*ÍNDIA OLINTA DE AZEVEDO QUEIROZ*

# Influência da Diabetes Mellitus na resposta tecidual e na capacidade de mineralização dos cimentos Sealapex®, MTA Angelus® e MTA Fillapex®

*Dissertação apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP como parte dos requisitos para obtenção do título de Mestre em Endodontia.*

**Orientador: Prof. Adj. João Eduardo Gomes Filho**

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*DEDICATÓRIA*

*Dedico esse trabalho, aos meus pais **João e Suelly**, e as irmãs **Indira e Indiane** que sempre me motivaram e apoiaram em todos os momentos da minha vida. Amo vocês!!!*

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# *RESUMO*

Queiroz, IOA. Influência da Diabetes Mellitus na resposta tecidual e na capacidade de mineralização dos cimentos Sealapex®, MTA Angelus® e MTA Fillapex®. Araçatuba, 2013. 71p. Dissertação (Mestrado em Endodontia) – Faculdade de Odontologia, Campus de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho".

# Resumo

O objetivo deste estudo foi avaliar a influência da diabetes mellitus na resposta tecidual e na capacidade de mineralização dos cimentos Sealapex®, MTA Angelus® e MTA Fillapex®. Vinte e quatro ratos Wistar foram divididos em dois grupos: diabéticos e não diabéticos. A indução de diabetes foi feita por meio da aplicação do Aloxano. Cada animal recebeu três implantes de tubos de polietileno contendo os materiais a serem testados e mais um tubo vazio como controle. Seis animais de cada grupo receberam injeções dos fluorocromos Calceína (7º dia), Alizarina (14º dias) e Oxitetraciclina (21º dias) durante a fase experimental. Os tempos operatórios foram 07 e 30 dias, quando os animais foram mortos e os espécimes processados para análise histológica de hematoxilina e eosina, Von Kossa, luz polarizada e fluorescência. **Resultados:** No 7º dia, observou-se uma resposta inflamatória moderada entre todos os grupos e no 30º dia, verificou-se uma resposta inflamatória leve. Ambos os grupos diabético e não diabético apresentaram, aos 07 e 30 dias, com todos os materiais testados áreas de mineralização e estruturas birrefringentes à luz polarizada. Sealapex® e MTA Fillapex® apresentaram uma maior intensidade de fluorescência do que MTA Angelus® ($p < 0,05$) para todos os fluorocromos no grupo não diabético e somente para alizarina e oxitetraciclina no grupo diabético. Não houve diferença entre o Sealapex® e o MTA Fillapex® para nenhum fluorocromo independente da condição diabética. A intensidade de fluorescência não se alterou com nenhum material nos ratos diabéticos. **Conclusão:** A diabetes mellitus não interferiu na resposta tecidual e na capacidade de mineralização do Sealapex®, MTA Angelus® ou MTA Fillapex®.

**Palavras-chaves**: Diabetes Mellitus, Cimentos Dentários, Endodontia.

# *ABSTRACT*

Queiroz, IOA. Influence of diabetes mellitus on tissue response and ability to mineralization of sealers Sealapex®, MTA Angelus® e MTA Fillapex®. Araçatuba, 2013. 71p. Dissertation (Master in Endodontics) – Dental School of Araçatuba, São Paulo State University “Júlio de Mesquita Filho”.

# Abstract

**Objective**: The aim of this study was to evaluate the influence of diabetes mellitus on tissue response and mineralization ability of Sealapex® MTA Angelus® and MTA Fillapex® sealers. **Methods**: Twenty-four Wistar rats were divided into two groups: diabetics induced by Alloxan and non-diabetics. The materials were placed in the polyethylene tubes and implanted into dorsal connective tissue of rats for 7 and 30 days. Six animals from each group received injection with calcein on day 7, alizarin on day 14 and oxytretacycline hydrochloride on day 21. The animals were killed after 7 and 30 days and the specimens were prepared for histologic analysis with hematoxylin and eosin, Von Kossa technique, and not stained for polarized light or fluorescence. **Results:** On 7 days, it was observed similar inflammatory reactions among all groups characterized by a moderate inflammatory response and on 30 days, it was found a mild inflammatory response. Both materials groups exhibited positive structures on Von Kossa and Polarized light analysis either on 7 and 30 days. Sealapex® and MTA Fillapex® showed more fluorescence intensity than MTA Angelus® ($p<0.05$) for all fluorochromes in non-diabetic group and for alizarin and oxytretacycline in diabetic group. It was not observed difference between Sealapex® and MTA Fillapex® for all fluorochromes independently on the diabetic condition. Fluorescence intensity was not changed with any material in diabetic rats. **Conclusion:** It was concluded that diabetes mellitus did not influence the tissue response or mineralization stimulated by Sealapex® or MTA Angelus® or MTA Fillapex®.

**Key Words:** Diabetic Mellitus, Sealers, Endodontic

# *LISTA DE ABREVIATURAS*

# *Lista de Abreviaturas*

DM – Diabetes Mellitus

IDF – Internacional Diabetes Federation

ADA – American Diabetes Association

AIDS – Síndrome da Imunodeficiência Adquirida

GDM – Diabetes Gestacional

MTA – Agregado Trióxido Mineral

GMA – Glicol metacrilato

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# *INTRODUÇÃO*

# *Introdução*

Diabetes mellitus (DM) é descrita como uma desordem de origem metabólica caracterizada por uma hiperglicemia crônica e alterações no metabolismo dos carboidratos, lipídios e proteínas resultantes da insuficiente secreção/ação do hormônio insulina (ADA, 2011).

A insulina é um hormônio produzido pelo pâncreas e normalmente liberado constantemente em pequenas quantidades no organismo, porém quando do consumo de alimentos, esta liberação é em maior quantidade. O organismo tem a capacidade de remover o excesso de glicose presente no sangue e estocá-la no fígado e músculo na forma de glicogênio ou convertê-la em gordura. Quando necessário, a glicose armazenada é liberada de volta à corrente sanguínea para ser levada as células com auxílio da insulina (Guyton, 2006). Em pacientes diabéticos, este processo encontra-se alterado, pois a glicose é encontrada em excesso na corrente sanguínea devido à liberação insuficiente de insulina pelo pâncreas ou pela resistência celular à insulina.

Dados do Internacional Diabetes Federation (IDF) mostram que a incidência dessa patologia vem aumentando de forma significativa. Estima-se que para o ano de 2030 existam 552 milhões de diabéticos em todo o mundo, sendo 12.9 milhões destes encontrados no Brasil (Whiting et al, 2011).

A American Diabetes Association (ADA) classifica a DM com base na etiologia e apresentação clínica da doença em: Diabetes do tipo I (insulino - dependente), Diabetes do tipo II (não-insulino dependente), Diabetes gestacional (GDM) e outros tipos específicos como: problemas genéticos na função das células beta, defeitos genéticos na ação de insulina, doenças do pâncreas (fibrose cística), endocrinopatias, ou por meio de drogas ou produtos químicos decorrente do tratamento de AIDS ou órgão após transplante (ADA, 2011).

A hiperglicemia decorrente da DM causa efeitos moleculares e celulares, que são resultantes do estresse oxidativo, do aumento da resposta pró-inflamatória e das alterações vasculares que irão predispor os indivíduos a complicações sistêmicas (ADA, 2013). Complicações estas que podem gerar

disfunções e falência em vários órgãos como os olhos, rins, nervos, o coração, os vasos sanguíneos e osso (Lu et al, 2003; ADA, 2011,Blakytny et al 2011).

É comum a ocorrência de alterações ósseas em pacientes diabéticos e estudos mostram a forte relação entre a densidade de mineralização óssea e o risco de fraturas envolvendo tanto indivíduos portadores de diabetes tipo I ou tipo II (Chen et al, 2008; Yaturu, 2009; Vestergaard 2007; Botushanov & Orbetzova, 2009; McCabe et al, 2011)

No diabetes tipo I encontramos muitas vezes um córtex mais fino em decorrência da redução da taxa de formação óssea, redução da massa óssea, do conteúdo mineral ósseo e diminuição da taxa de mineralização, além de casos de osteopenia e osteoporose (López-Ibarra et al, 2001; Janghorbani et al, 2007; Khazai et al, 2009; Blakytny et al 2011; McCabe et al, 2011). No diabetes tipo II encontramos um quadro misto, onde temos uma perda ou aumento da massa óssea, uma densidade mineral óssea aumentada e um elevado risco de fratura se comparado com o tipo I, uma vez que a qualidade do osso formado é pior (Vestergaard 2007; Janghorbani et al, 2007; Khazai et al, 2009; Blakytny et al 2011; McCabe et al, 2011).

As razões que levam a essas mudanças no estado de cicatrização óssea e suas fraturas ainda não são totalmente conhecidas. Sabe-se que o controle da inflamação é essencial para que ocorra o processo de reparação óssea. A inflamação sistêmica induz um aumento no fluxo de neutrófilos para a região da fratura implicando em uma estimulação, pelos neutrófilos, da condrogênese e inibição da osteogênese (Bastian et al, 2011). Assim, o aumento ou o prolongamento da inflamação prejudica a consolidação da fratura (Grundnes & Reikeraas, 2000).

A Inflamação sistêmica que está presente em pacientes com DM pode levar ao aumento no tempo de cicatrização das fraturas prejudicando, assim, seu processo de reparo (Claes et al, 2012). Além das complicações provocadas nesse processo, a inflamação decorrente da DM produz também alterações na cavidade oral (Lamey et al, 1988; Moore et al, 2001; Lamster et al, 2008; Saini et al, 2010).

A DM pode alterar a prevalência ou o prognóstico de alterações patológicas na cavidade bucal, inclusive as lesões de origem endodôntica (Segura-Egea et al, 2012). As infecções endodônticas são prevalentes e causadas por fatores

microbianos (Siqueira, 2002), sendo o tratamento endodôntico o elemento decisivo para o restabelecimento da normalidade dos tecidos apicais e periapicais.

O sucesso do tratamento endodôntico depende de uma limpeza profunda do sistema de canais radiculares, do controle de microorganismos patogênicos e do completo selamento tridimensional do canal radicular que é feito normalmente com guta-percha e cimentos obturadores.

Um cimento endodôntico ideal deve ser biocompatível, apresentar adesividade e estabilidade dimensional, não ser irritante aos tecidos periapicais, ser de fácil preparo, estéril, radiopaco e não ter capacidade de expansão (Grossman, 1976). A composição química desse cimento pode influenciar positiva ou negativamente o resultado da terapia endodôntica (Silva et al, 1997). Dessa forma, existem atualmente no mercado diversas pastas e cimentos com as mais variadas composições: óxido de zinco e eugenol, resina epóxica, ionômero de vidro, hidróxido de cálcio e mais recentemente Agregado Trióxido Mineral (MTA) (Leonardo, 2005).

O Sealapex® (Sybron Endo, Glendora, USA) é um cimento obturador que contém óxido de cálcio e tem a capacidade de induzir a formação de tecido duro no ápice após a obturação do canal radicular (Holland & Souza, 1985). É biocompatível, tem ação bactericida, favorece o reparo induzindo a formação de tecido duro, tem boa capacidade de selamento, viscosidade e escoamento bastante satisfatórios (Gomes-Filho et al, 2008; Silva-Herzog et al, 2011; Siqueira et al, 1995; Yücel et al, 2006; Tanomaru et al, 2008).

O MTA (Agregado Trióxido Mineral) é um cimento reparador que foi introduzido na década de 90 com o intuito de proporcionar o selamento de comunicações patológicas ou iatrogênicas entre o dente e sua superfície externa (Lee et al, 1993, Mente et al, 2010). Além disso, devido às suas excelentes propriedades passou a ser utilizado também em pulpotomias, capeamentos pulpares diretos, apicogêneses e apicificações (Naik & Hegde, 2005; Torabinejad & Chivian, 1999; Eskandarizadeh et al, 2011; Damle et al, 2012; Main et al, 2004)

Estudos mostram que o MTA é um material biocompatível, radiopaco, possui atividade antimicrobiana, apresenta boa capacidade de selamento marginal, induz dentinogênese, cementogênese e osteogênese (Tanomaru et

al, 2008; Fischer et al, 1998; Torabinejad et al, 1995; Thomson et al, 2003; Torabinejad et al, 1995a; Maroto et al, 2006; Holland et al, 1999).

No entanto, apesar das suas características favoráveis, o MTA não apresenta propriedades físicas necessárias para ser usado como um cimento endodôntico. Deste modo, surgiu recentemente no mercado o MTA Fillapex® (Angelus, Londrina, Brasil) que é um cimento obturador que apresenta em sua composição MTA (Agregado Trióxido Mineral), resina salicilato, resina diluente, resina natural, óxido de Bismuto, sílica nanoparticulada e pigmentos. Segundo o fabricante, é um cimento pasta-pasta, que apresenta uma alta radiopacidade, baixa solubilidade, é de fácil manuseio e tem um tempo de trabalho adequado.

Na literatura ainda são escassos os trabalhos sobre suas características físico-químicas e biológicas. Sabe-se que esse é um cimento biocompatível, possui ação antimicrobiana, promove bom selamento marginal prevenindo infiltrações e capaz de induzir mineralização (Morgental et al, 2011; Gomes-filho et al, 2012; Gomes-filho et al, 2012a; Salles et al, 2012; Silva et al, 2012).

Considerando que uma das finalidades desses materiais é participar da indução da mineralização nos tecidos onde é aplicado e que a DM é uma desordem metabólica que altera a resposta inflamatória, alterando assim o processo de mineralização, justifica-se o estudo da resposta tecidual de animais diabéticos a materiais endodônticos indutores de mineralização.

# *PROPOSIÇÃO*

# *Proposição*

O objetivo geral desse estudo foi avaliar a influência da diabetes mellitus na resposta tecidual e na capacidade de mineralização dos materiais endodônticos Sealapex®, MTA Angelus® e MTA Fillapex®, frente a implantes de tubos de polietileno em tecido subcutâneo de ratos normais e ratos diabéticos.

Objetivos específicos:

- ✓ Analisar e quantificar a resposta inflamatória;
- ✓ Observar por meio da Luz Polarizada e coloração de Von Kossa a presença de tecido mineralizado;
- ✓ Verificar os estágios da mineralização através da marcação por fluorocromos (Calceína, Alizarina e Oxitetraciclina).

*ARTIGO*

# Influence of diabetes mellitus on mineralization ability of Endodontic materials

## Abstract

**Objective**: The aim of this study was to evaluate the influence of diabetes mellitus on tissue response and mineralization ability of Sealapex®, MTA Angelus® and MTA Fillapex® sealers. **Methods**: Twenty-four Wistar rats were divided into two groups: diabetics induced by Alloxan and non-diabetics. The materials were placed in the polyethylene tubes and implanted into dorsal connective tissue of rats for 7 and 30 days. Six animals from each group received injection with calcein on day 7, alizarin on day 14 and oxytretacycline hydrochloride on day 21. The animals were killed after 7 and 30 days and the specimens were prepared for histologic analysis with hematoxylin and eosin, Von Kossa technique, and not stained for polarized light or fluorescence. **Results:** On 7 days, it was observed similar inflammatory reactions among all groups characterized by a moderate inflammatory response and on 30 days, it was found a mild inflammatory response. Both materials groups exhibited positive structures on Von Kossa and Polarized light analysis either on 7 and 30 days. Sealapex® and MTA Fillapex® showed more fluorescence intensity than MTA Angelus® ($p<0.05$) for all fluorochromes in non-diabetic group and for alizarin and oxytretacycline in diabetic group. It was not observed difference between Sealapex® and MTA Fillapex® for all fluorochromes independently on the diabetic condition. Fluorescence intensity was not changed with any material in diabetic rats. **Conclusion:** It was concluded that diabetes mellitus did not influence the tissue response or mineralization stimulated by Sealapex® or MTA Angelus® or MTA Fillapex®.

**Key Words:** Endodontic, Diabetic Mellitus, Sealers

## Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The effects of DM include long-term damage, dysfunction and failure of various organs as eyes, kidneys, nerves, heart, blood vessels and bone (1,2). These complications of DM have been associated with activation of the pathways that increase inflammation, oxidative stress, and apoptosis (3,4).

DM changes the immune response and increase the detection rate of anaerobic bacteria in the dental pulp of diabetic rats (5). Those alterations may render more susceptible to certain infections that are associated with that condition. Therefore, this systemic condition can predisposes a wide spectrum of oral manifestation including periodontal disease and pulp diseases such as endodontic infections (6-9).

Endodontic infection is highly prevalent and caused by microbial factors (10) and studies showed the relationship between endodontic infections and DM (6,7). The endodontic therapy is an important element in the renewal of apical and periapical tissues. The success of this therapy depends cleaning and shaping of the root canal system, the control of pathogenic microorganisms, as well as root canal filling with proper material such as gutta-percha and sealer.

Sealapex® (Sybron Endo, Glendora, USA) is a sealer that contains calcium oxide and has the ability to induce hard tissue formation at the apex after root canal obturation (11). It also has antimicrobial activity and degradation of bacterial lipopolysaccharides (12,13).

Mineral Trioxide Aggregate (MTA) is a biomaterial widely accepted for excellent properties: biocompatibility, antimicrobial activity, good sealing capacity, ability to induce mineralized tissue formation and the dentin bridge. It has been used for several dental applications (12,14,15). However, even with its favorable characteristics, MTA does not exhibit the physical properties needed to be used as an endodontic sealer.

MTA Fillapex® (Angelus, Londrina, Brazil) is an endodontic sealer compound by MTA, salicylate resin, natural resin, bismuth oxide, and silica. Studies showed that sealer is biocompatible, has antimicrobial activity and ability to induce mineralized tissue formation (16-18).

These materials have been investigated but not in a DM rat model, which can modify the inflammatory response. Thus, the aim of this study was to evaluate the influence of DM on tissue response and mineralization ability of Sealapex®, Angelus MTA and MTA Fillapex® in subcutaneous connective tissue of rats.

## Materials and Methods

Twenty four male 3 to 4 months-old Wistar Albino rats, weighing 250 to 280 g, were used in the study. The animals were divided in two groups: diabetic (12 rats) and non-diabetic (12 rats) and were housed in temperature-controlled rooms receiving water and food ad libitum. The care of the animals was performed according to Ethical Committee (protocol number 0177-2011).

For the Diabetes induction, the animals received 150 mg/kg of Alloxan monohydrate (Sigma Aldrich Corp., St. Louis, USA) in a single intraperitoneal dose (19). In the third day of the experiment the glucose blood was measured to confirm hyperglycemia. The animals were considered diabetic when showed glucose levels higher than 250 mg/dL and this day was considered the first day of the Diabetes disease.

Ninety-six sterile polyethylene tubes (Abbott Labs of Brazil, São Paulo, Brazil) with 1.0 mm internal diameter, 1.6 mm external diameter and 10.0 mm length were used. Seventy-two tubes were filled with the sealers tested. Sealapex®, Angelus MTA® and MTA Fillapex® were prepared according to the manufacturer's recommendations and inserted into the tubes with a lentulo spiral (Maillefer Dentsply, Tulsa, USA). Twenty-four tubes remained empty and were used as control.

At the surgical procedure, the animals were disinfected with 5% iodine solution, after that they were shaved under xylazine (10 mg/kg; Anasedan, Divisão Vetbrands Saúde Animal, São Paulo, Brazil) and ketamine a 5% (25 mg/kg; Cetamin Syntec do Brasil, São Paulo, Brazil) anesthesia. The shaved backs received a 2cm incision in a head-to-tail orientation with a number 15 Bard-Parker™ blade (Franklin Lakes, NJ, USA). The skin was reflected, creating four pockets 6 cm apart on each side of the incision, two in the cranial portion and two in the caudal portion. The tubes were implanted into the spaces

created with blunt dissection, and the skin was closed with 4/0 silk suture (Ethicon; Johnson& Johnson, São Paulo, Brazil)

At $7^{th}$, $14^{th}$ and $21^{th}$ days after implantation fluorescent markers: Calcein (Sigma Aldrich Corp., St Louis, USA), Alizarin Red S (Sigma Aldrich Corp., St Louis, USA) and Oxytretacycline hydrochloride (Sigma Aldrich Corp., St Louis, USA) were injected by intramuscular injection at concentration of 20 mg/kg respectively (20). These fluorochromes were used for evaluation of calcium deposition through by marked free calcium in tissue on the different times.

After $7^{th}$ and $30^{th}$ days of implantation period, six animals from each group were killed by an overdose of anesthetic. The tubes were removed with the surrounding tissues and fixed in 10% formalin solution with pH 7.0 (21). The tubes were then bisected transversely, and both halves were cut again longitudinally with the use of a sharp blade to allow the surfaces to be readily kept in contact with the processing solutions. The specimens were processed and embedded in glycol methacrylate (GMA), serial sections of 3 µm were made for hematoxylin and eosin staining (22). The sections of 10 µm were stained according to Von Kossa technique or remained without staining for examination under polarized light. Sections of 50 µm were used for fluorescence examination.

The reactions of the tissue in contact with the material at the opening of the tube were evaluated according to ISO/TR 7405-1997 (23) as: 0, no or few inflammatory cells and no reaction; 1, less than 25 cells and reaction light; 2 between 25-125 cells and moderate reaction; and 3, 125 or more cells and severe reaction. A thin fibrous capsule was considered when the thickness is <150 µm thick when> 150 µm. Necrosis and mineralization were recorded in $\mu m^2$ according to Leica Qwin software (Leica Microsystems, Wetzlar, Germany). The average number of cells for each group was obtained in 10 separate areas (magnification x400) (Leica Microsystems, Wetzlar, Germany). Fluorescence intensity was measured and analysis by examined under fluorescent light microscopy (Leica Microsystems, Wetzlar, Germany) (534 nm Calcein, 357 nm Alizarin, and Ultraviolet 368 nm Oxytretacycline). The zone tissue in contact with the sealers at the opening of the tube was determined by measuring an area (150µm X 700µm) in the middle of tube ending. The fluorescence intensity

of area was determined by software LAS v4.1 (Leica Applications Suite – version 4.1; Leica Microsystems, Wetzlar, Germany).

The results for inflammatory response were statistically analyzed by one-way ANOVA and Kruskal-Wallis tests. The results for mineralization, necrosis and fluorescence intensity were analyzed by ANOVA and Mann Whitney tests ($p < 0.05$).

## Results

### Control (Empty Tubes)

On the 7$^{th}$ and 30$^{th}$ days, in both diabetic and non-diabetic groups, it was observed a mild inflammatory cell infiltration consisting of macrophages and lymphocytes was present in the thin fibrous capsule surrounding the tube. (Fig. 1A and Fig. 2A)

The empty tubes were not positive to Von Kossa stain (Fig. 1B and Fig. 2B), not birefringents structures under the polarized light (Fig. 1C and Fig. 2C) and no fluorescence intensity for calcein, alizarin red and oxytretacycline was observed for the diabetic and non-diabetic groups (Fig. 1D, Fig. 1E, Fig.1F and Fig. 2D, Fig. 2E, Fig. 2F)

### Sealapex®

On the 7$^{th}$ day, in both diabetic and non-diabetic groups, it was observed a moderate inflammatory cell infiltration consisting of macrophages and lymphocytes was present in a thick fibrous capsule. Von Kossa stain and Granulations Birefringents to polarized light positive was showed for all groups.

On the 30$^{th}$ day, for non-diabetic group, it was observed a moderate inflammatory infiltrate with few chronic inflammatory cells and thin fibrous capsule (Fig. 1G). In the diabetic group, a moderate inflammatory cell infiltration with lymphocytes and macrophages was observed (Fig. 2G). Presence of thin fibrous capsule, Von Kossa positive (Fig. 1H and Fig.2H), Granulations birefringent to polarized light (Fig. 1I and Fig.2I) and fluorescence intensity were observed in both groups (Fig. 1J, Fig. 1K, Fig.1L and Fig. 2J, Fig. 2K, Fig. 2L).

**MTA Angelus®**

On the 7th day, in both diabetic and non-diabetic groups, it was observed a moderate inflammatory cell infiltration consisting of macrophages and lymphocytes was present in a fibrous capsule. The fibrous capsule surrounding the tube was thick. Granulations birefringent to polarized light and Von Kossa positivity were observed for all groups.

On the 30th day, for non-diabetic group, it was observed a mild inflammatory infiltrate with few chronic inflammatory cells and thin fibrous capsule (Fig. 1M). In the diabetic group, a moderate inflammatory cell infiltrate of lymphocytes and macrophages (Fig. 2M). Presence of thin fibrous capsule, Von Kossa positive (Fig. 1N and Fig.2N), granulations birefringent to polarized light (Fig. 1O and Fig.2O) and fluorescence intensity were observed in both groups (Fig. 1P, Fig. 1Q, Fig.1R and Fig. 2P, Fig. 2Q, Fig. 2R).

**MTA Fillapex®**

On the 7th, day in both diabetic and non-diabetic groups, it was observed a moderate inflammatory cell infiltration consisting of macrophages and lymphocytes was present in a fibrous capsule. Granulations birefringent to polarized light and Von Kossa positivity were observed for all groups.

On the 30th day, in booth groups, it was observed a mild inflammatory infiltrate with few chronic inflammatory cells (Fig. 1S and Fig. 2S). The fibrous capsule surrounding the tube was thick. Granulations birefringent to polarized light (Fig. 1U and Fig.2U) and Von Kossa positivity were observed near the tube openings (Fig. 1T and Fig. 2T). The fluorescence intensity was positive for calcein, alizarin and oxytretacycline (Fig. 1V, Fig. 1X Fig.1Z and Fig. 2V, Fig. 2X, Fig. 2Z).

**Comparison among the Groups**

The data were compared in each period of time for inflammatory response, necrosis, and presence of mineralization and fluorescence intensity in each period of time for Calcein, Alizarin or Oxytretacycline as shown in Table 1.

Table 1 – Number of samples in Each Group Categorized According to the Inflammatory Score and medium of samples in Each Group Categorized Necrosis, Presence of Mineralization and Fluorescence Intensity.

| Groups/ Time | Material | Inflammatory (Score) | | | | Necrosis | Presence of Mineralization | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | | VK | F | | |
| | | | | | | | | C | A | O |
| Non-Diabetic (7days) | Control | 0 | 6 | 0 | $0^{1}$ | $0^{1}$ | $0^{1}$ | - | - | - |
| | Sealapex | 0 | 2 | 4 | $0^{2}$ | $10197,1^{2}$ | $260652,1^{2}$ | - | - | - |
| | MTA Angelus | 0 | 1 | 5 | $0^{2}$ | $11602,3^{2}$ | $219899,5^{2}$ | - | - | - |
| | MTA Fillapex | 0 | 0 | 6 | $0^{2}$ | $2012,8^{2}$ | $152732,1^{2}$ | - | - | - |
| Diabetic (7days) | Control | 0 | 6 | 0 | $0^{1}$ | $0^{1}$ | $0^{1}$ | - | - | - |
| | Sealapex | 0 | 2 | 4 | $0^{2}$ | $6017,3^{2}$ | $158667,0^{2}$ | - | - | - |
| | MTA Angelus | 0 | 2 | 4 | $0^{2}$ | $65075,1^{2}$ | $113108,4^{2}$ | - | - | - |
| | MTA Fillapex | 0 | 1 | 5 | $0^{2}$ | $18873.0^{2}$ | $130204,3^{2}$ | - | - | - |
| Non-Diabetic (30 days) | Control | 0 | 5 | 1 | $0^{1}$ | $0^{1}$ | $0^{1}$ | $44,858^{a1}$ | $24,349^{a1}$ | $47,957^{a1}$ |
| | Sealapex | 0 | 1 | 4 | $1^{2}$ | $0^{1}$ | $523301,5^{2}$ | $122,145^{a2}$ | $120,351^{b2}$ | $80,986^{c2}$ |
| | MTA Angelus | 0 | 5 | 1 | $0^{2}$ | $0^{1}$ | $159156,8^{3}$ | $51,507^{a3}$ | $31,087^{a3}$ | $44,900^{a3}$ |
| | MTA Fillapex | 0 | 4 | 2 | $0^{2}$ | $0^{1}$ | $198983,3^{3}$ | $84,357^{a2}$ | $70,263^{a2}$ | $74,136^{a2}$ |
| Diabetic (30 days) | Control | 0 | 4 | 2 | $0^{1}$ | $0^{1}$ | $0^{1}$ | $43,469^{a1}$ | $24,349^{a1}$ | $30,895^{a1}$ |
| | Sealapex | 0 | 0 | 4 | $2^{2}$ | $0^{1}$ | $568201,8^{2}$ | $107,426^{a2}$ | $105,678^{b2}$ | $90,154^{c2}$ |
| | MTA Angelus | 0 | 0 | 6 | $0^{2}$ | $0^{1}$ | $191337,3^{3}$ | $62,283^{a3}$ | $39,508^{a3}$ | $56,706^{a3}$ |
| | MTA Fillapex | 0 | 4 | 2 | $0^{2}$ | $0^{1}$ | $347460,1^{3}$ | $94,227^{a2}$ | $96,031^{b2}$ | $74,048^{c2}$ |

MTA: Mineral Trioxide Aggregate; VK: Von Kossa; F: Fluorescence intensity; C: Calcein; A: Alizarin; O: oxytretacycline
Score: 0: none or few inflammatory cells and no reaction; 1: <25 cells and mild reaction;2: between 25 and 125 cells and moderate reaction; 3:125 or more cells and severe reaction.
The areas of mineralization and necrosis were measured in $\mu m^2$.
*Same letters and numbers indicate no statistical difference among the groups in row and columns respectively ($p > 0.05$).

The inflammatory response for all groups was not altered in diabetic rats in any period of time. There were no statistically significant differences among the materials for necrosis areas ($p>0.05$) that was absent in 30 days.

Mineralization areas for Sealapex®, MTA Angelus® and MTA Fillapex® were observed by presence of positive Von Kossa and birefringent structures in

booth groups on day 7 and 30. There was statistically significant difference between all sealers and control ($p<0.05$) independently on the diabetic condition. On 30$^{th}$ days, Sealapex® showed more Von Kossa positive areas than the other materials ($p<0.05$) independently on the diabetic condition.

It was found statistically significant difference for fluorescence intensity between each material and control group independently of the fluorochrome and diabetic condition ($p<0.05$). Sealapex® and MTA Fillapex® showed more fluorescence intensity than MTA Angelus® ($p<0.05$), for all fluorochromes in non-diabetic group and for alizarin and oxytretacycline in diabetic group. Moreover, there was no statistically significant difference between Sealapex® and MTA Fillapex® group for all fluorochrome ($p>0.05$) independently on the diabetic condition.

## Discussion

Experimental studies performed in type I and II diabetic rat models are used for studying of diabetic disease (24,25). In this study, the DM was induced by administration of Alloxan (19) The Alloxan exerts direct cytotoxic action on the pancreas eliminating the production of insulin and causing severe hyperglycemia in the animal (26). Hyperglycemia is associated with elevated levels of systemic markers of inflammation (27) and the elevated inflammatory state contributes oral complications such as endodontic infections (8,9,28).

DM may modulating factor of endodontic infections and may compromise the healing process of periapical tissues (29). Sealapex®, MTA Angelus® and MTA Fillapex® are endodontic materials with chemical properties that favor the healing process (11,14,18). The calcium oxide present in the materials reacts with tissue fluids to form calcium hydroxide, which in contact with water dissociate in calcium ions and hydroxyl ions**.** The calcium ions react with the carbon dioxide in the tissues and form calcium carbonate granulations, which would stimulate the deposition of hard tissue (14). These biologic activities can explain the presence Von Kossa positive areas, birefringent structures to polarized light and fluorescence intensity increasing.

The fluorochromes help to identify where and when the mineralization in the process of ossification occurs (30). These substances have the ability to bind to

calcium ions through a chelation reaction (31). Fluorochrome analysis revealed similar fluorescence intensity in the diabetic and non-diabetic groups and the Sealapex® showed more fluorescence intensity than MTA Fillapex® or MTA Angelus®. The high solubility of Sealapex® and the releasing of more calcium and hydroxyl ions may lead it to induce more formation of mineralized tissue. Previous studies clearly show significant high solubility of Sealapex (32,33) and this might explain these results.

In our study, the hyperglycemia caused by the DM disease did not influence the inflammatory response and mineralization ability of these sealers. There was not the direct correlation between mineralization tissue formation and inflammatory cell infiltration. Although the inflammatory state produces an unfavorable environment for angiogenesis, cellular proliferation and wound healing (6), our findings disagree with that studies once it was not observed alteration on the inflammatory response independently on the diabetic condition (6,7,34). By the other hand, it was already observed an increasing on the osteopontin expression caused by the hyperglycemic that improved the pulp calcification in diabetic animals (35). These conflicting results evidence that the relationship of DM and endodontic healing is not yet completely understood.

In conclusion, the results of this study did not allowed to suggest that DM influence the tissue response including mineralization stimulated by Sealapex®, MTA Fillapex® or MTA Angelus ®.

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**Figure 1**

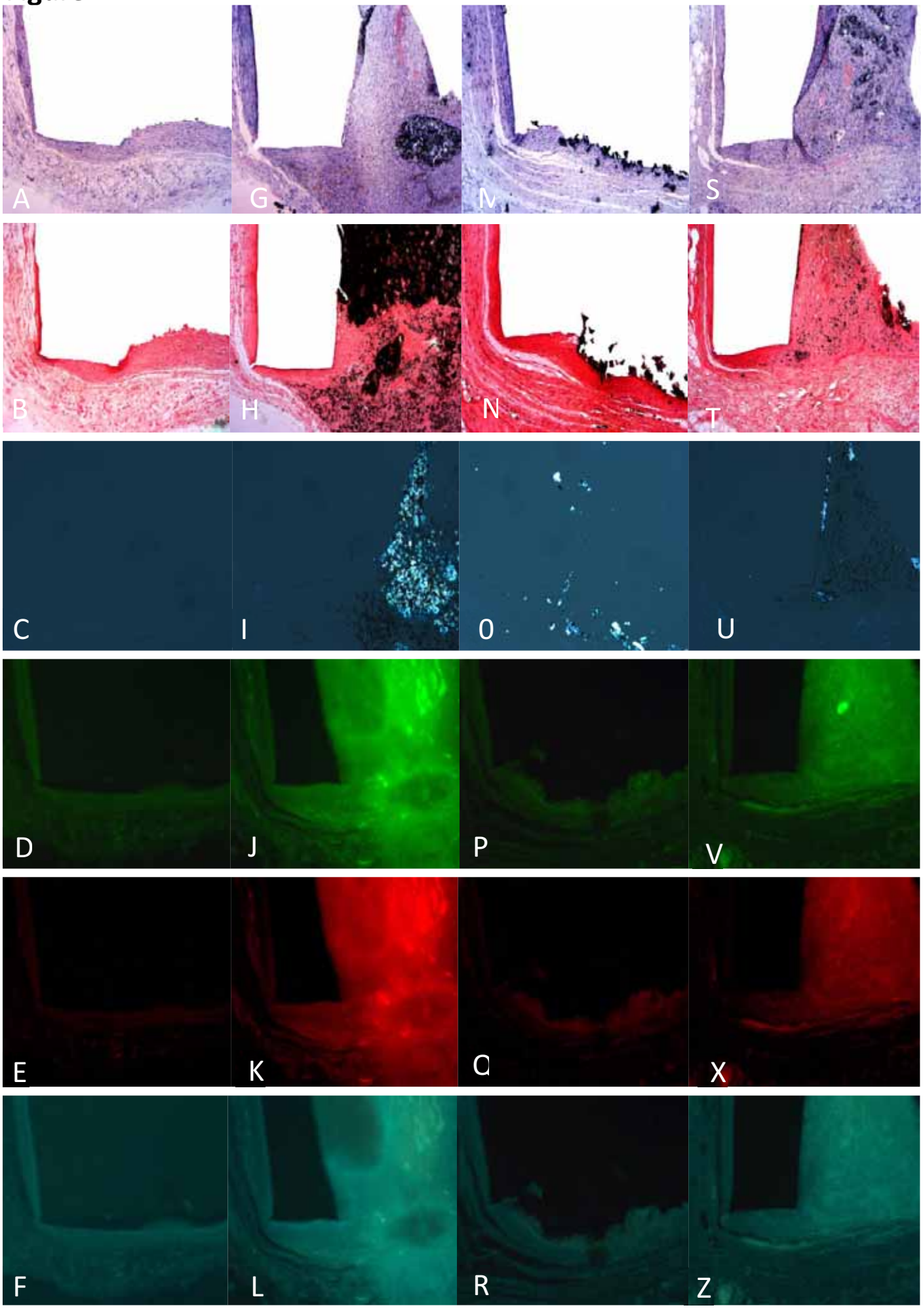


**Figure 1**: This image shows the response found for the non-diabetic group in time 30 days. Control: (A) a specimens showing thin fibrous capsule formation and mild inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10X); (B) mineralization absent (Von Kossa, 10X); (C) absent of birefringent structures to polarized light (Polarized light, 10X) (D-F) absence of calcification areas in close contact with the tube opening (fluorochrome analysis, 10X) for calcein, alizarin and oxytretacycline respectively. Sealapex®: (G) thick fibrous capsule formation and moderate inflammatory cell infiltration (hematoxylin-eosin, 10X); (H) presence of dystrophic calcification on the tube opening (Von Kossa, 10X); (I) presence of birefringent structures to polarized light (Polarized light, 10X); (J-L) calcification areas in close contact with the tube opening (fluorochrome analysis, X10) for calcein, alizarin and oxytretacycline respectively. MTA Angelus®: (M) presence of a mild inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10X); (N) dystrophic calcification on the tube opening (Von Kossa, 10X); (O) presence of birefringent structures to polarized light (Polarized light, 10X); (P-R) presence of calcification areas in close contact with the tube opening (fluorochrome analysis, X10) for calcein, alizarin and oxytretacycline respectively. MTA Fillapex®: (S) presence of a mild inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10X); (T) dystrophic calcification on the tube opening (Von Kossa, 10X); (U) presence of birefringent structures to polarized light (Polarized light, 10X); (V-Z) presence of calcification areas in close contact with the tube opening (fluorochrome analysis, X10) for calcein, alizarin and oxytretacycline respectively.

**Figure 2**

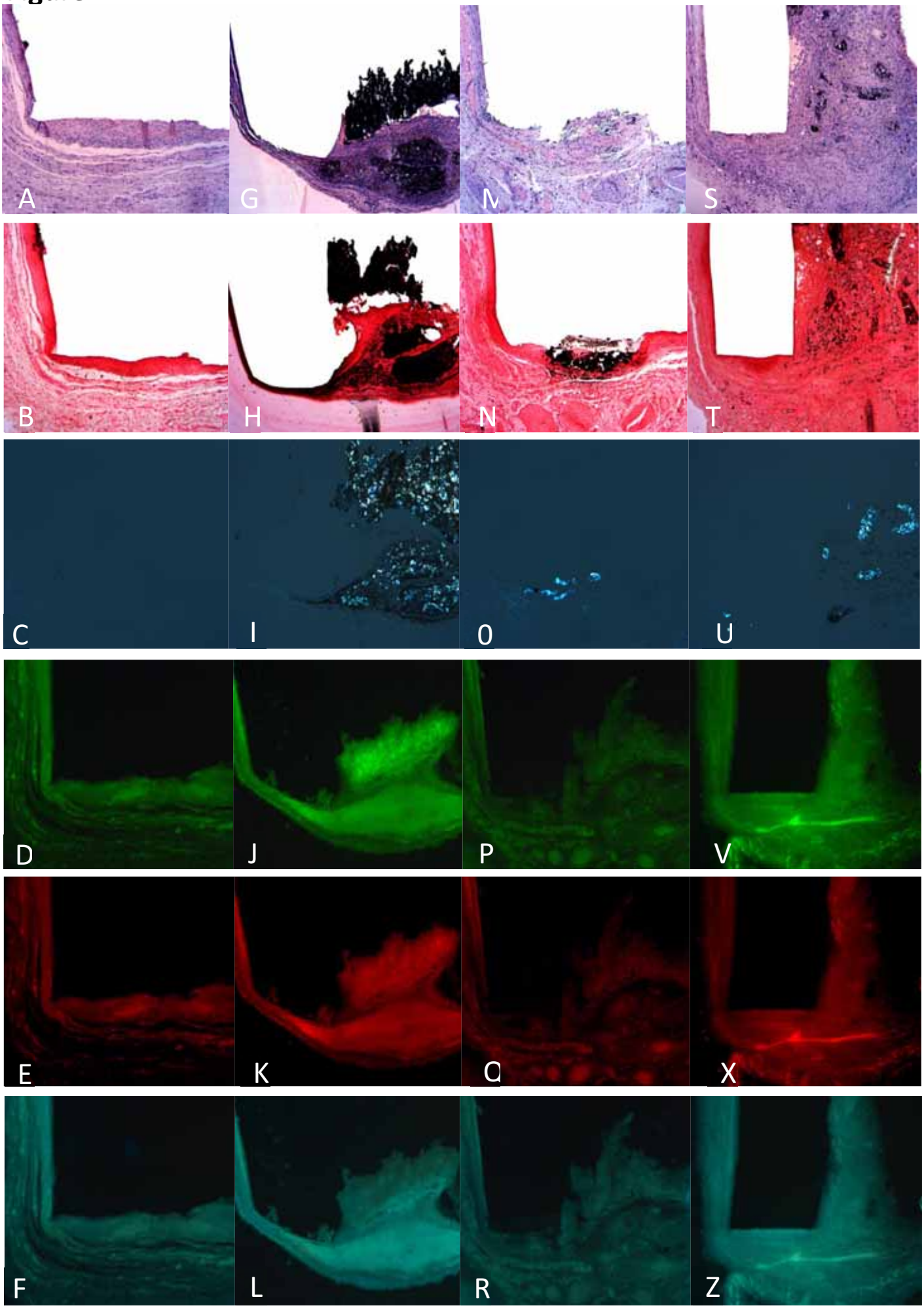


**Figure 2**: This image shows the response found for the diabetic group in time 30 days. Control: (A) a specimens showing thin fibrous capsule formation and mild inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10X); (B) mineralization absent (Von Kossa, 10X); (C) absent of birefringent structures to polarized light (Polarized light, 10X) (D-F) absence of calcification areas in close contact with the tube opening (fluorochrome analysis, 10X) for calcein, alizarin and oxytretacycline respectively. Sealapex®: (G) thick fibrous capsule formation and moderate inflammatory cell infiltration (hematoxylin-eosin, 10X); (H) presence of dystrophic calcification on the tube opening (Von Kossa, 10X); (I) presence of birefringent structures to polarized light (Polarized light, 10X); (J-L) calcification areas in close contact with the tube opening (fluorochrome analysis, X10) for calcein, alizarin and oxytretacycline respectively. MTA Angelus®: (M) presence of a moderate inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10X); (N) dystrophic calcification on the tube opening (Von Kossa, 10X); (O) presence of birefringent structures to polarized light (Polarized light, 10X); (P-R) presence of calcification areas in close contact with the tube opening (fluorochrome analysis, X10) for calcein, alizarin and oxytretacycline respectively. MTA Fillapex®: (S) presence of a mild inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10X); (T) dystrophic calcification on the tube opening (Von Kossa, 10X); (U) presence of birefringent structures to polarized light (Polarized light, 10X); (V-Z) presence of calcification areas in close contact with the tube opening (fluorochrome analysis, X10) for calcein, alizarin and oxytretacycline respectively.

# *CONCLUSÃO*

# *Conclusão*

Os resultados desse estudo sugerem, com base nas metodologias empregadas, que a resposta encontrada no tecido subcutâneo de ratos diabéticos e não diabéticos foi semelhante. De forma que, a diabetes mellitus não interferiu na resposta inflamatória e na capacidade de mineralização dos Sealapex ®, MTA Angelus ® e MTA Fillapex®.

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# *ANEXO 1*

# *Anexo 1*

## Tabela de controle de Glicemia

***Tabela 2:*** Tabela de controle glicêmico dos animais no período de 7 dias

| ***Animais*** | ***Glicemia antes da indução*** | ***Glicemia pós-indução/ 1º dia do experimento (realização do implante subcutâneo)*** | ***Glicemia/ Sacrifício*** |
|---|---|---|---|
| **I** | 102 | 382 | 402 |
| **II** | 99 | 418 | 418 |
| **III** | 86 | HI* | HI |
| **IV** | 104 | HI | HI |
| **V** | 87 | 454 | 454 |
| **VI** | 93 | 460 | 466 |

*HI: Glicemia acima de 600mg/dl

***Tabela 3:*** Tabela de controle glicêmico dos animais no período de 30 dias

| ***Animais*** | ***Glicemia antes da indução*** | ***Glicemia pós-indução/ 1º dia do experimento (realização do implante subcutâneo)*** | ***Glicemia/ Sacrifício*** |
|---|---|---|---|
| **I** | 100 | 501 | 501 |
| **II** | 94 | 375 | 418 |
| **III** | 110 | 414 | HI |
| **IV** | 96 | HI* | HI |
| **V** | 102 | HI | HI |
| **VI** | 114 | HI | HI |

*HI: Glicemia acima de 600mg/dl

# *ANEXO 2*

# *Anexo 2*

## Comissão de Ética no Uso de Animais – CEUA

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Araçatuba

Committee for Ethical Use of Animals (CEUA)

### CERTIFICADO

Certificamos que o Projeto **"Avaliação da resposta tecidual e da capacidade de mineralização dos cimentos Sealapex®, MTA Fillapex® e Ângelus MTA® e sua inter-relação entre a diabetes mellitus. Estudo histológico em ratos"** sob responsabilidade do Pesquisador JOÃO EDUARDO GOMES FILHO e colaboração de Índia Olinta de Azevedo Queiroz e Simone Watanabe está de acordo com os Princípios Éticos da Experimentação Animal (COBEA) e foi aprovado pelo CEUA, de acordo com o processo 01777-2011.

### CERTIFICATE

We certify that the research **"Evaluation of tissue response and mineralization ability of Sealapex®, MTA Fillapex® and Ângelus MTA® and their relationship with diabetes mellitus. Histological study in rats"**, protocol number 01777-2011, under responsibility of JOÃO EDUARDO GOMES FILHO and with collaboration of Índia Olinta de Azevedo Queiroz and Simone Watanabe agree with Ethical Principles in Animal Research (COBEA) and was approved by CEUA.

Prof. Dr. Fabiano A. Cadioli

CEUA Coordinator

Faculdade de Odontologia e Faculdade de Medicina Veterinária – **Departamento de Clínica, Cirurgia e Reprodução Animal** – Rua Clóvis Pestana, 793 CEP 16050-680 Araçatuba – SP
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# *ANEXO 3*

# *Anexo 3*

## Diretrizes para publicação de trabalhos no Journal of Endodontics

### Guidelines for Publishing Papers in the Journal of Endodontics

Writing an effective article is a challenging assignment. The following guidelines are provided to assist authors in submitting manuscripts.

1. The JOE publishes original and review articles related to the scientific and applied aspects of endodontics. Moreover, the JOE has a diverse readership that includes full-time clinicians, full-time academicians, residents, students and scientists. Effective communication with this diverse readership requires careful attention to writing style.

2. **General Points on Composition**

Authors are strongly encouraged to analyze their final draft with both software (e.g., spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery since their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision, or have unacceptable grammar. The following list represents common errors in manuscripts submitted to the JOE:

a. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not developthe theme of the paragraph (see also section “c”, below), or sentences with little to no transition within a paragraph.

b. Keep to the point. The subject of the sentence should support the subject of the paragraph. For example, the introduction of authors’ names in a

sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, “In 1983, Langeland et al., reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals” can be edited to: “Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al., 1983)”. In this example, the paragraph’s subject is sodium hypochlorite and sentences should focus on this subject.

c. Sentences are stronger when written in the active voice, i.e., the subject performs the action. Passive sentences are identified by the use of passive verbs such as “was,” “were,” “could,” etc. For example: “Dexamethasone was found in this study to be a factor that was associated with reduced inflammation”, can be edited to: “Our results demonstrated that dexamethasone reduced inflammation”. Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

d. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph.(This section was written deliberately with sentences of varying length to illustrate this point.)

e. Use parallel construction to express related ideas. For example, the sentence, “Formerly, Endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method”, can be edited to “Formerly, Endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation”. The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related.

f. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, “Accordingly, when conclusions are drawn from the

results of this study, caution must be used", can be edited to "Caution must be used when conclusions are drawn from the results of this study".

g. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.

**3. General Points on the Organization of Original Research Manuscripts**

Please Note: Starting in 2009, all abstracts should be organized into sections that start with a one-word title (in bold), i.e., Introduction, Methods, Results, Conclusions, etc., and should not exceed more than 250 words in length.

**a. Title Page**: The title should describe the major conclusion of the paper. It should be as short as possible without loss of clarity. Remember that the title is your advertising billboard–it represents your major opportunity to solicit readers to spend the time to read your paper. It is best not to use abbreviations in the title since this may lead to imprecise coding by electronic citation programs such as PubMed (e.g., use "sodium hypochlorite" rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at www.icmje.org).

**b. Abstract**: The abstract should concisely describe the purpose of the study, the hypothesis, methods, major findings and conclusions. The abstract should describe the new contributions made by this study. The word limitations (250 words) and the wide distribution of the abstract (e.g., PubMed) make this section challenging to write clearly. This section often is written last by many authors since they can draw on the rest of the manuscript. Write the abstract in past tense since the study has been completed. Three to ten keywords should be listed below the abstract.

**c. Introduction**: The introduction should briefly review the pertinent literature in order to identify the gap in knowledge that the study is intended to address. The purpose of the study, the tested hypothesis and its scope should be described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the JOE. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the introduction fails to provide sufficient detail. However, many

successful manuscripts require no more than a few paragraphs to accomplish these goals.

**d. Material and Methods**: The objective of the methods section is to permit other investigators to repeat your experiments. The three components to this section are the experimental design, the procedures employed, and the statistical tests used to analyze the results. The vast majority of manuscripts should cite prior studies using similar methods and succinctly describe the particular aspects used in the present study. The inclusion of a “methods figure” will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then the authors should carefully describe the method and include validation experiments. If the study utilized a commercial product, the manuscript should state that they either followed manufacturer’s protocol or specify any changes made to the protocol. Studies on humans should conform to the Helsinki Declaration of 1975 and state that the institutional IRB approved the protocol and that informed consent was obtained. Studies involving animals should state that the institutional animal care and use committee approved the protocol. The statistical analysis section should describe which tests were used to analyze which dependent measures; p-values should be specified. Additional details may include randomization scheme, stratification (if any), power analysis, drop-outs from clinical trials, etc.

**e. Results**: Only experimental results are appropriate in this section (i.e., neither methods nor conclusions should be in this section). Include only those data that are critical for the study. Do not include all available data without justification, any repetitive findings will be rejected from publication. All Figs./Charts/Tables should be described in their order of numbering with a brief description of the major findings.

**f. Figures**: There are two general types of figures. The first type of figure includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photo-, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (i.e., about 175 mm wide X 125 mm high when published in the JOE), you would be able to place about 12 panels of photomicrographs (or radiographs, etc.) as an array of four columns

across and three rows down (with each panel about 40 X 40 mm). This will require some editing on your part given the small size of each panel, you will only be able to illustrate the most important feature of each photomicrograph. Remember that each panel must be clearly identified with a letter (e.g., “A”, “B”, etc.), in order for the reader to understand each individual panel. Several nice examples of composite figures are seen in recent articles by Chang, et al, (JOE 28:90, 2002), Hayashi, et al, (JOE 28:120, 2002) and by Davis, et al (JOE 28:464, 2002). At the Editor’s discretion, color figures may be published at no cost to the authors. However, the Editor is limited by a yearly allowance and this offer does not include printing of reprints.

The second type of figure are graphs (i.e., line drawings) that plot a dependent measure (on the Y axis) as a function of an independent measure (usually plotted on the X axis). Examples include a graph depicting pain scores over time, etc. Graphs should be used when the overall trend of the results are more important than the exact numerical values of the results. For example, a graph is a convenient way of reporting that an ibuprofen treated group reported less pain than a placebo group over the first 24 hours, but was the same as the placebo group for the next 96 hours. In this case, the trend of the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

**Tables**: Tables are appropriate when it is critical to present exact numerical values. However, not all results need be placed in either a table or figure. For example, the following table may not necessary:

| % NaOCl | N/Group | % Inhibition of Growth |
|---|---|---|
| 0.001 | 5 | 0 |
| 0.003 | 5 | 0 |
| 0.01 | 5 | 0 |
| 0.03 | 5 | 0 |
| 0.1 | 5 | 100 |
| 0.3 | 5 | 100 |
| 0.001 | 5 | 0 |
| 0.003 | 5 | 0 |

Instead, the results could simply state that there was no inhibition of growth from 0.001-0.03% NaOCl, and a 100% inhibition of growth from 0.03-3% NaOCl (N=5/group). Similarly, if the results are not significant, then it is probably not necessary to include the results in either a table or as a figure. These and many other suggestions on figure and table construction are described in additional detail in Day (1998).

**g. Discussion**: The conclusion section should describe the major findings of the study. Both the strength and weaknesses of the observations should be discussed. What are the major conclusions of the study? How does the data support these conclusions? How do these findings compare to the published literature? What are the clinical implications? Although this last section might be tentative given the nature of a particular study, the authors should realize that even preliminary clinical implications might have value for the clinical readership. Ideally, a review of the potential clinical significance is the last section of the discussion.

**h. References**: The reference style follows Index Medicus and can be efficiently learned from reading past issues of the JOE. Citations are placed in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

# *ANEXO 4*

# *Anexo 4*

## Protocolo de indução de diabetes

Material: ALLOXAN MONOHYDRATE, 25 G
Referência do Produto: A7413
Marca: Sigma Aldrich

1. Realizar a pesagem e a dosagem da glicemia dos animais;

2. Preparo da solução de Aloxano de acordo com o número de animais utilizados para o experimento.

   Ex.: Para indução de 50 animais pesando 200g cada
   Em um recipiente coloca-se solução salina 0,9% estéril na proporção de 0,5ml para cada animal: 50 animais = 25 ml de solução salina
   Realiza a pesagem da droga para ser utilizada na proporção de 150mg/kg. Cada animal pesa 200g, logo 50 animais = 10000g

   150mg/kg: 150mg (droga) ---------- 1000g (droga)
   X mg (droga) _____ 10000g (peso total animais)

   X = 500mg (peso total da droga)

   Em béquer colocar 25 ml da solução salina e ir acrescentando aos poucos a droga (500mg) com agitação constante usando um agitador magnético até a completa diluição.

3. Realizar a injeção por via intraperitoneal na dose única de 150mg/kg, proporcional ao peso do animal com agulha e seringa de insulina;

   $$\text{Dose} = \frac{\text{peso do rato x volume solução (25ml)}}{\text{dose droga (500mg)}}$$

4. Após a indução deixar somente água e passadas 06 horas oferecer aos animais glicose a 10% (água com açúcar) e comida;

5. Retirar água com açúcar após 24hs.

6. Após o 3º dia realizar a dosagem da glicemia dos animais.

# *ANEXO 5*

# *Anexo 5*

## Protocolo para injeção dos Fluorocromos

Material: Calcein, 10 G
Referência do Produto: C0875
Marca: Sigma Aldrich

Alizarin Red S, 25 G
Referência do Produto: A5533
Marca: Sigma Aldrich

Oxytetracycline hydrochloride, 10 G
Referência do Produto: O5875
Marca: Sigma Aldrich

1. Realizar a pesagem dos animais;

2. Preparo da solução dos fluorocromos de acordo com o número de animais utilizados para o experimento.

   Ex.: Para indução de 10 animais pesando 300g cada
   Em um recipiente coloca-se solução salina 0,9% estéril na proporção de 1ml para cada animal: 10 animais = 10 ml de solução salina
   Realiza a pesagem da droga para ser utilizada na proporção de 20mg/kg. Cada animal pesa 300g, logo 10 animais = 3000g

   20mg/kg: 20mg (droga) ---------- 1000g (droga)
   X mg (droga) _____ 3000g (peso total animais)

   X = 60mg ou 0,0060g (peso total da droga)

   Em béquer colocar 10 ml da solução salina e ir acrescentando aos poucos a droga (60mg ou 0,0060g) com agitação constante usando um agitador magnético até a completa diluição.

3. Realizar a injeção por via intramuscular na dose única de 20mg/kg com agulha e seringa de insulina. Injetar 1ml da solução para cada animal.

# *ANEXO 6*

# *Anexo 6*

## Técnicas para inclusão em Glicol Metacrilato, coloração em Hematoxilina e Eosina, Von Kossa, Luz Polarizada e Fluorescência.

### Técnica para inclusão em Glicol Metacrilato (Leica – Historesin)

1) Lavar em água corrente no mínimo 5 horas;

2) Desidratar em álcool 70% por uma noite;

3) No dia seguinte:

A) Álcool 90% por 30 minutos;

B) Álcool 90% por 30 minutos;

C) Álcool 90% por 30 minutos;

D) Álcool 95% por 30 minutos;

E) Álcool 95% por 30 minutos;

F) Álcool 95% por 30 minutos;

4) Colocar as peças desidratadas na solução A ativada e deixar por 72 horas (temperatura ambiente);

5) Isolar os moldes de plástico próprio para inclusão com spray de teflon, colocar as peças no interior dos moldes, cobrir as peças com resina para inclusão (leica – historesin), depois de polimerizada, preencher com resina acrílica autopolimerizante e aguardar a polimerização.

### Técnica para Coloração em Hematoxilina e Eosina (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 3 µm.

1) Hidratar rapidamente em água destilada;

2) Colocar na Hematoxilina – 30 minutos;

3) Lavar em água corrente até remover o excesso de corante;

4) Colocar na Eosina – 5 minutos;
5) Lavar em água corrente até remover o excesso de corante;
6) Deixar secar na estufa, montar com Entelan e lamínula.

## Técnica para Coloração em Von Kossa (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 10 µm.
1) Hidratar rapidamente em água destilada;
2) Colocar sobre os cortes nitrato de prata 1% e deixar sob a luz solar por no mínimo 15 minutos;
3) Lavar em água corrente para remover o excesso de nitrato de prata;
4) Colocar no tiossulfato de sódio 5% - 30 segundos;
5) Lavar em água corrente - 1 minuto;
6) Colocar na safranina 0,5% - 30 segundos;
7) Lavar em água corrente para remover o excesso do corante.
8) Deixar secar na estufa, montar com Entelan e lamínula.

## Técnica para Luz Polarizada (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 10 µm.
1) Lâminas secas e montar com Entelan e lamínula.

## Técnica para Fluorescência (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 50 µm.
1) Após o corte, deixar secar por poucos minutos
2) Lâminas secas e montar com meio de montagem para fluorescência à base de glicerol e lamínula.
3) Armazenar em geladeira