A COMPARATIVE STUDY OF MUCIN HISTOCHEMISTRY IN MUCOUS CELLS OF SALIVARY GLANDS AND ODONTOGENIC CYSTS

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DECLARATION

I, Ridwaana Carim, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Dentistry in the branch of Oral Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

Signature of Candidate

Date: 18 July 2013

ABSTRACT

Introduction

Previous studies on the glandular odontogenic cyst (GOC) have largely focused on the application of immunohistochemistry for determining how the GOC lining epithelium compares with that of other odontogenic cysts. Studies on the histochemical composition of the mucous cells in the GOC are, however, lacking. This study therefore aimed to determine the mucin phenotype of the mucous cells in the GOC and compared these findings with the mucous cells in the epithelial linings of other odontogenic cysts and with normal salivary gland mucous acinar cells.

Materials and Methods

Twenty-seven cases made up of 10 GOCs, 9 dentigerous cysts (DC) with mucous cells and 8 radicular/residual radicular cysts (RC) with mucous cells were stained using the combined alcian blue pH 2.5-PAS (AB-PAS) histochemical technique. AB-PAS allows for differentiation between acidic- (type I mucous cells), neutral- (type II mucous cells) and mixed mucin-containing cells (type III mucous cells). Submandibular, sublingual and palatal salivary gland tissue was also subjected to AB-PAS staining. The odontogenic cysts and salivary glands were evaluated for the frequency of type I, II and III mucous cells in these tissues.

Results

There were significant differences between the level of type I, type II and type III mucous cells within each of the three cyst types; GOC (p=0.006), DC (p=0.0004), RC (p=0.0017). There were no significant differences in the cell counts for each mucous cell type between the 3 cyst types; type I mucous cells (p=0.54); type II mucous cells (p=0.73) and type III mucous cells (p=0.97). All 3 odontogenic cysts showed a predominance of type III mucous cells and this mirrored the mucin phenotype of the submandibular and sublingual salivary glands.

Conclusion

The mucin phenotype of the GOC is shared by DC and RC with mucous metaplasia. The overlapping mucin phenotypes of the different odontogenic cysts unfortunately does not support the use of the AB-PAS stain as a potential histochemical marker to distinguish between the GOC and other odontogenic cysts with mucous metaplasia. Similarities in the mucin phenotype between odontogenic cysts, submandibular and sublingual salivary glands may suggest a common ectodermal histogenetic origin for the mucous cells in odontogenic cysts and major salivary glands.

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CHAPTER 1

1. INTRODUCTION

The presence of mucin-producing epithelial cells (mucous cells) is one of the defining histological features of the glandular odontogenic cyst (GOC).[1,2] Mucous cells may also be encountered in the epithelial linings of other odontogenic cysts,[1,3-5] and more rarely in odontogenic tumours.[6-8] Those odontogenic cysts that show a tendency to exhibit mucous cells include the dentigerous cyst, the radicular cyst and the residual radicular cyst.[3,4,9] It has been shown that the frequency of occurrence of mucous cells in radicular cysts may be as high as 23.8%[1] and 39.6% of dentigerous cysts.[3]

The pathological basis for the transition from the usually non-mucinous odontogenic epithelium to cells that produce high molecular weight glycoproteins is poorly understood. On the basis of the presence of clear or vacuolated cells that are occasionally observed near the mucous cells in the epithelial linings of these cysts, it has been suggested that the mucous cells arise as a consequence of a metaplastic process in these cysts.[4] Other theories regarding the origin of mucous cells in the lining of odontogenic cysts include grafting of such cells from a contiguous epithelium, such as from the mucous cells in the lining of the antral sinus.[3] The third possibility has been attributed to the presence of embryological pluripotential cells in the epithelial residues from which these cysts arise.[10]

The morphological pattern of distribution of the mucous cells in the linings of these odontogenic cysts has been meticulously detailed in most previous publications on this

subject.[1,3,4] The mucous cells are usually present in the surface layer of the epithelial linings in numbers varying from occasional scattered cells to continuous rows of cells. In thickened epithelial linings, mucous cells may also be found in the spinous cell layer. Interestingly, clusters of mucous cells in the spinous layer may sometimes be arranged in an "acinar" type fashion or around a microcyst thereby imparting the impression of an intraepithelial gland-like structure.[1] Takeda *et al*.[1] further commented that the mucous cells in the epithelial linings of radicular and dentigerous cysts may resemble the glandular elements seen in the GOC. The key morphological criteria for the histological diagnosis of the GOC include a cyst that is lined with non-keratinised stratified squamous epithelium of varying thickness.[1,2] In more or less extensive areas, the superficial layer of the epithelium consists of eosinophilic cuboidal cells, columnar cells or mucous cells.[1,2] Within the thickness of the epithelium there are intraepithelial gland-like structures consisting of mucous cells and mucin-filled crypts or microcysts lined by cuboidal cells that are presumed to result from folding of the lining epithelium.[11]

Investigative histological studies on the GOC have largely focused on the application of immunohistochemistry for determining how the GOC lining epithelium compares with that of other odontogenic cysts.[12-15] Recent immunohistochemical findings on the GOC, in particular the cytokeratin profile of the cyst lining, advocate odontogenic differentiation within the GOC.[13,14] A literature survey revealed no previously published work on the histochemical nature of the mucous cells in the GOC. This study therefore aimed to histochemically characterise the mucous cells of the GOC, and to determine how this compares with the mucous cells of other odontogenic cysts and with normal salivary gland mucous acinar cells.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Mucin histochemistry

The study of mucins began in the middle of the nineteenth century, although much more is now known about the different types of epithelial mucin and their distribution in the human body. The type of mucin that is present within a particular cell type can be determined with the use of histochemistry. The latter denotes a laboratory technique in which a chemical reaction is involved in colouring tissue.[16]

Mucin histochemistry cannot be dissociated from the biochemical aspects of glycoproteins. For this reason, a brief reminder of mucin biochemistry and the histochemical classification of epithelial mucins is presented. Mucins are formed by a central protein core to which different carbohydrates are attached in the form of 4 oligosaccharide side chains.[16] These carbohydrates (70 to 90% by weight of mucins), vary in composition, length, branching, acidity and reactive groups.[16] In effect the various histochemical methods used for epithelial mucin identification react directly with these carbohydrates. Depending on the type of carbohydrates present, epithelial mucins are classified histochemically into two broad groups namely, acidic and neutral mucins.[16] Acidic mucins are divided into sialomucins (with carbohydrates containing sialic acid) and sulphomucins (with carbohydrates containing sulphate reactive groups).[17] Sialomucins are further subdivided into an N-acetyl form (sialidase or neuraminidase labile) and an O-acetyl form (sialidase or neuraminidase resistant). Sulphomucins comprise strongly and weakly sulphated acid. Neutral mucins contain free hexoses and are devoid of acid reactive groups

such as sialic acid or ester sulphate. Sialomucin contains sialic acid. Sialic acid was first isolated from bovine submandibular salivary gland, hence its name which is taken from the Greek word *(sialo)* for saliva.[17] Enzyme labile sialomucin is widely distributed and is typically found in the submandibular salivary gland, the bronchial submucosal glands and small intestine. It may occur alone, but more commonly as a mixture with other types of mucin (both acidic and neutral).[18]

The general principles of some of the most popular histochemical methods used for the routine demonstration of mucins are described below. *The Periodic Acid-Schiff reaction* (PAS), as generally employed, is a useful but non-specific indicator for the presence of mucins in tissues. PAS will react positively with a variety of mucins, such as mucins containing neutral mucin and enzyme-labile sialomucins. PAS-positive mucins are stained magenta. PAS will also stain glycogen but this effect can be abolished by prior treatment of sections with the enzyme diastase. Although mucicarmine also stains mucin, it does not offer an added benefit compared to PAS with diastase as both these stains are unable to distinguish between acidic and neutral mucins.[16]

Alcian blue (AB) is the most commonly used of the so-called cationic dyes for the demonstration of acidic mucins. At pH 2.5, AB will stain all sialomucins by reacting with their carboxyl groups, as well as staining most sulphomucins particularly those which are weakly sulphated. AB positive acidic mucins are stained a bright clear blue.[16]

Combined *alcian blue pH 2.5-Periodic acid-Schiff* (AB-PAS) is a more informative stain than mucicarmine, PAS-diastase and AB alone because it distinguishes between acidic mucins, neutral mucins and mixtures of acidic and neutral mucins.[19] With the combined

AB-PAS stain; acidic mucin stains blue, neutral mucin stains magenta and mixtures of acidic and neutral mucin stain purple.[19]

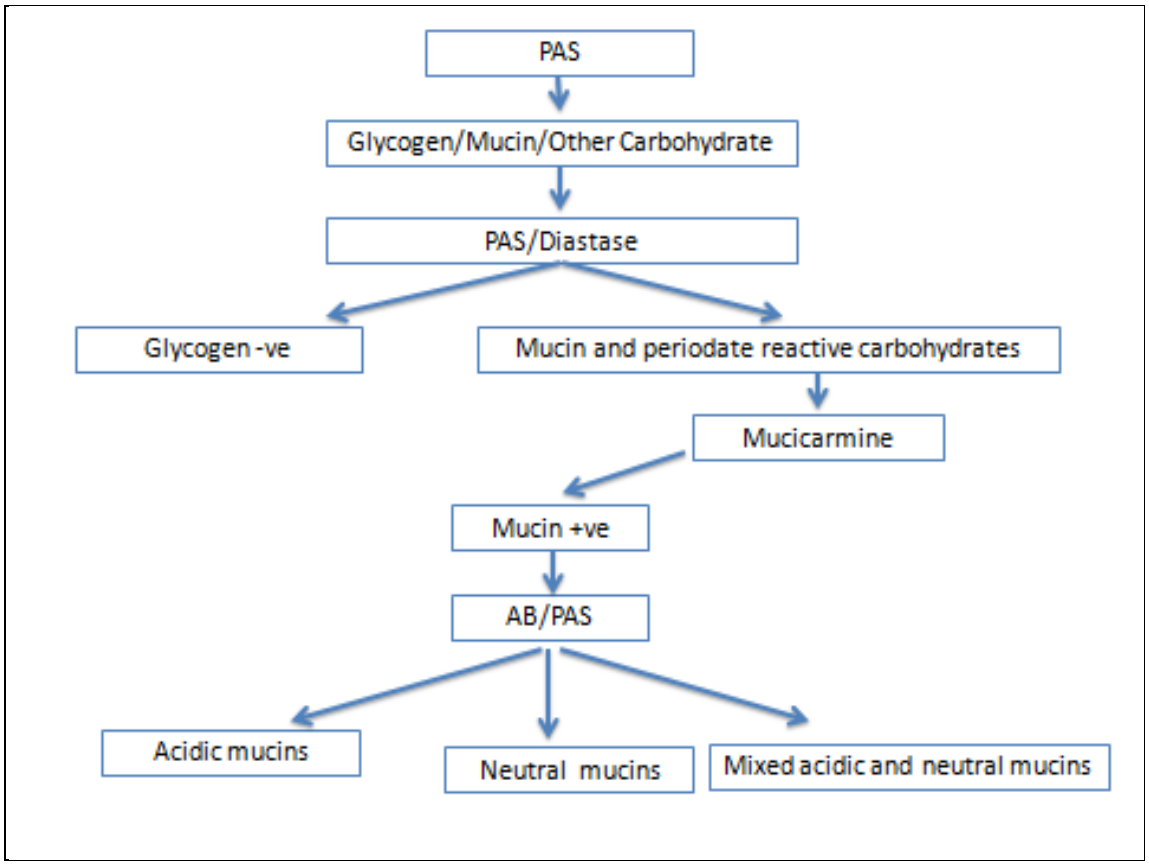

Figure 1. Algorithm depicting the sequence of histochemical stains for determining mucin composition.

2.1.1. Mucin histochemistry of normal salivary gland mucous acinar cells

One of the earliest studies that reports on the application of histochemistry for the study of salivary mucins was by Eversole.[20] In the former study the author systematically investigated the mucous cell-containing salivary glands in man, including both major and intraoral minor salivary glands. In the major sublingual salivary glands, the mucous acinar

cells yielded a positive result with the combined AB-PAS method. The sublingual gland showed a heterogenous composition of both acidic and neutral mucin containing mucous acinar cells. The mixed cell type, i.e. cells containing both acidic and neutral mucins and hence appearing purple on the AB-PAS stain, outnumbered the mucous cells containing only acidic mucin (blue staining cells) or only neutral mucin (magenta staining cells).[20]

Although the mucous acinar cells of the submandibular gland are less abundant than those of the sublingual gland they were found to be similar histochemically.[20] The submandibular gland also showed a heterogenous composition of both acidic and neutral mucin containing acinar cells. In this gland, the mixed cell type, i.e. cells containing both acidic and neutral mucins, was also found to be the predominant mucous cell type.[20]

In human minor salivary glands it was shown that the mucins present in mixed seromucous and pure mucous minor salivary glands are comparable to those encountered in the major sublingual and submandibular glands.[21] Mucous cells of the mixed seromucous glands of the lips, buccal mucosa and floor of the mouth synthesise both neutral and acidic mucins, which are present in varying amounts. The pure mucous glands of the hard and soft palate, and retromolar mucosal glands manifested comparable histochemical features.[21]

2.2. Prevalence of mucous cells in odontogenic cysts and tumours

The occurrence of mucous cells in odontogenic cysts and tumours is a rare but well recognised phenomenon.[1,3,8,22] Those odontogenic cysts that have a tendency for containing mucous cells in the cyst lining include the dentigerous cyst, radicular cyst, residual radicular cyst and by definition, the glandular odontogenic cyst (GOC).[1,4,9,23] Furthermore,

mucous cells have also been described in odontogenic tumours, in particular, in the ameloblastoma.[22]

In the context of odontogenic cysts, a literature survey revealed three studies that were specifically aimed at determining the prevalence of mucous cells in odontogenic cysts, with sample sizes of 200,[5] 205,[1] and 638,[3] respectively. Shear[5] reported an 18% prevalence of mucous cells in the epithelial linings of radicular cysts. Twenty-four of these were in the maxilla, 9 in the mandible and in 3 cases the location of the cyst was not stated.[5] Browne[3] reported a 30.9% prevalence of mucous cells in odontogenic cyst epithelial linings; with the overall prevalence of mucous cells being 38.6% of radicular cysts, 42% of dentigerous cysts, 20% of lateral periodontal cysts and 3.6% of odontogenic keratocysts. Statistical analysis showed no significant difference in the prevalence of mucous cells between the maxilla and the mandible for the radicular, dentigerous and lateral periodontal cyst, while there was a significant difference in the odontogenic keratocyst that showed a higher prevalence of mucous cells in the maxilla than the mandible.[3] Browne[3] commented on the close proximity of the antral and nasal linings to the alveolar bone of the maxilla that may result in collision of the two lining epithelia, hence making it difficult to determine the true origin of the mucous cells in the odontogenic keratocyst in his study.[3] However, the lack of a significant difference between the prevalence of mucous cells of the maxilla and mandible in the radicular, dentigerous and lateral periodontal cysts suggests that the close proximity of these cysts to the antral and nasal cavities may be of little significance. A study of radicular and residual radicular cysts by Slabbert *et al.*[4] showed an overall prevalence of 9.7% of mucous cells in the

epithelial linings of these jaw cysts. These two cysts were grouped together and no distinction in prevalence was made between them.[4]

In a study reported by Takeda *et al.*[1] mucous cells were found in the epithelial linings of 18% of radicular cysts, 23.8% of dentigerous cysts and 26.9% of primordial cysts, with an overall prevalence of 20.8% (75/361). The primordial cyst was defined as an intra-osseous developmental odontogenic cyst with a non-keratinised epithelial lining and no relationship to impacted teeth in the study by Takeda *et al.*[1] The prevalence of mucous cells in radicular cysts in the study by Takeda *et al.*[1] (18%) was similar to the findings of Shear[5] (18%) but lower than the prevalence reported by Browne[3] (39%) while the prevalence of mucous cells in dentigerous cysts (24%) was nearly twice less common than in the work reported by Browne[3] (42%). These discrepancies were found despite the large number of cases used in the studies by Takeda *et al.*[1] and Browne.[3] Takeda *et al.*[1] suggested that the difference may be due to the variations in tissue sampling for histological examination. Another difference noted between the studies of Browne[3] and Takeda *et al.*[1] is in the site distribution of radicular and dentigerous cysts with mucous cells. While Browne[3] found no significant difference in the occurrence of these cysts in the maxilla and mandible, Takeda *et al.*[1] found that the prevalence of mucous cells in radicular and dentigerous cysts was higher in the maxilla than the mandible. The distribution of radicular cysts with mucous cells in the study by Takeda *et al.*[1] was as follows; 21% (25/119) in the maxilla and 14% (12/86) in the mandible; and the distribution of dentigerous cysts with mucous cells was 28.8% (17/59) in the maxilla and 19.7% (14/71) in the mandible.

Among odontogenic tumours, mucous cells have been encountered in ameloblastomas. The ameloblastoma is the most common odontogenic epithelial neoplasm.[24] It occurs over a wide age range, with an average age of around 33 years and shows no gender predilection.[24] Most ameloblastomas occur in the mandible with a predilection for the molar-ramus area.[24] The histological hallmark of an ameloblastoma is ameloblastic differentiation as defined by the Vickers and Gorlin criteria.[24] The presence of mucous cells in the epithelial linings of ameloblastomas is a rare finding and only 9 cases of confirmed histological evidence of mucous cell differentiation have thus far been reported in the literature.[6-8,22,25-28]

Ameloblastomas with mucous cells have been reported to occur over a wide age range, with the youngest case reported in a 17-year old male,[7] and the oldest case reported in a 53-year old female.[22] There appears to be a distinct predilection for the anterior region of the mandible (7/9) in the reported cases.[6-8,26-29] There appears to be no gender bias. The histological subtypes of ameloblastomas wherein mucous cells have been identified include conventional solid ameloblastoma (n=3),[7,22,26] unicystic ameloblastoma (n=2)[8,25] and desmoplastic ameloblastoma (n=4).[6,7,27,28]

2.3. Theories regarding the histogenesis of mucous cells in odontogenic cysts and tumours

Metaplasia is the reversible replacement of one differentiated cell type with another mature differentiated cell type.[30] It is also known as transdifferentiation and is the result of a cell adapting to an abnormal stimulus. It can also be the result of stem cells that reprogramme

differentiation of cells by growth factors and cytokines in the cell's environment.[31] Interleukin-4 (IL-4) is a pleiotropic cytokine that can induce the differentiation of airway epithelial cells into mucous containing goblet cells. Expression of IL-4 in airway epithelium has been reported to be associated with airway inflammation.[32] In a model of asthma where IL-4 receptor signalling was blocked by treatment with an anti-IL-4 receptor antibody, both airway inflammation and goblet cell metaplasia were prevented.[32,33]

The pathological basis and stimulus for the transition from the non-mucinous odontogenic epithelium to cells that produce mucin is poorly understood. It is hypothesised that the origin of the mucous cells in odontogenic lesions may be due to the grafting of such cells from a contiguous epithelium normally containing mucous cells, such as the lining of the maxillary sinus, or to the presence of embryological pluripotential cells present in the cyst lining or to metaplasia of these cells in response to their micro-environment.[3,5,34]

The lack of a significant difference in the occurrence of mucous cells in radicular and dentigerous cysts of both the maxilla and the mandible in the study by Browne[3] makes the grafting theory unlikely. In the same study, mucous cells were also more frequently encountered in mandibular as opposed to maxillary lateral periodontal cysts.[3] Further, the varying frequencies of mucous cells in the different types of odontogenic cysts appear to make the hypothesis of origin from pluripotential cells unlikely since there seems to be a distinct preference for mucous metaplasia in dentigerous cysts, radicular cysts and residual radicular cysts as opposed to other odontogenic cysts. Some researchers therefore believe that the most likely hypothesis is metaplasia of the cells in response to their altered micro-environment.[3,5,7]

Browne[3] also reported an increasing prevalence of mucous cells in radicular and dentigerous cysts with an increase in age suggesting that the presence of mucous cells is also dependent on the age of the cyst lining thus favouring the origin of the mucous cells by metaplasia.[3] The mechanism of mucous metaplasia in odontogenic cysts is not clear. The presence of clear or vacuolated cells in the epithelial linings of odontogenic cysts frequently observed near the mucous cells is thought to represent a stage in the histogenesis of mucous cell metaplasia.[1,4] Slabbert *et al.*[4] hypothesised that in the initial process of metaplasia the keratinocytes become vacuolated. Within the vacuolated cells mucin granules may begin to accumulate leading to the formation of mucous cells.

Fell[35] described a process of mucous metaplasia in the skin of chickens in tissue culture under the influence of excess vitamin A. Excess vitamin A altered the environment of cells that were to keratinise. The cells were prevented from forming keratin, degenerated and were sloughed. The least differentiated cells switched to an alternative path and differentiated into a secretory epithelium.[35]

Mucous metaplasia has also been observed in areas of inflammation and necrosis.[36] Necrosis is, however, extremely rare in odontogenic lesions but inflammation has been considered to be a factor to induce metaplasia in some cysts.[37] Nevertheless no inflammation was reported in any of the reported cases of ameloblastoma with mucous cells.[7,25,28] In most of the reported cases of ameloblastomas with mucous cells close association between mucous cells with areas of cystic degeneration and areas of squamous metaplasia were demonstrated.[7,25,28] The mucous cells associated with areas of cystic

degeneration may imply that the cyst contents provide the stimulus for the metaplastic change.[7] Hodson[38] observed that a close relationship existed between mucous cells and squamous epithelial cells in ameloblastomas. Since the stratified squamous epithelium of odontogenic cysts appear to frequently undergo mucous metaplasia, the possibility exists that mucous cells in some odontogenic lesions arise from squamous epithelium.[3] However, the rarity of mucous cells compared with squamous metaplasia in ameloblastomas still needs to be explained.

2.4. Histological features of odontogenic cysts with mucous cells

The histological criteria for documenting the presence of mucous cells in odontogenic cysts has been when the epithelial cells show abundant pale-staining or vacuolated cytoplasm on conventional haematoxylin and eosin staining that stains positive with PAS and PAS-diastase.[4,39] Other authors have used alcian blue or mucicarmine.[1,9] Mucous cells have been described in all the epithelial layers of odontogenic cysts with the exception of the basal layer. The mucous cells may vary from occasional scattered cells to continuous rows of cells.[1,4,9] In thickened cyst epithelial linings, solitary mucous cells may be found scattered in the spinous layer in addition to the presence of mucous cells on the surface epithelial layer.[1] In the intermediate layers, clusters of cells are sometimes arranged in an acinar pattern around a microcyst that contains mucinous material.[4] The morphology of the mucous cells may vary from small and flat in the superficial layer to large and round in the intermediate layers. The mucinous material usually appears in a granular form and dispersed throughout the cytoplasm of the cell; the nucleus being often obscured by the densely packed mucin granules.[1,4,9]

Intra-epithelial gland-like structures lined with mucous cells have also been observed in the hyperplastic epithelial linings of radicular and dentigerous cysts.[1] These gland-like structures may resemble the glandular structures seen in the GOC.[1] Slabbert *et al*.[4] noted a gradual progression from clear cells that contained no PAS-positive material to vacuolated cells containing a delicate network of PAS-positive diastase resistant material to large round mucous cells.

2.5. The glandular odontogenic cyst

The glandular odontogenic cyst (GOC) is a rare developmental cyst of the jaws that was described by Gardener *et al*.[2] in 1988 as a distinct clinico-pathologic entity. This term was later adopted by the World Health Organisation who defined the GOC as a cyst arising in the tooth-bearing areas of the jaw, that is lined with cuboidal to columnar epithelial cells containing mucous and/or ciliated cells and that exhibits crypt-like and microcystic spaces often lined with mucous cells.[40] The GOC has also been referred to as sialo-odontogenic cyst and mucoepidermoid cyst in the literature.[23,41]

The histological diagnosis of the GOC may be challenging because of similarities in the microscopic appearance to other odontogenic lesions such as the radicular cyst and dentigerous cyst with mucous metaplasia, botyroid odontogenic cyst, surgical ciliated cysts and the central mucoepidermoid carcinoma.[42] To help with the histological diagnosis of GOC, Kaplan *et al*.[42] proposed a set of criteria, which have been divided into major and minor criteria. The presence of the major criteria are mandatory for the diagnosis while the minor ones need not be present for the diagnosis but may support it. The major criteria

include a non-keratinising squamous epithelial lining that has a flat interface with the connective tissue wall and lacks basal palisading. The epithelium exhibits variations in thickness along the cystic lining with or without epithelial 'spheres' or 'whorls' or focal luminal proliferation. Cuboidal eosinophilic cells or 'hob-nail' cells are present at the luminal aspect of the cyst lining. Mucous (goblet) cells with intra-epithelial mucous pools, with or without crypts lined by mucous-producing cells as well as intra-epithelial glandular, microcystic or duct-like structures are present within the epithelial lining. The minor histological criteria include papillary proliferation, ciliated cells, multicystic or multiluminal architecture and clear or vacuolated cells in the basal or spinous layers.[42]

Although histogenetic derivation of the GOC from intraosseous salivary gland tissue was initially suggested, immunohistochemical findings, in particular the cytokeratin profile of the cyst lining advocate an odontogenic origin.[23] Cytokeratins are a group of intermediate filaments that are expressed by epithelial cells.[12] They include a wide range of proteins that vary in molecular weight and acid-base composition. Immunohistochemical analysis of cytokeratin (CK) expression is regarded as a useful tool in identifying different epithelial types and origins. In two earlier isolated case reports on the GOC; Semba *et al*[12] and de Sousa *et al.*[13] reported that the GOC was positive for CK 19; but negative for CK 8, 18. In a later study by Pires *et al.*[43] the cytokeratin profile of 10 GOCs were analysed and compared to central and salivary gland mucoepidermoid carcinomas, odontogenic cysts and ameloblastomas. The GOC showed a similar cytokeratin profile to the other odontogenic lesions in the study being positive for CK 5, 7, 8, 13, 14 and 19; while central mucoepidermoid carcinomas showed the cytokeratin profile of salivary gland mucoepidermoid carcinomas and expressed CK 5, 7, 8, 14 and 18.[43] Whilst similarities in cytokeratin expression have been shown between the GOC and other odontogenic

lesions,[12,13,43] a literature survey reveals no published work on the histochemical nature of the mucous cells in the GOC and how this compares with the mucous cells in other odontogenic cysts, and with normal salivary gland mucous cells.

CHAPTER 3

3. AIM AND OBJECTIVES

3.1. Aim

The aim of this study is to determine the histochemical nature of the mucous cells in the glandular odontogenic cyst (GOC) and to compare these findings with the mucous cells in the epithelial linings of other odontogenic cysts and with normal salivary gland mucous acinar cells.

3.2. Objectives

3.2.1. Apply the combined alcian blue (pH 2.5)-PAS histochemical technique to normal salivary glands derived from the major and minor glands in order to determine the mucin phenotype of salivary gland mucous acinar cells.

3.2.2. Apply the combined alcian blue (pH 2.5)-PAS histochemical technique to GOCs, dentigerous cysts with mucous metaplasia, radicular/residual radicular cysts with mucous metaplasia in order to determine the mucin phenotype of these cysts.

3.3.3. Determine whether there are significant differences between the mucin phenotype of the GOC and odontogenic cysts with mucous metaplasia.

3.3.4. Determine whether there are significant differences between the mucin phenotype of the GOC and salivary gland mucous cells.

CHAPTER 4

4. MATERIALS AND METHODS

4.1. Tissue Samples

4.1.1. Normal salivary glands

From the University of the Witwatersrand Oral Pathology archived data retrieval base 30 normal salivary glands comprising 10 cases each of submandibular, sublingual and palatal salivary glands were initially selected. Among the minor salivary glands, the palatal glands were selected by virtue of their exclusive mucous cell composition. The respective haematoxylin and eosin (H&E) stained slides were examined histologically and four cases were subsequently excluded due to insufficient tissue and suboptimal representation of the tissue of interest on the slide. The normal salivary gland sample thus comprised of 9 submandibular glands, 10 sublingual and 7 palatal salivary gland tissue specimens that were selected for further histological study.

4.1.2. Odontogenic cysts

Ten cases each of GOC, dentigerous cyst with mucous cells and radicular/residual radicular cysts with mucous cells were selected from the Oral Pathology archived histopathology records, going back as far as 1975 from 2010. The corresponding H&E, PAS, PAS-diastase and/or mucicarmine stained slides were reviewed to confirm the initial diagnosis and the presence of mucous cells within the cyst linings. Cases with insufficient representation of cyst lining were excluded. The odontogenic cysts that were included in this study were purposely chosen from the mandible in order to negate the possibility that

the origin of the mucous cells may be the result of migration or grafting of such cells from the antral or nasal cavities. This criterion also avoided the inclusion of non-odontogenic cysts usually found in the maxilla and normally lined, at least in part, by mucous cells such as the nasopalatine duct cyst and of odontogenic cysts which had perforated into the maxillary sinus, and thus became lined secondarily with respiratory epithelium from the sinus. The GOCs and dentigerous cysts which were selected were devoid of inflammatory infiltrate in the cyst wall. By virtue of the nature of radicular and residual radicular cysts varying amounts of inflammation were invariably present in these cysts. After exclusion of unsuitable cases, the study sample comprised 27 cases made up of 10 GOCs, 9 dentigerous cysts (DC) with mucous cells, 3 radicular cysts with mucous cells and 5 residual radicular cysts with mucous cells. The radicular cysts and residual radicular cysts (RC) were collectively analysed thereby constituting 3 types of odontogenic cysts for analysis; namely GOC, DC with mucous cells and RC with mucous cells.

4.2. Combined alcian blue (pH 2.5)-PAS histochemical staining technique

Solutions:

a. Alcian blue (1g)

b. 3% acetic acid ($100cm^3$)

c. Schiff's reagent

Method:

Sections were dewaxed and rinsed with distilled water. Sections were then treated with alcian blue solution for 5 minutes and the washed in distilled water for 2 minutes. 1% aqueous periodic acid was applied for 5 minutes, and then rinsed well in distilled water. Schiff's reagent was applied for 15 minutes and washed in running tap water for 5 minutes. Nuclei were stained with haematoxylin. Sections were then washed in water, rinsed in absolute alcohol, cleared in xylene and mounted as usual. The staining characteristics of the alcian blue (pH 2.5)-PAS (AB-PAS) stain are shown in Table 1.

Table 1. Staining characteristics of the alcian blue (pH 2.5)-PAS stain

Method	**Staining**	**Interpretation**	**Control tissue**
AB-PAS pH 2.5	Blue	Acidic mucin	Cervix
	Magenta	Neutral mucin	
	Purple	Mixture of acidic and neutral mucin	

In this study mucous cells containing acidic mucins were designated as type I mucous cells, those containing neutral mucins as type II mucous cells and those mucous cells containing both acidic and neutral mucins as type III mucous cells.

4.3. Counting protocol for mucous cells in normal salivary glands and odontogenic cysts

4.3.1. Normal salivary glands

The combined AB-PAS histochemical stain at pH2.5 was used and the numbers of type I, II and III mucous cells were determined semi-quantitatively in the normal salivary glands as follows: 0: negative, 1: < 50% of cells positive and 2: ≥50% of cells positive for acidic (type I), neutral (type II) and/or mixed mucin (type III) containing cells.

4.3.2. Odontogenic cysts

As for the normal salivary glands, the odontogenic cysts were also subjected to staining with the combined AB-PAS technique at pH2.5. Since the odontogenic cysts contained fewer mucous cells compared to the salivary glands the total number of mucous cells present in the cyst lining was counted thereby yielding continuous (count) data.

Inter-observer reliability was established with a dual-headed microscope that enabled both observers (FM and RC) to count the data simultaneously by consensus. Every second specimen was then subjected to a recount, on a separate occasion to check the data and to establish the count-recount reliability.

4.4. Statistical analysis

All recorded data was entered into an Excel spread sheet. Data analysis was carried out in STATISTICA; StatSoft, Inc. version 7.1. The 0.05 significance level was used for all statistical tests, unless specified otherwise.

4.4.1 Salivary glands

4.4.1.1 Statistical tests of count-recount data for intra-observer reliability

The data were ordinal so the paired sample t-test could not be applied. The Stuart-Maxwell generalisation of the McNemar test (for more than two categories) was applied. *(For this test, a calculated value for χ^2 greater than the critical value for χ^2 implies a significant difference between the count and recount data.)* In some cases values in the cross-tabulation equalled zero on (at least) one category (Appendix C1-C3) and thus the McNemar test was applied. In these cases it was also found that the sum of the off-diagonal values of the cross-tabulation were below 10, in which case the McNemar test X^2 is not well approximated by the chi-squared distribution. Hence, a two-tailed exact test, based on the cumulative binomial distribution with p = q = 0.5 was used instead. *(For the McNemar and the exact test, a p-value below 0.05 implies a significant difference between the count and recount data).* The test was carried out for each type of salivary gland separately, as well as for the combined data for the 3 salivary gland types for each of the three mucous cell types (Appendix C4).

4.4.1.2. Comparison of mucous cell types within the submandibular, sublingual and palatal salivary glands

Since the data are categorical the chi-squared test was used.

4.4.2. Odontogenic cysts

Since the odontogenic cysts (GOC, DC, RC) contained fewer mucous cells compared to the salivary glands their actual counts were determined, so there is one value per mucous cell type for each cyst. (Appendix D)

4.4.2.1. Statistical tests of count-recount data for intra-observer reliability

For type II and Type III mucous cells, the paired t-test was used on the assumption that the paired differences (i.e. the count-recount values) were approximately normally distributed. Some of the frequency distributions (particularly because the sample size is small) could, however, not be said to be approximately normally distributed, so the paired t-test non-parametric equivalent, i.e. the Wilcoxon matched pairs test, which makes no such assumption about the distribution of the paired differences, was also carried out. (*For both of these tests, a p-value below 0.05 implies a significant difference between the count and recount data).* The test was carried out for each type of odontogenic cyst separately, as well as for the combined data for the three odontogenic cyst types.

4.4.2.2. Statistical tests for comparison of mucous cell types within the GOC, DC and RC

Since the data are count data and their frequency distribution was very positively skewed, the Kruskal-Wallis equivalent of the one-way ANOVA analysis was used, which is based on the ranks of the data rather than the actual data values.

4.5. Ethical consideration

Approval has been granted by the University of the Witwatersrand Human Research Ethics Committee to the Division of Oral Pathology for research conducted on archived paraffin wax embedded tissue blocks (Reference number: M080850; Appendix A).

CHAPTER 5

5. RESULTS

5.1. Normal salivary glands

The salivary gland tissue represented on each slide from each of the 26 normal salivary glands presented different numbers of lobules for determination of the respective amount of mucous cells. This was a function of the way in which the specimen was originally obtained and the way in which the section of specimen was cut for slide preparation. The data from the lobules for each sample were therefore not aggregated but were treated individually, i.e. the data for a particular salivary gland was considered to be made up of a number of lobules rather than a number of specimens (Table 2).

Table 2. Numbers of lobules examined for each salivary gland type

Histological examination	Salivary gland type		
	SM	SL	PAL
Total number of specimens	9	10	7
Total number of lobules	84	93	33
Average number of lobules per specimen	9.3	9.3	4.7

SM=submandibular salivary gland, SL=sublingual salivary gland, PAL=palatal salivary gland

5.1.1. Analysis of count-recount data for intra-observer reliability

The raw data used for establishing intra-observer reliability (count-recount data) is shown in Appendix B1-B3. The McNemar test showed no significant differences between the count and recount data for the salivary glands.

5.1.2. Comparison of mucous cell types within the submandibular, sublingual and palatal salivary glands

All three salivary gland types showed a significant association between the frequency and type of mucous cell within the glands ($p<0.001$). No type I mucous cells were identified in the submandibular and palatal salivary glands while in the sublingual gland type I cells were identified in only approximately 10% of the salivary gland lobules. Type II mucous cells were the predominant mucous cell type in the palatal salivary glands while in the submandibular and sublingual salivary glands type III mucous cells were the main mucous cell type (Table 3, Figures 2-8).

Table 3. Frequency of type I, II and III mucous cells in the salivary glands

Salivary gland type		**Submandibular gland**			**Sublingual gland**			**Palatal gland**		
Mucous-cell type		Type I	Type II	Type III	Type I	Type II	Type III	Type I	Type II	Type III
Count category	0	84	64	16	82	18	5	33	0	3
	< 50%	0	19	0	11	60	13	0	7	27
	≥ 50%	0	1	68	0	15	75	0	26	3
Total		84	84	84	93	93	93	33	33	33

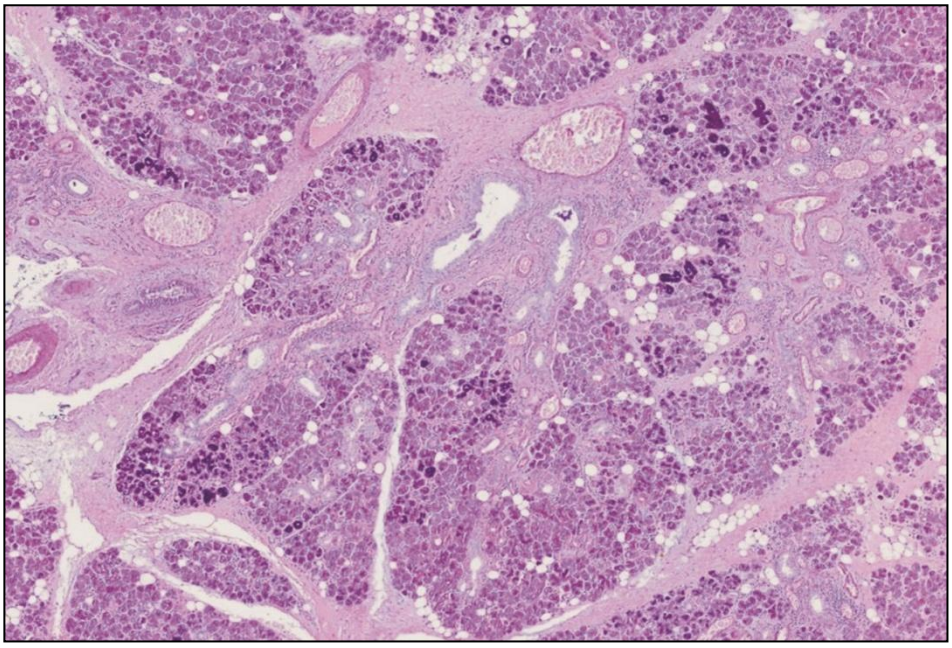

Figure 2. Low power photomicrograph of the submandibular salivary gland. Mucous cells comprise a minor proportion of the submandibular gland acinar cell population. (AB-PAS, original magnification X20)

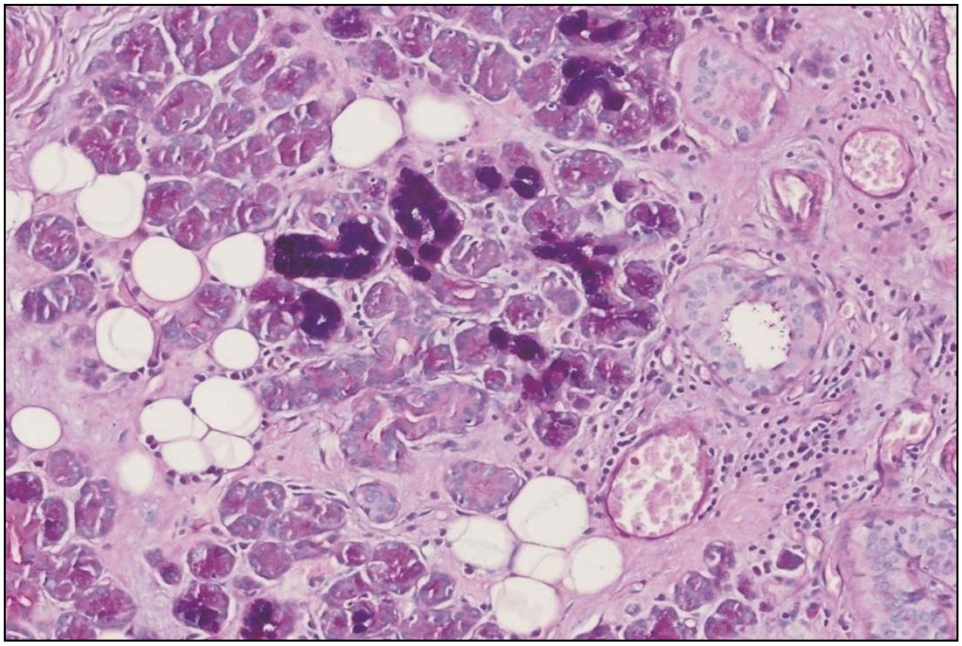

Figure 3. Medium power view of the submandibular salivary gland showing type III mucous cells (purple staining cells) amongst the serous acinar cells. (AB-PAS, original magnification X100)

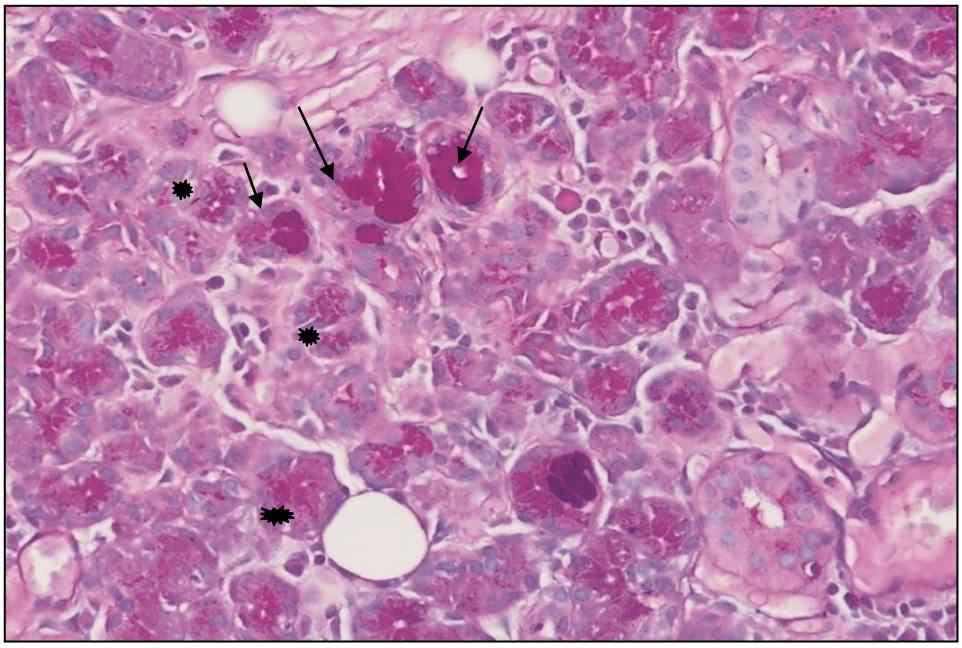

Figure 4. Photomicrograph showing type II mucous cells in the submandibular salivary gland, which stain magenta (arrows) on this stain. Note the different morphological appearance between the mucous acinar cells (arrows) and the serous acinar cells (asterisk) that contain zymogen granules. (AB-PAS, original magnification X200)

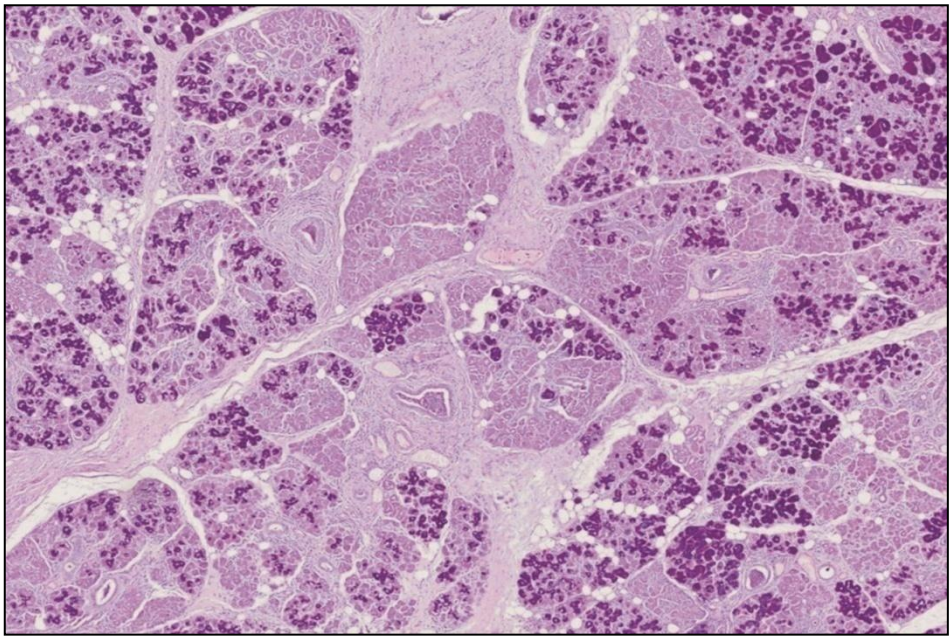

Figure 5. Low power view of the sublingual salivary gland showing a greater mucous acinar cell content than the submandibular salivary gland. (AB-PAS, original magnification X20)

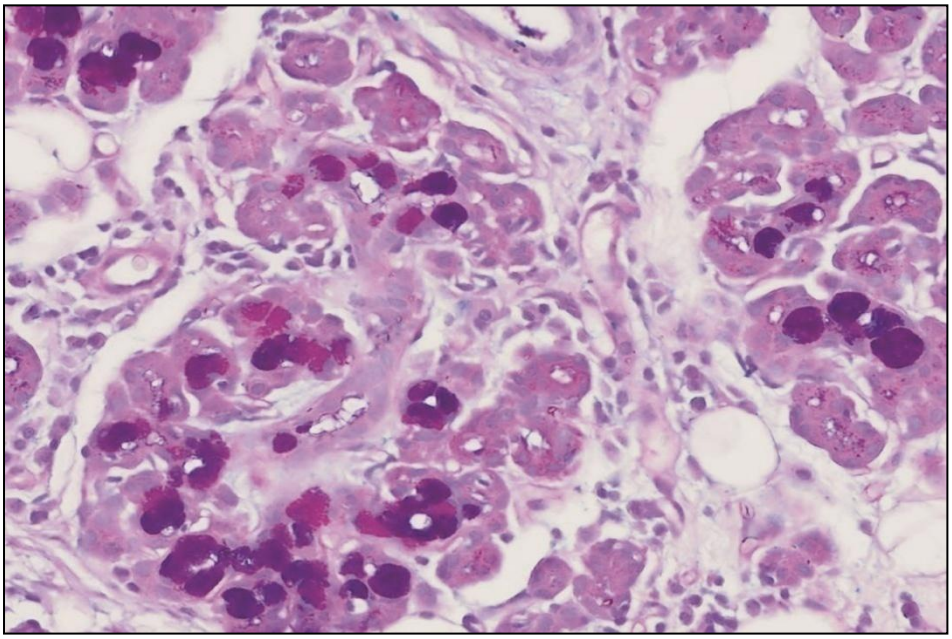

Figure 6. High power view showing a greater proportion of type III than type II mucous acinar cells in the sublingual salivary gland. (AB-PAS, original magnification X200)

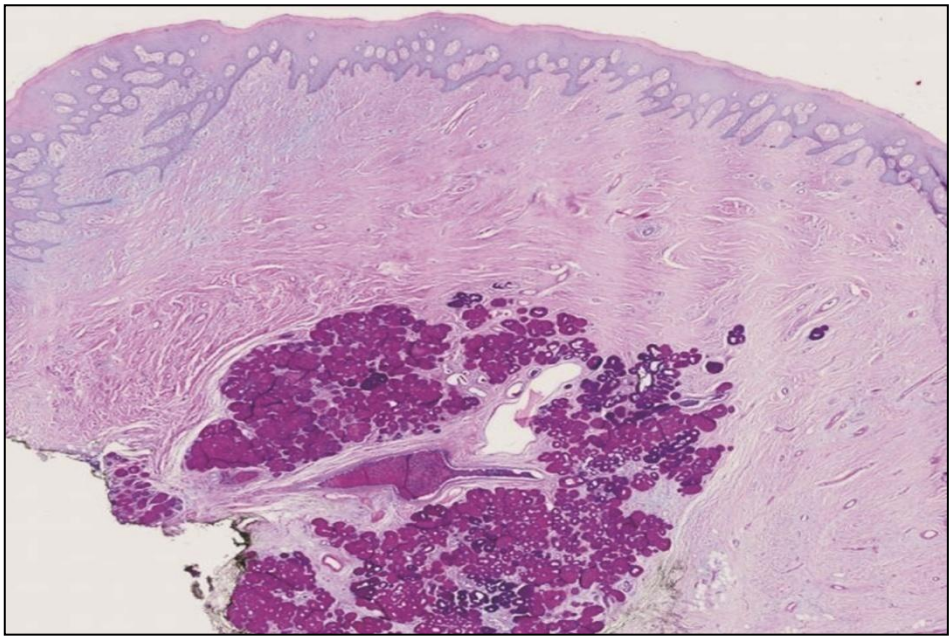

Figure 7. Low power view of a palatal minor salivary gland. (AB-PAS, original magnification X8)

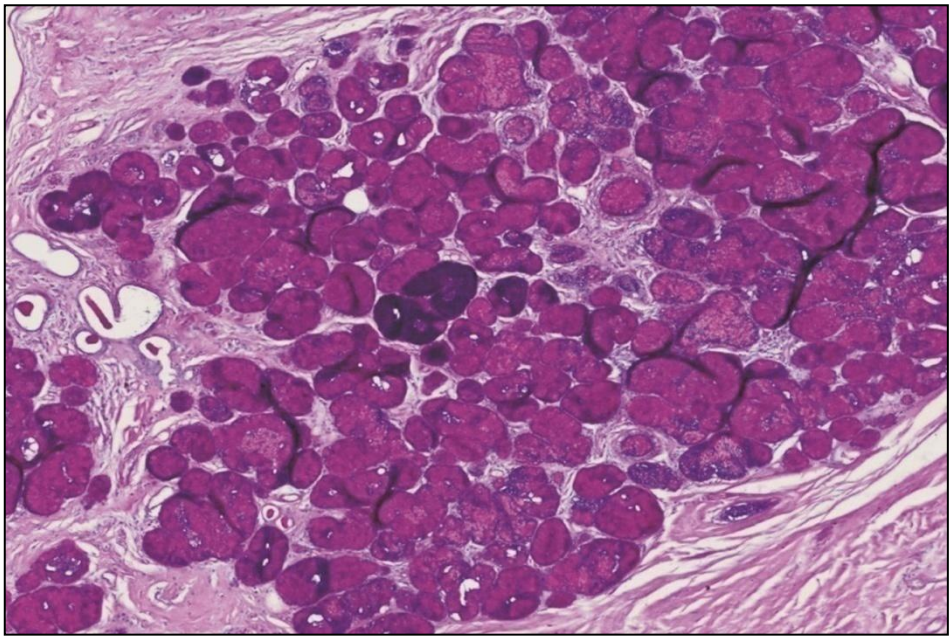

Figure 8. High power view of a palatal minor salivary gland showing a predominance of type II mucous cells. (AB-PAS, original magnification X40)

5.2. Odontogenic cysts

5.2.1. Analysis of count-recount data for intra-observer reliability

For type I mucous cells, across all 3 odontogenic cyst types, the count-recount differences were all zero, so without further analysis we could conclude that there was no significant difference between the count-recount data (Appendix D).

There were no significant differences between the count and recount data for the odontogenic cysts. We thus continued to analyse using the ‘count’ data and ignoring the ‘recount’ data.

5.2.2. Comparison of mucous cell types within the GOC, DC and RC

The frequencies of the mucous cell types within the GOC, DC and RC were similar with all 3 cyst types characterised by a trend of type I < type II < type III mucous cells (Figure 9). There were significant differences between the level of type I, type II and type III mucous cells within each of the three cyst types; GOC (p=0.006) (Figure 10, 11); DC (p=0.0004) (Figure 12-14); RC (p=0.0017) (Figure 15, 16). (Appendix F).

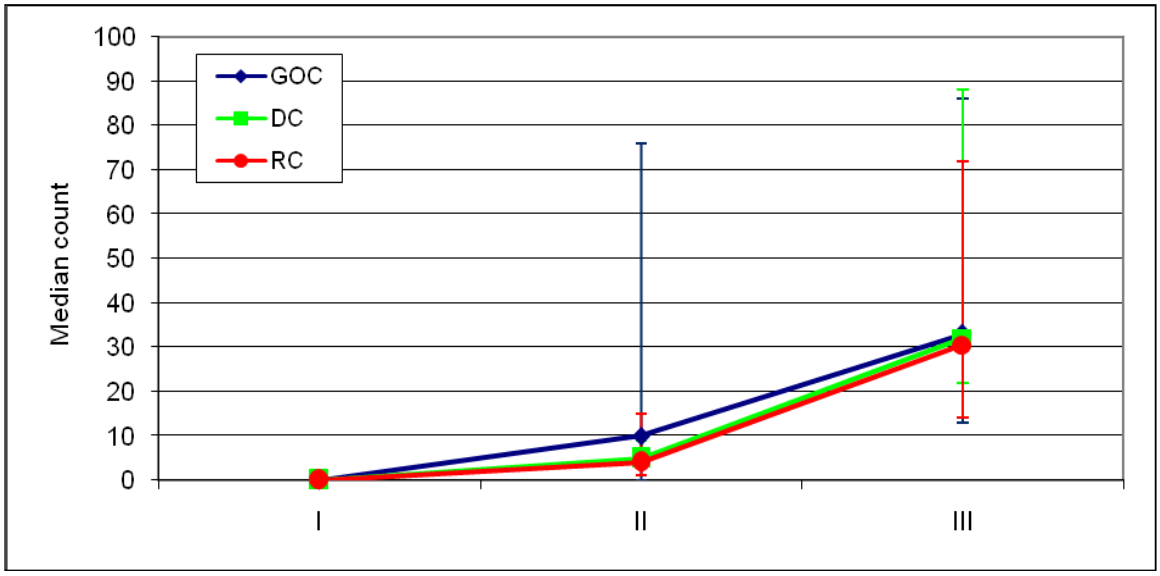

Figure 9. Median values for type I, II, III mucous cells in the glandular odontogenic cyst (GOC), dentigerous cyst (DC) and radicular/residual radicular cyst (RC).

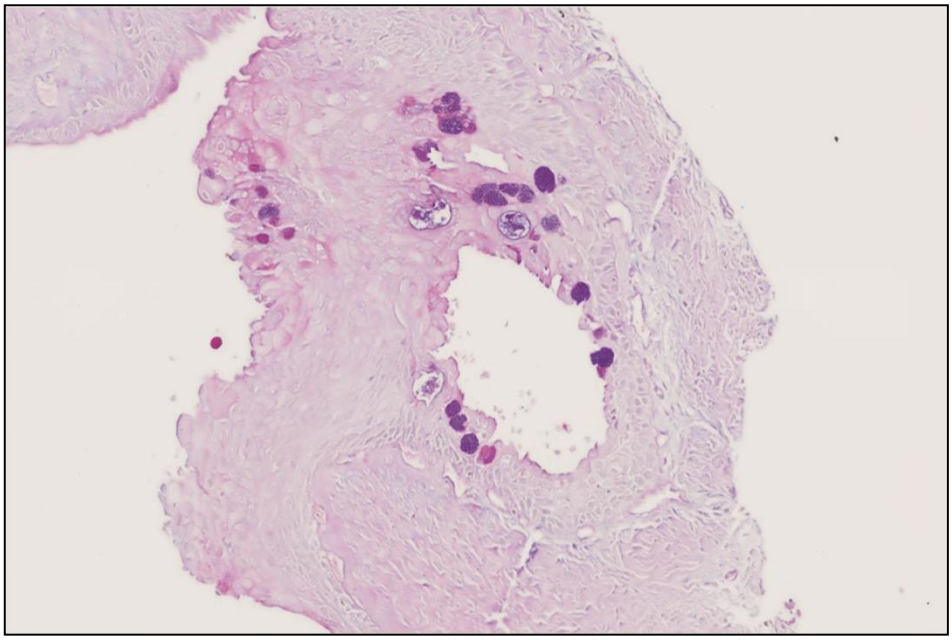

Figure 10. Photomicrograph of a glandular odontogenic cyst showing numerous mucous cells with a predominance of type III mucous cells scattered in the epithelium and lining the luminal aspect of an intra-epithelial crypt. (AB-PAS, original magnification X100)

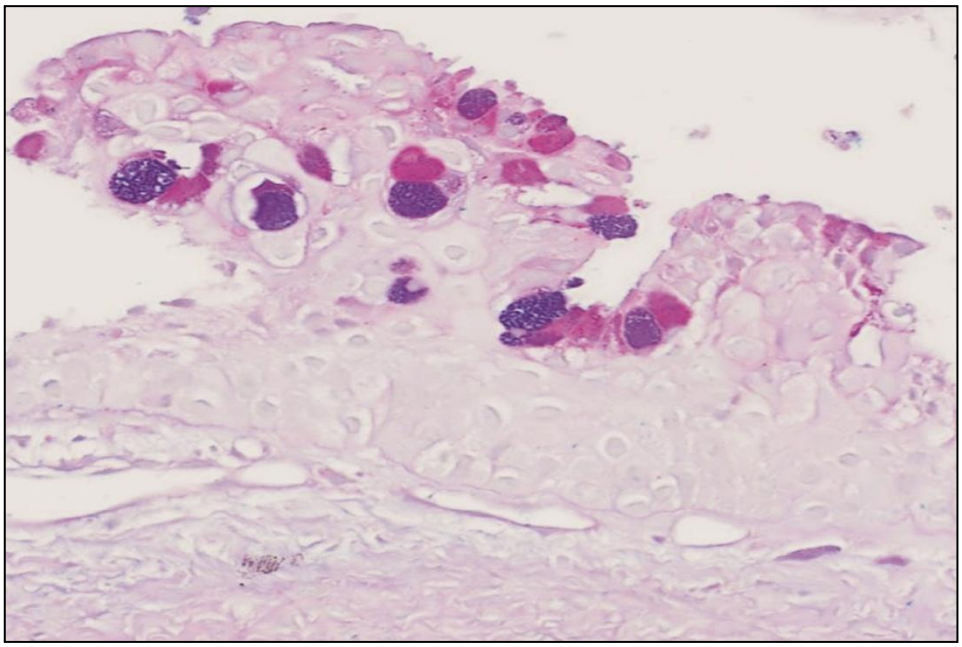

Figure 11. Photomicrograph of a glandular odontogenic cyst showing type II and type III mucous cells interspersed in the cyst lining epithelium. The surface layer of the epithelium shows a papillary surface and an intraepithelial invagination. (AB-PAS, original magnification X200)

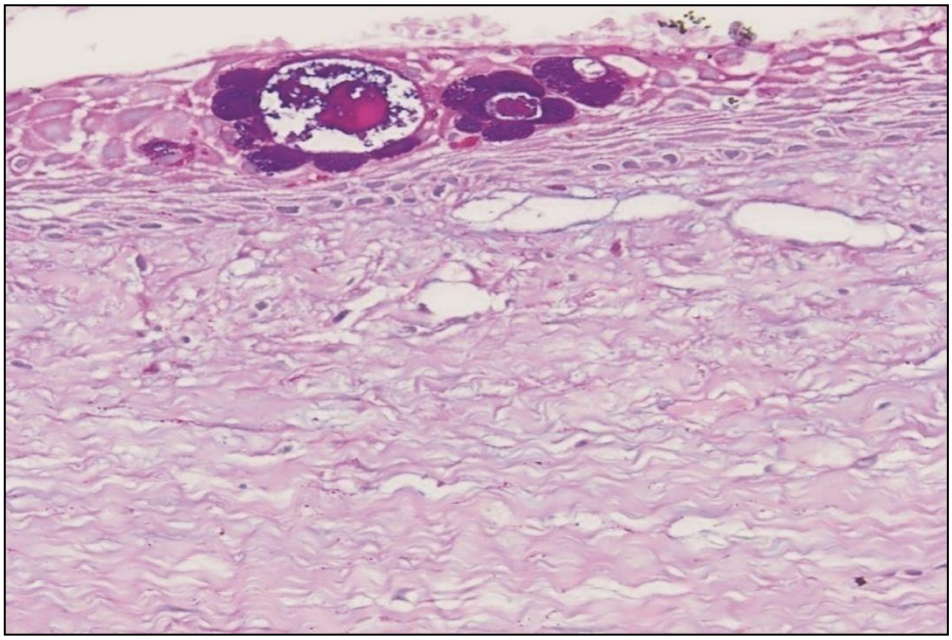

Figure 12. Photomicrograph of dentigerous cyst showing intraepithelial gland-like structures with adjacent acinar type arrangement of type III mucous cells. (AB-PAS, original magnification X200)

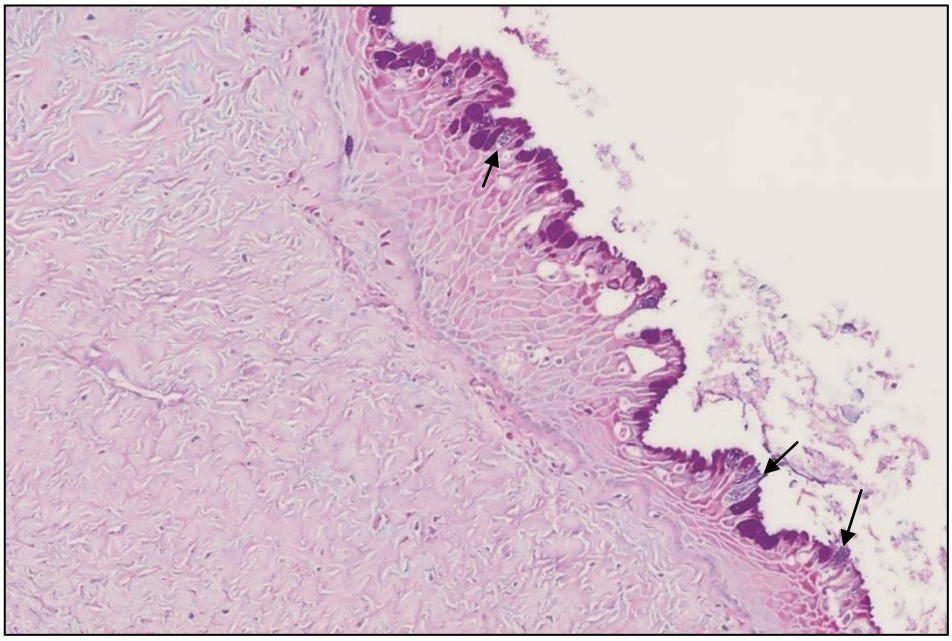

Figure 13. Photomicrograph of a dentigerous cyst showing type III mucous cells distributed in the superficial cyst lining. Scattered intervening type I mucous cell (arrows) are also seen. (AB-PAS, original magnification X100)

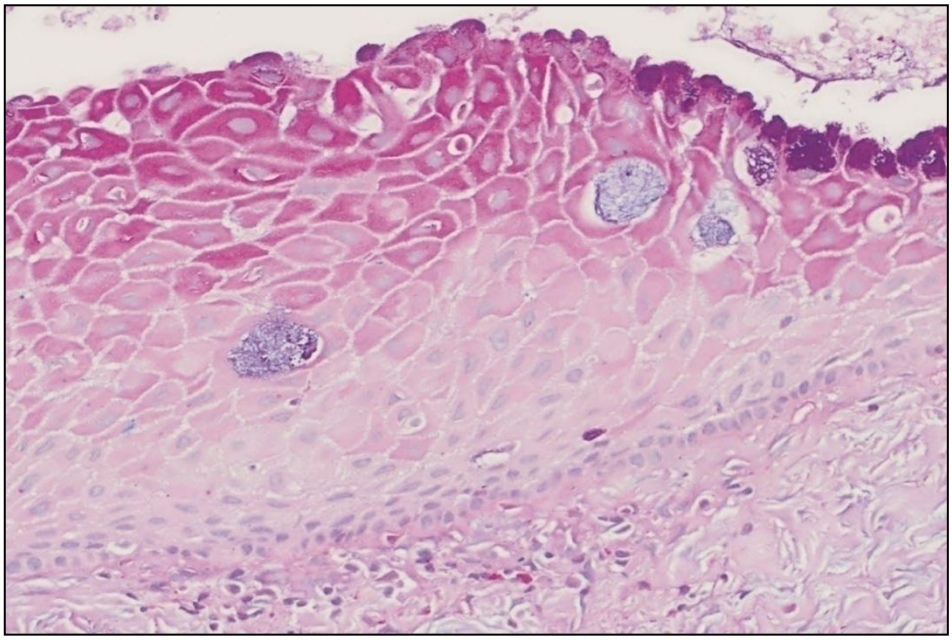

Figure 14. Dentigerous cyst with type I and type III mucous cells. (AB-PAS, original magnification X200)

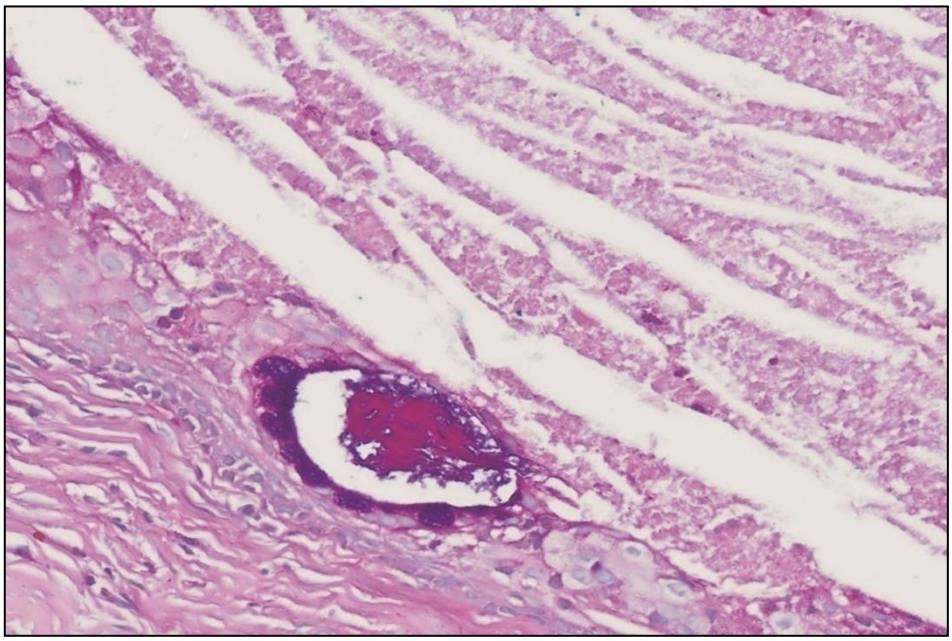

Figure 15. Photomicrograph of a residual radicular cyst showing an intraepithelial gland-like structure lined by type III mucous cells. (AB-PAS, original magnification X200)

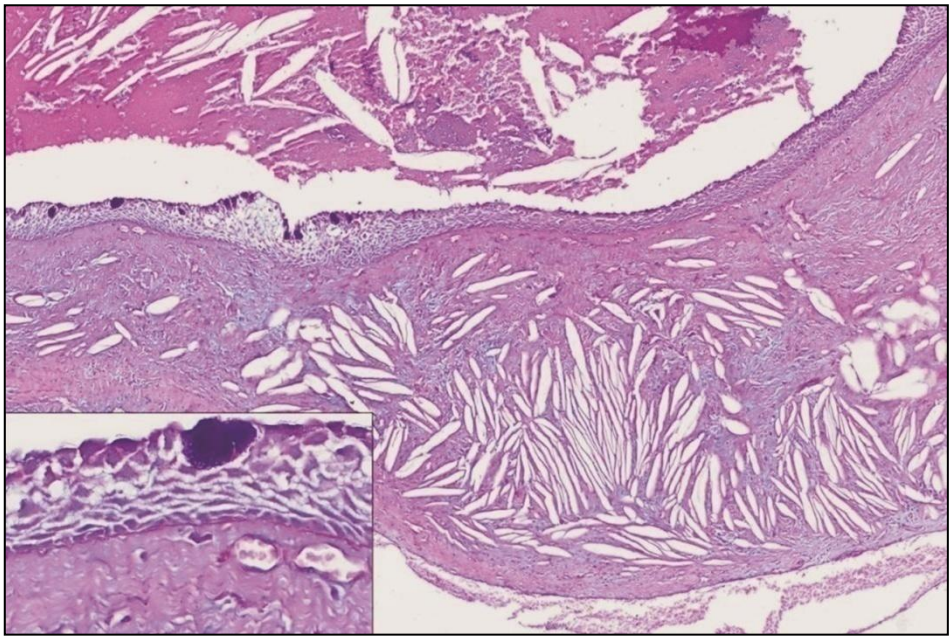

Figure 16. Residual radicular cyst with mucous cells distributed on the surface of the cyst lining. Cholesterol clefts visible in the connective tissue cyst wall. (AB-PAS, original magnification X40) *Inset:* High power magnification showing type III mucous cells on the surface. (AB-PAS, original magnification X400)

5.2.3. Comparison of mucous cell types between the odontogenic cysts

There were no significant differences in the cell counts for each mucous cell type between the 3 cyst types; type I mucous cells (p=0.54); type II mucous cells (p=0.73); type III mucous cells (p=0.97) (Appendix G). The mucin phenotype of the GOC is thus shared by DC and RC with mucous metaplasia.

5.2.4. Comparison of mucous cell types between odontogenic cysts and salivary gland mucous acinar cells

All 3 odontogenic cyst types had very low counts for type I mucous cells; this is mirrored by all 3 salivary gland types too. The type I < type II < type III mucous cell phenotype of the odontogenic cysts (Figure 9) is further probably best mirrored in this study by the submandibular (Figure 3) and sublingual salivary glands (Figure 6) but not the palatal salivary glands (Figure 8) as here type II outnumbered type III mucous cells.

CHAPTER 6

6. DISCUSSION

Studies on mucin histochemistry of odontogenic cysts with mucous cells are few. The nature and content of these mucins in odontogenic cysts are not well documented and explained. In the present study an attempt was made to determine the mucin phenotype of the mucous cells in the GOC, and in DC and RC with mucous metaplasia. This would allow the researcher to ascertain whether any similarities or differences exist between the mucin profiles of the 3 odontogenic cysts studied. The findings were further compared to the mucin composition of normal salivary gland mucous acinar cells.

A study by Eversole[20] on mucin histochemistry of the major submandibular and sublingual salivary glands and the minor palatal salivary glands revealed a heterogenous population of mucous cells within these glands. Histochemically, the major sublingual and submandibular salivary glands were found to be similar as both glands showed a predominance of mucous cells that contain mixtures of acidic and neutral mucins. Although the palatal salivary glands, which are pure mucous glands, also revealed a heterogenous population of mucous cells, the palatal glands showed a predominance of mucous cells that contain acidic mucin. The present study also revealed that a heterogenous population of mucous cells exists within the major sublingual and submandibular glands as well as the minor palatal glands. Similar to Eversole, mucous cells that contain mixtures of acidic and neutral mucins, denoted as type III mucous cells in this study, were found to be the dominant mucous cell type in the submandibular and sublingual salivary glands. Unlike the findings of Eversole, however, the palatal salivary gland mucous acinar cells in this study were found to elaborate a greater proportion of

neutral mucins as opposed to acidic mucins. The reason/s for the differences encountered are difficult to explain by a direct comparison of the findings between these two studies since the method of quantification of the different mucous cell types manifested in the palatal glands and statistical analysis thereof are not described in the study by Eversole.[21] The laboratory technique for the combined AB-PAS stain described by Mowry[19] at pH 2.5 was used in both studies and this therefore makes the laboratory method of tissue staining an unlikely cause for the different histochemical findings encountered in the palatal glands. Differences in staining characteristics has, however, been previously noted in the same minor salivary glands obtained from different subjects and it is thought that the differences observed may be indicative of different stages in secretory cycles of mucin.[21]

In the present study, the mucous cell types within the GOC, DC and RC were found to be similar within all 3 cyst types, which were characterised by a type I < type II < type III mucous cell phenotype. This mucous cell phenotype of the odontogenic cysts is further probably best mirrored in this study by the submandibular and sublingual salivary gland mucous cell phenotype. Of note, the mucous cell phenotype of the odontogenic cysts studied, irrespective of them being of developmental (glandular odontogenic cyst, dentigerous cyst) or inflammatory (radicular or residual radicular cyst) origin, were the same and mimicked the submandibular and sublingual salivary glands. Recent immunohistochemical findings on the GOC strongly suggest an odontogenic origin.[13,43] As far as we are aware, this is the first histochemical study undertaken to analyse the mucous cell phenotype in the GOC. The study findings suggest that the mucin phenotype of the GOC is shared by DC and RC with mucous metaplasia.

The similar mucin phenotype of odontogenic cysts and the major submandibular and sublingual salivary glands allows us to speculate on the histogenesis of odontogenic cysts with mucous metaplasia. It is well established that an ectodermal origin is conceded for most salivary glands. The failure of development or hypofunction of other ectodermally derived tissues in conditions characterised by major salivary gland aplasia suggest that the ectodermal germ layer is the most likely source of origin of the major salivary glands.[44] Similarities in the mucin phenotype between odontogenic cysts, submandibular and sublingual salivary glands, as demonstrated in this study, may suggest a common ectodermal histogenetic origin for the mucous cells in odontogenic cysts and major salivary glands.

As there are no previous studies that evaluated the mucin phenotype of the mucous cells in odontogenic cysts, the literature is limited in comparative data on this aspect of the present study. Future studies on larger numbers of cases of odontogenic cysts with mucous metaplasia are therefore needed to expand on the current findings and to improve our understanding of the histochemical composition of the mucous cells in odontogenic cysts. The GOC further shows overlapping histological features with the central mucoepidermoid carcinoma. By applying the combined AB/PAS histochemical technique used in this study, future studies could aim to determine whether or not type III mucous cells are also the predominating mucous cell type in central (intra-osseous) mucoepidermoid carcinomas.

CHAPTER 7

7. CONCLUSIONS

7.1. There was no significant difference in the histochemical composition of the mucous cells in the GOC, DC and RC with mucous metaplasia as determined with the use of the combined AB pH2.5/PAS stain.

7.2. The overlapping mucin phenotype of the GOC, DC and RC unfortunately does not support the use of the combined AB pH2.5/PAS stain as a potential histochemical marker to distinguish between the GOC and odontogenic cysts with mucous metaplasia.

7.3. The histochemical mucous cell phenotype of the odontogenic cysts examined in this study mirrored the mucous cell phenotype of the mucous acinar cells normally found in the submandibular and sublingual salivary glands but not the palatal minor salivary glands.

CHAPTER 8

8. APPENDIX

APPENDIX A. Ethics clearance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Altini

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080850**
PROJECT	Approval for Research Conducted on Archived Paraffin Wax Embedded Tissue Blocks (Original Class Approval M00/08/29)
INVESTIGATORS	Prof M Altini
DEPARTMENT	Dept of Oral Pathology
DATE CONSIDERED	08.09.10
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 23.09.08 **CHAIRPERSON** .....
pp (Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof M Altini

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B1. Raw data used for establishing intra-observer reliability in the submandibular salivary gland

Submand-ibular salivary gland case	Submand-ibular salivary gland lobule	Type I mucous cell count	Type II mucous cell count	Type III mucous cell count	Type I mucous cell recount	Type II mucous cell recount	Type III mucous cell recount
SM1	1	0	0	0	0	0	0
SM1	2	0	0	0	0	0	2
SM1	3	0	0	2	0	0	2
SM1	4	0	0	0	0	0	0
SM1	5	0	0	0	0	0	2
SM1	6	0	0	2	0	0	2
SM1	7	0	0	2	0	1	2
SM1	8	0	1	2	0	0	2
SM1	9	0	1	2	0	1	2
SM2	10	0	0	2			
SM2	11	0	0	2			
SM2	12	0	1	2			
SM2	13	0	0	2			
SM2	14	0	0	2			
SM2	15	0	1	2			
SM2	16	0	1	2			
SM2	17	0	1	2			
SM2	18	0	1	2			
SM2	19	0	1	2			
SM3	20	0	0	0			
SM3	21	0	0	2			
SM3	22	0	0	2			
SM3	23	0	0	2			
SM3	24	0	0	2			
SM3	25	0	0	2			
SM3	26	0	0	2			
SM3	27	0	0	0			
SM3	28	0	1	2			
SM3	29	0	1	2			
SM3	30	0	1	2			
SM3	31	0	0	2			
SM3	32	0	0	2			
SM3	33	0	0	2			
SM3	34	0	0	2			
SM3	35	0	0	2			
SM3	36	0	0	2			
SM4	37	0	0	0	0	0	0
SM4	38	0	0	2	0	0	2
SM4	39	0	0	2	0	0	0
SM4	40	0	0	2	0	0	2

SM4	41	0	0	2	0	0	2
SM4	42	0	0	2	0	0	2
SM4	43	0	0	2	0	0	2
SM4	44	0	0	2	0	0	2
SM4	45	0	0	0	0	0	2
SM4	46	0	0	2	0	0	2
SM4	47	0	0	0	0	0	2
SM4	48	0	0	2	0	0	2
SM4	49	0	0	2	0	0	0
SM4	50	0	0	2	0	0	2
SM4	51	0	0	2	0	0	2
SM4	52	0	0	2	0	0	2
SM5	53	0	0	2			
SM5	54	0	0	0			
SM5	55	0	0	2			
SM5	56	0	0	2			
SM5	57	0	0	2			
SM5	58	0	0	2			
SM5	59	0	0	2			
SM6	60	0	0	2			
SM6	61	0	1	2			
SM6	62	0	1	2			
SM6	63	0	1	2			
SM6	64	0	1	2			
SM6	65	0	0	0			
SM6	66	0	0	2			
SM6	67	0	0	2			
SM6	68	0	2	0			
SM7	69	0	1	2			
SM7	70	0	1	2			
SM8	71	0	0	2			
SM8	72	0	1	2			
SM8	73	0	1	2			
SM8	74	0	0	2			
SM8	75	0	0	2			
SM8	76	0	0	2			
SM9	77	0	0	2	0	0	2
SM9	78	0	0	0	0	0	0
SM9	79	0	0	0	0	0	2
SM9	80	0	0	0	0	0	2
SM9	81	0	0	0	0	0	0
SM9	82	0	0	2	0	0	2
SM9	83	0	0	2	0	0	2
SM9	84	0	0	2	0	0	2

APPENDIX B2. Raw data used for establishing intra-observer reliability in the sublingual salivary gland

Salivary gland case	Sublingual salivary gland lobule	Type I mucous cell count	Type II mucous cell count	Type III mucous cell count	Type I mucous cell recount	Type II mucous cell recount	Type III mucous cell recount
SL1	1	0	1	2			
SL1	2	0	1	2			
SL1	3	0	1	2			
SL1	4	0	1	2			
SL1	5	0	2	1			
SL1	6	0	1	2			
SL2	7	0	0	2	0	1	2
SL2	8	0	1	2	0	1	2
SL2	9	0	1	2	0	1	2
SL2	10	0	1	2	0	1	2
SL2	11	0	1	2	0	1	2
SL2	12	0	1	2	0	1	2
SL2	13	0	1	2	0	1	2
SL2	14	0	1	2	0	1	2
SL2	15	0	1	2	0	1	2
SL3	16	0	0	2			
SL3	17	0	1	2			
SL3	18	0	1	2			
SL3	19	0	1	2			
SL3	20	0	1	2			
SL4	21	0	2	1			
SL4	22	0	2	1			
SL4	23	0	2	1			
SL4	24	0	2	1			
SL4	25	0	2	1			
SL4	26	0	2	1			
SL4	27	0	2	1			
SL4	28	0	2	0			
SL4	29	0	2	0			
SL4	30	0	2	1			
SL4	31	0	2	1			
SL4	32	0	2	1			
SL4	33	0	1	2			
SL4	34	0	1	2			
SL4	35	0	1	2			
SL4	36	0	1	2			
SL4	37	0	1	2			
SL5	38	0	1	2			
SL5	39	0	0	2			
SL5	40	0	1	2			
SL5	41	0	1	2			
SL5	42	0	1	2			

SL5	43	0	1	2			
SL5	44	0	2	1			
SL6	45	0	0	0			
SL6	46	0	1	2			
SL6	47	0	1	2			
SL6	48	0	1	2			
SL6	49	0	1	2			
SL6	50	0	0	2			
SL6	51	0	1	2			
SL6	52	0	0	2			
SL6	53	0	1	2			
SL6	54	0	0	0			
SL6	55	0	0	2			
SL6	56	0	0	2			
SL6	57	0	0	0			
SL7	58	1	2	1			
SL7	59	1	1	2			
SL7	60	1	1	2			
SL7	61	1	1	2			
SL7	62	1	1	2			
SL7	63	1	1	2			
SL7	64	1	1	2			
SL7	65	1	1	2			
SL7	66	1	1	2			
SL7	67	1	1	2			
SL7	68	0	1	2			
SL7	69	1	0	2			
SL8	70	0	0	2			
SL8	71	0	0	2			
SL8	72	0	0	2			
SL9	73	0	1	2	0	1	2
SL9	74	0	1	2	0	1	2
SL9	75	0	1	2	0	1	2
SL9	76	0	1	2	0	1	2
SL9	77	0	1	2	0	1	2
SL9	78	0	1	2	0	1	2
SL9	79	0	1	2	0	1	2
SL9	80	0	1	2	0	1	2
SL9	81	0	1	2	0	1	2
SL9	82	0	1	2	0	1	2
SL9	83	0	1	2	0	1	2
SL9	84	0	1	2	0	1	2
SL9	85	0	1	2	0	1	2
SL9	86	0	1	2	0	1	2
SL10	87	0	0	2	0	1	2
SL10	88	0	0	2	0	1	2
SL10	89	0	0	2	0	1	2
SL10	90	0	0	2	0	1	2
SL10	91	0	1	2	0	1	2
SL10	92	0	1	2	0	1	2
SL10	93	0	1	2	0	1	2

APPENDIX B3. Raw data used for establishing intra-observer reliability in the palatal (PAL) salivary glands

Salivary gland case	Palatal salivary gland lobule	Type I mucous cell count	Type II mucous cell count	Type III mucous cell count	Type I mucous cell recount	Type II mucous cell recount	Type III mucous cell recount
PAL1	1	0	2	1	0	2	1
PAL2	2	0	2	1			
PAL2	3	0	2	1			
PAL2	4	0	2	1			
PAL2	5	0	2	1			
PAL2	6	0	2	1			
PAL2	7	0	2	1			
PAL3	8	0	2	0	0	2	0
PAL3	9	0	2	1	0	2	1
PAL4	10	0	2	1	0	2	1
PAL4	11	0	1	1	0	1	1
PAL4	12	0	2	1	0	2	1
PAL4	13	0	1	1	0	2	1
PAL4	14	0	2	1	0	2	1
PAL4	15	0	2	1	0	2	1
PAL4	16	0	2	1	0	2	1
PAL4	17	0	2	1	0	2	1
PAL4	18	0	2	1	0	2	1
PAL4	19	0	2	1	0	2	1
PAL4	20	0	2	1	0	2	1
PAL5	21	0	2	1	1	1	0
PAL5	22	0	2	1	0	2	1
PAL5	23	0	2	1	0	2	1
PAL5	24	0	1	1	0	1	1
PAL5	25	0	1	2	0	1	2
PAL5	26	0	1	2	0	1	2
PAL6	27	0	2	0			
PAL6	28	0	2	1			
PAL6	29	0	2	1			
PAL7	30	0	2	0			
PAL7	31	0	1	2			
PAL7	32	0	1	1			
PAL7	33	0	2	1			

APPENDIX C1. Frequency of type I, II and III mucous cells in the submandibular (SM) salivary gland (count-recount data)

SM / Type I Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	29	0	0	29
	< 50%	0	0	0	0
	> 50%	0	0	0	0
Total		29	0	0	29

Perfect agreement.

SM / Type II Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	26	1	0	27
	< 50%	1	1	0	2
	> 50%	0	0	0	0
Total		27	2	0	29

Exact test: p=0.099

SM / Type III Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	4	0	6	10
	< 50%	0	0	0	0
	> 50%	2	0	17	19
Total		6	0	23	29

Exact test: p=0.289

APPENDIX C2. Frequency of type I, II and III mucous cells in the sublingual (SL) salivary gland (count-recount data)

SL / Type I Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	27	0	0	27
	< 50%	0	0	0	0
	> 50%	0	0	0	0
Total		27	0	0	27

Perfect agreement.

SL / Type II Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	0	5	0	5
	< 50%	0	22	0	22
	> 50%	0	0	0	0
Total		0	27	0	27

Exact test: p=0.063

SL / Type III Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	0	0	0	0
	< 50%	0	0	0	0
	> 50%	0	0	27	27
Total		0	0	27	27

Perfect agreement.

APPENDIX C3. Frequency of type I, II and III mucous cells in the palatal (PAL) salivary glands (count-recount data)

PAL / Type I Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	19	1	0	20
	< 50%	0	0	0	0
	> 50%	0	0	0	0
Total		19	1	0	20

Exact test: p=1.0

PAL /Type II Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	0	0	0	0
	< 50%	0	4	1	5
	> 50%	0	1	14	15
Total			5	15	20

Exact test: p=1.0

PA L/ Type III Frequencies

		Recount			
		0	< 50%	> 50%	total
Count	0	1	0	0	1
	< 50%	1	16	0	17
	> 50%	0	0	2	2
Total		2	16	2	20

Exact test: p=1.0

APPENDIX C4. Frequency of type I, II and III mucous cells in the submandibular, sublingual and palatal salivary glands (count-recount data)

All salivary glands / Type I

		Recount			
		0	< 50%	> 50%	total
Count	0	75	1	0	76
	< 50%	0	0	0	0
	> 50%	0	0	0	0
Total		75	1	0	76

Exact test: p=1.0

All salivary glands / Type II

		Recount			
		0	< 50%	> 50%	total
Count	0	26	6	0	32
	< 50%	1	27	1	29
	> 50%	0	1	14	15
Total		27	34	15	76

Stuart-Maxwell test:

X2 (calc)	X2 (crit)
3.57	5.99

not significant

All salivary glands / Type III

		Recount			
		0	< 50%	> 50%	total
Count	0	5	0	6	11
	< 50%	1	16	0	17
	> 50%	2	0	46	48
Total		8	16	52	76

Stuart-Maxwell test:

X2 (calc)	X2 (crit)
3.00	5.99

not significant

APPENDIX D. Raw data used for establishing the intra-observer reliability in the RC, DC and GOC

Cyst type	Type I mucous cell count	Type I mucous cell recount	Type II mucous cell count	Type II mucous cell recount	Type III mucous cell count	Type III mucous cell recount
RC 1	0		0		20	
RC 2	0		5		28	
RC 3	0	0	3	4	95	75
RC 4	1		2		8	
RC 5	0	0	0	1	33	48
RC 6	0	0	36	49	0	0
RC 7	0	0	6	3	49	43
RC 8	0		24		324	
DC 1	0		52		32	
DC 2	0		5		22	
DC 3	0		0		3	
DC 4	0	0	50	63	23	22
DC 5	0	0	0	0	165	157
DC 6	0		5		2	
DC 7	3	3	3	2	56	49
DC 8	0	0	8	12	107	112
DC 9	0	0	9	1	88	105
GOC 1	0		289		599	
GOC 2	0	0	1	0	86	79
GOC 3	0	0	69	32	83	99
GOC 4	0	0	0	0	42	40
GOC 5	0		0		24	
GOC 6	0		97		448	
GOC 7	0		0		14	
GOC 8	0	0	4	3	0	3
GOC 9	0	0	76	56	10	11
GOC 10	0	0	16	17	13	20

APPENDIX E. Reliability tests for GOC, RC and DC

GOC/ Type I mucous cells, DC/ Type I mucous cells and RC/ Type I mucous cells paired differences all = 0: thus no test can be done and we conclude no difference between count and recount data.

GOC/ Type II

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
GOC Count	27.666667	35.26282								
GOC Recount	18.000000	22.42320	6	9.666667	15.56492	1.521267	5	0.188677	-6.66771	26.00105

Wilcoxon matched pairs test	Valid	T	Z	p-value
GOC Count & GOC Recount	5	2.000000	1.483240	0.138012

GOC/ Type III

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
GOC Count	39.00000	37.92624								
GOC Recount	42.00000	38.95639	6	-3.00000	7.924645	-0.927293	5	0.396332	-11.3164	5.316405

Wilcoxon matched pairs test	Valid	T	Z	p-value
GOC Count & GOC Recount	6	6.500000	0.838628	0.401679

DC/ Type II

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
DC Count	14.00000	20.45727								
DC Recount	15.60000	26.93139	5	-1.60000	7.700649	-0.464598	4	0.666381	-11.1616	7.961619

Wilcoxon matched pairs test	Valid	T	Z	p-value
DC Count & DC Recount	4	4.000000	0.365148	0.715001

DC/ Type III

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
DC Count	87.80000	53.70940								
DC Recount	89.00000	53.61436	5	-1.20000	10.25671	-0.261612	4	0.806539	-13.9354	11.53538

Wilcoxon matched pairs test	Valid	T	Z	p-value
DC Count & DC Recount	5	7.000000	0.134840	0.892738

RC/ Type II

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
RC Count	11.25000	16.68083								
RC Recount	14.25000	23.20022	4	-3.00000	6.928203	-0.866025	3	0.450185	-14.0243	8.024317

Wilcoxon matched pairs test	Valid	T	Z	p-value
RC Count & RC Recount	4	3.000000	0.730297	0.465209

RC/ Type III

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
RC Count	44.25000	39.50844								
RC Recount	41.50000	31.03224	4	2.750000	14.50000	0.379310	3	0.729714	-20.3227	25.82274

Wilcoxon matched pairs test	Valid	T	Z	p-value
RC Count & RC Recount	3	2.000000	0.534522	0.592980

All cysts / type II

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
C All	18.73333	26.10546								
RC All	16.20000	22.46648	15	2.533333	12.26416	0.800019	14	0.437065	-4.25833	9.324997

Wilcoxon matched pairs test	Valid	T	Z	p-value
C All & RC All	13	39.50000	0.419314	0.674987

All cysts / Type III

Paired t-test	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p	95% LCL	95% UCL
C All	56.66667	46.86556								
RC All	57.53333	45.82711	15	-0.866667	10.16202	-0.330307	14	0.746059	-6.49421	4.760873

Wilcoxon matched pairs test	Valid	T	Z	p-value
C All & RC All	14	50.50000	0.125553	0.900086

APPENDIX F. Kruskal-Wallis ANOVA test for statistical comparison of mucous cell types within the GOC, DC and RC

Glandular odontogenic cyst

Depend.: GOC	Kruskal-Wallis ANOVA by Ranks; GOC (RC data) Independent (grouping) variable: BMP Kruskal-Wallis test: H (2, N=30) = 14.87399 p = .0006			
	Code	Valid N	Sum of Ranks	Mean Rank
B	1	10	75.0000	7.50000
M	2	10	175.5000	17.55000
P	3	10	214.5000	21.45000

Depend.: GOC	Multiple comparisons z' values ; GOC (RC data) Independent (grouping) variable: BMP Kruskal-Wallis test: H (2, N=30) = 14.87399 p = .0006		
	B R:7.5000	M R:17.550	P R:21.450
B		2.552703	3.543304
M	2.552703		0.990601
P	3.543304	0.990601	

Dentigerous cyst

Depend.: DC	Kruskal-Wallis ANOVA by Ranks; DC (RC data) Independent (grouping) variable: BMP_DC Kruskal-Wallis test: H (2, N=27) = 15.70380 p = .0004			
	Code	Valid N	Sum of Ranks	Mean Rank
B	101	9	57.0000	6.33333
M	102	9	135.0000	15.00000
P	103	9	186.0000	20.66667

Depend.: DC	Multiple comparisons p values (2-tailed); DC (RC data) Independent (grouping) variable: BMP_DC Kruskal-Wallis test: H (2, N=27) = 15.70380 p = .0004		
	B R:6.3333	M R:15.000	P R:20.667
B		0.061632	0.000383
M	0.061632		0.389712
P	0.000383	0.389712	

Radicular cyst

Depend.: RC	Kruskal-Wallis ANOVA by Ranks; RC (RC data) Independent (grouping) variable: BMP_RC Kruskal-Wallis test: H (2, N=24) = 12.73484 p = .0017			
	Code	Valid N	Sum of Ranks	Mean Rank
B	101	8	49.5000	6.18750
M	102	8	104.0000	13.00000
P	103	8	146.5000	18.31250

Depend.: RC	Multiple comparisons p values (2-tailed); RC (RC data) Independent (grouping) variable: BMP_RC Kruskal-Wallis test: H (2, N=24) = 12.73484 p = .0017		
	B R:6.1875	M R:13.000	P R:18.313
B		0.161989	0.001814
M	0.161989		0.398825
P	0.001814	0.398825	

B = (type I mucous cells), M = (type II mucous cells), P = (type III mucous cells)

APPENDIX G. Kruskal-Wallis ANOVA test for statistical comparison of mucous cell types between the GOC, DC and RC

Type I mucous cells

Depend.: B	Kruskal-Wallis ANOVA by Ranks; B (RC data) Independent (grouping) variable: OC Kruskal-Wallis test: H (2, N = 27) = 1.223291 p = .5425			
	Code	Valid N	Sum of Ranks	Mean Rank
GOC	101	10	130.0000	13.00000
DC	102	9	131.0000	14.55556
RC	103	8	117.0000	14.62500

Type II mucous cells

Depend.: M	Kruskal-Wallis ANOVA by Ranks; M (RC data) Independent (grouping) variable: OC Kruskal-Wallis test: H (2, N = 27) = .6422611 p = .7253			
	Code	Valid N	Sum of Ranks	Mean Rank
GOC	101	10	153.0000	15.30000
DC	102	9	126.5000	14.05556
RC	103	8	98.5000	12.31250

Type III mucous cells

Depend.: P	Kruskal-Wallis ANOVA by Ranks; P (RC data) Independent (grouping) variable: OC Kruskal-Wallis test: H (2, N = 27) = .0571713 p = .9718			
	Code	Valid N	Sum of Ranks	Mean Rank
GOC	101	10	142.5000	14.25000
DC	102	9	128.0000	14.22222
RC	103	8	107.5000	13.43750

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