed with a Zeiss light microscope. The smear was classified, according to Kent's staging (1945), as being either in the oestrus, metoestrus, early dioestrus, mid-dioestrus, late dioestrus or in the proestrus part of the oestrus cycle.

Females were allowed to progress through three normal 4-day oestrus cycles and were then classified as ready for mating. On the day before oestrus of their 4^{th} cycle, a female was placed in a breeding cage (drop tray) with a fertile male. The animals were caged overnight. The presence of a vaginal plug the following morning indicated that copulation had taken place. A vaginal smear was made to ascertain whether any sperm were present. The day the vaginal plug was found, as well as positive evidence of sperm in the vagina when a vaginal smear was taken, was recorded as day 0 of pregnancy.

7.5 Experimental groups

Forty-two dams were divided into six groups.

1 Control groups: A (n=7) and B (n=7). These animals were given an intraperitoneal injection of a solution of 0.9% sterile NaCl. This solution

was also the vehicle that was used to deliver the creatine to the experimental groups. (For times of injections, see section 7.4.1).

2 Experimental groups: A1, A2, B1 and B2

a) A1 (n=7) and B1 (n=8): Animals in these groups were injected intraperitoneally with 53.5 mg creatine/250 g body weight made up in a 0.9% sterile NaCl saline solution (also referred to as 15g/day or low dose group (see sections 7.4 and 7.5.1 for rationale)).

b) A2 (n=7) and B2 (n=8): Animals in these groups were injected intraperitoneally with a solution 107 mg creatine/250 g body weight made up in a 0.9% sterile NaCl saline solution (also referred to as 30g/day or high dose group (see section 7.4 and 7.5.1 for rationale)).

Groups will now be referred to as control group A or B and experimental groups A1, A2, B1 and B2.

7.5. Dosage of creatine

Dosages were calculated from the amount of creatine consumed by a normal physically active person (70kg male)(Feldman, 1999). The dosage was then recalculated to the amount that an adult female rat of approximately 250 grams would consume. Women athletes usually take a loading dose or a maintenance

dose of creatine for at least a week. Dosages were calculated to a normal maintenance dose (15g/day) or a loading dose (30g/day)(Feldman, 1999).

One millilitre of the creatine-saline solution was injected intraperitoneally into the experimental and control groups. Usually, when exogenous creatine is ingested, the creatine is made up in 500ml of water per 70 kg individual. Recalculation to rat weight estimated that an amount of 1.7ml of the solution would be needed. After careful deliberation with the veterinarian in the Central Animal Unit (Dr. L. Sinclair), it was decided that approximately 2 ml of the solution might cause intestinal distress and intraperitoneal discomfort to the animal, thus jeopardizing the experiment. A volume of 1 ml of the creatine-saline solution was thus decided on. This would deliver sufficient creatine for the purpose of the experiment.

7.5.1 Rationale for the dosages used

Creatine is fully soluble in saline. The 53.5mg/250g body weight dose was chosen as it is equivalent to a maintenance dose of 15g/70kg in the daily training of athletes (Wyss and Kadurrah-Daouk, 2000, Persky and Brazeau, 2001; Brudnak, 2004). The 107mg/250g body weight is equivalent to a loading dose used by athletes when they wish to boost their creatine levels (30g/70kg daily) (Wyss and Kadurrah-Daouk, 2000, Persky and Brazeau, 2001; Brudnak, 2004). Control group A, as well as experimental groups A1 and A2, were injected consecutively from day 7 to 13 of embryonic development (for rationale of time

periods, see section 7.5.3). Control group B, as well as experimental groups B1 and B2, received two injections: the first on day 9, and the following on day 11 of embryonic development. Days 9 and 11 were chosen due to the critical stages of organogenesis, which occur at this time (see section 7.5.3).

7.5.2 Route of administration

Usually creatine is ingested orally, but in the current experiment, an intraperitoneal injection of the solution was used. The rationale for using an intraperitoneal route rather than an oral route was: 1) it would be difficult to determine the amount of creatine that the rat ingested if the creatine was mixed with the food and 2) intraperitoneal injections were favoured as the solution would be absorbed into the bloodstream relatively fast. The intact molecule of creatine passes through the intestinal wall when ingested orally and thence into the portal hepatic system that would take it directly to the liver (Wu and Meininger, 2000; Wyss and Kadurrah-Daouk, 2000). With intraperitoneal injections, the injected creatine would reach the liver through the hepatic portal vein.

7.5.3 Organogenesis on selected days of rat embryo/foetus development with particular reference to specific organs and structures

Days 7-13 (group A) and days 9 and 11 (group B) of embryonic development were chosen due to specific development of critical organs at this time (Baker *et al.*, 1980). See Appendix D for events of organogenesis during these stages of development.

7.6 Sacrificing of animals

All dams were killed on day 20 of embryonic development. As birth occurs on day 21, and in order to avoid the killing of individual foetuses, day 20 was selected for termination of pregnancy. All the major organs will have reached completion by this time. An intraperitoneal injection of Sodium Pentobarbitone (Eutha-nase) (1ml/350gram rat) was injected into the dams.

7.7 Collection and processing of foetuses

A mid-abdominal section was made and the uterine horns were exposed and excised. The number of foetuses present was counted, as well as the number of resorbed foetuses. The foetuses were then dissected free of the uterine horns and of their placentas. Measurements were carried out to determine if prolonged doses of creatine affected overall growth of the foetuses. The weight of each

foetus was estimated to the nearest 0.05g using a Sartorius scale. Crown-rump (length) measurements of each foetus were taken. A vernier calliper (calibrated to read 0.05 of 1 mm) was used for this measurement. All foetuses were examined with a stereomicroscope to see whether any obvious external abnormalities were present.

7.7.1 Preparation of foetuses for light microscopy

Foetuses were placed in Allen's fixative (Gray, 1953)(See Appendix B for method) for at least 72 hours. Fixed foetuses were immersed in a decalcification solution (Bancroft and Gamble, 1998). The decalcification solution consisted of a 90% formic acid-formalin solution (Bancroft and Gamble, 1998) into which foetuses were placed for three days. Foetuses were then put into a fresh solution of 90% formic acid-formalin at the beginning of the fourth day, for a further two hours. The fresh solution will remove any calcium that is still present in the bone and cartilage. An "endpoint" test was carried out to determine whether all the calcium had been removed (Bancroft and Gamble, 1998). This was done by slowly adding concentrated ammonia to 5ml of the 90% formic acid-formalin solution, slowly until a pH of 7 was reached (determined by using litmus paper). If there was no cloudiness in the solution, this meant that all the calcium had been removed from the foetuses. The foetuses were then ready for processing for light microscopy, which was carried out in a Sanford tissue-processing machine. The processing machine was programmed to take the foetuses through a graded

series of alcohol starting with 75% alcohol (2hrs), 90% alcohol three times (3hrs each time) and then three changes of absolute alcohol (3hrs each change). The foetuses were immersed in chloroform twice (4hrs each change) and lastly placed into warm paraffin wax twice (6hrs each). After processing, the foetuses were embedded in fresh paraffin wax.

Foetuses were sectioned on a sledge microtome at five μm. Sections were then placed on clear glass slides and dried in an oven for two hours at 90°C. Prior to staining, sections were dewaxed in xylene for 5 minutes. The sections were dehydrated through a graded series of alcohols and then washed in running water (10 min) to remove the picric acid residue (yellow pigment) in the sections. The sections were then placed in haematoxylin (5 min), washed in water (30 seconds), differentiated in acid alcohol (3 dips) and again rinsed in water. Sections were immersed in eosin (1min), rinsed in running water, and taken through a graded series of alcohols to dehydrate. The sections were then placed in xylene. Entellan was used to mount the coverslips on the sections. Sections were viewed using a Zeiss light microscope to determine any histological abnormalities.

7.7.2 Skeletal staining using Alizarin red S and Alcian Blue

Foetuses from both the control and experimental groups were used (n=44). An Alizarin red S and Alcian Blue technique (Menegola *et al.*, 2001)(Appendix B)

was used to demonstrate development of cartilage and bone in the foetus. After the foetuses had been weighed and their length taken, they were placed into a solution of 4% NaCl overnight. The following day the foetuses were taken out of the solution, eviscerated, and skinned. They were then immersed in an acidic staining solution (ph 2.7)(Alcian blue (Sigma Aldrich) and Alizarin red S (Sigma Aldrich)) for 24 hours, followed by 96% ethanol to dehydrate. Foetuses were then placed in a basic staining solution of 0.7% KOH and Alizarin red S for a further 30 hours. The basic staining solution caused maceration of the soft tissues. A clearing solution, which consisted of 70% ethanol, glycerine and benzyl alcohol was used. The foetuses were then preserved in a 70% alcohol: glycerine solution.

7.7.3 Immunocytochemistry (ICC)

One section of each pancreas was stained with haematoxylin and eosin for routine histological examination.

This section underwent dewaxing in xylene. Normal haematoxylin and eosin histological staining technique was followed (see section 7.8.1). The section from each group was then examined with a light microscope to confirm that the tissue was present. After confirmation of pancreatic tissue, the other sections then underwent immunocytochemistry. Immunocytochemical localization for insulin

and glucagon was carried out on pancreatic tissue from randomly selected foetuses of each control and experimental group (n=4/group).

Sections of pancreatic tissue were cut (5μm) and placed on clean glass slides and allowed to dry for two hours at 90°C in an oven. An indirect immunoenzyme procedure (see Appendix B) was used to detect whether α - and β-cells were present. Insulin antiserum (Euro Diagnostech)(1:800) was raised to detect any immunoreactivity against natural guinea-pig insulin, while glucagon antiserum was raised against natural rabbit glucagon (Euro Diagnostech)(1:1000) to test for immunoreactivity against glucagon. Antisera (30μl/section) were applied to 12 randomly selected sections of each pancreas respectively.

Sections for immunolocalization were dewaxed in xylene and hydrated through a graded series of alcohols. All the sections were then immersed in distilled water. After rinsing the sections at least twice in distilled water, the sections were then placed in Tris/saline (see Appendix B). The sections were submerged in a 3% hydrogen peroxide/distilled water solution and left to stand for 10 minutes. The sections were washed again for 5 minutes in Tris/saline. Non-specific localization was then blocked by adding 10% swine serum (see Appendix B) on to the sections. The sections were then left in a damp chamber for 10 minutes (room temperature). Sections were drained of swine serum and were carefully wiped around the section to remove all the remaining serum. The sections were then

incubated with 30 μl primary antibody at a concentration of 1: 800 for insulin (Euro Diagnostech) and 1:1000 for glucagon (Euro Diagnostech) at 4^0C.

The following day the sections were drained and washed for 15 minutes in 1% horse serum (3x5min, each time renewing the solution). Sections were drained of the horse serum and placed in the damp chamber. The secondary antibody was then pipetted onto the section. Anti-guinea pig secondary antibody (1:20)(DAKO) was used for insulin, while anti-rabbit secondary antibody (1:20)(DAKO) was used for those sections that were immunolocalized with glucagon. The sections were then left in the damp chamber for 1 hour (room temperature). Sections were drained again and washed for 15 minutes (3x5min) in a Tris/saline solution, renewing the solution every 5 minutes. Sections were then incubated with a diaminobenzidine (DAB) solution (see Appendix B) and timed individually for 5 minutes in order to avoid background overstaining. Sections were then placed in distilled water and immersed in gently running tap water for a further 5 minutes. Sections were then dehydrated through a graded series of alcohols and cleared in xylene. Coverslips were mounted with Entellan.

7.7.3.1 Immunocytochemical controls

Absorption controls were established by substituting the primary antisera with the primary antiserum which had been pre-absorbed with its own antigen (natural guinea pig insulin at 20μg/ml, Euro Diagnostech; natural rabbit glucagon at 40μg/ml, Euro Diagnostech) overnight at 4°C. Sections adjacent to sections showing immunolocalization for insulin and glucagon respectively, were then incubated with the antibody-antigen complex for one hour at room temperature.

Sections adjacent to the sections that showed immunolocalization of insulin and glucagon were used as negative immunocytochemical controls. In these sections, either the primary antiserum or secondary antiserum was substituted with Tris/Saline or a nonimmune serum of the relevant species.

Sections of pancreas from an adult female rat were used as positive controls in this study. After the immunocytochemical procedure, definite α- and β- cell immunolocalization could be observed in the pancreatic sections.

7.8 Statistical methodology (see Appendix E for raw data, calculations and graphs)

In order to determine whether the data was normally distributed, the weight and length of foetuses were sorted from the smallest value to the biggest value in

each group and the frequency determined. The interval in the weight category was equal to one (one gram interval), while an interval of five was used with the calculation of the length frequency. From the frequency table, an observed value could be determined, while an expected value was calculated using the formula $y=(N \times i/sd\sqrt{2\Pi})e^{-(x-m_x)2/}2sd^2$ (personal communication Prof J.C. Allan). The observed and expected values were then used to draw graphs and to determine whether there was a normal distribution in the data presented. χ^2-tests were also carried out to determine if there were significant differences between the observed and expected values. These tests showed that there was no significant difference in values in the length group (see section 8.6.2). It was thus decided on the advice of a statistician (Prof. J.C. Allan) to use Students "t"-test to determine whether there were significant differences between the experimental groups. As the chi-square test showed a significant difference in the normal distribution for weight between the observed and expected values, it was decided to make use of a non-parametric test. The Mann-Whitney test for large samples of uncorrelated data was used to determine if there were any significant differences between the experimental and control groups in relation to weight.

7.9 Photography

Photos of abnormal macroscopic development were taken with a digital camera. Unfortunately a scale was not included in the photographs.

8. RESULTS

8.1 General

Three hundred and forty foetuses were collected from 40 pregnancies. One hundred and sixteen absorption sites were found. Four out of forty dams were pseudopregnant. Pseudopregnancy, as defined by Salamonsen *et al.* (1996), is when an animal exhibits all the necessary signs of pregnancy (vaginal plug, weight increase), but when killed, has no foetuses or absorption sites present.

8.2 Surviving foetuses vs. absorbed foetuses

Three hundred and forty foetuses were collected in total. The percentage of surviving foetuses vs. absorbed foetuses across all the groups is 25.4%. The highest number of absorption sites was found in the B2 experimental group (high dose, injected days 9 and 11). More absorption sites were found in the B-groups (experimental) when compared to the A-groups (experimental). Figure 2 depicts the survival: absorption ratio for the different groups.

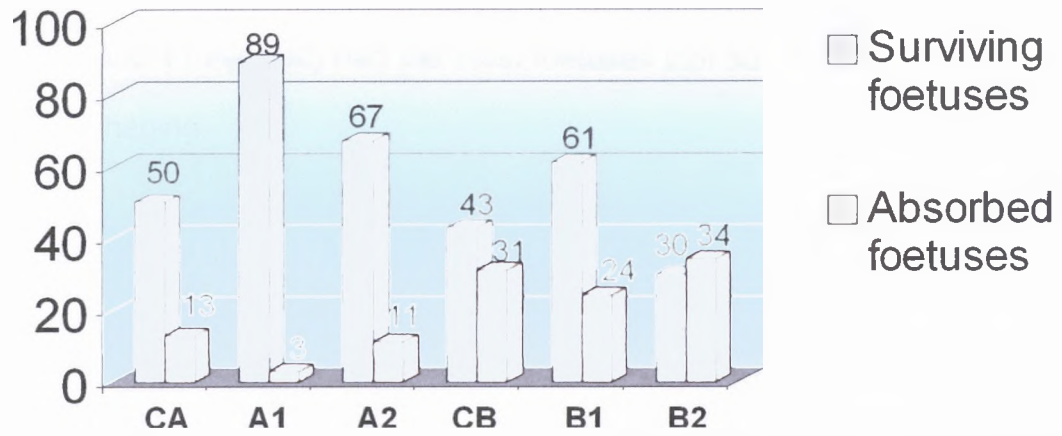


Figure 2. Number of surviving foetuses vs. absorbed foetuses in control and experimental groups.

In the A-group (CA, A1 and A2)(injected for 7 days consecutively) the respective percentages were 21% (13/63) absorbed foetuses (control, saline), 3% (3/92) absorbed foetuses (A1, low dose) and 14% (11/78) of foetuses absorbed (A2, high dose). In the B-groups (CB, B1 and B2)(injected on days 9 and 11 of embryonic development), there was a marked increase in the number of absorbed sites. Control group B showed a 42% (31/74)) absorption percentage, while the two experimental groups (B1 (low dose) and B2 (high dose)) each showed a 28% (24/85) and 53% (34/64) absorption rate respectively.

8.3 Subcutaneous haemorrhaging and underdevelopment of foetuses

Foetuses with severe subcutaneous haemorrhaging (figure 3) were observed, particularly in the groups that were treated with creatine. The number of foetuses observed in each group which suffered from subcutaneous haemorrhaging was 2/50 in control group A (4%), 1/89 in A1 (1.1%), 2/67 in A2 (3%), 1/43 in control group B (2%), 4/61 in B1 (6.5%) and 2/30 in group B2 (6.6%) (figure 4). Of the

groups that were treated with creatine, the B1 experimental group (low dose, days 9 and 11 injected) had the most foetuses that suffered from subcutaneous haemorrhaging.

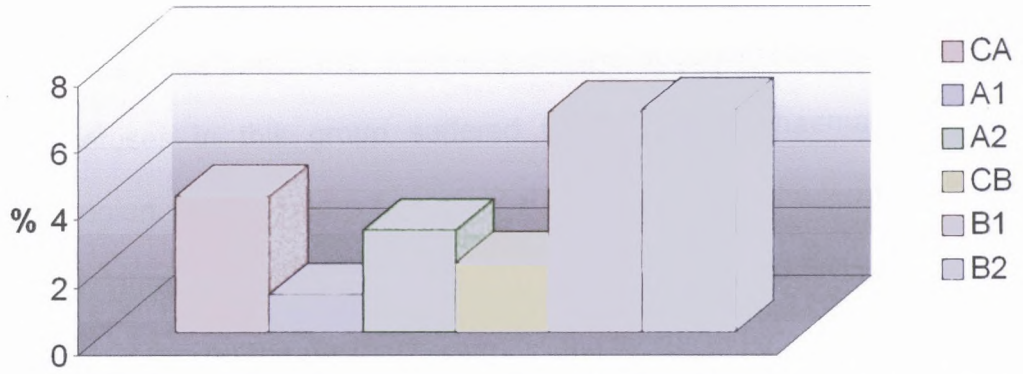


Figure 4: Subcutaneous haemorrhaging in percentage of foetuses from control and experimental groups.

In one animal in the B1 experimental group, the whole body of the foetus was covered with a subcutaneous haematoma which stretched from the pelvic area to the cranial region. In severe cases, the haematoma covered the whole of the cranium as well (figure 5).

8.4 Macroscopic abnormalities

8.4.1 Control group A (saline, consecutive injections)

This group contained fifty foetuses. Two foetuses from this group were found to suffer from subcutaneous haemorrhaging. In addition, 14 underdeveloped (underdeveloped = behind normal developmental stage, weighed significantly

less than group mean and was of a shorter length than the mean length of the group) were noted. The most severely affected foetus displayed crystal-like structures in the abdominal area (figure 6).

8.4.2 Experimental group A1 (low dose, continuous injections)

This group had one foetus that showed extensive subcutaneous haemorrhaging. All the foetuses in this group suffered from extensive haematomas in the temporal region. Figure 7 depicts abnormal development of the temporal artery in one foetus. Normally the temporal artery would be anterior to the ear and runs in a vertical direction on the desmocranium. In the foetus in figure 7, the artery runs posterior to the ear and in a more horizontal direction on the desmocranium. The abnormal development of this artery may be related to a temporal haematoma.

This experimental group also had a higher incidence of underdeveloped foetuses when compared to the other experimental groups and control group B. Group B1 (low dose, days 9 and 11 injected) and control group A (saline, continuous injections) had the most underdeveloped foetuses.

8.4.3 Experimental group A2 (high dose, continuous injections)

Although this group showed the highest mean weight (4.797 grams) of all the experimental groups (groups A1, A2, B1 and B2)(see Appendix C), it was found that this group also had the most severe subcutaneous haemorrhaging (figure 8) which tended to spread over the whole of the body of the foetus.

Temporal bleeding in this group was also found to be the most severe of all the experimental groups (figure 9).

Foetuses from this experimental group, which suffered from subcutaneous haemorrhaging, also had a high incidence of crystal-like structures forming on the abdominal surface. When these foetuses were eviscerated, it was observed that the crystal-like structures were also present **in** the abdominal cavity (figures 6 and 10). Crystal-like structures were also found in the temporal region of certain foetuses in this particular group.

8.4.4 Control group B (saline, days 9 and 11 injected)

The foetuses in this group did not demonstrate extensive subcutaneous haemorrhaging. Out of 43 foetuses, haemorrhaging was observed in five foetuses (11.6%). Haemorrhaging was localized to the abdominal area and sometimes extended into the thorax. No temporal or extensive subcutaneous haemorrhaging was observed in the animals of control group B [(compare figure 6; (control foetus with mild abdominal haemorrhaging) to figure 5 (experimental foetus with severe haemorrhaging)].

8.4.5 Experimental group B1 (low dose, days 9 and 11 injected)

This group had 4/61 foetuses displaying extensive subcutaneous bleeding (figures 11 and 12) as well as subcutaneous abdominal haemorrhaging. Two foetuses displayed signs of extensive temporal haemorrhaging (figure 12).

This experimental group had the second most underdeveloped foetuses (figure 13)(19.7%) of all the control and experimental groups; besides control group A (saline, consecutive injections). It was observed that these underdeveloped embryos were at least 3-4 days behind average development with regard to length and weight data compared to control groups at the same stage. Crystal-like structures were also found in one foetus that suffered from extensive subcutaneous haemorrhaging.

8.4.6 Experimental group B2 (high dose, days 9 and 11 injected)

Experimental group B2 had two foetuses (2/30; 6.7%), which suffered from extensive subcutaneous haemorrhaging and subcutaneous haemorrhaging in the abdominal area. Temporal bleeding was observed in one foetus. One foetus from the total group (1/340) did not develop a superficial temporal artery (figure 14).

8.5 Statistical analysis

8.5.1 Weight and length averages

Means of weight and length from the foetuses in the different experimental and control groups were calculated. It was found that control group B had the highest overall weight (figure 15), while experimental group A2 had the highest overall length of all the groups (figure 16).

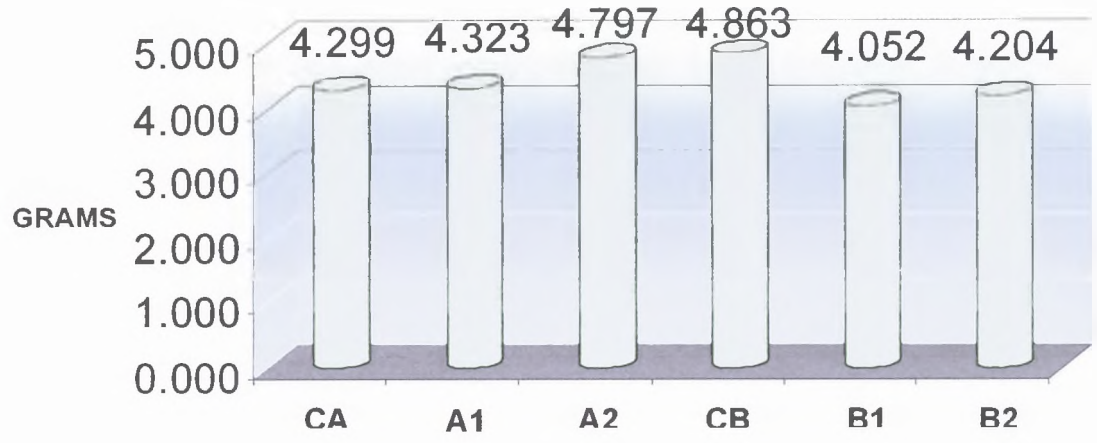


Figure 15. Average weight of foetuses in experimental groups.

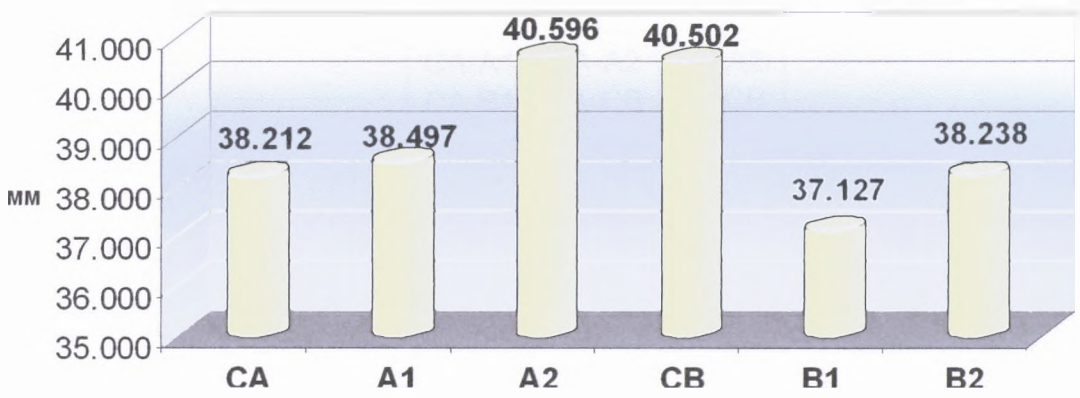


Figure 16. Average length of foetuses in experimental groups.

Experimental group B1 had both the smallest mean weight and length, while control group A and experimental group B2 had almost similar values in both the weight and length charts (figures 15 and 16).

8.5.2 Length statistics

Groups were compared (see section 7.9 Statistical methodology) using Student's "t"-test. An α-level of 0.05 ($P \leq 0.05$) was used as an indicator to determine

whether there would be a probable chance of significance between group A and group B. Control groups were compared with their respective experimental groups. When compared, it was found that CA-A1 groups were not significantly different (P>0.05). Groups CA-B1, CA-B2, A1-B2, A2-CB and B1-B2 were also not significantly different. Groups CA-A2, CA-CB and B2-B1 had a P≤0.01 value, while the groups A1-A2, A1-CB, A1-B1, A2-B1, A2-B2, CB-B1 and CB-B2 all had a significant P value either equal to 0.001 or less than 0.001. This would mean that there is an almost certain probability that there is a significant difference between the groups just described. (Refer to table 1).

P≥0.05	P≤0.01	P≤0.001
CA-A1	CA-A2	A1-A2
CA-B1	CA-CB	A1-CB
CA-B2	B2-B1	A1-B1
A1-B2		A2-B1
A2-CB		A2-B2
B1-B2		CB-B1
		CB-B2

Table 1. Groups in each column that were not statistically significant or were statistically significantly different pertaining to length

8.5.3 Weight statistics

The foetuses from the control and experimental groups were compared with regard to their weight (refer to table 2). Groups which had a P<0.05 were CA-A1, CA-CB, CA-B1, CA-B2, A1-B1, A1-B2, A2-CB and B1-B2 (refer to table 2).

CA-A2 and CB-B2 were between 0.05>P>0.01. The groups A1-CB, A2-B2 and CB-B1 all had a p-value that was smaller than 0.01, but bigger than 0.001

(0.01>P>0.001). Groups A1-A2 and A2-B1 had P-values smaller than 0.001 (P<0.001).

P>0.05	P<0.05	P<0.01	P<0.001
CA-A1	CA-A2	A1-CB	A1-A2
CA-CB	CB-B2	A2-B2	A2-B1
CA-B1		CB-B1	
CA-B2			
A1-B1			
A1-B2			
A2-CB			
B1-B2			

Table 2. Groups in each column that were not statistically significant or were statistically significant pertaining to weight.

8.6 HISTOLOGICAL STUDY OF PARTICULAR ORGANS

A histological study was undertaken on the kidney, skeletal muscle, liver and pancreas. These organs are active in creatine synthesis, creatine transport or utilization of creatine in the adult. Any effects that may occur in these organs/tissues in the developing foetus would be due to the administration of creatine.

8.6.1 Kidney

When kidney sections from the different groups were compared at the light microscopic level, it was found that there were no differences in tissue or cellular structure between the control and experimental groups, whether they were injected consecutively or only on the two selected days. A cortex and medulla

could be clearly distinguished. The nephrons and collecting duct system appeared normal (figure 17).

8.6.2 Skeletal muscle

Skeletal muscle had undergone normal development in the foetuses of control groups A (figure 18) and B (figure 19) for that particular stage of development. Skeletal muscle from experimental group A1 (low dose, continuous injections) showed similar features to that of the control animals. The striation pattern (experimental group A1, figure 20) was very clear and at the same stage of development as that of the control group. However, when the muscle of experimental animals in group A2 (high dose, continuous injections) was compared with the muscle of control animals and of experimental animals (group A1), it was observed that the skeletal muscle fibres appeared to be more densely "packed" than in both the control group and experimental group A1. It appeared that there were more fibres per section area in animals from experimental group A2 (quantitation not carried out). There also appeared to be more striations in the muscle fibres from experimental group A2 than from group A1 or the control group (figure 21). Muscle fibres from the foetuses of the B1 (low dose, day 9 and 11 injected) (figure 22) and B2 (high dose, day 9 and 11 injected)(figure 23) experimental groups appeared to be immature when compared to control groups A and B. Banded striation patterns (experimental group B1) were visible, but were not as densely compacted compared to the A-groups (experimental). The muscle fibres from foetuses form both the B1 and B2 groups (experimental) also

appeared disorganized. Striations in muscle from the foetuses of the B2 experimental group were faint to almost non-existent, when compared to control groups A and B and experimental groups A1, A2 and B1.

8.6.3 Pancreas

Absorption (figure 24), negative (figure 25) and positive (figure 26) immunocytochemical control gave satisfactory results indicating that the antisera used were specific.

In control groups A and B, large islets containing both insulin-secreting cells (figure 27) and glucagon-secreting cells (figure 28) were observed. These had the typical mammalian pattern of a mantle of α-cells and a core of β-cells. In experimental group A1 (low dose, continuous injections), the α- and β-cells were not as intense as in the α- and β-cells in the pancreata of the A and B control groups.

Islets [containing both α- (figure 29) and β-cells (figure 30)] although not quantitated, appeared to be more numerous in pancreata of animals from group A2 (experimental, figure 31), compared to the other experimental and control groups.

In the B1 experimental group, α- and β-cells (figures 32 and 33) displayed intense localization. The B2 experimental group also displayed α- and β-cell

immunolocalization (figures 34 and 35), but this was not as intense as that in control groups A and B and the other experimental groups. Although not quantitated, the B1 group appeared to have the most numerous β-cell population.

8.6.4 Liver

An abundance of liver cord cells (precursors to normal hepatocytes) were observed in control groups A (figure 36) and B. Numerous precursor blood cells were also observed. Sinusoids in the control groups appeared to be relatively wide.

In experimental group A1 (low dose, consecutive injections), there was a general decrease in the number of liver cord cells and the sinusoids were distended (figure 37) compared to the tissues of the control group A. Experimental group A2 (high dose, consecutive injections) also displayed relatively wide sinusoids (figure 38) compared to control groups A and B. Although not quantitated, an increase in liver cord cells was observed in sections of liver from experimental group A2 when compared to liver sections from experimental group A1.

Sinusoids in experimental group B1 (low dose, days 9 and 11 injected) were narrow and filled with erythrocytes (figure 39). Megakaryocytes were also present in the sinusoids. In addition, an increase in the number of blood vessels was observed. None of the control groups or other experimental groups displayed this particular characteristic. A decrease in the number of liver cord cells

(experimental group B1), compared to the controls, and was also noted. Megakaryocytes were also observed in the sections of liver from the B2 experimental group (high dose, day 9 and 11 injected). Liver cord cells (B2 experimental group) appeared disorganized with distended sinusoids between them (figure 40).

8.7 Skeletal development

Following skeletal staining, foetuses were examined with a stereomicroscope. Differences were found between the animals in the control and in the experimental groups.

8.7.1 Desmocranium of the rat foetus

Differences in bone thickness and cartilage formation were observed between the experimental and control groups (Table 3). The most obvious difference in thickness of bone in the skull region was the development and the thickness of the desmocranium. For descriptive purposes, the foetuses were either classified as having "thick" desmocrania (well developed, thick bone) or "thin" desmocrania (less well developed, thin bone). In control group A all the foetuses displayed thin desmocrania (figure 41) (100%) (4/4)(Table 3), while 62.5% (5/8) of foetuses in control group B displayed thin desmocrania. Two foetuses from control group B also displayed signs of immature bone development (bone did not develop). Group A1 (experimental) had 73% (11/15)(Table 3) of foetuses with thin

desmocrania, while the remaining 27% (4/15) of foetuses displayed thicker bone of the desmocranium (figure 42). Experimental group A2 (high dose, continuous injections) displayed 60% (3/5) of foetuses with thick desmocrania, while the remaining 40% (2/5) of foetuses had thin desmocranial plates. In group B1 (experimental) 4/8 foetuses (50%)(Table 3) were classified as having thick desmocrania. One foetus (experimental group B1) showed very immature bone development in the cranial region (figure 43). In experimental group B2, 100% (4/4) of foetuses displayed thick desmocranial plates.

8.7.2 Cartilage development in the nose

None of the foetuses in control groups A and B had cartilage present in the nasal region. In foetuses from experimental groups, it was observed that cartilage was present in the nose. Foetuses with cartilage were from experimental groups A1 (13%), A2 (40%) and B2 (75%). No foetuses in experimental group B1 displayed signs of cartilage development in the nose.

	CA	A1	A2	CB	B1	B2
DESMOCRANIUM:						
THIN	100	73	40	62.5	50	0
THICK	0	27	60	37.5	50	100
CARTILAGE DEVELOP-MENT IN THE NOSE	0	13	40	0	0	75
PRESENCE OF CARTILAGE:						
INTENSE	0	0	40	0	0	75
MODERATE	0	47	20	37.5	0	25
EXTREMITIES	0	40	0	25	0	0
NO STAINING	100	13	40	37.5	100	0
VERTEBRAE:						
ALMOST FUSED	0	53	100	25	62.5	100
WIDE OPEN	100	47	0	75	37.5	0
IMMATURE BONE	0	7	0	25	50	0

Table 3. *Summary of bone and cartilage development in control and experimental foetuses.*

8.7.3 Presence of cartilage in the skeleton of the developing foetus

Cartilage development was divided into four categories. The foetuses were classified as having cartilage present throughout the axial skeleton (figure 44), present in pelvis, ribs and sternum only, present in the extremities or no cartilage present at all (figure 45).

No cartilage was present in foetuses of control group A. Thirty seven and a half percent (3/8) of foetuses in control group B had no cartilage in the axial skeleton, while 37.5% (3/8) had cartilage in the ribs. The remaining foetuses (25%, 2/8) in

control group B showed cartilage in the lower limbs only. Forty-seven percent (7/15) of foetuses in experimental group A1 (low dose, continuous injections) presented with cartilage in the ribs, while 13% (2/15) of foetuses did not exhibit any cartilage development. The remaining 40% (6/15) of foetuses in the A1 experimental group only had cartilage in the distal parts of the limbs. In experimental group A2, 40% (2/5) of foetuses presented with cartilage development in the whole axial skeleton, while another 40% (2/5) of foetuses presented with no cartilage. The remaining 20% (1/5) presented with cartilage in the ribs. The remaining foetuses of this group showed no cartilage development. In experimental group B1 (low dose, days 9 and 11 injected) 100% (8/8) of foetuses showed no cartilage development. In experimental group B2 (high dose, two injections) 75% (3/4) of foetuses showed cartilage throughout the whole body, while the remainder of foetuses (25%, 1/4) presented with cartilage in the ribs only.

8.7.4 Vertebrae

The vertebral columns of all of the foetuses were compared to see whether creatine had an effect on their development. Foetuses were divided into either 1) an almost fused vertebral column (where the pedicles of the vertebral body were almost fused to form the neural arch)(figure 46) or 2) a wide/open column (figure 47) in which the pedicles are widely seperated.

In control group A, the pedicles of the vertebral column were widely spaced in all of the foetuses (4/4). Twenty five percent (2/8) of foetuses in control group B, 53% (8/15) of foetuses in the A1 group, 62.5% (5/8) of foetuses in B1 group and 100% of foetuses in A2 (5/5) and B2 (4/4) dispayed pedicles that were almost fused.

8.7.5 Rudimentary ribs

Osteogenic cell clusters developed in 25% (1/4) of foetuses from control group A (figure 48) at the base of the 14th thoracic vertebra where the head of a rib usually develops. Sixty two and a half percent (5/8) of foetuses in control group B displayed osteogenic cell clusters in this position. In experimental group A1, 60% (9/15) of foetuses displayed cell cluster development in this position, while one foetus developed a full 14th extra rib on the left (figure 49). Osteogenic cell clusters were observed in 80% (4/5) of foetuses in the A2 experimental group at the T14 vertebral level, in 37.5% (3/8) of foetuses in experimental group B1, and in 50% (2/4) of foetuses from experimental group B2. One foetus in this group (B2) also developed an extra 14th left rib.

Derangement in the shape of the ribs occurred in 43% of foetuses in experimental group A1 (figure 50). No other foetuses (albeit the control or other experimental groups) were observed with the same derangement in the shape of the ribs. Usually ribs 6-12 were the most affected.

Fig 3. Control foetus (group A) displaying no subcutaneous haematoma. Discolouration in abdominal region is due to normal development of the liver. Experimental foetus (group A2) displays haematoma covering most of the body.

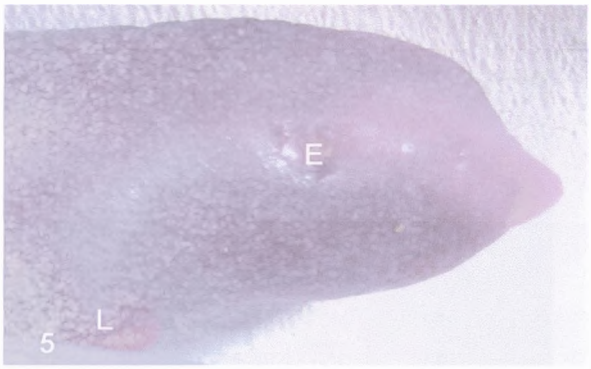


Fig 5. Excessive haemorrhaging in the cranial region of embryo from experimental group B1 (L=limb, E=ear). 6.4x

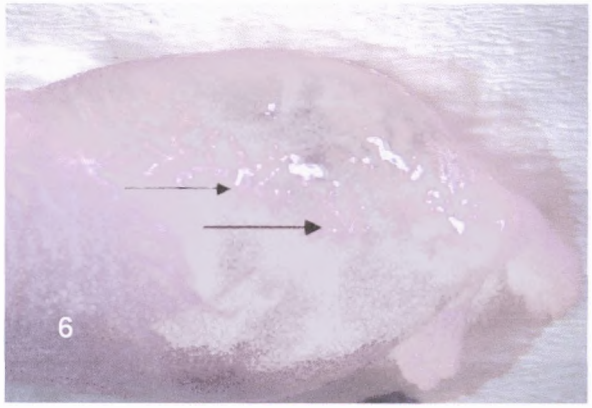
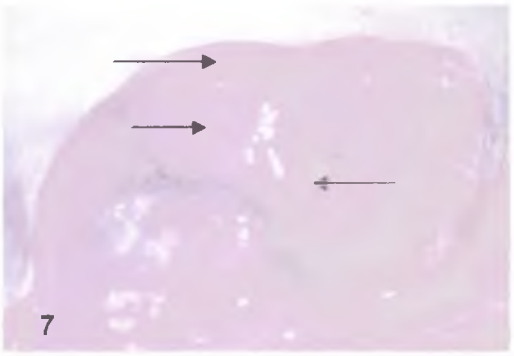


Fig 6. Crystal-like structures (arrows) spread over dorsum and abdomen of foetus in control group A. 6.4x stereomicroscope.



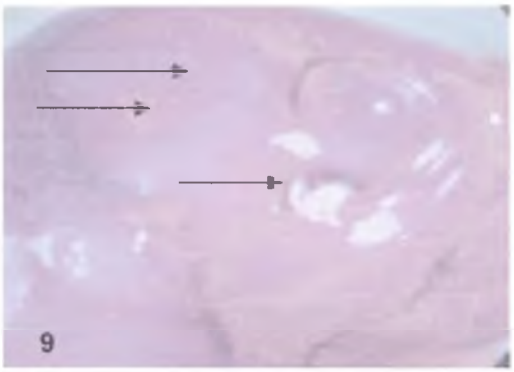


Fig 7. Subcutaneous haemorrhaging in temporal region of foetus from experimental group A1 (arrows). 16x stereomicroscope.
Fig 8. Comparison of subcutaneous haemorrhaging between foetuses from control group A (right) and experimental group A2 (left).
Fig 9. Abnormal subcutaneous haemorrhaging in cranial region of foetus from experimental group A2 (arrows). 40x stereomicroscope.

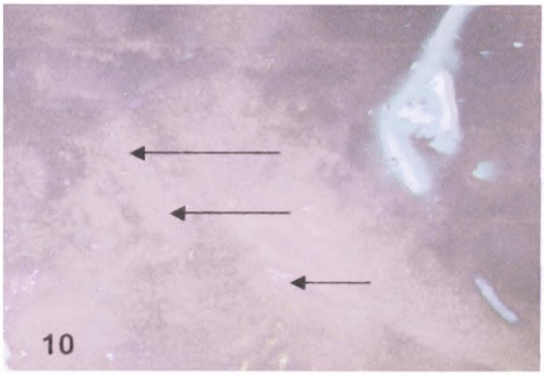


Fig 10. Crystal-like structures (arrows) inside **abdomen** of foetus in A2 experimental group. 40x stereomicroscope.
Fig 11. Comparison of foetuses from control group B vs experimental group B1.Note difference in body size.
Fig 12. Abdominal and temporal subcutaneous haemorrhaging (arrows) in foetus from experimental group B1. 16x stereomicroscope.

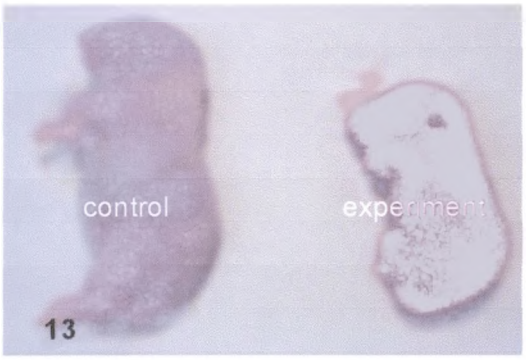


Fig 13. Underdeveloped foetus (right) from experimental group B1 compared to foetus from control group B (left) at the same age. Note excessive subcutaneous haemorrhaging in experimental foetus. No haematoma was noted in the control foetus.

Fig 14. Absence of temporal artery (white line) in foetus from experimental group B2. Note abnormal superficial subcutaneous haemorrhaging in cranial region (arrows). 16x stereomicroscope.

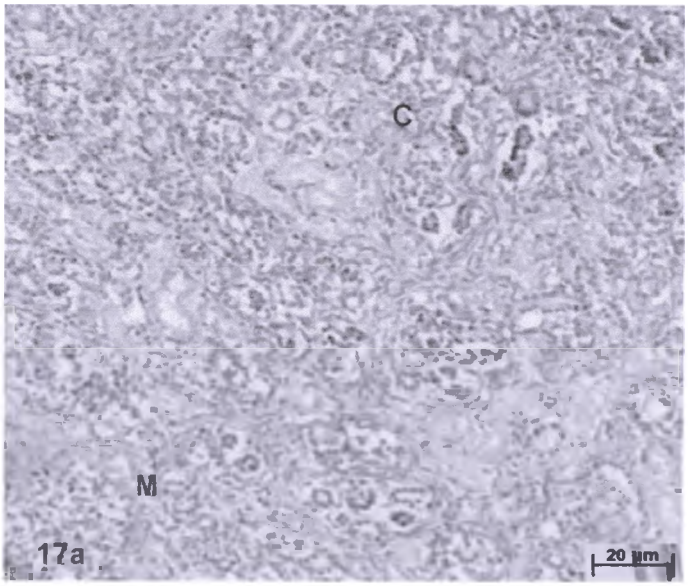


Fig. 17a. Representative section of the cortex (C) and medulla (M) of the kidney from a foetus in control group A. x100 Haematoxylin and Eosin.

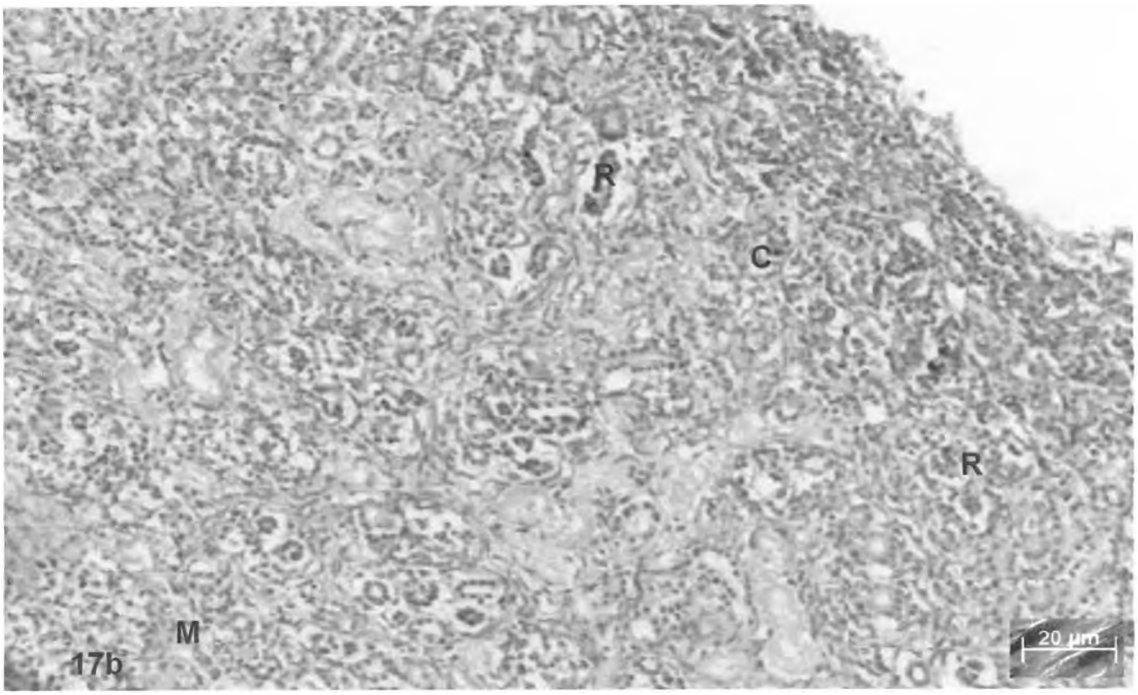


Fig 17b. Representative section of the cortex (C) and medulla (M) of the kidney from a foetus in experimental group A2. R= renal corpuscle. 100x Haematoxylin and Eosin.

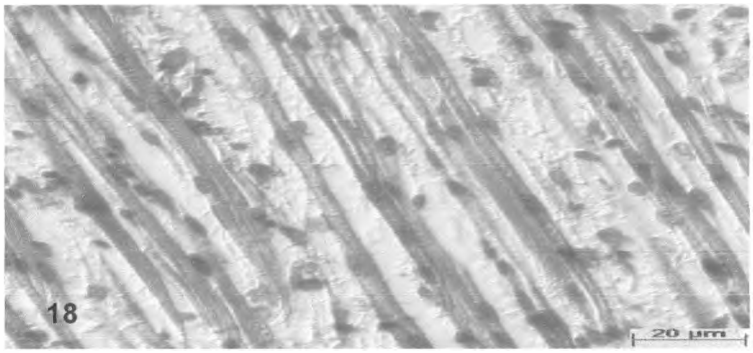


Fig 18. Representative section of skeletal muscle obtained from a foetus in control group A. x400, Iron haematoxylin.

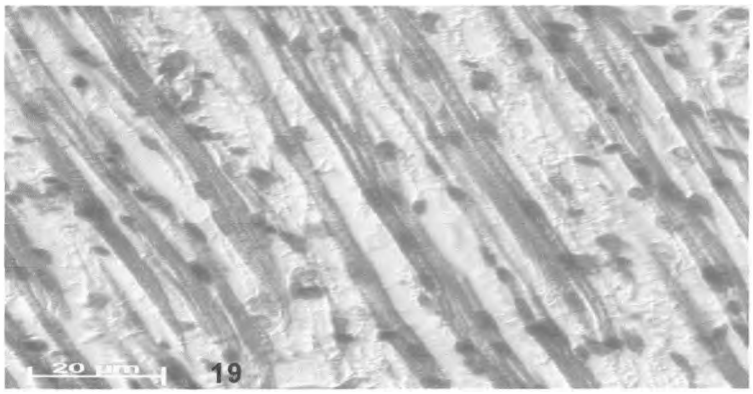


Fig 19. Representative section of skeletal muscle obtained from foetus in control group B. Both figures 18 and 19 display normal skeletal development for foetuses at the particular stage of development. x400, Iron haematoxylin.

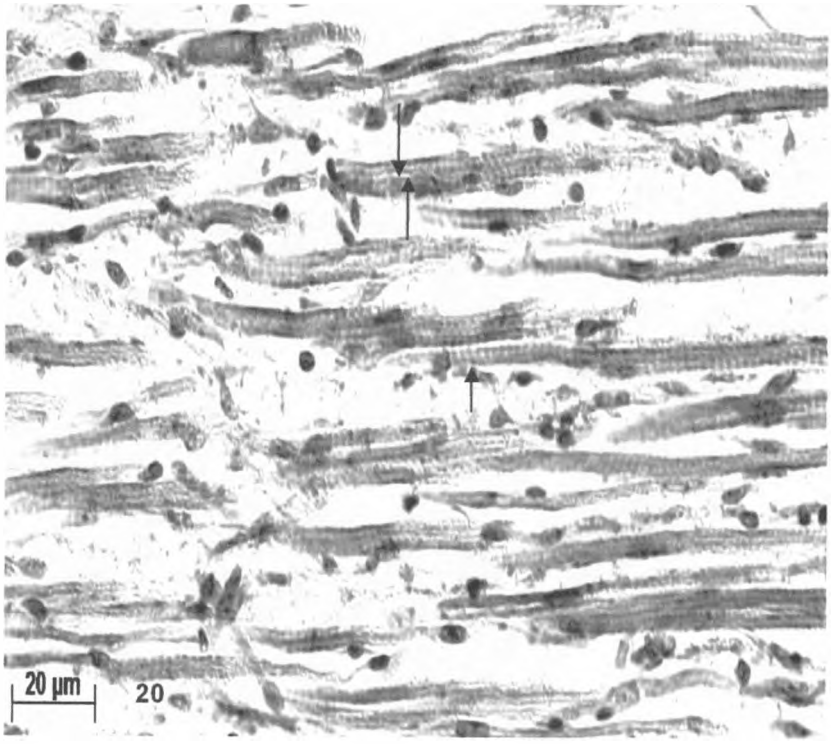


Fig 20. Representative section of skeletal muscle from foetus in experimental group A1. Note striations (arrows). X400, Iron haematoxylin.

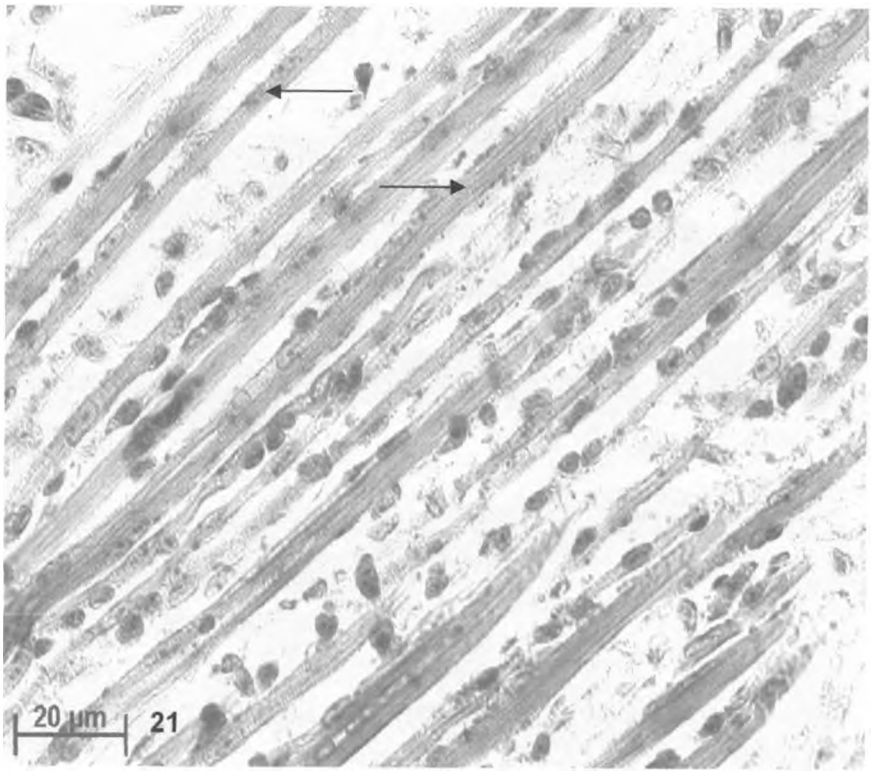


Figure 21. Representative section of skeletal muscle from foetus in A2 experimental group. Fine striations (arrows) can be observed. x400. Iron haematoxylin.

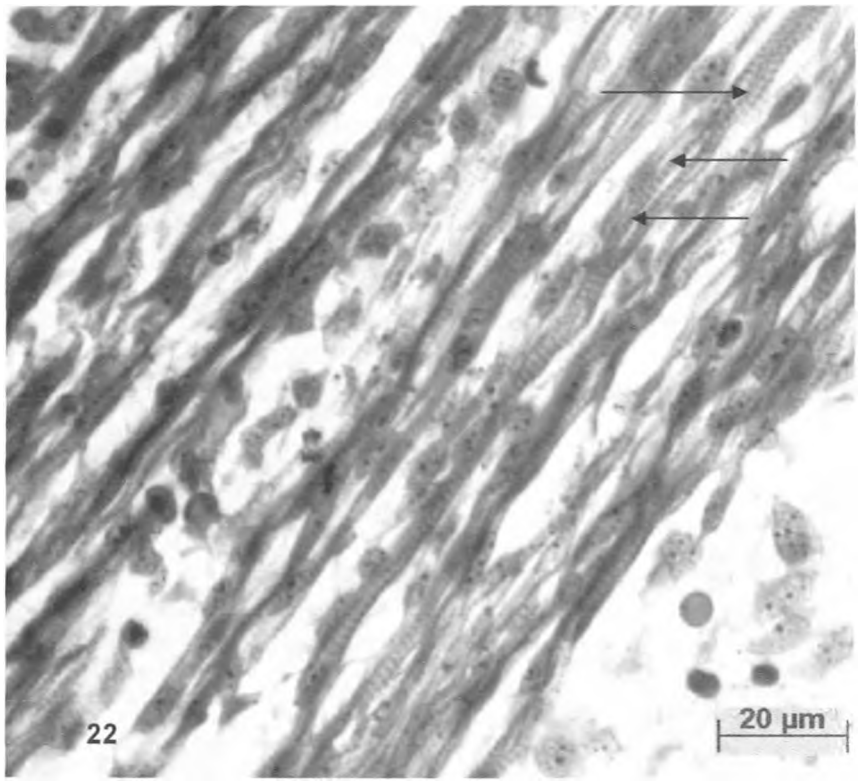


Fig 22. Representative section of skeletal muscle from foetus in experimental group B1. Some striations (arrows) can be identified. Tissue looked immature and not well developed, if compared to the A experimental groups. X400 iron haematoxylin.

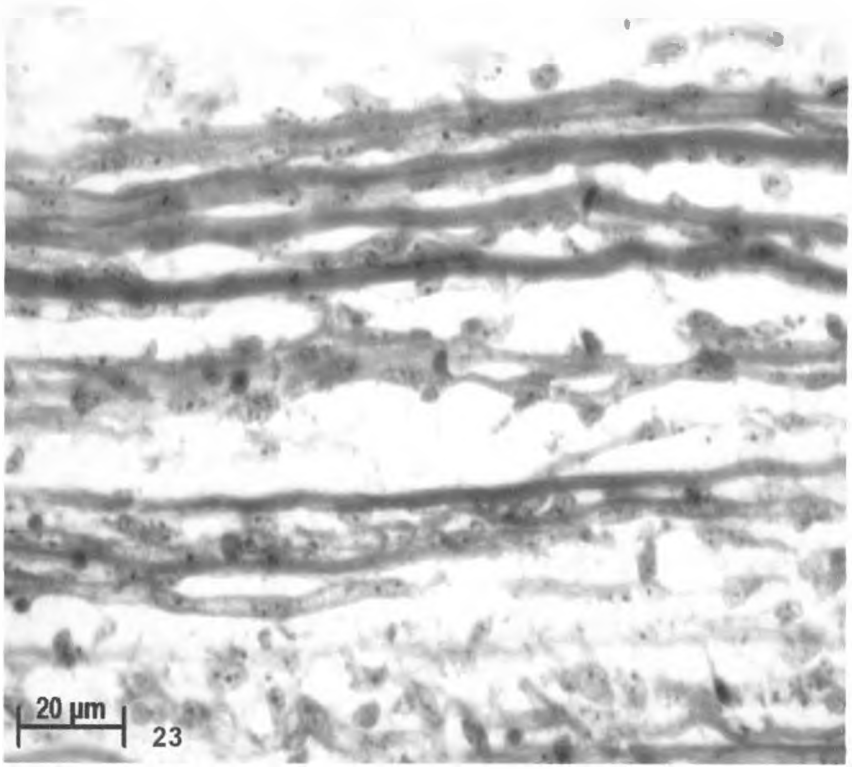


Fig 23. Representative section of skeletal muscle from foetus in experimental group B2. Note the immature developed muscle fibres as well as disorganized myoblasts. No apparent striations were observed, if compared to group A1 (experimental). X400 iron haematoxylin.

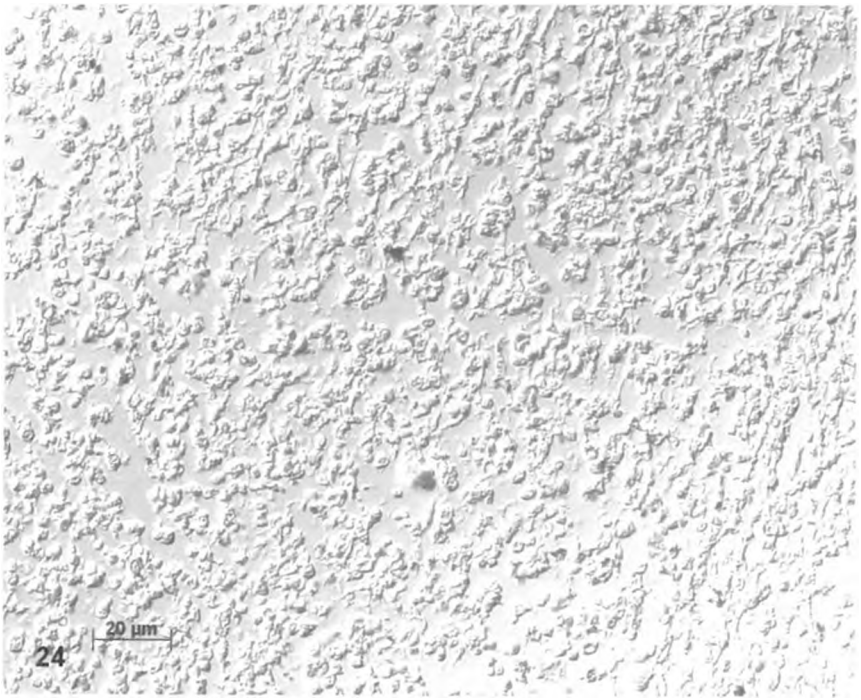


Fig 24. Representative section of pancreas from foetus in control group displaying absorption control. Anti-insulin serum pre-absorbed with insulin. X100.

Fig 25. Representative section of pancreas (negative control) from foetus in control group A. x100.

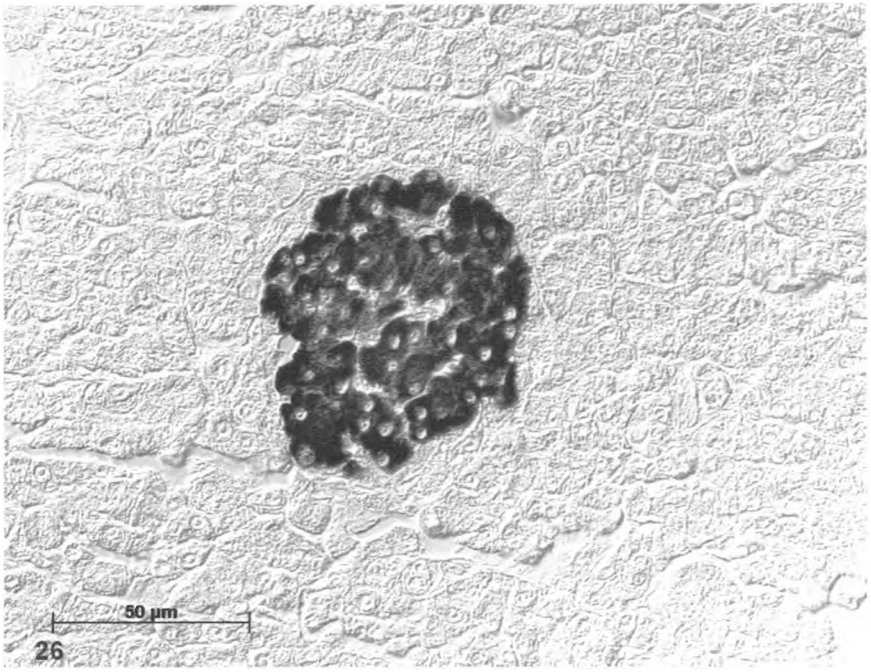


Fig. 26. Section of pancreas from adult female rat that was used as positive control (insulin). X100.

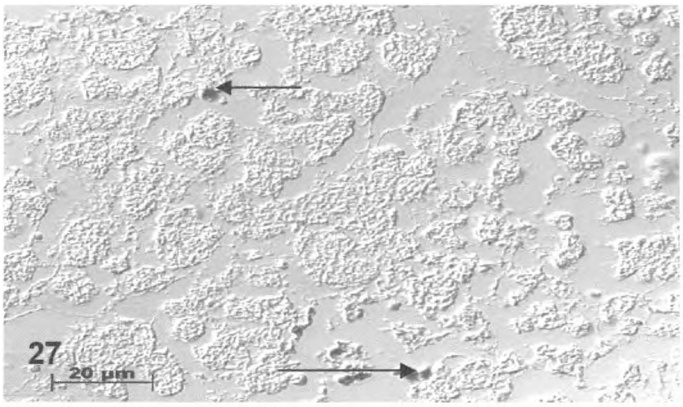


Fig 27. Section of pancreas from foetus in control group A showing localization of B-cells (arrows). These cells will only develop into endocrine islet within the next day or two. Interference contrast, x100.

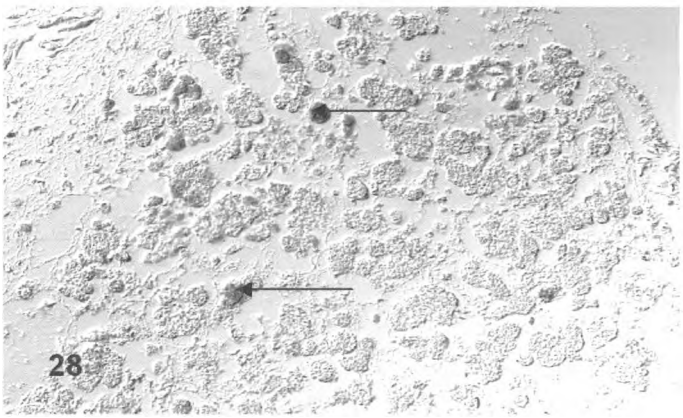


Fig 28. Localization of alpha cell s (arrows) in section of pancreas obtained from foetus in control group A. Interference contrast, x100.

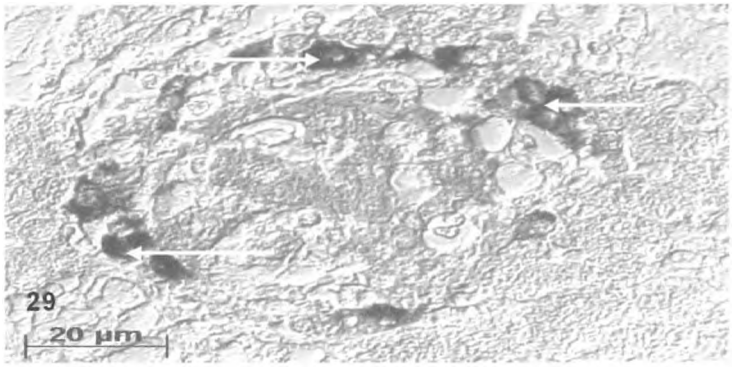


Fig 29. Localization of alpha cells (white arrows) around the periphery of an endocrine islet, found in the pancreas of a foetus from the A2 experimental group. Interference contrast, x400.

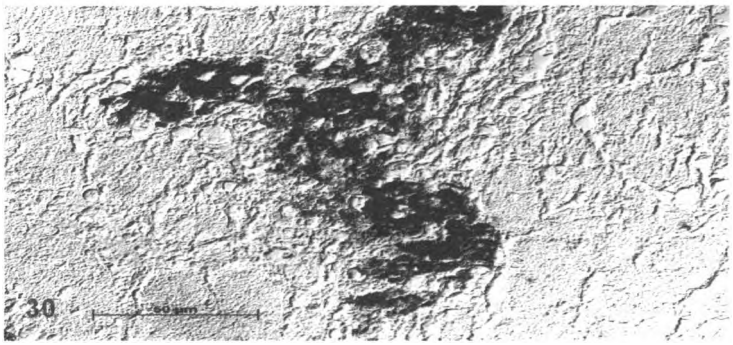


Fig 30. Intense localization of beta cells from foetus in experimental group A2. Note endocrine islet formed, especially if compared to the pancreas of foetus in figure 27 (control group) that was of the same age. Interference contrast, x400.

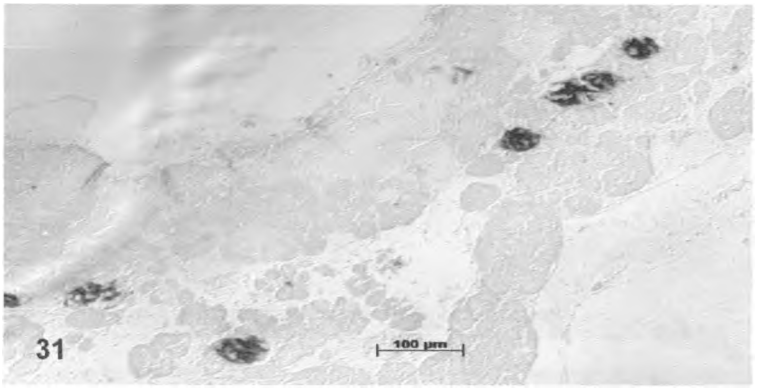


Fig 31 Lower magnification of section (in figure 28) of pancreas from foetus in experimental group A2. Note amount of endocrine islets (beta cells) present in the small piece of pancreas. x100.

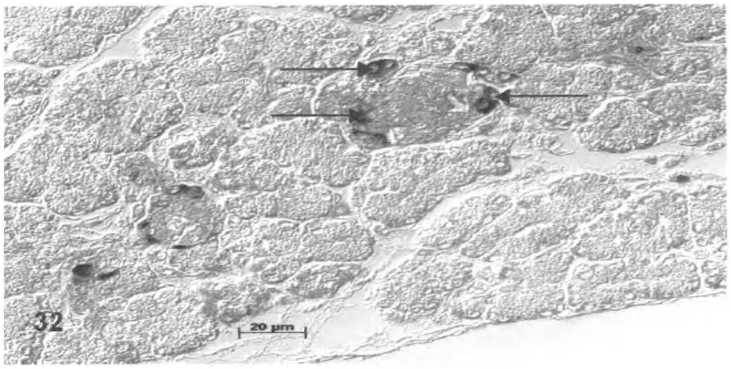


Figure 32. Alpha cell immunolocalization (arrows) around periphery of endocrine islet (B1 experimental group). Interference contrast, x100.

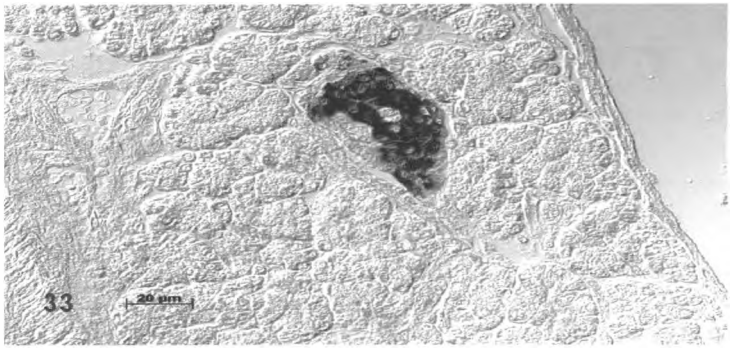


Figure 33. Intense immunolocalization of Beta cells in the endocrine islet (B1 experimental group). Interference contrast, x100.

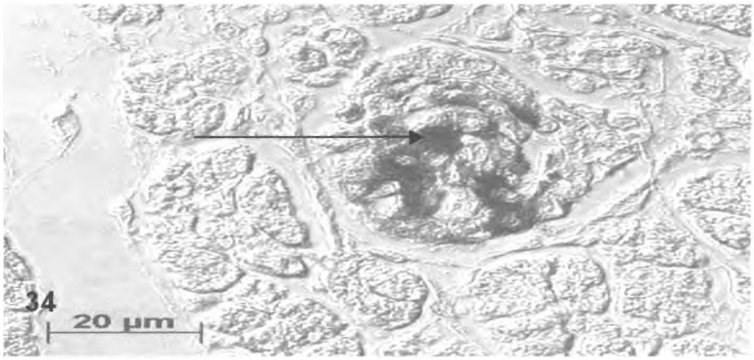


Figure 34. Beta cell immunolocalization in B2 experimental animal pancreas. Interference contrast, x100.

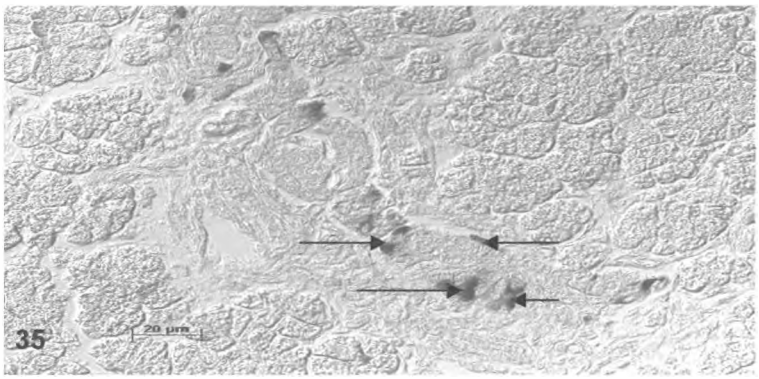


Figure 35. Forming of alpha cells (arrows) around periphery of endocrine islets in B2 experimental animal. Interference contrast, x100.

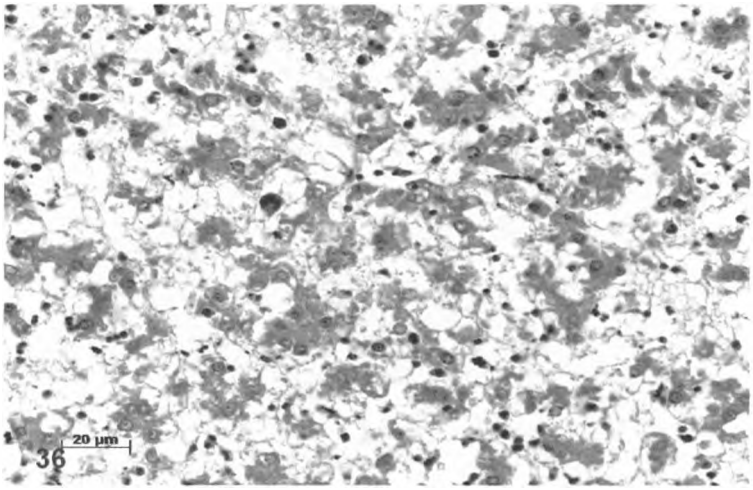


Figure 36. Representative section of liver taken from foetus in control group A. x400, Haematoxylin and Eosin.

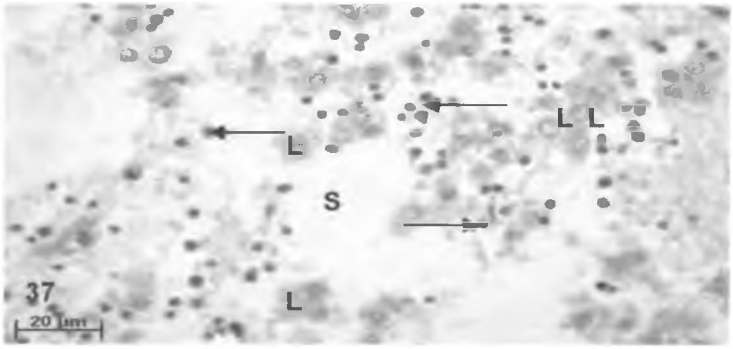


Fig 37. Representative section of liver taken from foetus in experimental group A1. Note distended sinusoids (S) and disorganized liver cord cells (L). Arrows=precursor blood cells. X400, Haematoxylin and Eosin.

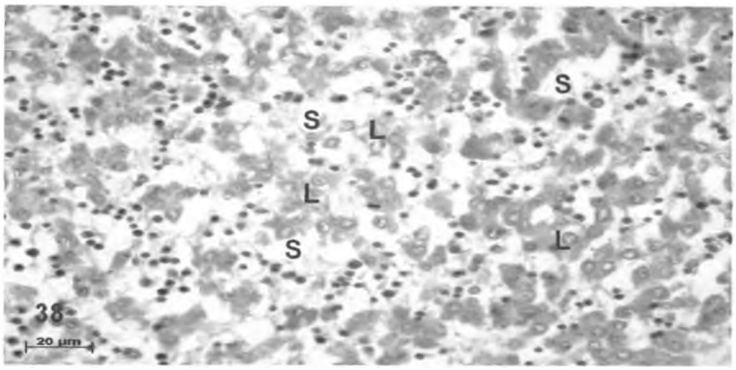


Fig 38. Section of liver from foetus in experimental group A2. Note the increase in number in liver cord cells (L), precursor blood cells and wider sinusiods (S). X400, Haematoxylin and Eosin.

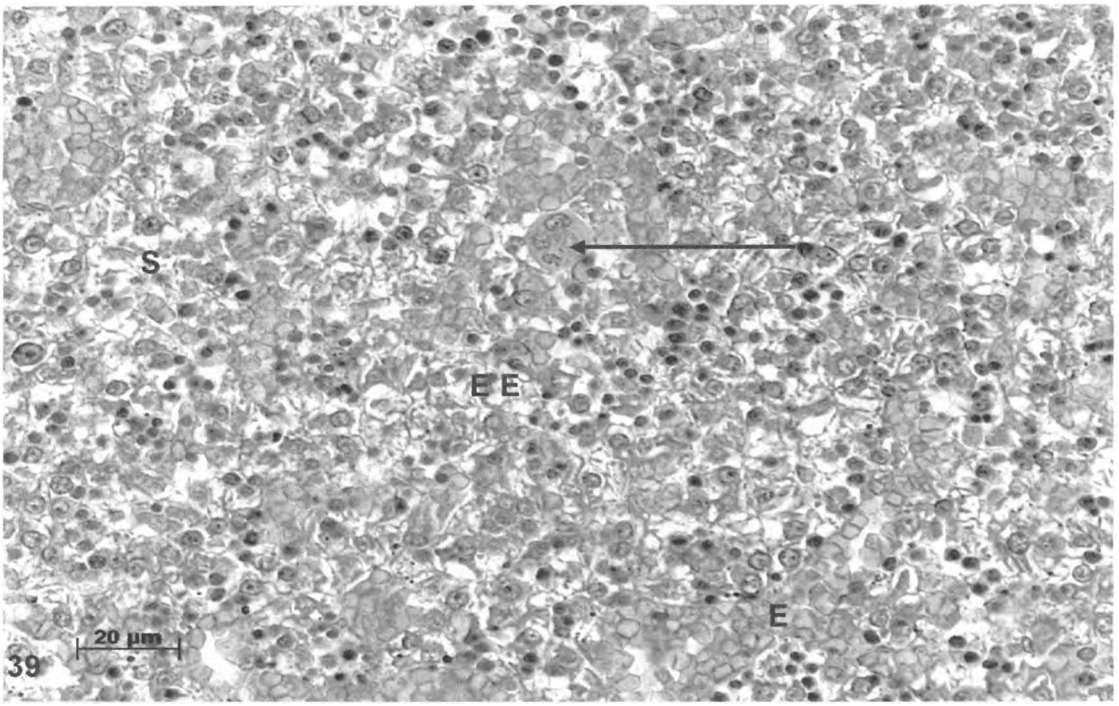


Fig 39. Representative section of liver from foetus in experimental group B1. Very narrow sinusoids (S), megakaryocyte (arrow) and erythrocytes (E) in sinusiods (S). x400, Haematoxylin and Eosin.

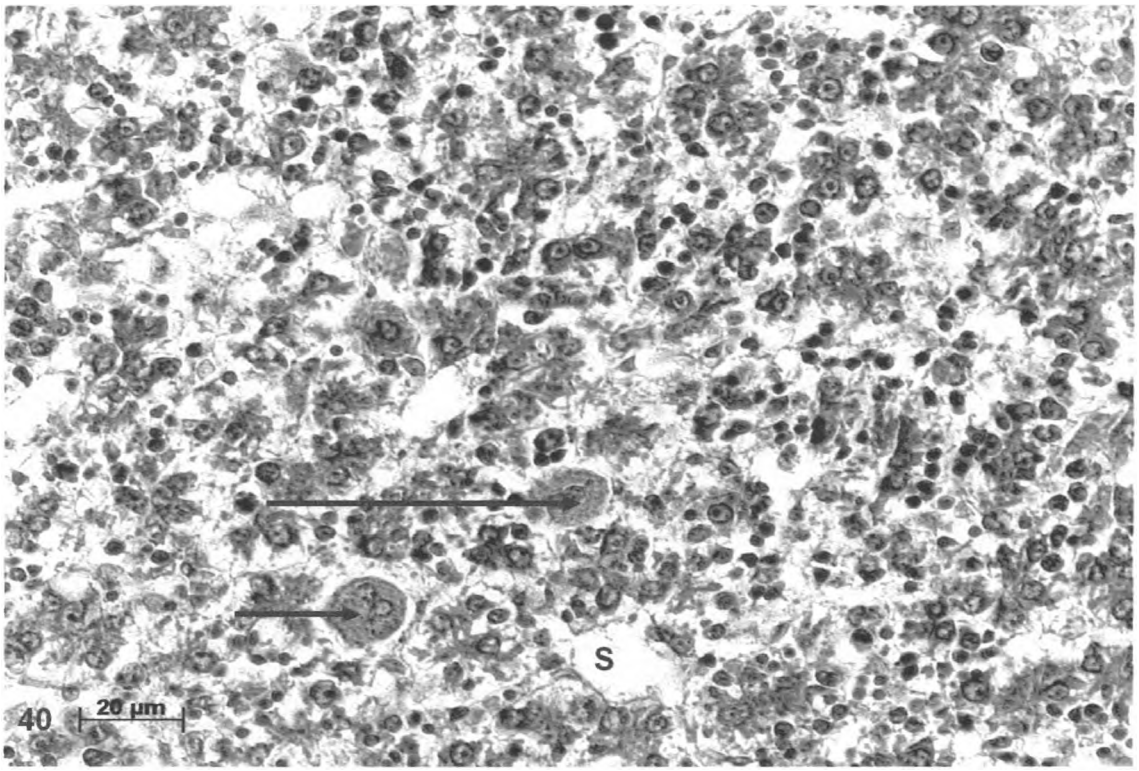


Fig 40. Representative section of liver from foetus in experimental group B2. Arrows indicate megakaryocytes.Sinusoid (S). x400, Haematoxylin and Eosin.

Figure 41. Foetus from control group A that displays typical less well developed (thin) desmocranium.Note the difference in bone development between this foetus and foetus in figure 39 (black arrow). 16x stereomicroscope. Alizarin red S and Alcian Blue.

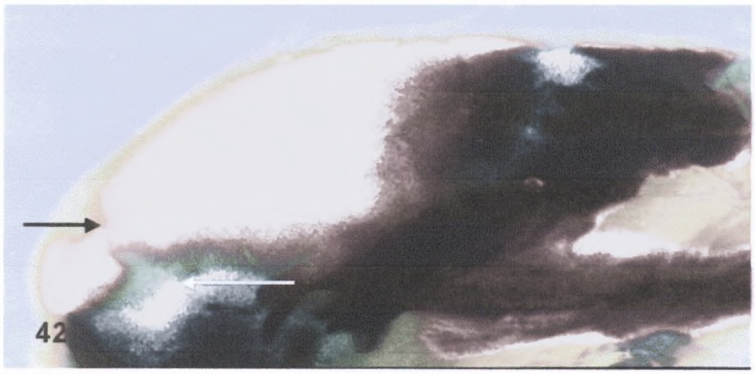


Figure 42. Foetus from experimental group displaying well developed (thick) desmocranium compared to foetus in figure 38 (black arrow). Cartilage is present (white arrow). 16x stereomicroscope. Alizarin red S and Alcian Blue.

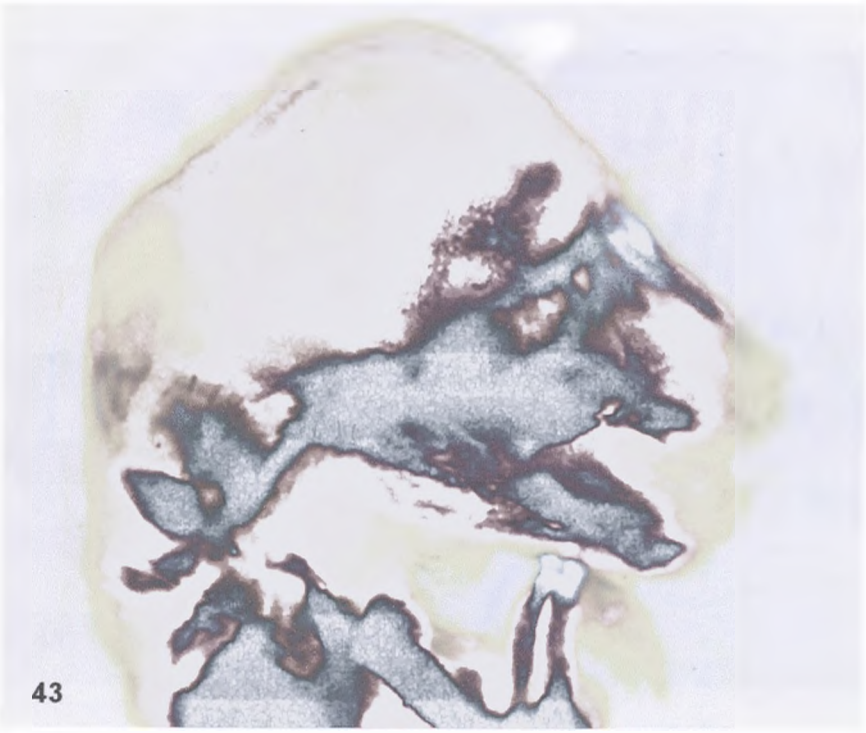


Figure 43 shows foetus from experimental group B1, which is at a comparable age to foetuses in figures 41 and 42, with immature bone development. 16x stereomicroscope . Alizarin red S and Alcian Blue.

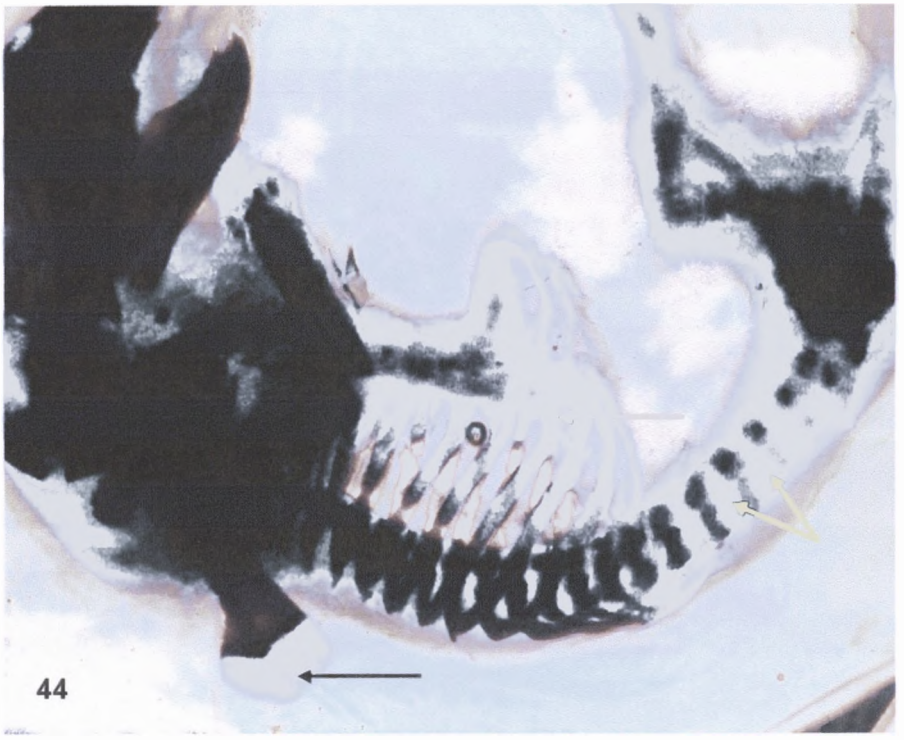


Figure 44. Foetus from A2 experimental group. Presence of cartilage (blue stained) very prominent in the ribs (red arrow), intervertebral discs (yellow arrows) and scapula (black arrow). Bone is stained red. 6.4x stereomicro-scope. Alizarin red S and Alcian Blue.

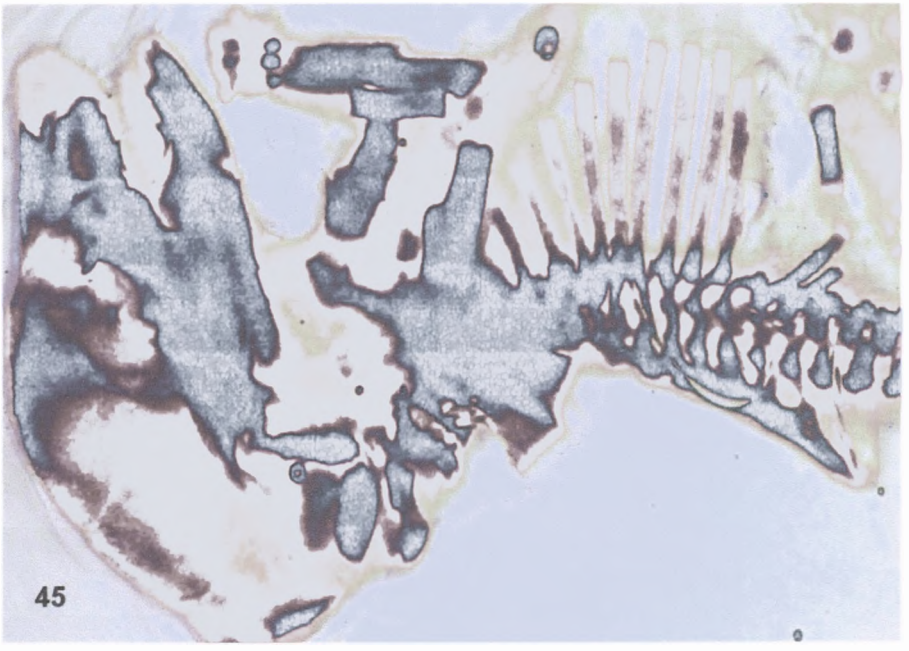


Figure 45 shows foetus from a control group A. No cartilage was present in foetuses of this group. 6.4x stereomicro-scope. Alizarin red S and Alcian Blue.

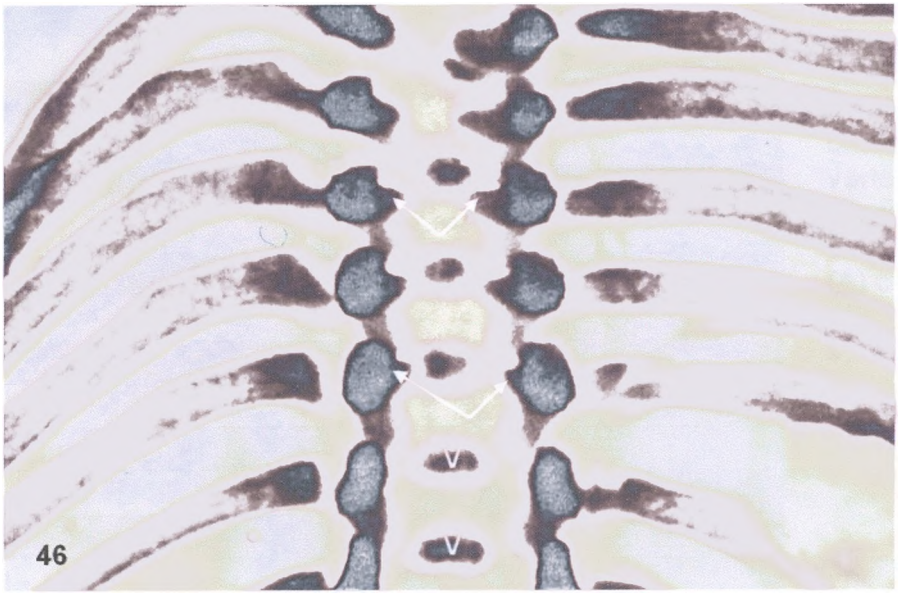


Figure 46. Dorsal view of vertebral column of foetus in experimental group A2. Vertebral pedicles almost fused (white arrows). Vertebral bodies (V) can be observed. 40x stereomicro-scope. Alizarin red S and Alcian Blue.

Figure 47. Dorsal view of vertebral column from foetus in control group A Note the vertebral pedicles (white arrows). 40x stereomicroscope. Alizarin red S and Alcian Blue.

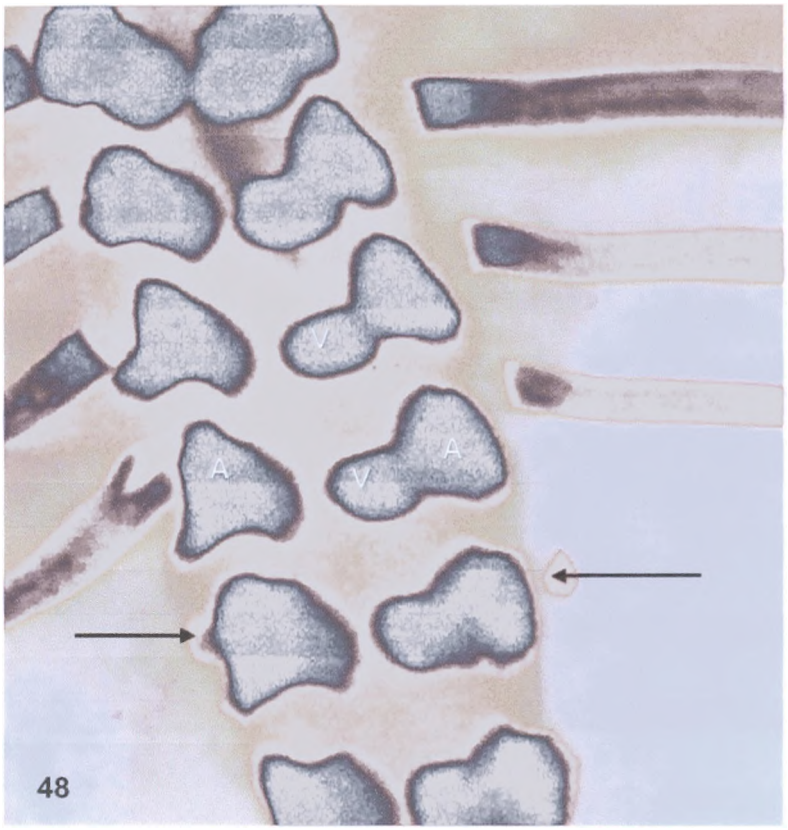


Figure 48. Red stained bone (osteogenic) cell clusters (dorsal view) observed at the level of the 14th thoracic vertebra (black arrows). Vertebral bodies (V) and arches (A) can be observed. 40x stereomicroscope. Alizarin red S and Alcian Blue.

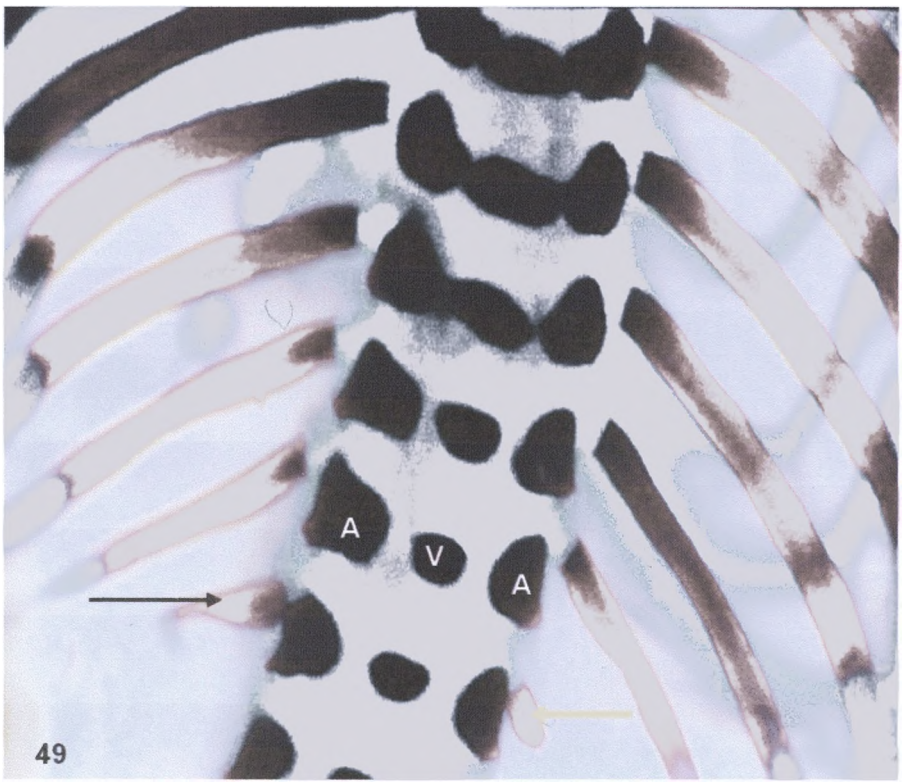


Figure 49. Foetus from A1 experimental group that developed 14th rudimentary rib right (yellow arrow), and 14th extra rib left (black arrow). Vertebral bodies (V) and arches (A) can be observed. 40x stereomicro-scope. Alizarin red S and Alcian Blue.

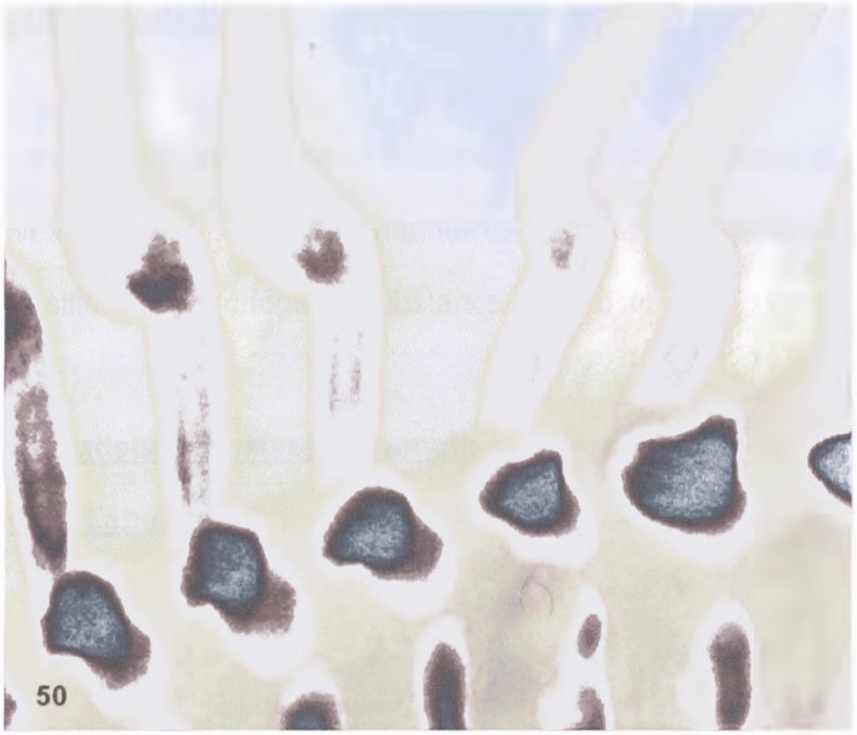


Figure 50. Foetus in A1 experimental group displaying derangement of the ribs (dorsal view). 40x stereomicroscope. Alizarin red S and Alcian Blue.

9. DISCUSSION

In this study, the effect of creatine on the development of the rat foetus was investigated to determine whether this substance is safe or whether it could be classified as a teratogenic substance when used during pregnancy.

9.1 Statistical analysis of length and weight of foetuses from the different control and experimental groups

9.1.1 Length

When a low dose of creatine was injected over a prolonged period (day 7-13) of development (experimental group A1), it did not have a statistically significant effect ($P>0.05$) on the growth and development of the foetuses when compared to foetuses in control group A. Foetuses that were exposed to a high dose (experimental group A2) of creatine over a prolonged period (day 7-13 of development) of time, had statistically significantly increased length when compared to foetuses in the A control group. However, when foetuses from control group A were compared to foetuses from control group B it was found that there was a statistical significant difference in length ($P<0.01$) between these groups. It appears that repeated injections of saline through days 7-13 of development had a negative influence on the development of the foetuses in the A control group. Foetuses in control group B, in which saline was injected on days 9 and 11 of development were significantly longer ($P<0.01$) than foetuses in

the A control group. The foetuses in the B control group did not seem to be affected by the injections of saline. There was no statistically significant difference ($P>0.05$) between control groups A and experimental groups B1 (low dose, day 9 and 11 injected) and B2 (high dose, days 9 and 11 injected).

Experimental group A1 foetuses (low dose) were statistically significantly shorter in length ($P<0.001$) compared to experimental group A2 foetuses (high dose). Although both the experimental groups had the same amount of time of exposure to creatine, it appears as though the different dosages have different effects on the development of the foetus. An increase in dosage, surprisingly, had an effect on the length of the foetus, thus suggesting that if creatine is administered near the time of implantation, creatine does seem to have beneficial effect on the development of the foetus.

There are two possibilities that may promote the growth of the foetus. The one possibility is that nitric oxide may play a significant role in increasing growth hormone, while the other possibility is the effect of insulin on the developing foetus. It is well documented that nitric oxide (NO) stimulates growth hormone production and release (Wu and Meininger, 2000). Since arginine (Arg) is a substrate to NO as well as to creatine, an increase in creatine concentration may shift the balance of Arg in a way that more NO is formed. This is because more Arg is available for NO production, since creatine is supplemented exogenously. Due to exogenous creatine supplementation, an increase in creatine

concentration is believed to have occurred (Wyss and Kadurrah-Daouk, 2000). More Arg was thus used in the production of NO, which in turn stimulated an increase in growth hormone. This explains why the foetuses in experimental group A2 had a statistically significant increase in length over that of foetuses from the other groups with the exception of control group B. The role of insulin on the developing foetus is discussed in section 9.1.3.

Foetuses from experimental group A1 showed a statistically significantly increase in length over that of foetuses from experimental group B1 ($P<0.001$). This may indicate that repeated injections and thus possible sustained, high levels of creatine played a role in the increase in length of the foetus.

Experimental group A2 was the group with the highest mean length. The only group that was not statistically significantly different in length ($P>0.05$) from group A2 was control group B. These results indicates that when a high dosage of creatine is administered over a prolonged period of time at repeated dosages, a probability exists that foetuses will grow more and show an increase in length. It is possible that an increase in growth hormone would lead to this effect (Wu and Meininger, 2000). The elevation in hormone levels is caused by an increase in NO, which can be brought about by elevated creatine levels due to creatine supplementation.

Foetuses in control group B were statistically significantly longer than foetuses in experimental groups B1 and B2 ($P<0.001$). The reason why the foetuses were smaller in experimental groups B1 and B2 is that creatine may cause harm to certain metabolic processes during organogenesis. The NO-growth hormone hypothesis may not come into play when creatine is given at specific stages of organogenesis. Because creatine was supplemented on specific days i.e. days 9 and 11 of development, and not repeatedly sustained (as in the A experimental groups), the foetus and the female rat may have had produce their own creatine endogenously. Arg may thus have been used in the production of creatine and not in the production of NO. This lead to the conclusion that there was no increase in growth hormone levels in these foetuses during this time. This data correlates well with the study that was undertaken by Badenhorst and Vorster (2000, unpublished data), which indicated that creatine did have a negative effect on the development of the chick embryo if administered during organogenesis.

9.1.2 Weight

When the weights of foetuses from different groups are compared to determine whether there are statistically significant differences between the control and experimental groups, it was found that creatine did not have as much influence on weight fluctuation as it had on length fluctuation. No significant difference in weight was found between control groups A and B, indicating that administration of saline over a prolonged period of time or during certain stages of organogenesis did not affect the mean weight of foetuses in those particular

groups. When control group A was compared to experimental group A1, it was found that creatine at a low dose did not have a significant effect on the weight of foetuses ($P>0.05$). When control group A was compared to experimental group A2, a probable significant increase in weight ($0.05>P>0.01$) was found. This indicates that high doses of creatine given from day 7-13 of intrauterine life increased the weight of the foetuses. This however, does not mean that creatine is beneficial to the development of the embryo or foetus, since certain foetuses in this group also suffered either from subcutaneous haemorrhaging (foetuses were heavier than average) or were underdeveloped (foetuses were lighter than average). Control group A was also not significantly different ($P<0.05$) from experimental groups B1 and B2.

The mean weight of the foetuses in experimental group A1 was however significantly lighter than the mean weight of foetuses in experimental group A2 ($P<0.001$). Thus, a higher dose of creatine over a prolonged time period appears to increase the weight of the foetuses significantly when compared to foetuses that received a low dose of creatine supplementation. Experimental group A1 was not statistically significantly different from experimental group B1. This indicates that when foetuses are given the same dose of creatine over a prolonged period or during specific stages of organogenesis, no statistically significantly differences in weight occur.

Experimental group A2, as mentioned above, was statistically significantly heavier in weight than control group A and experimental group A1. When experimental group A2 is compared to experimental group B2, a statistically significantly difference in weight was found ($0.01>P>0.001$). It may be speculated from these results that the dosage of creatine appeared to have played a definite role. The closer to the time of implantation and the longer the creatine is administered, the heavier the foetus will be at term.

When experimental groups B1 and B2 are respectively compared to control group B, it can be seen that there was a statistically significantly difference in weight between control group B and B1 ($P<0.01$) and control group B and B2 ($P<0.05$), with control group B having the highest mean weight. This would suggest that when creatine is administered during certain stages of organogenesis, independent of the dosage administered, the weight of the foetuses is decreased. It appears that creatine might have a delaying effect on development of the foetus if creatine is administered during particular stages of organogenesis.

It is well known that insulin plays an anabolic role during development and growth of the foetus (Korgun *et al.,* 2003). Insulin also acts as an anabolic hormone that may stimulate protein synthesis (Augustin *et al.*, 2003). If creatine is supplemented exogenously, the increase in creatine concentration can elicit an insulin response in the mother (Wyss and Kadurrah-Daouk, 2000). An increase in

maternal insulin levels increases the rate of cleavage and development of the blastocyst as well as increasing the chances of the blastocyst to implant successfully (Augustin *et al.*, 2003). It has also been shown that there is an increase in insulin receptors in the uterus, during the implantation period, and in the embryo (Kaye and Gardner, 1999). If the pregnant female has artificially enhanced insulin levels (due to exogenous creatine supplementation), the increased insulin and insulin receptors may trigger increased growth in the foetus (due to the anabolic properties of insulin). Although the foetus only starts to produce its own insulin on day 14 of development, the insulin serum (produced by the embryo) is too weak to have a significant effect on growth and development of the embryo (Louvi *et al.*, 1997). The embryo (and later foetus) thus utilizes insulin from the maternal source. This mechanism enables the mother to control the placental and foetal growth (Korgun *et al.*, 2003), until the foetus has established its own endocrine system just before birth.

Since creatine was supplemented from day 7-13 of intrauterine development, an increase in maternal insulin levels promoted implantation and post-implantation growth of the blastocyst. Repeated injections of creatine kept the insulin levels of the female elevated. The elevated insulin (being an anabolic hormone) levels promoted growth in the embryo through the organogenesis period. However, in groups B1 and B2, creatine supplementation on days 9 and 11 of intrauterine development, may not have affected the insulin levels of the female significantly, thus not promoting enhanced growth compared to the foetuses in the A

experimental groups. However, it appears that creatine did have a negative effect on the developing embryo if injected on these particular days of organogenesis. The timing of the injection may explain why the foetuses from experimental groups A1 and A2 had higher mean lengths and weights compared to the other control and experimental groups.

9.2 Surviving versus absorbed embryos

Three hundred and forty foetuses were collected from 40 pregnant adult females. An additional 116 absorbed foetal sites were counted in the uterine tubes of these pregnant females.

It was found that 21% (13/63)(control group A), 3% (3/92)(A1 experimental group) and 14% (11/78)(A2 experimental group) of foetuses were absorbed while in the B-groups 42% (31/74)(control group B), 28% (24/85)(B1) and 53% (34/64)(B2) of foetuses were absorbed (figure 2). It appears that prolonged administration of creatine (A experimental groups) (whether it is a low or high dose that is administered) causes less absorption than saline which was administered to control group A. In contrast, when creatine was administered on days 9 and 11 of development (B experimental groups), a definite increase in absorptions compared to the A groups occurred. This may indicate that creatine administration over a prolonged period of time (due to the effect of increased maternal insulin levels) increases the probability of successful events associated

with the peri-implantation period (Kaye and Gardner, 1999). Compared to the B groups, administration of creatine during certain critical periods of organogenesis tends to increase the probability of mortality *in vivo* and absorption of a foetus.

Kimmel and Wilson (1973) intraperitoneally injected adult female pregnant rats with water on day 9 and on day 11 of development respectively. They observed that there was a 9% mortality and absorption rate of foetuses of pregnant female rats that were injected on day 9 and a 22% mortality rate of foetuses of pregnant female rats injected on day 11. They concluded that even a simple substance like water might induce absorptions. If these results are compared to control group A, it can clearly be seen that the saline group (20.6%, day 7-13 injected) has a decreased percentage in mortality in relation to the work of Kimmel and Wilson (1973). However, when comparing the work of Kimmel and Wilson (1973) to control group B (injected days 9 and 11) a 200% increase in resorption sites was found in the current experiment. Thus, it appears that when any solution is injected at a particular time of organogenesis, that solution might have a significant effect on the survival of the embryo/foetus. Kimmel and Wilson (1973) also injected pregnant female rats with Actinomycin D (10% of foetuses absorbed), Acetazolamide (6% of foetuses absorbed) and sodium Salicylate (26% of foetuses absorbed). When the data from Kimmel and Wilson (1973) is used as a measure to compare absorption rates of foetuses that were exposed to creatine, it appears that prolonged administration of creatine is not as harmful as Actinomycin D or sodium Salicylate to the foetus (A1, A2 experimental groups). It

however, has a harmful effect on the foetus when injected on days 9 and 11 of development (B1, B2 experimental groups) (developmental time period of eye, brain and heart)(Baker *et al.*, 1980).

Experimental group B2 (high dose, days 9 and 11 injected) had a 188% increase in foetal absorptions when compared to experimental group B1 (low dose, days 9 and 11 injected). Experimental group B2 also had a 377% increase in foetal absorptions when compared to experimental group A2 (high dose, days 7-13 injections) that had the same creatine dosage. This is a very high absorption percentage, suggesting that creatine might act once again as a teratogen on the developing embryo if given at a specific time during organogenesis. An increase in dose may also act is a teratogen to induce mortality. The higher the dose that is administered, the greater the chance that a foetus may be absorbed. Miller *et al.* (1993) found that cyclocreatine (a creatine analogue) can be lethal to the developing chick and rat. They found that increased dosages of cyclocreatine, administered to developing chick embryos and pregnant female rats, induced a higher mortality rate (chicks and rats foetuses) and a higher absorption rate (rat foetuses). When the present study is compared with the study of Miller *et al.* (1993), the results from the B groups, mirrors Miller's findings. However, the results obtained from the A groups (control and experimental) suggest that creatine might be beneficial to the growth and development of the embryo/foetus if administered around the time of implantation. This increased and then

sustained creatine concentration might have lead to an increase in insulin, which would benefit and enhance the growth of the developing embryo and foetus.

9.3 Macroscopic abnormalities

9.3.1 Subcutaneous haemorrhaging

Twelve foetuses from the total foetal group (340 foetuses) (0.04%) were found to suffer from abnormal subcutaneous haemorrhaging. In control group A it was observed that 4% of foetuses suffered from subcutaneous haemorrhaging. Experimental groups A1 and A2 each had 1.1% and 3% respectively of foetuses that suffered from subcutaneous haemorrhaging. In control group B, 2% of foetuses suffered from subcutaneous haemorrhaging, while 6.5% and 6.6% suffered from subcutaneous haemorrhaging in experimental groups B1 and B2 respectively.

A possible explanation for why a small number of foetuses developed subcutaneous haemorrhaging may be attributed to the fact that arginine inhibits vasoconstriction (Wu and Meininger, 2000) by being a substrate to nitric oxide (NO). It is well documented that NO promotes angiogenesis, but also inhibits the aggregation of platelets and adhesion molecules of the vascular cells (Wu and Meininger, 2000). NO further inhibits the formation of smooth muscle and endothelin-1 release and plays a crucial role in vasodilatation (British Pharmacopoeia, 1953). Wyss and Kaddurah-Daouk (2000) found that NO inhibits

the endothelial nitric oxide synthase (eNOS) process that is necessary for the maintenance of endothelial arterial walls. Inhibition of this process might lead to hypertension. Since arginine is a substrate for NO as well as for creatine, it is possible to speculate that a sudden increase in creatine concentration might shift the balance of Arg, with more NO being formed. This possible increase in creatine leads to a multitude of factors that might cause the subcutaneous haemorrhaging in the foetuses. Experimental groups B1 and B2 (injected on days 9 and 11 of embryonic development) might have underwent such an Arg-NO shift, with NO inhibiting eNOS. The increase in subcutaneous haemorrhaging that this shift brought along can clearly be seen in these groups (experimental groups B1 and B2). It thus appears that creatine supplementation may play a critical role in Arg-NO synthesis as well as NO metabolism. When this metabolism-synthesis equation is disturbed at certain stages of development, it has the potential to "induce" subcutaneous haemorrhaging in the embryo/foetus.

Foetuses from the different groups were compared and it was found that with an increase in the concentration of creatine, the incidence of subcutaneous haemorrhaging increased. No haemorrhaging was observed in the control groups. In the few foetuses that displayed signs of subcutaneous haemorrhaging, it was mostly localized in and around the abdominal area. However, foetuses from experimental group A1 had extensive subcutaneous haemorrhaging which stretched from the abdominal area into the thoracic area. These foetuses also displayed signs of haematomas in the temporal region, especially in the region

where the superficial temporal artery is located. If the foetuses from group A1 are compared to the foetuses from group B1 (both groups had the same dose, but different times of injections), foetuses in experimental group B1 displayed subcutaneous haemorrhaging. Foetuses from experimental group B1 had larger areas of haemorrhaging, especially in the temporal region. It thus appears that an increase in dosage and repeated exposure to creatine increases the probability that a foetus will suffer from subcutaneous haemorrhaging.

When groups A2 (experimental) and B2 (experimental) are compared to each other, one would expect an increase in the severity of haemorrhaging (due to the fact that creatine supplementation would increase NO production, which in turn would inhibit eNOS production). In fact, it was observed that the A2 experimental group was the most affected. In this group, it was found that the haemorrhaging reached over the entire trunk and limbs, including the cranium. Although experimental group B2 had an increased number of foetuses (6.6%, 2/30) that were affected by the haemorrhaging, none of those foetuses displayed the same severity of haemorrhaging as those foetuses in experimental group A2. Again, it can be speculated that according to Miller *et al.* (1993), creatine and creatine analogs may have a toxic effect that induces subcutaneous haemorrhaging in the developing foetus.

Crystal-like structures were found in some of the foetuses. These structures were not found in control group B or experimental groups A1 and B2. However, control

group A had one foetus (abdominal area) and experimental group B1 had one foetus that displayed these structures in the abdominal area. All the foetuses from the experimental A2 group that suffered from subcutaneous haemorrhaging also displayed crystal-like structures. In two of the most severe cases (experimental group A2) the crystal-like structures were also found in the temporal area. This may be a "precipitate" from one of the solutions used in preparation of the foetuses. However, the same solutions were made up fresh and used throughout the experiment. It appears that some internal mechanism was disrupted and caused these crystal-like structures. No explanation for this was found after an extensive literature search. Further research needs to be carried out in this regard to determine whether creatine disrupts any particular metabolic event.

While subcutaneous haemorrhaging was primarily observed in the abdominal area, it was also observed in the temporal area and cranium. The bigger the surface of the trunk that was covered with subcutaneous haemorrhaging, the more severe and excessive the haemorrhaging became. While no cases of temporal bleeding were observed in groups A and B (controls), there were two foetuses and one foetus respectively in groups B1 and B2 (experimental) that suffered from temporal bleeding. One foetus was found which lacked a temporal artery. All of the foetuses that suffered from subcutaneous haemorrhaging in experimental groups A1 and A2, also suffered from temporal bleeding. It may be postulated that when there is an increase in NO due to the increase in arginine,

the excessive NO produced inhibits eNOS. eNOS is very important in maintaining arterial wall elasticity. Inhibition of eNOS by NO could have an artherosclerotic effect on the arterial walls. These arteries may lose their ability to handle the stress of hypertension and "burst", thus creating a small haemorrhagic spot. NO also inhibits platelet aggregation (Wu and Meininger, 2000) thus causing that the foetus might bleed to death *in vivo*.

As the dosage of creatine increases, it seems that subcutaneous haemorrhaging affects the whole foetus from abdomen to cranium.

9.3.2 Underdeveloped foetuses

A number of underdeveloped foetuses (underdeveloped = behind normal developmental stages and which weighed significantly less than the group mean and was of a shorter length than the mean length of the group) were observed. Interestingly, it was control group A that had the most underdeveloped foetuses (28%) of all the groups, followed by experimental group B1 (19.7%) and experimental group A1 (4.5%). Control group B and experimental groups A2 and B2 did not have any underdeveloped foetuses. It could be argued that the underdeveloped foetuses are just at the extreme end of the range. However, a pilot study done by Badenhorst and Vorster (2000, unpublished data) showed that creatine inhibited growth in the developing chick embryo, indicating the involvement of creatine in inhibition of embryonic growth.

While group B2 did not display underdeveloped foetuses, it is possible that those foetuses that were not developing well were eventually absorbed as 53% (34/64)

of foetuses were absorbed in this group. The work of Miller *et al.* (1993) illustrated that an increase in dosage of a creatine analogue increased *in vivo* foetal mortality rates.

9.4 The effect of creatine on histological structure

Histology was carried out on the kidneys, skeletal muscle, pancreas and liver of foetuses from all groups. These organs were chosen since they are primary production sites of creatine (Walker, 1979; Horn *et al.*, 1998). Skeletal muscle was also utilized since it stores 95% of the total creatine content of the body (Persky and Brazeau, 2001). Each organ was studied and comparisons were made between the experimental and control groups.

9.4.1 Kidneys

The renal corpuscles of the kidney in the different groups (groups A and B, experimental and control) were well developed, as were the collecting tubules. Anecdotal reports (Robinson et al., 2000; Wyss and Kaddurah-Daouk, 2000; Brudnak, 2004) claim that creatine might influence the filtration apparatus of the kidney and might result ultimately in kidney failure. If creatine had a significant effect on the filtration apparatus and cellular structure of the kidney, it would manifest itself especially during organogenesis of the kidney. It appears that creatine did not have a significant effect on the development of the kidney in the

foetuses of this study, although the ultrastructure of the cells and the filtration barrier was not studied.

9.4.2 Skeletal muscle

Control groups A and B had normal striations and well developed muscle fibres. Very precise "A" bands were observed in these groups as well as the interlinking "I" bands. When skeletal muscle sections from control animals were compared with those from experimental group A1, the tissue appeared to be very similar. However (although not quantitated), when experimental group A2 was compared to control group A, it appeared that there was an increase in the number of muscle fibres in group A2. Skeletal muscle creatine content is boosted by factors that play a role in glucose metabolism (Weiss *et al.*, 2002). Factors such as insulin and insulin-like growth factor (IGF) are involved. It appears that creatine administered over a period may cause the increased formation of myofilaments, thus leading to the increase in striations in the developing myoblasts. This could be attributed to the anabolic effects of insulin (Augustin *et al.*, 2003). Mujika and Padilla (1997) found that an increase in creatine content and creatine concentration (due to creatine supplementation) induces increased protein synthesis in skeletal muscle.

The skeletal muscle fibres of experimental groups B1 and B2 were not as well developed as those of experimental groups A1 and A2. Although not quantitated, more immature fibres were observed in experimental groups B1 and B2.

Once again, it is postulated that the sudden increase in creatine concentration (due to creatine supplementation) in the myoblasts at critical stages (days 9 and 11) of development might cause a shift in the Arg-NO metabolism. This in turn may increase the amount of NO produced. NO inhibits mitochondrial function by destabilizing the mitochondrial membrane potential (Gross *et al.*, 1996), thus probably explaining why the myoblasts in experimental groups B1 and B2 were underdeveloped. The reason why the muscle fibres in A1 and A2 were better developed than their counterparts in the B groups could be attributed to the anabolic properties of insulin (Augustin *et al.*, 2003), as well as the increase in growth hormone (due to excessive NO production). Mujika and Padilla (1997) found that exogenous creatine supplementation increased muscle fibre diameter, whereas creatine analogs fed to rats for a period of time, decreased fast-twitch muscle fibre diameter (Wyss and Kadurrah-Daouk, 2000).

9.4.3 Pancreas

When the histology of the pancreas of the control groups was compared to those of the experimental groups (A and B), it was found that there was a more intense localization of the contained protein in the α- and β cells in experimental groups than in the control groups.

Rall *et al.* (1973) found that rat foetal pancreas starts to produce its own insulin after 14 days of development. However, the serum levels of insulin are very low between days 14 and 18 of development. Ninety five percent of insulin is

accumulated in the β cells between days 18 and 22 (birth)(Rishi *et al.,* 1969; Rall *et al.,* 1973). The serum concentration of insulin is increased threefold during these four days (Kervan and Gerard, 1974). The rat embryo thus receives its insulin for the whole period of gestation from the maternal source. Exogenous creatine supplementation elicits a direct insulin response (Wyss and Kadurrah-Daouk, 2000), thus the pregnant mother would have elevated insulin levels circulating in her blood (Wyss and Kadurrah-Daouk, 2000). The purported raised insulin levels in the female of this study may have an enhancing effect on the development of the pancreas, causing the pancreas to be "ahead" of development. In this study, foetuses from experimental groups had pancreata that were ahead of development, while the pancreata of control group foetuses were at the normal stage of development. This may explain why the experimental groups had a more intense localization of insulin protein in the β- cells than the control groups. More insulin may have been accumulating in the pancreata of the animals in the experimental groups during days 18 to 20 of development because the development of the pancreata of foetuses in the experimental groups was enhanced by the anabolic properties of insulin.

It is in the very late stages of rat pancreatic development that typical islet formation is seen, with β cells in the core of the islet and other endocrine cells spread around the periphery (Herrera *et al.*, 1991; Louvi *et al.,* 1997). When The present study is compared to the that of Herrera *et al.* (1991) and Louvi *et al.* (1997), typical islets had not formed in the pancreata of control foetuses, while

the pancreata of the experimental groups appeared to be "ahead" of their stage of development. This is possibly due to the effect of maternal insulin on the developing pancreas. It thus appears also that creatine supplementation has a positive effect on the development of the pancreas, especially the endocrine cells.

9.4.4 Liver

In a toxicology study, the liver would be the first organ usually affected at a cellular level, due to its function as a detoxifying agent. The histology of the liver from control groups A and B appeared to be similar, with an abundance of liver cord cells as well as relatively wide-open sinusoids. Precursor blood cells were found in the sinusoids. When the control groups were compared to the experimental groups, it was found that creatine appeared to negatively affect the development of the liver. In all the experimental groups (although not quantitated), there was a decrease in the number of liver cord cells compared to control groups. Fewer precursor blood cells were present in the experimental groups compared to the control groups. The sinusoids in the experimental groups were also wider relative to those of the control groups. This may be attributed to the fact that there is a decrease in number of liver cord cells, thus making the sinusoidal spaces look wider. An exception to the abovementioned statement was the liver of animals from experimental group B1, which showed a number of altered features. Erythrocytes were found in the sinusoids and it appeared that there was an increase in blood vessels in the sections of liver examined. The

sinusoids of this group, contrary to the other experimental groups, were very narrow. Megakaryocytes were found in the liver sections of experimental groups B1 and B2, but no megakaryocytes were observed in any of the other groups. In the B2 group (experimental) the liver cord cells appeared to be disorganized. The present research supports work done by Hatano *et al.* (1996) and Kanazawa *et al.* (1998). Both groups of researchers injected mice with D-galactosamine to induce hepatic injury. Hatano *et al.* (1996) found that the effects of D-galactosamine were reduced if mice were supplemented with creatine. Kanazawa *et al.* (1998) observed that 48 hours after D-galactosamine administration, 80% of mice supplemented with creatine were alive, compared to the 100% mortality rate of mice in the group not supplemented with creatine. It appears that the longer the developing liver was exposed to high creatine concentration levels in this study, the less damage there was to the liver.

9.5 SKELETAL DEVELOPMENT

The desmocranium of the foetuses from all groups were compared to see whether creatine had a significant impact on the development of bone. Thin desmocrania were observed in control groups A and B. When the control groups were compared to the experimental groups, thicker desmocrania were observed in all the experimental groups. Thus, it appears as if creatine may increase the rate of bone growth. The A1 group (experimental) had a 27% (4/15) increase in the number of foetuses with thick desmocrania and the A2 group (experimental)

a 60% (3/5) increase in the number of foetuses that presented with thick desmocrania. B1 and B2 groups [50% (4/8)and 100% 4/4) respectively] exhibited the same pattern as was found in the A experimental groups. From this data, it can be speculated that creatine appears to influence the development of the cranial plates and in particular, the desmocranium, in such a way as to stimulate bone growth in the developing rat foetus. In addition, the time of creatine administration does not play a role in this regard, since foetuses in both the A2 and B2 groups had thicker desmocrania.

Endochondral and intramembranous ossification takes place to form bone in the rat foetus from early stages of development until birth (Baker *et al.*, 1980). At the particular stage of skeletal development observed in foetuses in this study, cartilage is not yet present in the ribs, vertebral bodies or limbs of the foetus. Cartilage formation (in the ribs, vertebral bodies, limbs) takes place from day 20 of development until birth (Louvi *et al.*, 1997; Menegola *et al.*, 2001). Thus, at the particular stage of development represented by the foetuses in this study, an axial skeleton would be present, but no cartilage would yet exist in the ribs and sternum.

The development of cartilage was divided into four categories for this study. When the foetuses from the different groups were compared, the foetuses were classified as having cartilage throughout the axial skeleton, present in the pelvis, present in the ribs and sternum only, present in the extremities or no cartilage

present at all. In control group A, none of the foetuses (4/4) exhibited any cartilage, while in control group B only 37.5% (3/8) of foetuses had no cartilage present. The remainder of foetuses in this group showed cartilage in the ribs and sternum (37.5%, 3/8) and in the extremities (25%, 2/8).

There was a definite increase in the amount of cartilage from experimental group A1 to experimental group A2 and from B1 to B2. There was no cartilage present at all in the foetuses (8/8) in experimental group B1.

It was consistently found that in the control groups, the pedicles of vertebrae were widely spaced and not fused, while in experimental groups A1 and B1 the pedicles of the vertebrae were almost fused in 53% (8/15) and 62.5% (5/8) of the foetuses respectively. In experimental groups A2 and B2, all the foetuses observed had pedicles of vertebrae that were much closer to fusion or fused. In this particular part of the study, the time of administration did not play a significant role, but rather the dosage of creatine that was administered appears to have affected development. The higher the dosage that was administered, the higher the incidence of closely placed pedicles.

The fact that 50% (4/8) of foetuses in experimental group B1 showed immature bone development underlines the fact that some internal mechanism may have been disrupted. Due to the fact that 50% of the foetuses displayed immature bone development, it also explains why 100% (8/8) of the foetuses in this

particular group did not display any presence of cartilage. Foetuses with immature/delayed bone development were found in control group B (25%, 2/8) and experimental groups A1 (7%, 1/15) and B1 (50%, 4/8).

Kimmel and Wilson (1973) did a study to determine whether a substance could induce extra rib formation (rats have 13 pairs of ribs). They defined a 14^{th} rudimentary rib as one that is less than half the length of the 13^{th} rib. A 14^{th} rib that is longer than 50% of the 13^{th} rib would be termed an extra rib (Kimmel and Wilson, 1973). In the present experiment, small groups osteogenic cells were observed at the level of the 14^{th} thoracic vertebra, where the head of the rib would normally attach to the vertebra. In control group A, 25% (1/4) of foetuses showed osteogenic cell cluster at this level, compared to 62.5% (5/8) of foetuses in control group B. No extra 14^{th} rib was observed in control group A, while one foetus in control group B had an extra 14^{th} rib. Interestingly, experimental groups A1 and A2 also displayed 60% (9/15) and 80% (4/5) respectively of foetuses that had an osteogenic cell cluster. Forty percent (6/15) of foetuses in experimental group A1 also developed an extra 14^{th} rib. In experimental groups B1 and B2 it was observed that each displayed a 36% and 50% formation of cell clusters respectively, while experimental group B2 had 25% of foetuses that formed a 14^{th} extra rib. According to this information and the data collected by Kimmel and Wilson (1973), rats do have the ability to form a 14^{th} rib if exposed to a foreign substance, which in this case could also have been induced by saline (see control group B).

Creatine analogs (i.e. cyclocreatine) inhibit cartilage formation given to rat foetuses *in vivo* and chicken chondrocytes *in vitro* (Wyss and Kadurrah-Daouk, 2000). Creatine might stimulate tumour growth (Wyss and Kadurrah-Daouk, 2000), since it has been shown that creatine analogs can inhibit tumour growth (Miller *et al.,* 1993). It thus appears that creatine administration increased the rate of cartilaginous growth in the present study. In a study done by Louvi *et al.* (1997), they cross bred mice until they had a wild type group, an Ir (insulin receptor deficient) group, an Igr1r (insulin-like growth factor 1 receptor deficient) group and an Igf2r (insulin-like growth factor 2 receptor deficient) strain. Females from each group were made pregnant and sacrificed at certain stages of development; foetuses were collected and underwent skeletal staining. It was found that mice foetuses that had no insulin receptors, as well as the mice foetuses that were deficient in insulin-like growth factor 1 receptors, suffered from delayed growth and skeletal development. The wild type foetuses were the biggest and most advanced in skeletal development. They concluded that if there were a decrease in insulin or insulin receptors, skeletal growth would be delayed. However, an increase in insulin concentration (maternally, due to exogenous creatine supplementation) may induce enhancement of growth of the foetus, especially the skeletal system. This may explain the results of the present study in which creatine exposure (repeated or day 9 and 11 injected) may have increased insulin levels (maternally), thus influencing the growth of the foetus positively. This may have led to an enhanced rate of growth of the foetal skeletal system. To explain this further, when the foetuses from control and experimental

groups were compared, it was observed that normal skeletal ossification took place in the region of the ribs, indicated by a prominent primary ossification centre. However, it was observed that the experimental groups (A and B) had extensive costochondral cartilage formation while costochondral cartilage formation in the control groups was absent. When these observations are compared to the work done by Louvi *et al.*, (1997), it appears that the experimental groups had enhanced cartilage formation and were at least one to two days ahead of development of that of the control groups. It was observed that there was an increase in the number of osteogenic cell clusters (at T14 level) from the control group A to experimental groups A1 and A2; supporting the fact that creatine did have an enhancing effect on bone development. This may indicate that exogenous creatine supplementation may have elicited an insulin response and that the elevated insulin levels may have enhanced skeletal growth in these foetuses.

10. CONCLUSION

It appears that creatine has both a positive effect and a negative effect on the developing rat foetus. Negative effects include subcutaneous haemorrhaging, underdevelopment of foetuses, delay skeletal muscle development (B1 and B2 experimental groups) as well as a decrease in cord cells in the liver of animals from the experimental groups. However, in the adult, creatine stimulates protein synthesis in skeletal muscle. Which is contrary to what was found in experimental

groups B1 and B2. Further research needs to be carried out in order to explore the opposite effects on skeletal muscle in the foetus and the adult.

Positive effects of creatine include an increase in bone (thickness) and cartilage formation, enhanced β-cell formation in the pancreata of animals from experimental groups, as well as increased mean weights and lengths.

The positive effects of creatine may be attributed to increased levels of insulin in the pregnant female rat (due to creatine supplementation) and the anabolic properties of insulin. This effect could be studied further by investigating serum levels of insulin and creatine in the pregnant rats undergoing creatine supplementation. It appears that some mechanisms, however, may have been disrupted during certain early stages of development (day 9 and 11 injected foetuses) thus inducing the negative effects. These mechanisms, however, cannot be speculated on here.

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APPENDIX A

All necessary documentation pertaining to animal ethics are presented in this appendix.

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

2003	48	3

APPLICANT: Henk Badenhorst

DEPARTMENT: School of Anatomical Sciences

PROJECT TITLE: The Effect of Creatine on the Developing Rat Foetus

Species	Number	Expiry Date
Sprague dawley	40	2005

i) Approval is hereby given for the experiment described in the above application.

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

APPROVED subject to:
-using sterile injections and medium ⊛
-demonstrating the injection technique to the CAS staff
-discussing the route of administration with Professor Norton
-providing information on the method of euthanasia to be used
- the fate of 4females (to CAS staff)

SIGNED D. A. Gray DATE: 2 June 2003
(Chairman: ~~Animal Ethics Screening Committee~~)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1)(c) of the Veterinary and Para-veterinary Professions Act (19 of 1982)

SIGNED _____ DATE: 2 June 2003
(Registered Veterinarian)

NOTE:

First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available, and the procedures required by the CAS for the carrying out of experiments.

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "J", please use the back of this form.

The effect of creatine on the developing rat foetus

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: HENK BADENHORST

b. Department: ANATOMICAL SCIENCES

c. Experiment to be modified / extended

AESC NO:		
2003	48	3

d. Project Title: THE EFFECT OF CREATINE ON THE DEVELOPING RAT FOETUS

e. Number and species of animals **originally approved**:

40	SD rats 36 female 4 male

f. Number of additional animals **previously allocated** on M&Es:

g. Total number of animals **allocated** to the experiment to date:

20	SD rats 16 females 4 male

h. Number of animals **used** to date:

20	SD rats

i. Specific modification / extension requested:

We would like to carry out vaginal smears on the female rats each day to confirm the stage of the oestreous cycle.

j. Motivation for modification / extension:

More accurate way to determine in which stage of oestreous the female is in and when the best time is to mate female with male.

Date: ..... Signature: 13/11/2003

RECOMMENDATIONS:

Approved: use of vaginal smear, daily.

Date: 13/11/03 Signature: D. A. Gray

Chairman, AESC

102

November 2000

Please note that only typewritten applications will be accepted. Should additional space be required for section "i" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

The effect of creatine on the developing rat foetus

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: F. H. BADENHORST

b. Department: ANATOMICAL SCIENCES

c. Experiment to be modified / extended

AESC NO: 2003 | 48 | 3

d. Project Title: THE EFFECT OF CREATINE ON THE DEVELOPING RAT FOETUS

e. Number and species of animals **originally approved**:

SD	4 MALES 36 FEMALES

f. Number of additional animals **previously allocated** on M&Es:

g. Total number of animals **allocated** to the experiment to date:

SD	4 MALES 36 FEMALES

h. Number of animals **used** to date:

SD	4 MALES 36 FEMALES

i. Specific modification / extension requested:

LOOK ATTACHED PAPER

j. Motivation for modification / extension:

LOOK ATTACHED PAPER

Date: 25/5/04 Signature: [signature]

RECOMMENDATIONS:

Approved: 16 additional rats.

Date: 25/05/04 Signature: D. A. Gray

Chairman, AESC

ANIMAL ETHICS EXTENSION ON NR 2003/48/3

SPECIFIC MODIFICATION/EXTENSION

16 SD females required

MOTIVATION FOR MODIFICATION/EXTENSION

New literature suggests that the normal maintenance dose of creatine is anything between 3-7 grams per day. In the current experiment I am using a maintenance dose of 15 grams per day, according to older literature. I would like to investigate what the difference between the two doses could be and for this I would require 15 animals. One female is also needed as one excisting female was accidentally injured by a male and had to be euthanased.

APPENDIX B

Shorr staining technique (Drury and Wallington, 1980)

500 ml 50% ethyl alcohol

2.5g Biebrich scarlet

1.25g Orange G

0.375g Fastgreen FCF

7g Phosphohomolybdic acid

5ml Glacial acetic acid

Allen's fixative (Gray, 1953)

75ml saturated aqueous picric acid

25ml (40%) concentrated formalin

5ml glacial acetic acid

1gr urea/100ml fixative

Combine solutions to form 100ml of fixative. Mix urea into solution and dissolve urea completely.

A derivative of this method is Allen's fixative, where 1gr urea/100ml fixative is dissolved in the fixative. The reason for use of the urea is that it can prolong the

fixation period indefinitely without serious harm (swelling, hardening) to the tissue.

Formic acid-formalin decalcification (Bancroft and Gamble, 2002)

10ml 90% stock formic acid

5ml 40% formalin

Distilled water to make up to 100ml.

Immerse embryo in solution and leave for at least 72 hours for solution to penetrate embryo fully. After 72 hours, prepare a fresh solution and immerse the embryo in the fresh solution. Two hours after immersion of the embryo into the fresh solution, can an endpoint test be done. If the solution turns cloudy during the endpoint test, there is still calcium present in the foetus. If the solution stays clear, all the calcium has been dissolved and the embryo is ready for processing.

Formic acid is a weak acid and was used in this experiment to decalcify the foetal bone. Formic acid does not cause extensive swelling or harm to the bones or tissue like strong acids (HCl or nitric acids), but takes longer to decalcify the specimen.

Alcian Blue and Alizarin red S skeletal staining method (Menegola *et al.*,2001)

Acid staining solution (pH 2.8)

0.14% Alcian blue dissolved in ethanol (70%), 5 parts

0.12% Alizarin red S dissolved in ethanol (96%), 1 part

Glacial acetic acid, 8 parts

Ethanol (70%), 50 parts

Basic staining solution

0.7% KOH dissolved in distilled water, 250 parts

0.5% Alizarin red S dissolved in distilled water, 1 part

Clearing solution

Ethanol (70%), 2 parts

Glycerine, 2 parts

Benzyl alcohol, 1 part

Conservation solution

Ethanol (70%), 1 part

Glycerine, 1 part

Foetuses were immersed in 4% NaCl overnight. The following day foetuses were then skinned and eviscerated. Dorsal muscles were gently removed as well as the heat pad that is situated between the scapulae.

Embryos were then immersed in acid staining solution (24 hours) and then dehydrated in 96% alcohol (24 hours). Embryos were then immersed in the basic

staining solution for at least 30 hours. The solution must be renewed at least three times. The KOH causes maceration of the soft tissue.

After the basic staining solution, the embryo was then placed in the clearing solution. This solution clears and hardens the tissue. A skeletal structure can now be seen. In the final step, the foetus was then placed in conservation solution.

Immunocytochemistry

Notes

Primary and secondary antibodies were made up in a special diluent (see diluent for antiserum).

Each section required approximately 30µl of antibody.

Diluent for antiserum

100 ml Tris/saline

0.25g sodium azide (this must be added to Tris in fume cupboard)

5mg bovine serum albumin

40 mg E.D.T.A.

1ml swine serum

Tris solutions

1.Tris/HCl (0.05 M Tris buffer pH 7.6)

Stock solutions that will be needed is 1 M Tris and 1 N HCl.

Mix 100ml (Tris) to 76.8ml of the HCl. The pH must then be adjusted to 7.6 (it might be necessary to add more HCl). Add distilled water until the solution is made up to 2 litres.

Tris/saline (0.95% NaCl. 0.05 M Tris/HCl at pH 7.6)

Dissolve 42.75g NaCl in 4.5 litres distilled water. Add 500ml Tris/HCl to make up 5 litres of solution.

DAB

1mg of DAB weighed into new bijou bottle.

When second day starts, put 29ml (distilled water) in fridge (4°C).

Add 2ml of Tris/HCl to DAB in bijou bottle.

Add 1ml of 30% Perhydrol (Hydrogen peroxide) to the 29ml distilled water to make up a 30 ml 1% peroxide solution. Mix well.

Add 20µl of 1% perhydrol to DAB/Tris/HCl solution and mix well.

Iron haematoxylin

Iron Alum solution

5-gram iron alum

100 ml distilled water

Haematoxylin

0.5gram haematoxylin

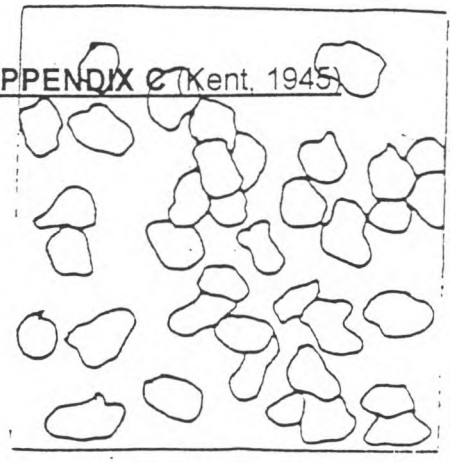
100ml 90% ethanol

Notes

Dewax sections in xylene and rehydrate to water. Place in iron alum (mordant) for 1 hour. Rinse in distilled water. Immerse sections into 0.5% haematoxylin (depending on the fixative) for at least one hour (Bouin's = one hour, Helly's = three hours). Wash sections in running tap water and put in distilled water. Differentiate each section separately (microscopically) in 5% iron alum until structures are clearly desired. Wash in tap water for ten minutes. Dehydrate through graded series of alcohols to absolute alcohol, immerse in xylene and mount in entellan.

A OESTROUS

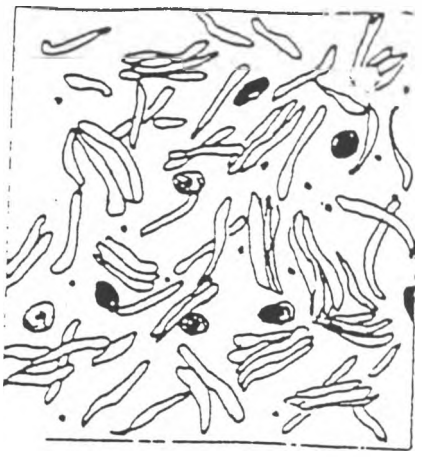
APPENDIX C (Kent, 1945)



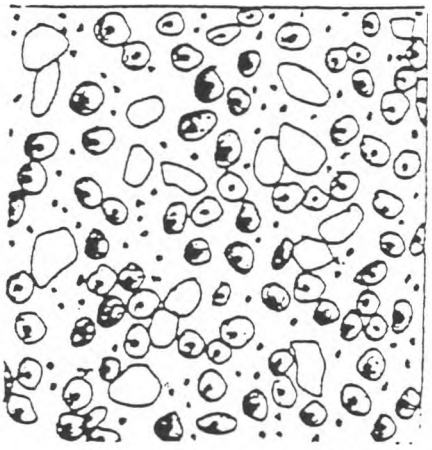
dance of cornified polygonal cells.

OESTRUS

B. METOESTRUS



Abundance of elongated cornified cells.
Some nucleated cuboidal cells.
Occasional leucocytes.



(i)

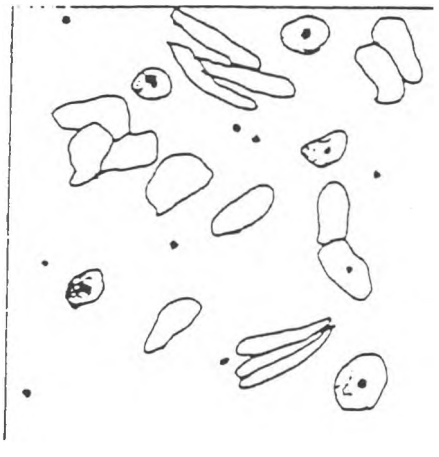
rly Dioestrus

merous nucleated cuboidal cells.
e cornified polygonal cells.
undance of leucocytes.

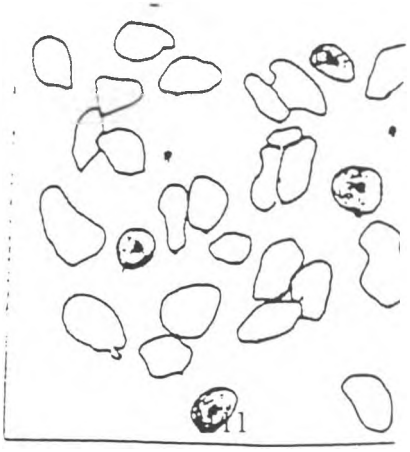
(ii) *Mid-Dioestrus*

Numerous nucleated cuboidal cells.
Occasional elongated-polygonal cornified cell
some leucocytes.

D. PROESTRUS



(iii)



Polygonal cornified cells numerous.
Some nucleated cuboidal cells.
Very few leucocytes.

APPENDIX D

Days and events of organogenesis (Baker *et al.*, 1980)

Day 7

Gastrulation stage: implantation of the zygote almost completed.

Cardiac region: mesodermal cardiogenic primordia (paired) develop.

Day 8

Primitive streak: amniotic and ectochorionic cavities connected, primitive streak develops.

Circulatory system: mesodermal cardiogenic primordia (paired) are developing.

Day 9

Digestive system: Initiation of development of foregut.

Circulatory system: fusion of cardiogenic primordial, formation of endothelial cells between splanchnic mesoderm and endoderm. Initiation of myocardial contractions in partly fused heart (still tubular). Primitive bulboventricular rudiment formed from cardial myoblasts and initiation of myocardial contractions.

Day 10

Neurula: Formation of somites 5-12 and first visceral arch and regression of the peripheral yolk sac. Embryo is bent dorsally. Formation of visce-

ral arches I and II (recognizable), placenta of yolk sac. Embryo folds ventrally.

Digestive system: rupturing of oral membrane and formation of liver primordium. Yolk stalk tapers down to slim tube.

Circulatory system: Tubular heart in S-shape, formation of atrial and bulbo-ventricular zones and aortic arch I-III, development of sinus venosus, ventricular loop, umbilical veins and atrium. Heart starts pumping, circulation through body established.

Day 11

Neurula: Lower thoracic somites form and yolk stalk closes (15 somite level). Buds of arm and leg develop and primitive streak recedes

Digestive system: Liver and dorsal pancreas develop together with laryngo-tracheal groove. Closure of vitteline duct and formation of cloaca and tailgut. Stomach is longitudinal and trabeculae forms in liver. Formation of cloacal membrane.

Circulatory system: Definite aortic arches I-III and formation of interventricular sulcus. Aortic arch I start regressing, aortic arch IV develops. Left and right heart chambers just recognizable as separated. Aortic arch IV still small while aortic arch I regress more. Aortic arch II starts regressing. End of day 11, aortic arches III and IV well developed

while irregular aortic arch V appears.

Muscular system: Myotomes made up of longitudinally arranged lengthened myoblasts.

Day 12

Tail bud embryo: Formation of somites 32-40 as well as deep cervical sinuses, olfactory pits and umbilical herniation.

Complete embryo: Vascularization of limb buds and development of brachial nerves in upper limb.

Digestive system: Pharyngeal pouches undergo changes. Numbers. 1 and 3 contact ectoderm while number 2 bursts into the visceral groove. Ultimobranchial body develops in pouches 4-6. Development of stomach, hepatopancreatic duct and duodenum. Ventral and dorsal pancreas (with duct) develops. Tongue develops while the trachea separates from oesophagus. Liver with deep cut lobes while stomach takes on oblique shape. Regression of tailgut.

Circulatory system: Formation of atrial septum and atrioventricular canal, large aortic arches IV and V present. Aortic arches I and II completely disappeared. Interventricular septum forming together with atrio-ventricular cushions and septa in the heart. Pulmonary vein opens into left atrium. Aortic arch V regresses, collection of vitteline and umbilical veins into hepatic vein. Fusion of ventral and dorsal atrio-ventricular cushions. Atrial septum still incomplete, but forming foramen ovale.

Muscular system: Development of spindle-shaped myoblasts.

Endocrine glands: Formation of dorsal and ventral pancreatic buds. Pancreatic ducts develop.

Day 13

Metamorphosing embryo: Cartilage beginning to develop in distal part of limbs.

Digestive system: Fusion of ventral and dorsal pancreas while primary duct disappears. Single intestinal loop recognizable.

Circulatory system: Aortic trunk beginning to separate into pulmonary and systemic parts, aortic arches same as day 12. Foramen ovale present and formation of auricles occurring. Left and right separation of heart nearly complete.

Endocrine glands: Dorsal and ventral pancreata fuse, complete with islets and acini. Pancreas also sprouts tubules.

It must be mentioned in that cartilage development in the distal parts of the limbs only start on day 13 of development and skeletal cartilage of the ribs on day 14 of development, while crystallization of the cartilage to bone only starting to occur from day 17 and onwards.

APPENDIX E

Statistical analysis

All statistical calculations as well as all raw data is included in this section.

An alpha level of ($P \leq 0.05$) was used in all the calculations to determine whether there is a significant difference between all the groups.

LENGHT OF FOETUSES (millimeter)

Group A (day 7-13 consecutive injections)

Foetus nr	Control A Saline
E1-1	34.65
E2-1	47.45
E2-2	41.25
E2-3	45
E2-4	40.85
E2-5	41.85
E2-6	42.6
E2-7	43.5
E2-8	40.15
E2-9	47.25
E2-10	46.95
E2-11	47.15
E2-12	46.25
A5-1	40.6
A5-2	42.25
A5-3	41.55
A5-4	42.5
A5-5	42.5
A5-6	39.45
A5-7	44.05
A5-8	39.2
A5-9	41.4
A5-10	43.95
A5-11	40.15
A5-12	37.25
B5-1	31.75
B5-2	27.75
B5-3	32
B5-4	31.4
B5-5	30.45
B5-6	30.65
B5-7	29.15
B5-8	32.2
B5-9	31.25
B5-10	34.85
B5-11	31.25
B5-12	31.9
B5-13	31.1
B5-14	24.95
D1-1	39.6
D1-2	38.6
D1-3	37.2
D1-4	39.2
D1-5	33.9
D1-6	34.2
D1-7	40.1
D1-8	38.8
D1-9	38.75
D1-10	37.65
D1-11	42.2

Foetus nr	Exper. A1 [15g/day]
A1-1	42
A1-2	43
A1-3	43.8
A1-4	39.2
A1-5	36
A1-6	41.8
A1-7	43
A1-8	40
A1-9	40.8
A1-10	42.1
A1-11	42.7
E3-1	38.2
E3-2	39.9
E3-3	41
E3-4	38.55
E3-5	40.05
E3-6	41
E3-7	38.47
E3-8	38.9
E3-9	37.3
E3-10	42.8
E3-11	38.55
E3-12	42.25
E3-13	39.6
E3-14	39.55
E3-15	37.85
E3-16	38.45
E3-17	38
H3-1	33.6
H3-2	37.9
H3-3	38.45
H3-4	39.45
H3-5	26.5
H3-6	38.9
H3-7	42.1
H3-8	38.7
H3-9	34.35
H3-10	38.15
H3-11	38.45
H3-12	40.9
H3-13	37.15
H3-14	37.7
H3-15	40
H3-16	36.35
H3-17	37.85
H3-18	38.6
C5-1	37.7
C5-2	37.5
C5-3	38.45
C5-4	36.4

Foetus nr	Exper. A2 [30g/day]
G3-1	39.2
G3-2	36.1
G3-3	39.1
F1-1	36.05
F1-2	39
F1-3	37.2
F1-4	41.35
F1-5	38.1
F1-6	37.2
F1-7	39.55
F1-8	38.9
F1-9	38.15
F1-10	37.25
F1-11	41.25
F1-12	39.05
F1-13	38.25
F1-14	39.2
C3-1	45.4
C3-2	42.7
C3-3	42.35
C3-4	44
C3-5	46.05
C3-6	47
C3-7	41.35
C3-8	45.2
C3-9	47.5
C3-10	43.85
C3-11	42.1
C3-12	46.2
C3-13	44.15
A4-1	42.5
A4-2	45.4
A4-3	44.55
A4-4	44.8
A4-5	42.05
A4-6	42.25
A4-7	42
A4-8	44.65
A4-9	45.8
A4-10	41.9
A4-11	44.3
A4-12	46.25
A4-13	41.35
E5-1	40.5
E5-2	41.6
E5-3	38.1
E5-4	40.1
E5-5	40.45
E5-6	40.75
E5-7	38.95

SUM	1910.6
AVE	38.212
STDEV	5.691

C5-5	36.1
C5-6	36.85
C5-7	36.15
C5-8	37.25
C5-9	36.35
C5-10	36.2
C5-11	35.5
C5-12	33.95
C5-13	35.05
D5-1	40.1
D5-2	39.55
D5-3	41.25
D5-4	27.9
D5-5	36.4
D5-6	39.2
D5-7	37.85
D5-8	39.05
D5-9	37.8
D5-10	39.65
D5-11	39.4
D5-12	40.15
D5-13	41.6
G1-1	35.5
G1-2	38.25
G1-3	40.65
G1-4	41.20
G1-5	36.75
G1-6	38.85
G1-7	37.9
G1-8	35.8
G1-9	40
G1-10	38.7
G1-11	38.6
G1-12	37.6
G1-13	36.45
G1-14	40.75
G1-15	36.85
G1-16	38.9
G1-17	40.2
SUM	3426.22
AVE	38.497
STDEV	2.766

E5-8	38.95
E5-9	39.6
E5-10	38.95
E5-11	38.4
E5-12	41.45
I1-1	32.15
I1-2	39.6
I1-3	36.55
I1-4	40.00
I1-5	34.95
I1-6	38.9
I1-7	37.35
I1-8	36.2
I1-9	36.15
I1-10	38.75
I1-11	32.45
I1-12	39.15
SUM	2719.95
AVE	40.596
STDEV	3.430

LENGHT OF FOETUSES (millimeter)

Group B (day 9 and day 11 injection)

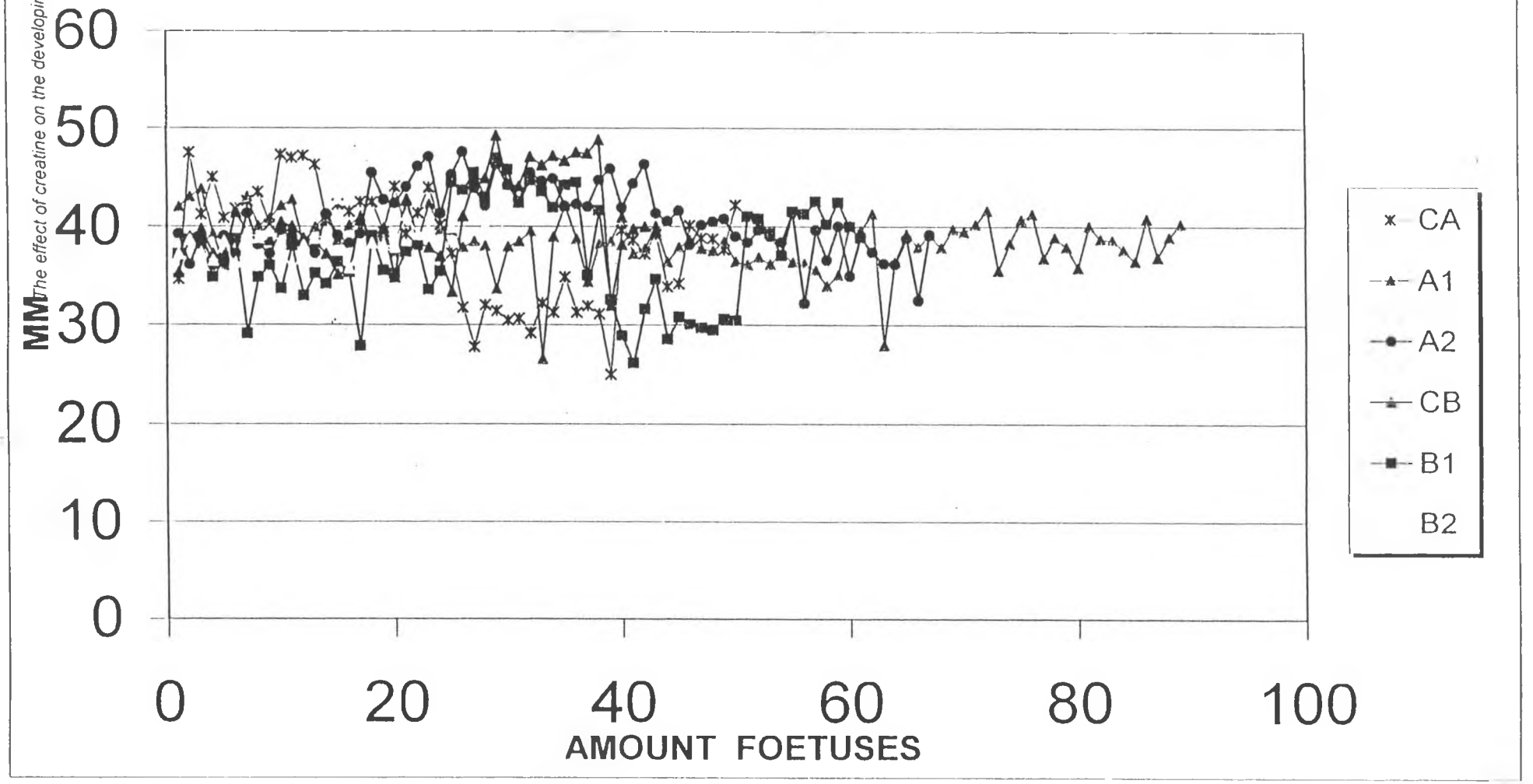
Foetus nr	Control B Saline
B3-1	35.3
B3-2	38.9
B3-3	39.7
B3-4	37.2
B3-5	37
B3-6	41.4
B3-7	39.5
B3-8	38.3
B3-9	38.55
B3-10	40.4
B3-11	40
B3-12	38.8
B3-13	38.4
H2-1	37.15
F3-1	35.1
F3-2	35.4
F3-3	40.55
F3-4	38.8
F3-5	39.8
F3-6	34.8
F3-7	42.6
F3-8	38.4
F3-9	37.8
F3-10	36.95
F3-11	33.3
F3-12	41
D4-1	44.7
D4-2	44.85
D4-3	49.2
D4-4	44.25
D4-5	44.05
D4-6	47
D4-7	46.15
D4-8	47.1
D4-9	46.6
D4-10	47.45
D4-11	47.35
D4-12	48.7
G5-1	31.9
H5-1	38.1
H5-2	39.7
H5-3	39.9
H5-4	39.5
SUM	1741.6
AVE	40.502
STDEV	4.344

Foetus nr	Exper. B1 [15g/day]
E4-1	37.15
E4-2	38.65
I3-1	38.15
C2-1	34.8
C2-2	36.45
C2-3	38.7
C2-4	29.05
C2-5	34.8
C2-6	36
C2-7	33.6
C2-8	38
G2-1	32.9
G2-2	35.2
G2-3	34.15
G2-4	36.35
G2-5	35.3
G2-6	27.8
G2-7	39
G2-8	35.5
G2-9	35.15
G2-10	37.35
G2-11	38
G2-12	33.5
B4-1	35.4
H4-1	44.4
H4-2	43.65
H4-3	45.4
H4-4	42.95
H4-5	46.85
H4-6	45.7
H4-7	42.4
H4-8	44.6
H4-9	43.5
H4-10	41.9
H4-11	44.15
H4-12	44.4
H4-13	35
H4-14	41.6
A6-1	32.5
A6-2	28.85
A6-3	26.05
A6-4	31.55
A6-5	34.6
A6-6	28.5
A6-7	30.75
A6-8	30
A6-9	29.65
A6-10	29.45
A6-11	30.55
A6-12	30.4

Foetus nr	Exper. B2 [30g/day]
B1-1	37.15
B1-2	38.6
B1-3	37.25
B1-4	36.5
B1-5	38.4
B1-6	36.3
B1-7	39.45
B1-8	38.8
B1-9	39.45
B1-10	38.3
B1-11	35.1
I4-1	38.05
I4-2	38.75
I4-3	39.1
I4-4	42.8
C4-1	35.75
C6-1	37.95
C6-2	38.25
C6-3	38.05
C6-4	37.85
C6-5	41.05
C6-6	39.15
C6-7	39.6
C6-8	34.25
C6-9	39.9
C6-10	36.4
C6-11	34.8
C6-12	40.1
C6-13	37.25
C6-14	42.8
SUM	1147.15
AVE	38.238
STDEV	2.036

I5-1	40.95
I5-2	40.75
I5-3	39.25
I5-4	37
I5-5	41.5
I5-6	41.25
I5-7	42.5
I5-8	40.15
I5-9	42.400
I5-10	39.9
I5-11	38.8
SUM	2264.75
AVE	37.127
STDEV	5.242

FOETUS LENGHT IN ALL GROUPS



WEIGHT OF FOETUSES (grams)

Group A (day 7-13 consecutive injections)

Foetus nr	Control A Saline
E1-1	3.25
E2-1	6.7
E2-2	6.04
E2-3	6.17
E2-4	6.02
E2-5	6.57
E2-6	6.04
E2-7	6.08
E2-8	4.3
E2-9	6.81
E2-10	6.38
E2-11	6.59
E2-12	6.38
A5-1	4.8
A5-2	4.76
A5-3	4.32
A5-4	4.82
A5-5	4.83
A5-6	4.28
A5-7	5.35
A5-8	4.42
A5-9	4.81
A5-10	5.07
A5-11	4.6
A5-12	3.26
B5-1	2.51
B5-2	2.42
B5-3	2.56
B5-4	2.59
B5-5	2.52
B5-6	2.32
B5-7	2.09
B5-8	2.56
B5-9	2.75
B5-10	2.74
B5-11	2.68
B5-12	2.72
B5-13	2.45
B5-14	1.98
D1-1	4.5
D1-2	4.11
D1-3	3.6
D1-4	4.39
D1-5	2.65
D1-6	2.77
D1-7	4.73
D1-8	4.55
D1-9	4.04
D1-10	5.41
D1-11	6.67

Foetus nr	Exper. A1 [15g/day]
A1-1	6.39
A1-2	6.18
A1-3	5.91
A1-4	5.62
A1-5	4.65
A1-6	5.94
A1-7	6.21
A1-8	6.3
A1-9	5.47
A1-10	5.84
A1-11	5.69
E3-1	4.28
E3-2	4.17
E3-3	4.56
E3-4	4.56
E3-5	4.13
E3-6	4.35
E3-7	4.46
E3-8	4.25
E3-9	4.31
E3-10	4.19
E3-11	4.09
E3-12	4.77
E3-13	4.72
E3-14	4.68
E3-15	3.56
E3-16	4.21
E3-17	4.85
H3-1	3.67
H3-2	4.74
H3-3	4.05
H3-4	4.47
H3-5	2.05
H3-6	4.02
H3-7	4.54
H3-8	4.15
H3-9	2.72
H3-10	3.89
H3-11	4.15
H3-12	4.47
H3-13	3.90
H3-14	4.08
H3-15	4.18
H3-16	3.39
H3-17	4.24
H3-18	4.18
C5-1	4.12
C5-2	3.87
C5-3	3.88
C5-4	3.71

Foetus nr	Exper. A2 [30g/day]
G3-1	4.34
G3-2	3.72
G3-3	4.31
F1-1	3.87
F1-2	2.92
F1-3	4.4
F1-4	4.53
F1-5	3.77
F1-6	4.06
F1-7	4.8
F1-8	4.18
F1-9	4.21
F1-10	3.52
F1-11	4.41
F1-12	4.56
F1-13	3.32
F1-14	3.99
C3-1	6.36
C3-2	5.82
C3-3	5.07
C3-4	6.15
C3-5	5.61
C3-6	6.07
C3-7	5.88
C3-8	5.75
C3-9	6.5
C3-10	5.71
C3-11	4.95
C3-12	5.75
C3-13	5.79
A4-1	6.08
A4-2	6.61
A4-3	6.64
A4-4	5.92
A4-5	5.94
A4-6	4.97
A4-7	6.02
A4-8	5.94
A4-9	6.28
A4-10	4.75
A4-11	5.76
A4-12	6.41
A4-13	7.62
E5-1	4.23
E5-2	4.63
E5-3	3.88
E5-4	4.2
E5-5	3.93
E5-6	4.35
E5-7	4.26

SUM	214.96
AVE	4.299
STDEV	1.507
C-DENCE	0.418

C5-5	4.11
C5-6	3.82
C5-7	3.75
C5-8	3.95
C5-9	3.98
C5-10	3.56
C5-11	3.95
C5-12	2.92
C5-13	4.01
D5-1	4.40
D5-2	4.68
D5-3	4.45
D5-4	2.08
D5-5	4.44
D5-6	4.13
D5-7	4.34
D5-8	4.46
D5-9	4.53
D5-10	4.21
D5-11	4.29
D5-12	4.34
D5-13	4.53
G1-1	4.19
G1-2	4.3
G1-3	4.13
G1-4	4.38
G1-5	3.8
G1-6	4.45
G1-7	4.32
G1-8	4.05
G1-9	4.46
G1-10	4.07
G1-11	4.09
G1-12	4.32
G1-13	3.94
G1-14	4.33
G1-15	4.11
G1-16	3.8
G1-17	4.26
SUM	384.74
AVE	4.323
STDEV	0.754

E5-8	4.68
E5-9	4.56
E5-10	4.26
E5-11	4.08
E5-12	4.48
I1-1	3.5
I1-2	4.27
I1-3	4.58
I1-4	4.13
I1-5	3.23
I1-6	3.91
I1-7	4.37
I1-8	3.44
I1-9	3.75
I1-10	4.1
I1-11	2.68
I1-12	4.71
SUM	321.42
AVE	4.797
STDEV	1.056

WEIGHT OF FOETUSES [grams]

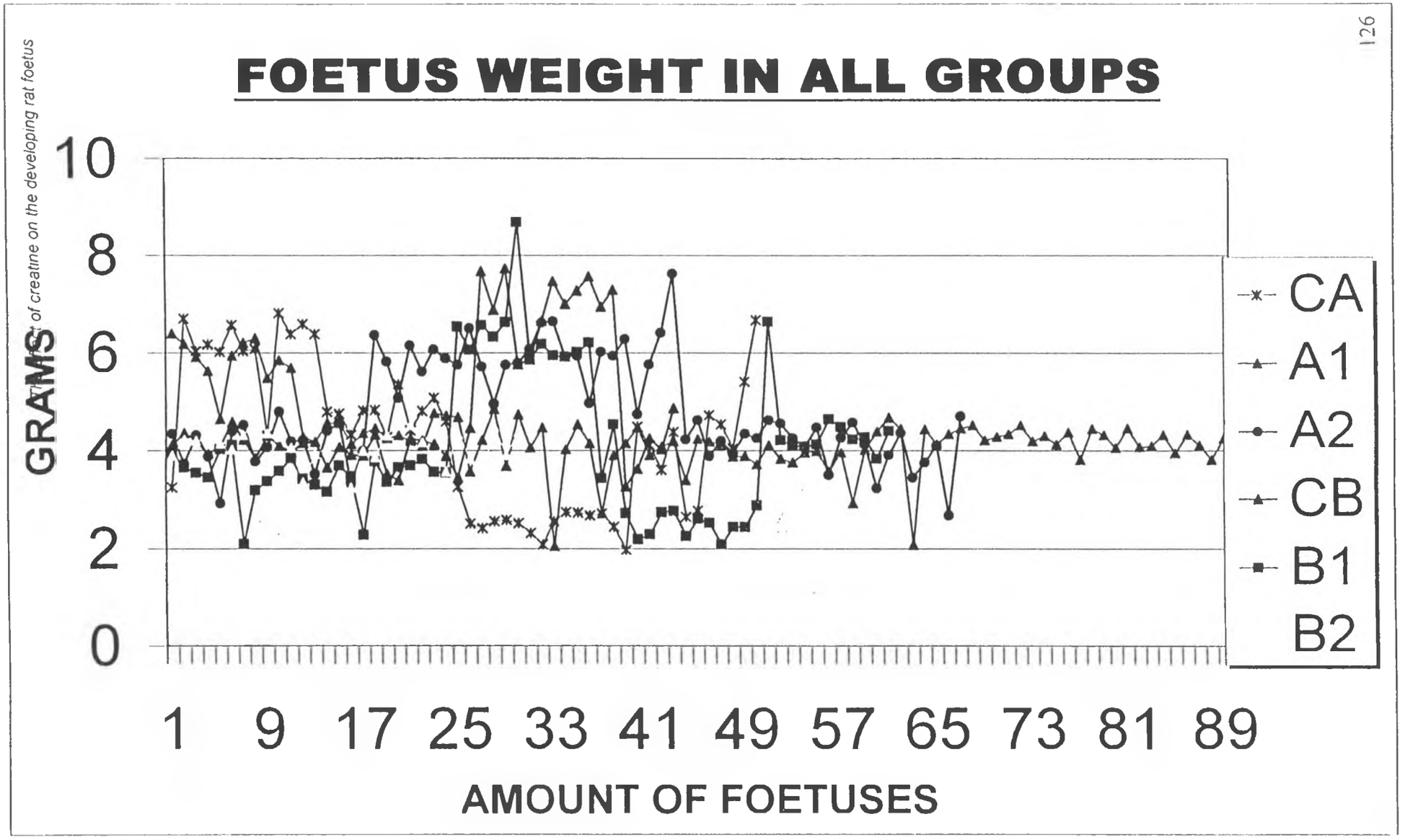
Group B (day 9 and day 11 injection)

Foetus nr	Control B Saline
B3-1	4.11
B3-2	4.36
B3-3	4.2
B3-4	3.92
B3-5	4.09
B3-6	4.58
B3-7	4.22
B3-8	3.83
B3-9	4.31
B3-10	4.09
B3-11	3.96
B3-12	4.16
B3-13	4.17
H2-1	3.65
F3-1	4.06
F3-2	3.91
F3-3	3.89
F3-4	4.32
F3-5	3.52
F3-6	3.39
F3-7	4.25
F3-8	4.2
F3-9	4.13
F3-10	3.88
F3-11	3.44
F3-12	4.46
D4-1	7.66
D4-2	6.87
D4-3	7.72
D4-4	5.76
D4-5	5.86
D4-6	6.65
D4-7	7.46
D4-8	6.99
D4-9	7.26
D4-10	7.55
D4-11	6.94
D4-12	7.28
G5-1	3.26
H5-1	3.62
H5-2	4.25
H5-3	4.02
H5-4	4.87
SUM	209.12
AVE	4.863
STDEV	1.417

Foetus nr	Exper. B1 [15g/day]
E4-1	3.94
E4-2	3.65
I3-1	3.53
C2-1	3.44
C2-2	4.02
C2-3	4.11
C2-4	2.1
C2-5	3.18
C2-6	3.37
C2-7	3.57
C2-8	3.83
G2-1	3.41
G2-2	3.29
G2-3	3.15
G2-4	3.68
G2-5	3.46
G2-6	2.28
G2-7	3.77
G2-8	3.35
G2-9	3.65
G2-10	3.68
G2-11	3.81
G2-12	3.55
B4-1	3.54
H4-1	6.53
H4-2	6.06
H4-3	6.56
H4-4	6.33
H4-5	6.62
H4-6	8.67
H4-7	5.88
H4-8	6.18
H4-9	5.95
H4-10	5.92
H4-11	6.01
H4-12	6.21
H4-13	3.42
H4-14	4.54
A6-1	2.72
A6-2	2.19
A6-3	2.29
A6-4	2.74
A6-5	2.76
A6-6	2.25
A6-7	2.61
A6-8	2.52
A6-9	2.09
A6-10	2.44
A6-11	2.44
A6-12	2.88

Foetus nr	Exper. B2 [30g/day]
B1-1	3.89
B1-2	4.22
B1-3	4.2
B1-4	4.23
B1-5	4.15
B1-6	4.02
B1-7	4.32
B1-8	4.58
B1-9	4.36
B1-10	4.28
B1-11	4.05
I4-1	3.34
I4-2	3.92
I4-3	4.04
I4-4	7.6
C4-1	3.24
C6-1	3.99
C6-2	3.86
C6-3	4.31
C6-4	4.52
C6-5	4.53
C6-6	4.17
C6-7	4.42
C6-8	3.56
C6-9	4.33
C6-10	3.83
C6-11	3.37
C6-12	4.49
C6-13	3.91
C6-14	4.39
SUM	126.12
AVE	4.204
STDEV	0.732

I5-1	6.63
I5-2	4.21
I5-3	4.1
I5-4	4.08
I5-5	4.13
I5-6	4.65
I5-7	4.49
I5-8	4.23
I5-9	4.28
I5-10	3.82
I5-11	4.41
SUM	247.2
AVE	4.052
STDEV	1.438


FOETUS WEIGHT IN ALL GROUPS
GRAMS
10
8
6
4
2
0
1
9
17
25
33
41
49
57
65
73
81
89
AMOUNT OF FOETUSES
CA
A1
A2
CB
B1
B2

AMOUNT OF EMBRYOS VS RESORBED EMBRYOS IN EACH GROUP

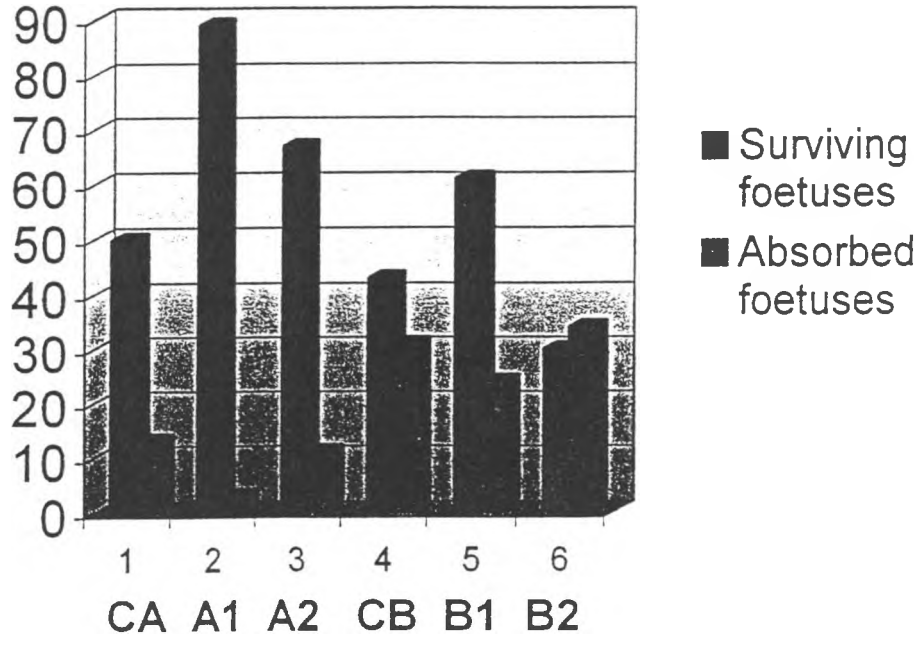
7 DAYS CONSECUTIVELY

SALINE	15G	30G
A2	**A1**	**A4**
PP	11E	13E+3R
D1	**C1**	**C3**
11E	PP	13E
E1	**E3**	**F1**
1E+2R	17E	14E
E2	**G1**	**G3**
12E	17E+1R	3E+5R
H1	**H3**	**I1**
8R	18E	12E+3R
A5	**C5**	**E5**
12E+2R	13E+2R	12E
B5	**D5**	**F5**
14E+1R	13E	PP
50E+13R	**89E+3R**	**67E+11R**
50	89	67
13	3	11

DAY 9 AND 11 INJECTIONS

SALINE	15G	30G
B3	**B4**	**B1**
13E	1E+5R	11E
D4	**C2**	**C4**
12E	8E+1R	1E+7R
F3	**E4**	**F2**
12E	2E+7R	9L+2R
F4	**G2**	**G4**
7R	12E+1R	8R
H2	**H4**	**I2**
1E+4R	14E+1R	11R
G5	**I3**	**I4**
1E+7R	1E+7R	4E+6R
H5	**I5**	**B6**
4E+13R	11E	PP
	A6	**C6**
	12E+2R	14E+2R
43E+31R	**61E+24R**	**30E+34R**
43	61	30
31	24	34

SURVIVING FOETUSES VS ABSORBED FOETUSES



DISTRIBUTION NUMBERS

WEIGHT

CA	A1	A2	CB	B1	B2
2	2	3	3	2	3
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7	4	6		5	
7	4	6		5	
7	4	6		6	
7	4	6		6	

LENGTH

CA	A1	A2	CB	B1	B2
25	27	32	32	26	34
28	28	32	33	28	35
29	34	35	35	29	35
30	34	36	35	29	36
31	34	36	35	29	36
31	35	36	35	29	36
31	36	36	37	30	37
31	36	37	37	30	37
31	36	37	37	30	37
32	36	37	37	31	37
32	36	37	38	31	38
32	36	37	38	32	38
32	36	38	38	33	38
34	36	38	38	33	38
34	36	38	38	34	38
35	36	38	39	34	38
35	36	38	39	34	38
37	36	38	39	35	39
37	37	39	39	35	39
38	37	39	40	35	39
39	37	39	40	35	39
39	37	39	40	35	39
39	37	39	40	35	39
39	37	39	40	35	39
39	38	39	40	35	40
39	38	39	40	36	40
40	38	39	40	36	40
40	38	39	41	36	41
40	38	39	41	36	43
40	38	40	41	37	43
41	38	40	43	37	
41	38	40	44	37	
41	38	40	44	38	
41	38	40	45	38	
42	38	40	45	38	
42	38	41	46	39	
42	38	41	47	39	
42	38	41	47	39	
43	38	41	47	39	
43	38	41	47	39	
43	38	41	47	40	
44	38	41	49	40	
44	38	42	49	41	
44	39	42		41	
45	39	42		41	
46	39	42		42	
47	39	42		42	
47	39	42		42	
47	39	42		42	
47	39	43		42	

4	6	6
4	6	6
4	6	6
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4	6	6
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4	6	7
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39	43	43
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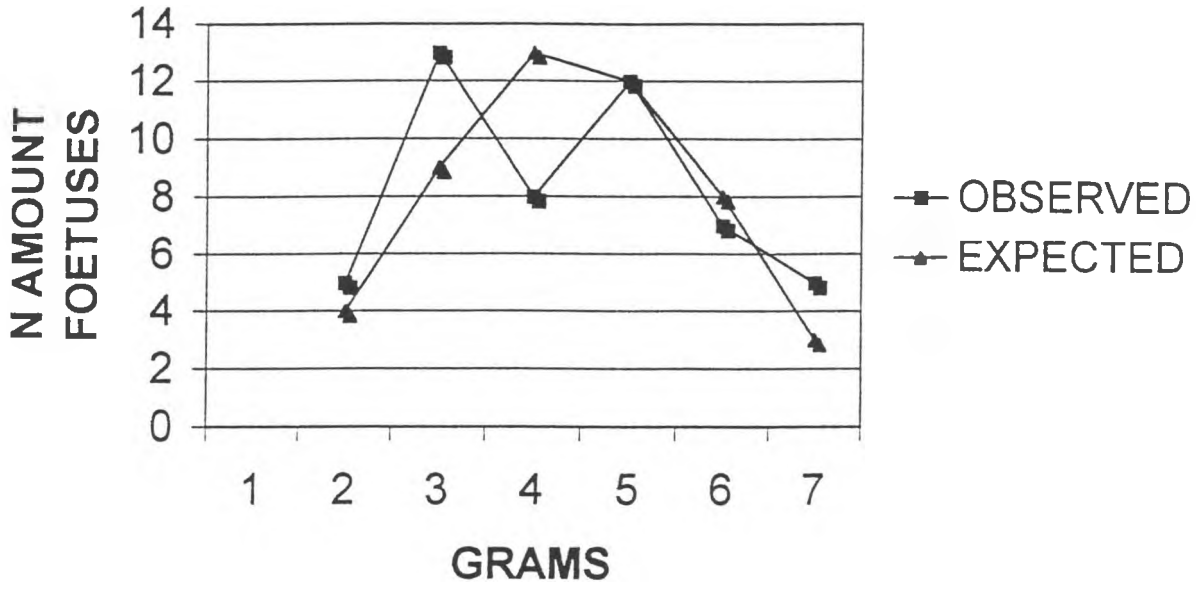
NORMAL DISTRIBUTION AND CHI-TEST DERIVED FROM FOETAL WEIGHT

	CA		A1		A2		CB		B1		B2	
	OB	EX	OB	EX	OB	EX	OB	EX	OB	EX	OB	EX
GRAM												
2	5	4	2	1					8	6	3	6
3	13	9	3	11	5	6	3	5	15	13	23	14
4	8	13	62	42	28	18	26	10	23	16	3	8
5	12	12	13	30	12	23	2	12	2	13		
6	7	8	9	4	18	14	2	9	8	7		
7	5	3			3	0	7	4	4	3		
8					1	1	3	1			1	0
9									1	0		
CHI TEST		3.5		17.04		13.8		44.15		13.7		8.32

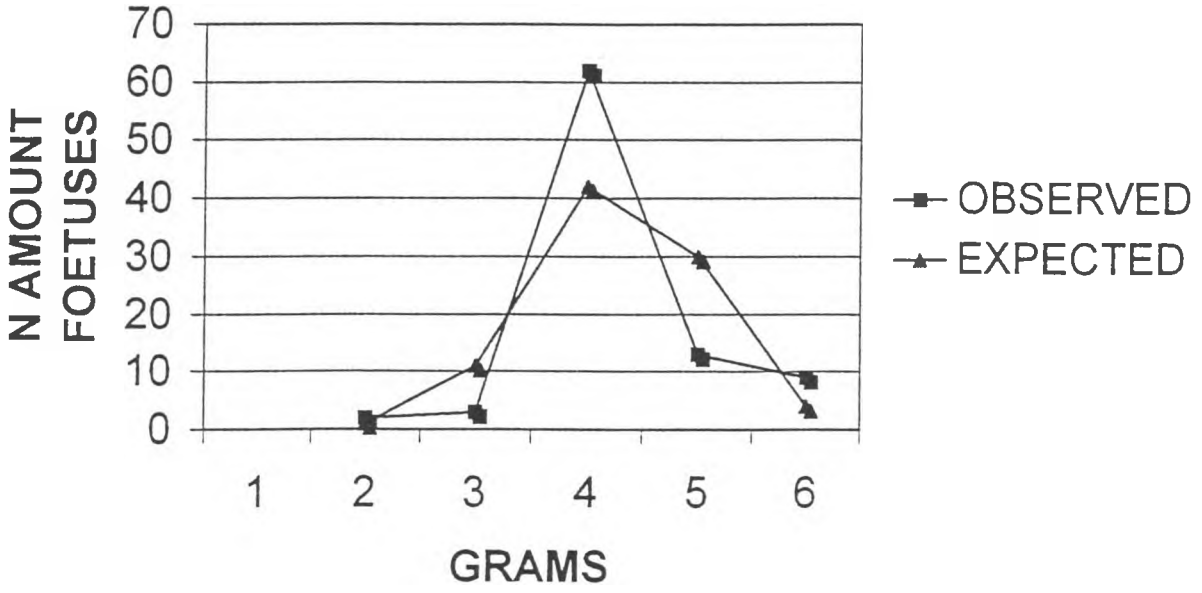
NORMAL DISTRIBUTION AND CHI-TEST DERIVED FROM FOETAL LENGTH

	CA		A1		A2		CB		B1		B2	
	OB	EX	OB	EX	OB	EX	OB	EX	OB	EX	OB	EX
MM												
27	3	2	2	0					6	4		
32	12	10	3	8	2	3	2	2	11	14	1	1
37	11	17	53	52	27	23	17	14	23	23	23	25
42	18	14	31	28	26	32	14	19	17	15	6	5
47	6	5			12	9	10	7	4	4		
CHI TEST		3.98		1.12		2.37		3		0.13		0.29

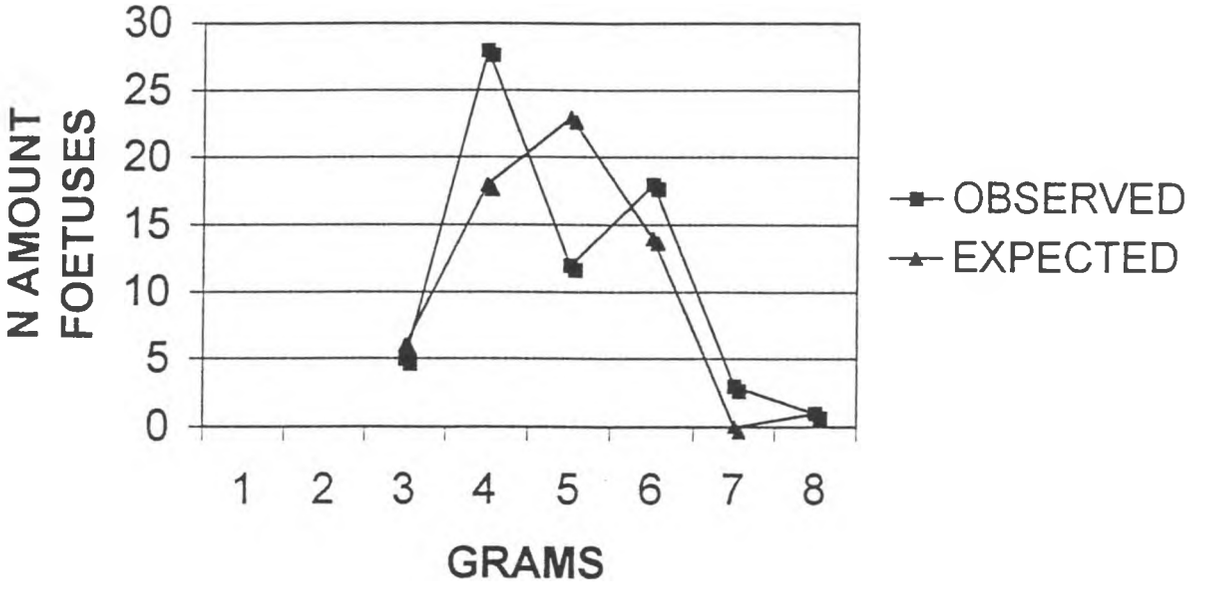
NORMAL DISTRIBUTION OF CA WEIGHT



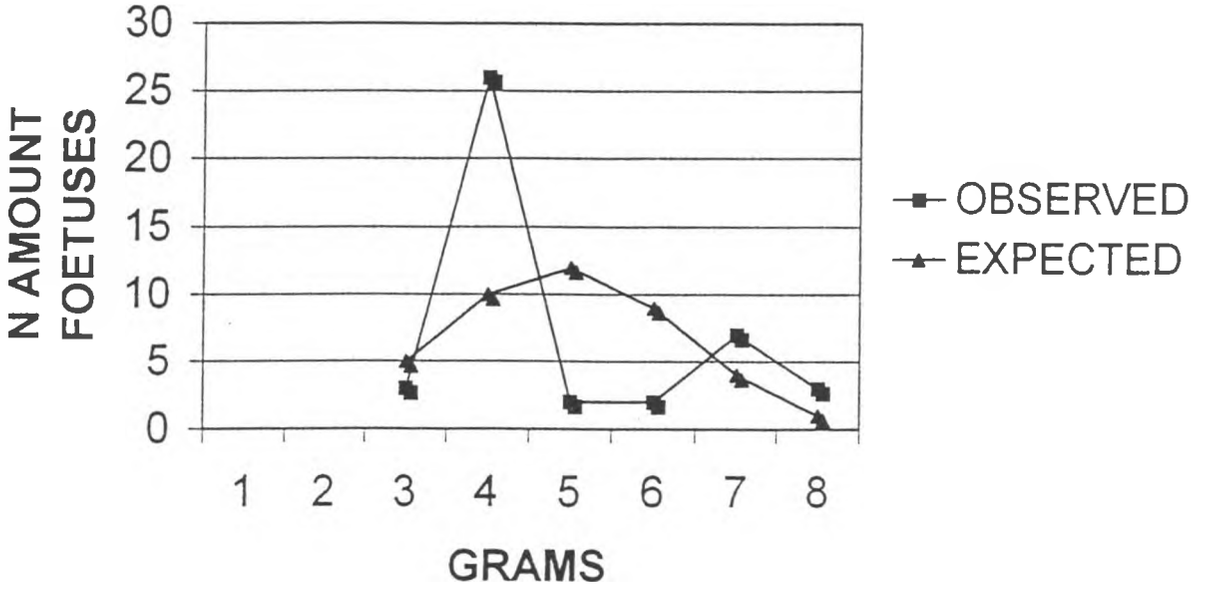
NORMAL DISTRIBUTION OF A1 WEIGHT



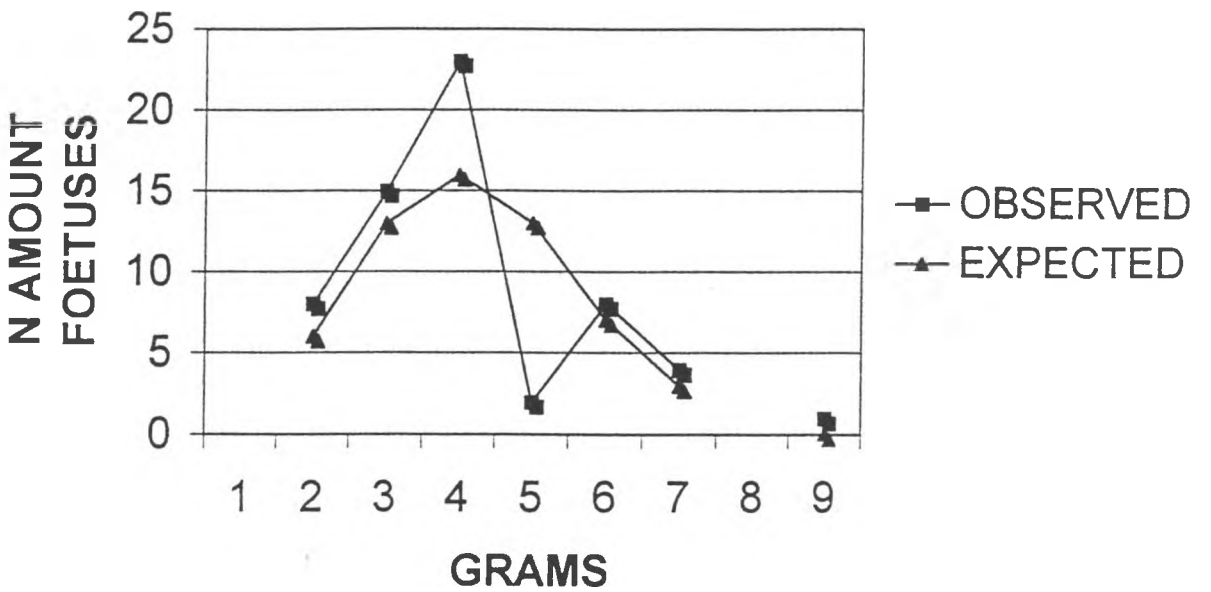
NORMAL DISTRIBUTION OF A2 WEIGHT



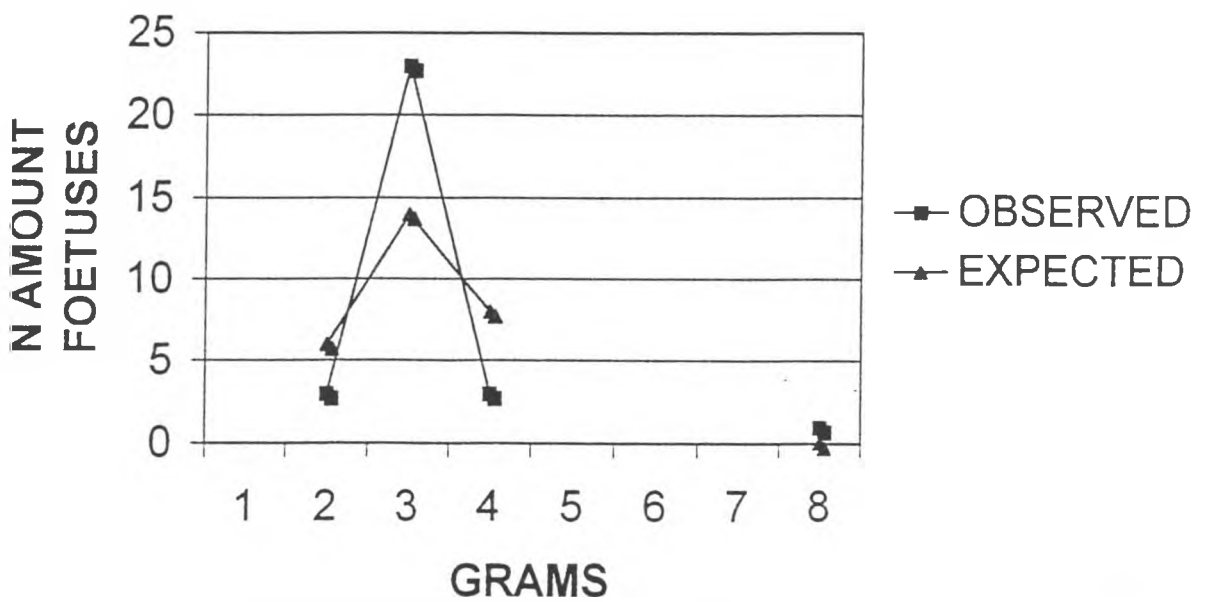
NORMAL DISTRIBUTION OF CB WEIGHT



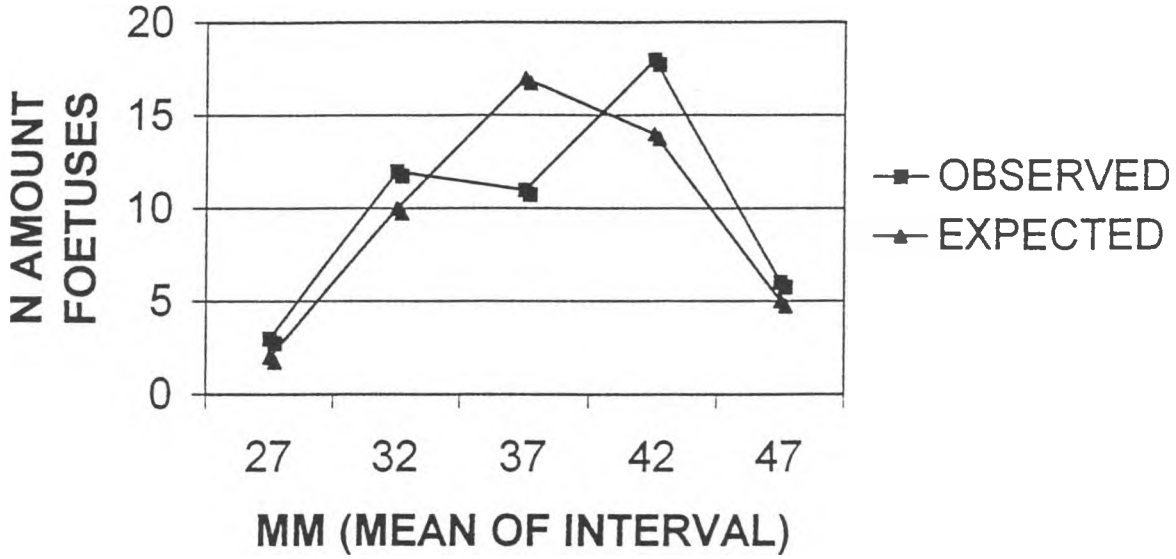
NORMAL DISTRIBUTION OF B1 WEIGHT



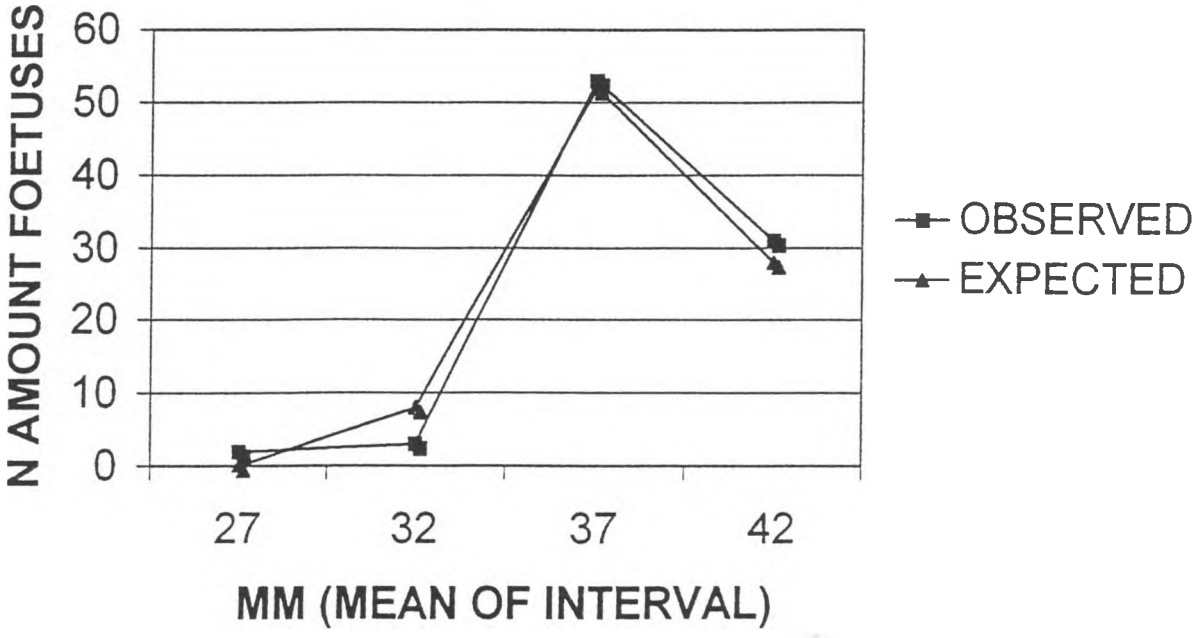
NORMAL DISTRIBUTION OF B2 WEIGHT



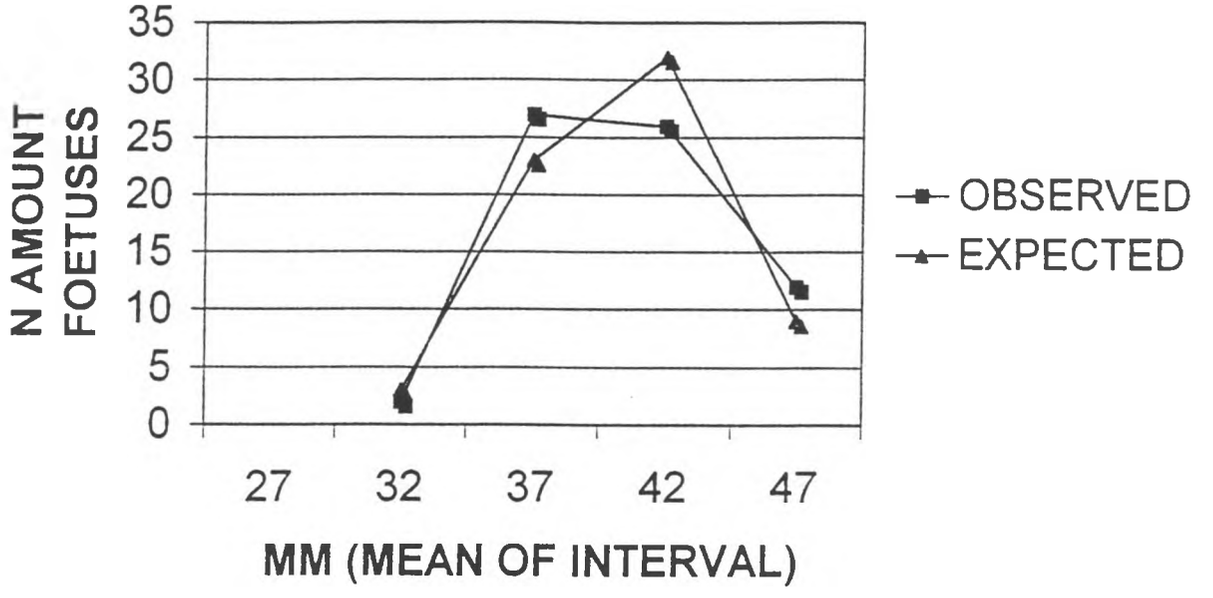
NORMAL DISTRIBUTION OF CA LENGTH



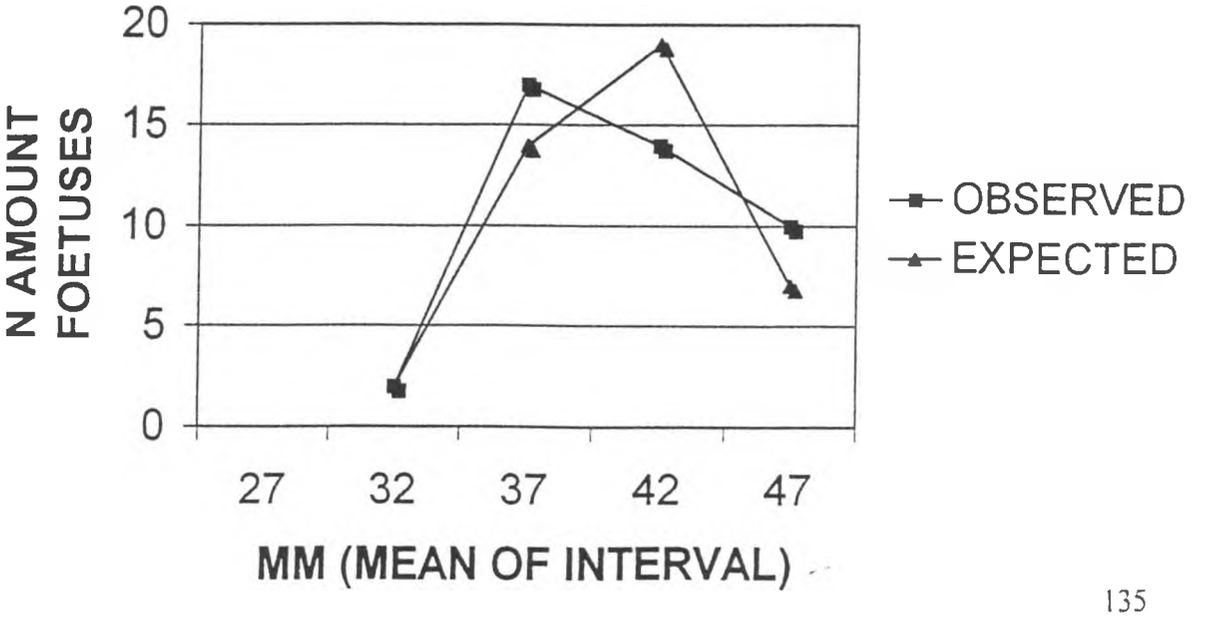
NORMAL DISTRIBUTION OF A1 LENGTH



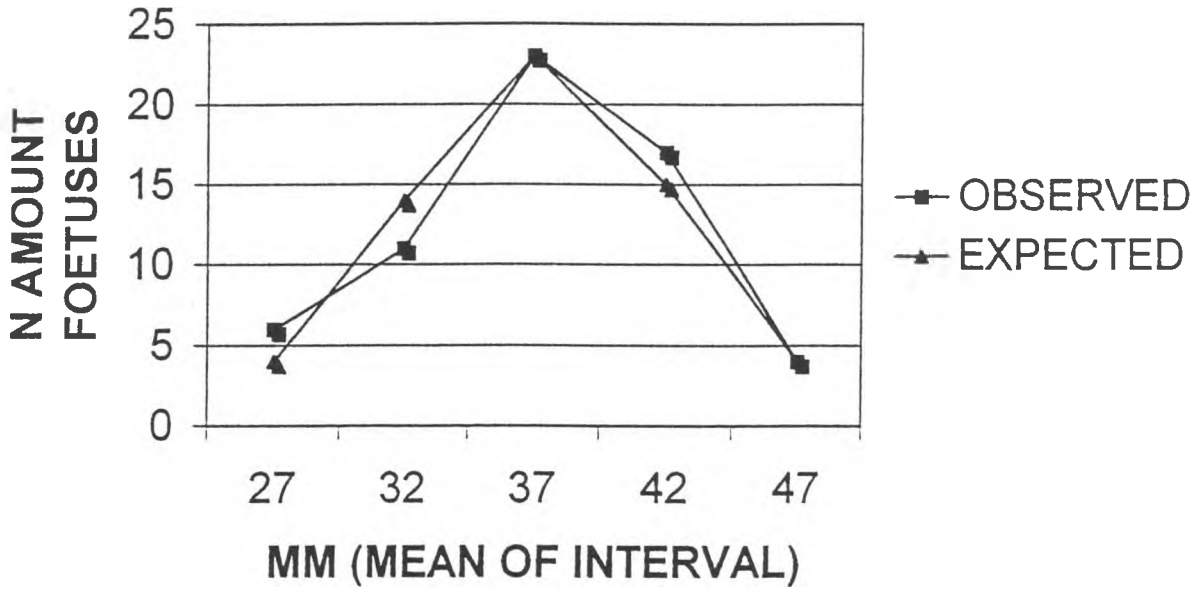
NORMAL DISTRIBUTION OF A2 LENGHT



NORMAL DISTRIBUTION OF CB LENGHT



NORMAL DISTRIBUTION OF B1 LENGHT



NORMAL DISTRIBUTION OF B2 LENGHT