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Comparative Efficacy of Modified Gallego's Stain (MGS) in the Differential Diagnosis of Mineralized Components in Oral Pathologies

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ABSTRACT:
Accurate diagnosis and early detection of **oral pathologies** are fundamental to effective treatment planning and determining prognosis. While **Hematoxylin and Eosin (H&E)** staining remains the gold standard for morphological analysis of **formalin-fixed, paraffin-embedded (FFPE)** tissues, its efficacy is often limited when faced with the complex, composite hard and soft tissue nature of many oral lesions. Specifically, the definitive identification and differentiation of various **mineralized components**—such as enamel, dentin, bone, and cementum—can be ambiguous with H&E alone, necessitating the use of specialized staining techniques. **Immunohistochemistry (IHC)**, though highly specific, is constrained by its high cost, technical sensitivity, and potential for equivocal results. This study advocates for the use of **Modified Gallego's Stain (MGS)**, an economical, reliable, and differential staining method derived from Lille's stain. MGS utilizes a combination of dyes (hematoxylin, carbol fuchsin, and aniline blue) that distinctly color hard tissue integrants: cementum as deep red, dentin and bone as green, and enamel as pink. Employing a **retrospective design** on **35 diverse oral lesions** and **15 control samples** (normal teeth and bone), this research aims to systematically validate MGS's role as an invaluable adjunct to H&E by distinctly delineating dissimilar hard tissue components. MGS has the potential to resolve diagnostic challenges, particularly in overlapping histomorphological spectra like the **fibro-osseous lesions**, thereby simplifying and confirming the precise nature of calcifications.

1. Introduction: The Diagnostic Imperative in Oral Pathology

The cornerstone of successful clinical management in oral medicine hinges upon the valid diagnosis and early detection of pathological lesions. This diagnostic accuracy is not only essential for designing a pertinent treatment plan but is also indispensable for the determination of the patient's long-term prognosis. While the tools of molecular biology are increasingly being integrated into the examination of both hard and soft tissue pathologies within the oral cavity, the histological inspection of tissues remains the primary diagnostic modality. Specifically, the analysis of FFPE tissues stained with H&E constitutes the linchpin of modern cancer diagnosis and pathological staging in routine clinical practice.

1.1 H&E: The Standard and Its Limitations

Introduced in the mid-1800s, H&E has earned its status as the universally accepted standard technique among pathologists due to its remarkable versatility. It is compatible with a wide array of fixatives and provides a broad visualization of crucial cellular features, including cytoplasmic details, nuclear morphology, and the extracellular matrix. Hematoxylin, a basic dye, imparts a deep blue-purple hue to basophilic structures, primarily staining the nucleus and nucleic acids. The patterns of heterochromatin condensation within the nucleus are critical morphological markers used to identify various cell types and malignancy types, carrying outstanding diagnostic significance. Conversely, Eosin, an acidic dye, stains basic proteins and the cytoplasm a distinct pink color. [1]

However, the diagnostic process in the oral cavity is frequently complicated by the presence of a heterogeneous mixture of hard and soft tissues. Oral tumors often involve a complex concoction of mineralized matrices (such as enamel, dentin, bone, and cementum) that are challenging to distinctly identify and differentiate using the routine H&E stain alone. This lack of clear differential staining for mineralized components can render complex lesions "like chalk and cheese" to accurately interpret, thereby potentially obscuring a definitive diagnosis. [2]

1.2 The Role of Special Stains

To ameliorate the limitations of H&E, various special stains have been developed and refined over time. These stains employ diverse groups of dyes and procedures to specifically highlight particular structures, tissue types, or even pathogens, assisting pathologists in tissue-based diagnosis. Their use is often deemed ineluctable alongside routine methods when H&E fails to provide sufficient diagnostic detail. The steps of special staining involve complex chemical interactions between tissue components and reagents, where staining intensity and color depend upon the tissue's configuration, chemical affinity, and

reciprocity with the dye molecules. IHC, another diagnostic technique, offers high specificity but is constrained by being high-priced, technique-sensitive, and frequently yielding contentious results in certain states of affairs. In contrast, there remains a demand for specialized histochemical methods that are cost-effective, dependable, and robust for routine laboratory use.

1.3 Modified Gallego's Stain: A Differential Approach

Among the effective and economical alternatives, the MGS, obtained by modifying Lille's stain, stands out. MGS is particularly valuable as a differential counterstain for the hard tissues of the teeth and for pathological lesions pervaded microscopically with a fibrous stroma containing mineralized material (enamel, dentin, bone, and cementum-like material). It consists of basic reagents, including hematoxylin, carbol fuchsin, and aniline blue. These contradictory hard tissue components are typically a product of secretory material within many oral pathological lesions that undergo subsequent calcification. MGS is considered an avaricious, dependable, and less technique-sensitive special stain.

The distinct mechanism of MGS lies in its ability to differentially stain these mineralized tissues based on their chemical composition and the principles of molecular competition and permeability:

- **Cementum** stains **deep red** (attributed to the strong retention of the basic dye, carbol fuchsin, due to the slightly acidic nature of cementum's Type I collagen and Non-Collagenous Proteins).
- **Dentin** and **Bone** stain **green** (assigned to their affinity for the anionic dye, aniline blue).
- **Enamel** stains **pink** (due to its high inorganic content and weak retention of the basic carbol fuchsin).

This differential coloring, especially the ability to distinguish cementum from bone, is of extreme importance in diagnosing lesions with an overlapping histomorphologic spectrum, such as the **fibro-osseous lesions**. The exact nature of the mineralizing component in such lesions cannot be reliably identified by H&E alone, which only provides a diagnosis based on morphological features that may not always be correct.[3]

1.4 Research Hypothesis and Aim

Given the documented advantages and the diagnostic challenges posed by mineralized oral lesions, this study hypothesizes that MGS can serve as a superior and cost-effective differential stain compared to H&E in identifying and characterizing various hard tissue components.

The study aims to determine the nature of dissimilar hard tissue components in variegated oral pathological lesions by using Modified Gallego's Stain (MGS) to facilitate a more precise and effortless final diagnosis.

3. Materials and Methods

3.1 Ethical Clearance and Study Design

This **retrospective study** was conducted at the Department of Oral and Maxillofacial Pathology, Sri Sai College of Dental Surgery, Vikarabad. **Ethical clearance** for the study was obtained from the Institutional Ethical Clearance Committee.

3.2 Sample Selection

A total of **50 samples** were included in the study, comprising both pathological cases and control tissues.

Study Group (n=35): Oral Pathological Lesions with Calcifications

The study group consisted of **35 samples** of previously histopathologically diagnosed lesions, selected based on the presence of hard tissue components or calcifications:

- **Calcifying Epithelial Odontogenic Tumor (CEOT):** 2 cases
- **Ameloblastic Fibro Odontoma (AFO):** 3 cases
- **Compound Odontome:** 2 cases
- **Calcifying Odontogenic Cyst (COC):** 2 cases
- **Odontogenic Keratocyst (OKC):** 1 case
- **Peripheral Ossifying Fibroma (POF):** 3 cases
- **Fibrous Dysplasia:** 2 cases
- **Focal Cemento Osseous Dysplasia (FCOD):** 2 cases
- **Juvenile Ossifying Fibroma (JOF):** 2 cases (Trabecular variant)
- **Juvenile Ossifying Fibroma (JOF):** 2 cases (Psammomatoid variant)
- **Central Giant Cell Granuloma (CGCG):** 1 case
- **Osteomyelitis:** 5 cases
- **Fibroma with Calcifications:** 2 cases
- **Fibroepithelial Hyperplasia with Calcifications:** 2 cases
- **Sialolithiasis:** 1 case
- **Sclerosing Angioma:** 3 cases

Control Group (n=15)

- **Normal Teeth (Ground Sections):** 10 samples
- **Normal Bone Tissue (FFPE Sections):** 5 samples

3.3 Inclusion and Exclusion Criteria

Category	Criterion
Inclusion Criteria	Soft tissue lesions of the oral cavity bearing some type of calcifications were included.
Exclusion Criteria	Soft tissue lesions with no hard tissue component were excluded.

3.4 Armamentarium and Reagent Preparation

MGS utilizes three key staining solutions

Reagent	Composition
Harris Hematoxylin	(Proprietary/Commercial preparation)
Mordant	200 ml Distilled Water + 1.5 ml Nitric Acid + 1 ml 40% Formaldehyde + 1.5 ml Ferric Chloride
Carbol Fuchsin Solution	4 ml Carbol Fuchsin + 50 ml Distilled Water + 0.2% Acetic Acid
Aniline Blue Picric Acid Solution	1.18 g Picric Acid + 100 ml Distilled Water + 0.01% Aniline Blue

3.5 Staining Protocol for Modified Gallego's Stain (MGS)

Formalin-fixed, paraffin-embedded sections of pathological lesions and normal bony tissue, along with ground sections of normal teeth, were subjected to the following MGS technique:

1.**Deparaffinization:** Sections were deparaffinised in Xylene and rehydrated through graded alcohols.
2.**Hematoxylin Staining:** Stained in Hematoxylin for **8–12 minutes**.
3.**Rinsing:** Rinsed in distilled water.
4.**Sensitization (Mordant):** Sensitized in the prepared mordant solution for **2 minutes**.
5.**Rinsing:** Rinsed in distilled water.
6.**Carbol Fuchsin Staining:** Stained with 4 ml of carbol fuchsin diluted in 50 ml of 0.2% acetic acid (Carbol Fuchsin Solution) for the specified time.
7.**Rinsing:** Rinsed in distilled water.
8.**Washing (Mordant):** Washed in the mordant solution for **1–2 minutes**.
9.**Aniline Blue Staining:** Stained with 0.01% aniline blue in saturated picric acid solution for **30 seconds**.
10. **Dehydration/Clearing/Mounting:** Dehydrated, cleared with xylene, and mounted using DPX mounting media.

3.6 Histological Interpretation and Statistical Analysis

All prepared slides were viewed under **10x magnification** of a light compound microscope (Photograph 2 in original data). The interpretation of the hard tissue components was strictly adhered to the established staining pattern based on prior studies (Sandhya et al., 2014):

Hard Tissue Component	Interpretation (MGS Color)
Enamel	Pink
Dentin	Green
Cementum	Red
Bone	Green

The **intensity of the color** was noted as varying according to the **degree of mineralization** of the hard tissue component. The presence, distribution, and nature of all hard tissue deposits were meticulously documented for each case.
All calculations and data organization were performed using **MS Excel 2010**.

RESULTS AND OBSERVATION:

The present study was undertaken at Sri Sai College of Dental Surgery, Vikarabad in the Department of Oral and Maxillofacial Pathology, with an aim of determining the nature of hard tissue components in various oral pathological lesions using MGS.

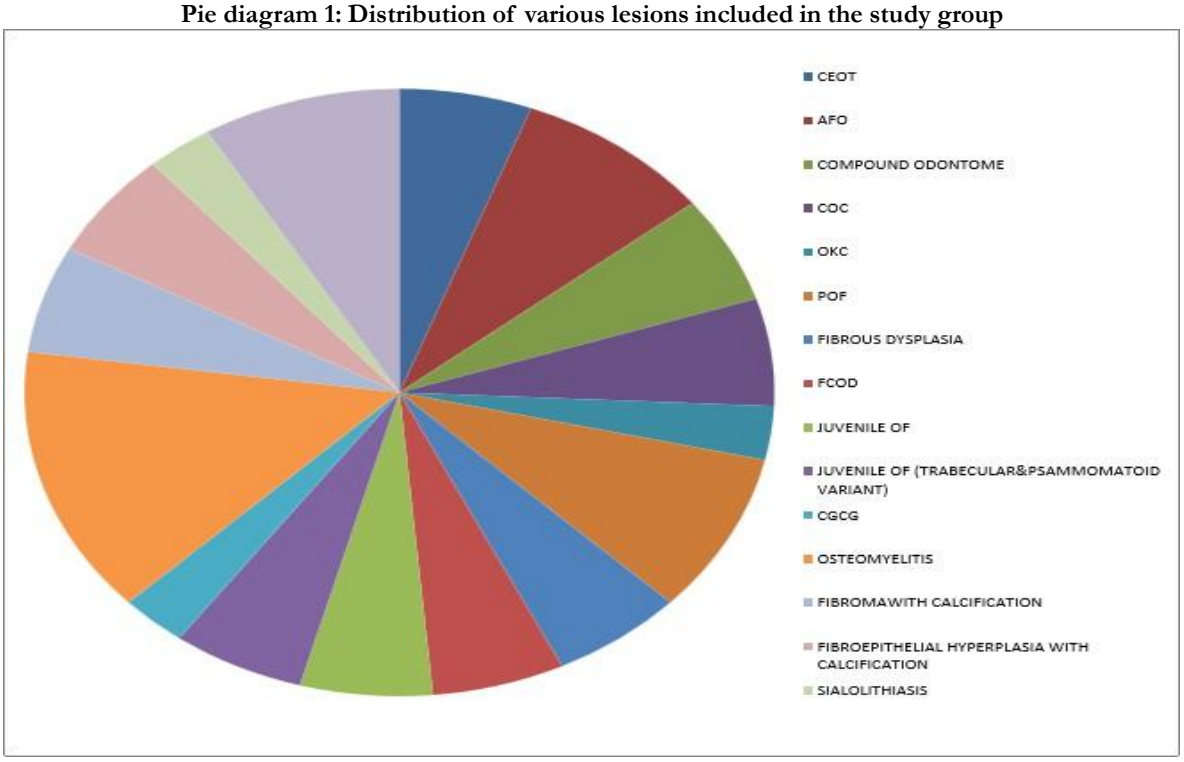
Pathological tissue	Number of cases	Colour obtained	Interpretation
CEOT	2	Green	Dentin- like/ Bone-like
AFO	3	Pink & Green	Enamel- like & Dentin - like/Bone – like
Compound Odontome	2	Green	Dentin – like/ Bone - like
COC	2	Green	Dentin – like/ Bone - like
OKC	1	Green	Dentin like/ Bone- like

POF	3	Green	Bone- like
		Green	Bone – like
		Green- Red	Bone like & Cementum like
Fibrous dysplasia	2	Green	Bone- like
FCOD	2	Green	Bone- like
		Green & Red	Dentin- like/ Bone like & Cementum like
Juvenile OF	2	Green	Bone- like
Juvenile OF (psammatoid & trabecular variant)	2	Green & Red	Bone – like & Cementum like
CGCG	1	Green	Bone- like
Osteomyelitis	5	Green	Bone – like
Fibroma with calcification	2	Green	Bone- like
Fibro epithelial hyperplasia with calcificatoin	2	Green	Bone- like
Sialolith	1	Green	Bone – like
Sclerosing angioma	3	Green	Bone – like

Interpretation of nature of Secretion using MGS in Pathological Tissues

Pathological tissue	**Calcification observed**
CEOT	Liesgang rings
AFO	Sheets of hard tissue and hyalinized droplets of hard tissue
Compound odontome	Uniform sheet of hard tissue
COC	Foci of hard tissue
OKC	Multiple small bits of hard tissue in the connective tissue
POF	Haphazard arrangement of multiple bits of hard tissues
Fibrous dysplasia	Irregular bony trabeculae
FCOD	Haphazard arrangement of hard tissue at a foci
Juvenile OF	Haphazard arrangement of multiple bits of hard tissue
Juvenile OF (trabecular and psammomatoid variant)	Haphazard arrangement of hard tissue at a foci and globules of calcifications scattered in connective tissue
CGCG	Haphazard arrangement of hard tissue at a foci in the connective tissue stroma
Osteomyelitis	Haphazard arrangement of multiple bits of hard tissue
Fibroma with calcification	Haphazard arrangement of hard tissue at a foci in the connective tissue stroma
Fibro epithelial hyperplasia with calcificatoin	Haphazard arrangement of hard tissue at a foci
Sialolithiasis	Haphazard arrangement of hard tissue
Sclerosing angioma	Haphazard arrangement of hard tissue at a foci

Pie diagram 1: Distribution of various lesions included in the study group



DISCUSSION:

The complex histopathology of the oral cavity frequently involves the presence of calcified structures derived from the dental apparatus—**enamel, dentin, and cementum**—and bone. In numerous **odontogenic and fibro-osseous tumors**, the deposition and differentiation of these hard tissue components are critical diagnostic features. However, distinguishing between these varied mineralized matrices poses a significant challenge using standard **H&E** staining alone, often leading to diagnostic ambiguity in lesions with an overlapping morphologic spectrum.

To address this challenge, specialized histochemical techniques are employed. While single-purpose stains exist (e.g., Von Kossa for bone, Picrothionin for dentin), the identification of all dental hard tissues typically requires a combination of methods. **MGS**, introduced by Gallego in 1954, overcomes this limitation by combining dyes such as carbol fuchsin and aniline blue to differentially stain hard tissues based on molecular size and permeability. This stain is distinctive because it colors **cementum red**, **dentin and bone green**, and **enamel pink**, offering unparalleled distinction in a single section. [3]

This study was undertaken to evaluate MGS's utility across an **unprecedentedly diverse range of 35 oral pathologies**, including various odontogenic tumors (**CEOT, AFO, Odontome, COC, OKC**), fibro-osseous lesions (**POF, FCOD, Juvenile OF**), bone infections (osteomyelitis), and rare connective tissue and vascular lesions (**Fibroma with calcifications, Sclerosing Angioma**). Normal teeth and bone served as controls, confirming the expected staining pattern (enamel: pink; dentin/bone: green; cementum: red), consistent with original studies.

Key findings demonstrated MGS's ability to specify calcification type:

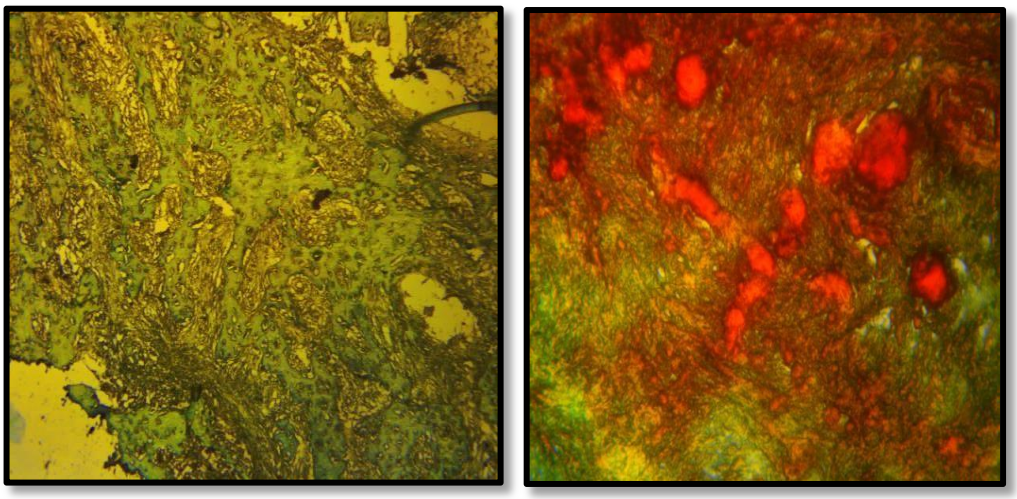
- In **CEOT**, calcifications (Liesegang rings) consistently stained **green**, suggesting a dentin-like rather than bone-like origin, supporting its odontogenic nature. [3,4]
- In **AFO**, hyalinized deposits stained **pink (enamel)** and sheets stained **green (dentin)**. This is in accordance to the case report published by **LK Surej Kumar et al in 2014.** [5]
- Crucially, in **POF** and **FCOD**, MGS revealed instances of mixed **green and red** areas, indicating the coexistence of bone and cementum in varying stages of mineralization—a differentiation often impossible with H&E alone. According to **Tamgagde S (2014)** immature hard tissue depositions stains in the shades of red and green[2]. According to **Dhouskar S (2019),** admixture of green and red represents various stages of bone and cementum deposition. They hypothesized that the admixture of green and red could be attributed to their degree of mineralization. \

In their study, the bone was green in color and covered by osteoid tissue which is less mineralized and has the composition similar to cementum. Therefore, osteoid might have appeared red as cementum at this stage, which they observed in their cases as combination of both tissues[6]. Similar hypothesis could be validated in our case and we can attribute the mixture of these colours as immature hard tissue deposits which are in various stage of their mineralization. [7]

- Similarly, **psammomatoid bodies** in Juvenile OF stained **red**, confirming their cementum-like composition.
- Long standing fibrous hyperplasias may show foci of calcifications due to metaplastic changes in the connective tissue stroma. In our study, foci of multiple hard tissue deposits were seen in the connective tissue towards periphery in fibroma and fibroepithelial hyperplasia. These deposits stained green suggesting it to be bone like material. The calcified structure

had lacunae of osteocytes and osteoblastic rimming in the periphery which is suggestive of bone. We have not come across any study which included these lesions till date.
Sialolithiasis is the presence of one or more oval or round calcified structures in a duct of a major or minor salivary gland. In our study, haphazard arrangement of foci of hard tissue was seen in the periphery of the lesion. These calcifications stained green suggesting it to be bone like material. As it is already stated, MGS stains green for dentin like and bone like components. Sialolithiasis is known to not have origin from the odontogenic apparatus, hence, the stained calcifications suggest to be bone like material. This lesion too, has not been included in any study till now.

These results validate MGS as an invaluable, single-step adjunct to H&E, capable of resolving complex diagnostic dilemmas posed by heterogeneous mineralized components in a wide spectrum of oral lesions.



Bony trabeculae stained green in Juvenile OF (10x) . Cementum like deposits suggestive of psammomatoid bodies stained red in juvenile OF .

SUMMARY AND CONCLUSION:

Hence, we conclude that MGS has proved to be useful in differentiating between different hard tissue components and can be used as an adjuvant to H&E in histopathological laboratories. Because of ease of staining, it should be considered as an alternative before the pathology moves on to more advanced methods like IHC. The stain gives an added insight into the character of the lesion and therefore it is advantageous to use it at times to reach a diagnosis or confirm diagnosis of a lesion. Further studies with greater sample size could be conducted to establish the effectiveness of this stain.

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