Pedro Henrique Chaves de Oliveira

INFLUÊNCIA DAS SUPLEMENTAÇÕES DE ÔMEGA-3 E MELATONINA SOBRE A RESPOSTA TECIDUAL DE DIFERENTES CIMENTOS ENDODÔNTICOS

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Dissertação apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção de título de Mestre em Ciência Odontológica - Área de Concentração: Endodontia.

Orientador: Prof. Assoc. Luciano Tavares Angelo Cintra

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Dedicatória

Pedro Henrique Chaves de Oliveira

Dedico este trabalho,

Ao meu avô, **Pedro Chaves**

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Epígrafe

"O mundo é um lugar perigoso não por causa daqueles que fazem o mal, mas por causa daqueles que veem e deixam o mal ser feito"

Albert Einstein

Resumo Geral

Oliveira, PHC. **Influência das suplementações de Ômega-3 e Melatonina sobre a resposta tecidual de cimentos endodônticos.** 2020**.** Dissertação de Mestrado - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2020.

RESUMO

O objetivo deste estudo foi avaliar a influência das suplementações de ômega-3 e melatonina sobre a resposta tecidual de cimentos endodônticos. Foram utilizados 72 ratos Wistar alocados em 3 grupos principais: ratos controle tratados com água (TA), ratos tratados com ômega-3 (TO) e ratos tratados com melatonina (TM). Em cada rato foram implantados 4 tubos de polietileno, sendo 1 tubo vazio para controle, e os demais contendo um dos cimentos endodônticos: Sealapex®, AH Plus® e Endofil®. Os períodos de avaliação foram de 5, 15 e 30 dias (n=8/tempo). Após eutanásia, os tubos foram removidos e processados para análise microscópica. A inflamação foi avaliada em coloração de H.E. e técnica imuno-histoquímica para IL-6 e TNF-α, o colágeno pela coloração de Picrosírios red e a deposição de cálcio, pela técnica de von Kossa e luz polarizada. A estatística foi realizada de acordo com cada tipo de análise, com nível de significância de 5% ($p < 0,05$) e considerando cada tipo de suplementação comparado ao controle. Pode-se observar infiltrado inflamatório reduzido com o tempo e de menor intensidade nos grupos TO ou TM comparado a TA, assim como a imunomarcação para IL-6 e TNF-α. O cimento Sealapex induziu menor inflamação aos 30 dias, tanto em TO quanto em TM. Em relação ao colágeno, foi observado maior expressão de fibras imaturas no grupo TO aos 15 e 30 dias, entretanto, no grupo TM, foi observado maior quantidade de fibras maduras aos 5 dias, ambos comparados a TA. O cimento Sealapex foi único a apresentar marcação positiva para cálcio e cristais de calcita birrefringentes à luz polarizada, independente das suplementações. Pode-se concluir que a suplementação com ômega-3 ou melatonina modularam a inflamação, diminuindo o infiltrado inflamatório e a imunomarcação de IL-6 e TNF-α, estimularam o reparo tecidual e não influenciaram no processo de deposição de cálcio.

Palavras-chave: Inflamação, ácidos graxos, ômega-3, melatonina

Oliveira, PHC. **The influence of the supplementation of Omega 3 and melatonin on tissue response of different endodontic sealers.** 2020. Dissertação Mestrado – Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2020.

General Abstract

The aim of this study was to evaluate the influence of omega-3 and melatonin supplements on the tissue response of endodontic sealers. 72 Wistar rats were allocated in 3 main groups: control rats treated with water (TW), rats treated with omega-3 (TO) and rats treated with melatonin (TM). In each rat, 4 polyethylene tubes were implanted, 1 empty tube for control, and the others containing one of the endodontic sealers: Sealapex®, AH Plus® and Endofil®. The evaluation periods were of 5, 15 and 30 days (n = 8 / time). After euthanasia, the tubes were removed and processed for microscopic analysis. Inflammation was evaluated in H.E. staining and immunohistochemical technique for IL-6 and TNF-α, collagen by Picrosírios red staining and calcium deposition by von Kossa and polarized light technique. Statistics were performed according to each type of analysis, with a significance level of 5% ($p < 0.05$) and considering each type of supplementation compared to the control. It is possible to observe a reduced inflammatory infiltrate over time and less intensity in the TO or TM groups compared to TW, as well as the immunolabeling for IL-6 and TNF-α. Sealapex sealer induced less inflammation at 30 days, both in TO and TM. Regarding collagen, a greater expression of immature fibers was observed in the TO group at 15 and 30 days, however, in the TM group, a greater amount of mature fibers was observed at 5 days, both compared to TW. Sealapex sealer was the only one to present positive marking for calcium and birefringent calcite crystals under polarized light, regardless of supplementation. It can be concluded that supplementation with omega-3 or melatonin modulated the inflammation, reducing the inflammatory infiltrate and the immunolabeling of IL-6 and TNF-α, they stimulated tissue repair and did not influence the calcium deposition process.

Lista de Abreviatura

LISTA DE ABREVIATURAS

TA/TW – Grupo tratado com água

TO – Grupo tratado com ômega

TM – Grupo tratado com melatonina

IL-6 – Interleucina – 6

TNF- α – Fator de necrose tumoral alfa

ISO - International Organization for Standardization (Organização Internacional de Normalização)

NF-Kb - factor nuclear kappa B (Fator nuclear Kappa B)

H.E. – Hematoxilina e Eosina

VK – von Kossa

POL – Polarizada

PSR – Picrosirius red

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A endodontia é a especialidade da odontologia que tem como objetivo o diagnóstico e o tratamento das infecções ou traumas do endodonto. Em casos de necrose pulpar, o tratamento endodôntico promoverá a remoção de conteúdos necróticos e infecciosos dos canais radiculares (Tanomaru et al. 2003; Gomes-Filho et al. 2011). Após limpeza e desinfecção, o sucesso da terapia endodôntica dependerá de um selamento adequado, impedindo a penetração de fluidos e microrganismos (Begotka et al. 1996; Demoor et al. 2000). Os cimentos endodônticos desempenham uma função seladora de extrema importância, pois são eles que anulam os espaços vazios promovendo um selamento hermético do sistema de canais radiculares (Holland & Souza 1985; Sadr et al. 2015).

Os componentes dos cimentos determinam reações teciduais de caraterísticas e intensidades diferentes podendo gerar efeitos adversos interferindo no reparo periapical (Geurtsen et al. 2001; Kim et al. 2010). Estes materiais são divididos em diferentes grupos, de acordo com o principal composto, sendo os tipos mais comuns os cimentos à base de óxido de zinco e eugenol, à base de resina epóxica, e os cimentos que contêm o hidróxido de cálcio. Independentemente do tipo, o cimento deve apresentar-se biocompatível, ser atóxico, facilmente aceito pelos tecidos e não promover sequelas inflamatórias (Guttuso 1963; Morse et al. 1984).

As reações teciduais de materiais que entram em contato com os tecidos vivos devem ser verificadas segundo as normas ISO para testes de avaliação das propriedades biológicas. Dentre estes testes, temos o de avaliação do efeito local, utilizando o método de implantação em tecido subcutâneo de ratos (ISO 10993-6:2007). Como a resposta inflamatória para todos os tecidos conjuntivos é semelhante, esta é uma técnica adequada para avaliarmos a reação tecidual de um material endodôntico (Orstavik 1988; Farhad et al. 2011).

A biocompatibilidade pode ser avaliada pela observação do processo inflamatório em cortes teciduais corados com hematoxilina e eosina (H.E.) (Gomes-Filho et al. 2009, 2011; Cintra et al. 2017a). Por outro lado, a utilização da técnica imunoistoquímica nos oferece a possibilidade de compreendermos os mecanismos

celulares e moleculares envolvidos com a resposta inflamatória de forma mais precisa. Os marcadores IL-6 e TNF-α são frequentemente utilizados em trabalhos que visam uma maior compreensão de fenômenos que envolvem uma resposta tecidual inflamatória.

A interleucina-6 (IL-6) é uma citocina pleomórfica que está presente na resposta imunológica e na hematopoese (Nibali et al. 2012; Azuma et al., 2014). É uma citocina secretada por células T, monócitos, fibroblastos, células epiteliais e macrófagos em resposta a antígenos e outras citocinas, como o TNF-α, além de atuar estimulando a diferenciação e recrutamento de osteoclástos (Heinrich et al. 2003). Está envolvida na regulação da fase aguda em resposta à uma injúria ou infecção, além de atuar na hematopoese, regeneração neuronal e do fígado, desenvolvimento embrionário e processos de resposta imune (Heinrich et al. 2003). Sabendo que a diminuição da IL-6 é um indicativo de redução de inflamação para cimentos (Silva et al. 2015), se torna importante avaliar essa citocina por meio da técnica de imunohistoquímica.

A produção de fator de necrose tumoral TNF-α atua de forma importante na resposta ao tecido danificado ou infecção por promover sinais da inflamação, recrutamento de linfócitos e monócitos, além de estimular células endoteliais a expressar moléculas de adesão e secretar quimiocinas (Pfeffer 2003, Tansey & Szymkowski, 2009). Estudos mostram que durante um processo inflamatório, TNF-α tem efeito pró-inflamatório inicial, seguido por um efeito regulador nos estágios tardios da inflamação, reduzindo a atividade imune (Zakharova & Ziegler 2005). Assim, como a IL-6, o TNF-a está relacionado com a resposta inflamatória frente a materiais endodônticos (Chang et al. 2015; da Silva et al. 2017, Andrade et al. 2018).

Após a inflamação ser controlada, o processo de reparo é iniciado com a diferenciação, migração e proliferação fibroblástica (Clark 1996), seguida da deposição e posterior maturação do colágeno. (Diegelmann et al, 2004). O colágeno pode ser avaliado por meio da coloração de Picrosirius red (Cintra et al. 2017b), cuja birrefringência permite distinguir as fibras maturas de imaturas (Melo et al. 2011). Alguns tipos de cimentos podem induzir a deposição de cálcio que pode ser avaliada pela técnica de Von Kossa e por meio da luz polarizada (Gomes-Filho et al. 2011; Cintra et al. 2017a). A coloração pela técnica de Von Kossa é utilizada para detectar estruturas mineralizadas do tecido, que na análise microscópica aparecem coradas

em preto. A observação em luz polarizada permite observar estruturas birrefringentes que possuem cristais de carbonato de cálcio originados a partir da combinação dos íons cálcio do material e o gás carbônico do tecido (Gomes-Filho et al. 2008). A presença dessas partículas de cálcio, podem indicar um possível início do processo mineralizador (Cintra et al. 2017).

Existem substâncias capazes de reduzirem ou controlarem a inflamação, como o ômega-3 (Azuma et al. 2017). O ácido poli-insaturado ômega-3 (ω-3 PUFAs) é uma substância que foi aceita como terapia coadjuvante às inflamações crônicas como a artrite reumatóide e doenças cardiovasculares (Lee et al, 2012 e Jain et al, 2015) e são citados como redutores da reabsorção óssea (Maggio M et al, 2009).

A literatura suporta que o ácido graxo ômega-3 possui potentes efeitos imunológicos, p (Serhan et al. 2014), principalmente o que se encontra em doses de 60% de EPA e 40% de DHA (Calder et al. 2013), considerando a existência de outros tipos de Ômega-3 com concentrações diferentes desses compostos, que podem estar mais relacionados com diminuição, por exemplo do estresse oxidativo (Vardar- Segül et al. 2006; Calder et al. 2015) e que a suplementação com essa substância, é capaz de reduzir a inflamação, interrompendo a cascata inflamatória, por meio da inibição do ácido araquidônico, modulando o processo inflamatório, diminuindo a proliferação de linfócitos e a produção de citocinas pró-inflamatórias como o fator de necrose tumural alfa TNF-α, além de serem capazes de regularem as respostas das células polimorfonucleares (Alam et al. 1991; Fernandes et al. 1993; Azuma et al. 2017).

O ômega-3 também está relacionado com o processo de mineralização do tecido ósseo (Fong et al. 2012), porém seus mecanismos não estão claramente definidos (Azuma et al. 2017). Especula-se que seu efeito controlador da reabsorção se deve a capacidade de inibirem a cascata inflamatória pelo ácido araquidônico, diminuindo a produção de mediadores pró-inflamatórios, como a prostaglandina E2, Interleucina L-1β, TNF-α (Wallace et al. 2003), células polimorfonucleras e linfócitos, mediadores químicos e células que estão associados com a osteoclasteogênese e supressão de osteoblastogênese (Calder et al. 2006; Mukaro et al. 2008; Körner et al. 2017, Azuma et al. 2017). Portanto, como ω-3 PUFAs regula a expressão dessas substâncias, a reabsorção óssea tende a ser diminuída. Assim, uma primeira hipótese seria que a suplementação com o ômega-3 poderia influenciar na resposta inflamatória e na biomineralização de cimentos endodônticos. (Calder et al. 2013)

Uma outra substância que comumente vem sendo utilizada como supressora do processo inflamatório, é a melatonina. Essa molécula (N-acetil-5-metoxitriptamina) é uma indolamina endógena encontrada em diversos organismos (Hardeland et al. 2003; Slominski et al. 2012). Esta substância geralmente é derivada da glândula pineal (Reiter et al. 2009; Iriti et al. 2010), embora haja produção da mesma em órgãos do trato gatro-intestinal, retina, glândulas salivares, até mesmo linfócitos (Acuña-Castroviejo et al. 2014; Zhang & Zhang 2014). A literatura suporta que a melatonina além de antioxidante, possui também propriedades anti-inflamatórias (Mauriz et al. 2013; Dong et al. 2016). Alguns genes da inflamação podem ser estimulados devido à ativação do fator de transcrição nuclear - kappa B (NF-κB) (Reiter et al. 2000). Após a ativação, NF-kB transloca para o núcleo e se liga a elementos específicos que modulam a transcrição de genes pró-inflamatórios (Baeuerie et al.1996; Mauriz et al. 2013). Diversos estudos afirmam que a melatonina é capaz de modular a via de sinalização do NF-KB (Vriend et al, 2014; Favero et al, 2017) e de modular as citocinas pró-inflamatórias e anti-inflamatórias em condições fisiopatológicas diferentes (Mauriz et al. 2013, Habtemariam et al 2017). A capacidade anti-inflamatória da melatonina, portanto é comprovada, sendo evidenciado em estudos que após a suplementação dessa referida substância, houve redução de citocinas pré e pro-inflamatórias, como prostaglandinas, IL-6 e a TNF-α (Carrillo-Vico et al. 2003; Zhou W et al. 2016; Chahbouni M et al. 2017).

Assim como o ômega-3, a melatonina também está relacionada com o processo de mineralização, atuando na proteção do tecido ósseo (Suzuki et al. 2008), bem como aumentando a formação óssea (Ping et al. 2017). Assim, a segunda hipótese seria que a suplementação com a melatonina poderia influenciar na resposta inflamatória e na formação de depósitos de cálcio que pode ser gerada por cimentos endodônticos.

Considerando ainda, como abordado anteriormente, que o tipo de cimento endodôntico determina reações teciduais de caraterísticas e intensidades diferentes, a terceira hipótese do estudo é que a suplementação com o ômega-3 e melatonina podem influenciar de forma diferente a resposta referente a deposição de cálcio. Dentre os cimentos selecionados para o estudo, o cimento Sealapex, possui capacidade de induzir a mineralização nos tecidos por possuir óxido de cálcio, que quando em contato com os fluidos tissulares, formam o hidróxido de cálcio, capaz de promover a mineralização de tecidos, além disso, é biocompatível e osteoindutor.

(Bueno et al. 2016). A dissociação iônica do cálcio presente no hidróxido de cálcio e a dissociação do dióxido de carbono nos tecidos, formam os cristais de calcita, que irão promover a mineralização (Holland et al. 2002).

O AH Plus é um dos cimentos mais utilizados no mundo, sendo considerado o padrão ouro pela literatura científica, pois possui excelentes propriedades físicas (Cintra et al. 2017b), além da biocompatibilidade (Silva et al. 2013; Cintra et al. 2017a). Apesar de se mostrar citotóxico em estudos in vitro, sua citotoxicidade é apontada nos períodos iniciais, sofrendo uma redução significante com o passar do tempo (Cintra et al. 2017a). Após uma semana em contato com os tecidos já se apresenta um material biocompatível (Silva et al. 2013). Por outro lado, o cimento Endofil, um cimento a base de óxido de zinco e eugenol, possui uma resposta inflamatória mais exacerbada quando comparada aos outros cimentos, além de ser mais citotóxico, característica provavelmente desencadeada pela presença do eugenol em sua composição, mantendo um processo inflamatório significante mesmo após os estudos de períodos mais longos. (Martins et al. 2013; Morgental et al. 2011; Cintra et al. 2017a). Apesar de suas características inflamatórias negativas, esse cimento possui um grande histórico de uso e ainda vem sendo muito utilizado por cirurgiões-dentistas (de Oliveira Mendes et al. 2003).

Os períodos de observação são fundamentais para o estudo de materiais de uso odontológico. De acordo com a FDI os períodos de observação para o estudo de cimentos endodônticos são: 7, 15, 30, 60 e 90 dias pós-operatórios (American National Standards Institute 1979, Federation Dentaire International 1980). Diversos trabalhos empregam apenas os períodos de 7 e 30 dias pois a maioria dos cimentos não apresentam mais inflamação considerável nos períodos de 60 e 90 dias (Cintra et al. 2017; Benetti F et al. 2019). No presente estudo optamos pelos períodos de 5, 15 e 30 dias, pois o objetivo principal é investigar a influência das suplementações sobre o processo inflamatório, e, para a maioria dos cimentos, é mais expressivo nos períodos iniciais.

Como já sabemos, os cimentos devem possuir propriedades que não atrapalhe o processo de reparo, induzindo menos inflamação (Kim et al. 2010). Portanto, temos como intenção, estudarmos o efeito da suplementação do ômega-3 e da melatonina e observar se o contato dessas substâncias com o organismo pode diminuir a resposta tecidual que os cimentos. Dessa forma, caso os suplementos

influenciem positivamente nas repostas induzidas pelos cimentos, a suplementação poderia ser avaliada como uma terapia coadjuvante na endodontia.

Referencias da Introdução Geral

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Artigo 1

Influence of supplement administration on the tissue response of endodontic sealers. Part 1: Supplementation with Omega 3

Journal of Endodontics

Influence of supplement administration on the tissue response of endodontic sealers. Part 1: Supplementation with Omega 3

Abstract

Introduction: The aim of this study was to evaluate the influence of omega 3 supplementation on tissue's response of different endodontic sealers. **Methods:** Polyethylene tubes were implanted in the subcutaneous tissue of 48 rats. One tube was empty for control and three filled with endodontic sealers (Sealapex, AH Plus and Endofill). Half of the animals was treated with omega 3 (TO) and the remaining received water (TW) for 15 days before implantation until euthanasia. After 5, 15 and 30 days (n=8), the tubes and surrounding tissue were removed and processed for analysis of inflammation, immunolabeling for IL-6 and TNF-α, collagen maturity and calcium deposition. Results were statistically analyzed ($p<0.05$). **Results:** The inflammatory infiltrate and immunolabeling for IL-6 and TNF-α decreased over time. The TO group showed lower inflammatory infiltrate at 15 and 30 days ($p<0.05$) compared to the TW group. Sealapex sealer had a mild inflammation at 30 days in the TO group ($p<0.05$). Among TW groups, it was observed that the Endofill evoked a more intense inflammatory infiltrate compared to the AH Plus and control group in the 30-day period ($p<0.05$). However, among TO groups, there was no difference between all groups in all periods ($p>0.05$). It was observed lower immunolabeling in TO group at 15 and 30 days for IL-6 and in all periods for TNF-α, as compared with TW group ($p< 0.05$). A higher percentage of immature fibres was observed at 15 and 30 days in the TO group, as compared with TW group ($p< 0.05$). The deposition of calcium particles was observed only by Sealapex sealer in all periods of analysis, despite the suplemmentation. **Conclusion:** It can be concluded that omega 3 supplementation influence on the inflammatory response of endodontic sealers, modulating the inflammation, the immunolabeling of IL-6 and TNF-α, the repair process and it does not interfere on calcium deposition.

KEY WORDS: tissue response; inflammation; biomineralization; omega 3

Endodontic sealers are materials found in different formulations that when in contact with tissues promote a biological response and may favor, delay, or impair the periapical repair (Geurtsen et al. 2001, Kim et al. 2010). These materials are divided in different groups, according to their main compound. Commonly, there are the sealers based on calcium's silicates, epoxi resins, oxid zinc and eugenol and those that contain in their composition calcium hydroxide (Morse et al. 1984, Bueno et al. 2016). The base compound can induce different tissue's response, being more tissue aggressive, such as the oxide zinc-based sealers or less irritating like the bioceramics ones (Zmener et al. 2012, Benetti et al. 2019). Independently of the formulation, an ideal sealer should have characteristics such as biocompatibility, be nontoxic and do not promote tissue sequelae, just as it should enable biological sealing (Bueno et al. 2016, Cintra et al. 2017).

The biological sealing will happen if the conditions that the tissues are exposed favor their repair, for that, the endodontics sealers inflammation ability should be ideal, being the material considered biocompatible (Cintra et al. 2017). The literature shows natural substances able to reduce or control inflammation, such as omega 3 (Azuma et al. 2017). Omega 3 is a polyunsaturated acid (ω-3 PUFAs) that acts interrupting the arachidonic acid cascade, modulating the inflammatory process, controlling the production of pre and proinflammatory cytokines, as well as the expression of immune cells; Also, it can strengthen cells membrane structures, favoring their resistance against the inflammatory mechanisms, modulating the expression of pre and pro inflammatory mediators and immunologic cells proliferation (Vardar-Sengül et al. 2006, Serhan et al. al 2014, Azuma et al 2018). Some studies, in animals and in humans show that the fatty acid also play a local role in areas such as the eye (Barabino et al. 2017). Consequently, omega 3 was accepted as an adjunctive therapy for chronical diseases such as rheumatoid arthritis and cardiovascular disfunctions and it is commonly taken by patients worldwide (Lee et al. 2012. Jain et al. 2015). Moreover, it is cited as potential bone resorption reducers (Maggio et al, 2009). In summary, according to all the scientific evidence, the Omega 3 is considered an anti-inflammatory substance, specially in the formulation of of 60% EPA + 40% DHA), since other composition can activate other mechanisms such as the oxidative stress (Vardar-Sengül et al. 2006, Calder et al. 2015). One of its actions is strengthening the cells' membrane structures, favoring their resistance against the inflammatory mechanisms, such as inflammatory cells attacks (Vardar et al. 2006, Azuma et al.

2017,2018); Also it can modulate the expression of pre and pro inflammatory mediators (Körner et al. 2008; Wallace et al. 2013).

Therefore, knowing the importance of a good interaction between sealers and tissues, associated with the fact that omega 3 can regulate the inflammation, the aim of this study was to evaluate the influence of omega 3 supplementation on the tissue's response of endodontic sealers. Null hypothesis: omega 3 supplementation would not influence on the tissue's response of different endodontic sealers.

MATERIAL AND METHODS

Experimental Animals

Forty-eight male Wistar albino rats weighing approximately 250-280g and three to four months of age were used. The sample size for the animal experiment was established based on previous studies involving analysis of materials in subcutaneous tissue of rats (Cintra et al. 2017, Benetti et al. 2019). The animals were kept in collective cages, in a controlled temperature and light cycle environment (22 ± 1 ° C, 70% humidity at 12 hours light and 12 hours dark). They were fed throughout the experimental period with solid diet and ad libitum water, except for the first 24 hours after the intervention. The experimental procedures were approved and performed according to the guidelines of the ethical committee (process n^{o}. 00229-2017).

Omega 3 supplementation

The animals were divided in group treated with omega 3 (TO) and group treated with water (TW). They received daily omega 3 and vehicle supplementation by gavage. The dose of omega 3 was of 40mg/kg (60% EPA + 40% DHA) diluted in 1ml of water (Vardar-Sengül et al. 2006, Azuma et al. 2017a). The supplementation was divided into prophylactic (15 days before the tube implantation procedure) and therapeutic (throughout the experimental period for the different analysis periods). The water supplementation group received 1ml of water instead of omega 3.

Subcutaneous Implant

Polyethylene tubes (Abbot Labs of Brazil, São Paulo, Brazil) with 1.0-mm internal diameter, 1.6-mm external diameter, and 10.0-mm length (ISO 10993-6: 2007), sterilized in ethylene oxide (ISO 10993-7: 1995). They were filled with endodontic Sealapex, AH Plus or Endofill prepared according to the manufacturer's

recommendations; empty tubes were used as a control. The surgical procedure was performed following previous studies (Cintra et al. 2017a). The rats were anesthetized, their dorsa were shaved, and a 2.0-cm incision was made in a head-to-tail orientation with a #15 Bard-Parker blade (BD, Franklin Lakes, NJ). The skin was reflected to create 2 pockets on the right side and 2 pockets on the left side of the incision. After the tubes were randomly implanted into the pockets and the skin was sutured.

After 5, 15 and 30 days (n = 8 rats / period), the animals were euthanised by an over-dose of anesthetic solution. The implanted tubes, along with the surrounding tissues, were removed and fixed in 10% formalin solution at a pH of 7.0. The fixed specimens were processed and embedded in paraffin and serially sectioned into 5-μm cuts for staining with hematoxylin-eosin, picrosirius red (PSR), and immunohistochemistry for IL-6 and TNF-α. It was also obtained 10-μm cuts for the von Kossa staining and some slices were kept unstained for polarized light analysis (Gomes-Filho et al. 2012, Bueno et al. 2016, Benetti et al. 2018).

Histological and immunohistochemical analysis

Histological and immunohistochemical analysis were performed by a single calibrated operator in a blinded manner under light microscopy under light microscope (400x, DM 4000 B; Leica, Wetzlar, Germany). To evaluate the inflammatory infiltrate that was graded in scores from 1 to 4 (1 - no or few inflammatory cells and no reaction; 2 - less than 25 cells and little reaction; 3 - between 25 and 125 cells and moderate reaction; 4 - 125 or more cells and severe reaction). The scores for the immunolabeling of IL-6 and TNF-α were graded also from 1 to 4 (1 - Absence of immunostaining in the region of interest; 2 - Low immunolabeling pattern, about ¼ of labeling of cells and poor labeling in extracellular matrix; 3 - Moderate immunolabeling pattern, about ½ of the immunolabeled cells and moderate labeling in the extracellular matrix; 4 – High immunolabeling pattern, about ¾ of immunolabeled cells and high labeling in the extracellular matrix (Benetti et al. 2018).

Picrosirius Red staining technique was used to analyze the maturation levels of collagen fibers under polarized light microscopy. The LAS program was used (400X magnification, Leica LAS, Leica Microsystems), allowing the selection of corresponding colors for each type of collagen fiber in subcutaneous tissues. After color selection, the program automatically calculated the marked area. Greenish yellow fibers were considered immature and thin, while yellowish red fibers were

considered mature and thick (Cintra et al. 2017a). The calcium deposition was evaluated in the identification of calcium carbonate in von Kossa staining or birefringent crystals on Polarized light (Cintra et al. 2017a).

Statistical Analysis

The data were analyzed using SigmaPlot 12.0 ™ program (Chicago, IL, USA) and the normality of the data set was verified for all analyses. The Kruskal-Wallis test or the Mann Whitney test was performed for nonparametric data. The analysis of variance followed by Tukey post hoc test was performed for parametric data. The level of significance was 5%.

RESULTS

Inflammatory infiltrate

Representative images of the different specimens from all groups are shown in Figure 1. Overall, H.E. staining showed a severe inflammatory infiltrate in the early periods for the inflammatory response of all sealers in the different groups, presenting at 5 days an intense inflammatory infiltrate, except for the response induced by the Sealapex that was considered moderate; At 15 days, in the omega 3 supplemented groups, the control tube and the other sealers presented a mild inflammatory response, whereas in groups that received water supplementation the inflammation was moderate. At 30 days, a general reduction in inflammation is observed for all groups. Among the water supplemented groups, it was observed that the Endofill evoked a more intense inflammatory infiltrate compared to the AH Plus group and control group in the 30-day period ($p=0.009$). However, among the omega 3 supplemented groups, there was no difference between all groups in any period of analysis ($p>0.05$). According to the Mann-Whitney statistical tests between all groups with omega 3 supplementation versus all groups with water supplementation, it can be observed a lower inflammatory infiltrate in the supplemented group at 15 days ($p=0.007$) and 30 days ($p<0.001$) (Table 1). Finally, comparing each group as a function of the supplementation with omega 3 or with water, there are differences for Endofill ($p=0.001$) and Sealapex ($p=0.005$) sealers in the 30-day period, presenting lower inflammatory infiltrate in the animals that had been treated receiving the supplementation (Table 1).

Table 1. Scores observed for inflammatory response of endodontic sealers and oral supplementation.

Period of analysis	Score	H.E. Staining for Groups								Statistical analysis
		Control	Control + Omega	AH Plus	AH Plus + Omega	Endofill	Endofill + Omega	Sealapex	Sealapex+ Omega	
5 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p=0.451
	2	2/8	2/8	1/8	2/8	1/8	2/8	2/8	3/8	
	3	3/8	3/8	3/8	3/8	3/8	3/8	3/8	2/8	
	4	3/8	3/8	4/8	3/8	4/8	3/8	3/8	3/8	
	Median**	3,5[a]	3,5[a]	4[a]	3,5[a]	4[a]	3,5[a]	3,5[a]	3[a]	
15 days	1	1/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p=0.007
	2	1/8	5/8	2/8	4/8	1/8	4/8	2/8	5/8	
	3	4/8	3/8	5/8	3/8	4/8	3/8	6/8	3/8	
	4	2/8	0/8	1/8	1/8	3/8	1/8	0/8	0/8	
	Median**	3[a]	2[a]	3[a]	2[a]	3[a]	2[a]	3[a]	2[a]	
30 days	1	3/8	7/8	2/8	2/8	0 /8	3/8	0/8	5/8	*Mann-Whitney p<0.001
	2	3/8	1/8	3/8	5/8	1/8	4/8	4/8	3/8	
	3	2/8	0/8	3/8	1/8	5/8	1/8	4/8	0/8	
	4	0/8	0/8	0/8	0/8	2/8	0/8	0/8	0/8	
	Median**	1,5[a]	1[a]	2,5[a]	2[a]	3[a]	2[b]	2,5[a]	1[b]	

* Mann-Whitney test between all groups with omega supplementation versus all groups with water supplementation
** Superscript lowercase letters for two groups analysis (Control versus Control + omega; AH Plus versus AH Plus + omega; Endofill versus Endofill + omega; Sealapex versus Sealapex + omega)

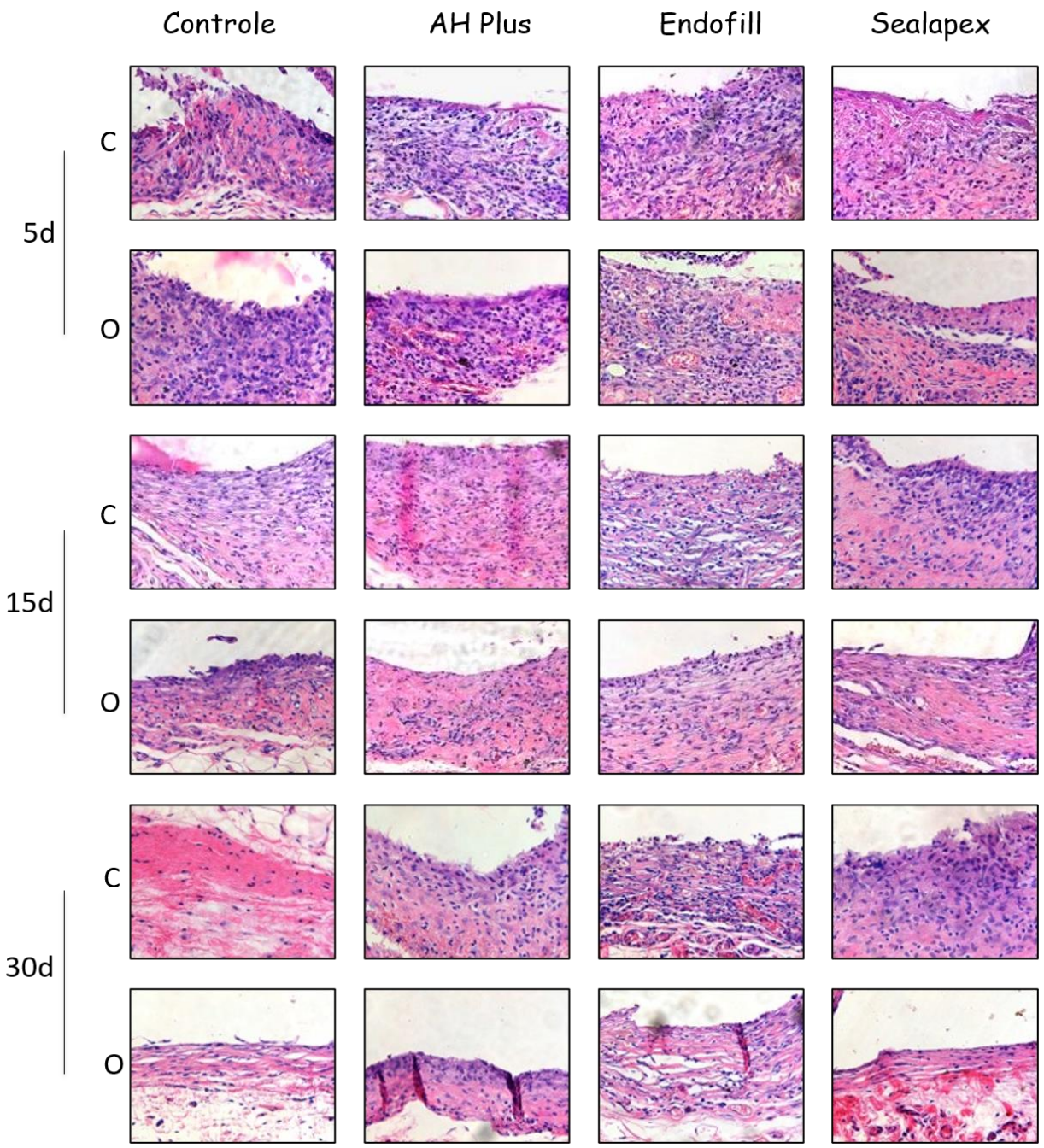

Figure 1. Representative images from the subcutaneous tissue reactions for TW groups (A-D. E-H; I-L) and TO groups (a-d. e-h, i-l) for each kind of sealer and the control tube used at 5, 15 and 30 days (A,a; E,e. I,I – Control; B,b; F,f;J,j – AH Plus; C,c; G,g;K,k – Endofill and D,d, H,h;L,l – Sealapex). At 5 days: intense inflammatory response for every sealers in both groups of analysis (A-D; a-d); At 15 days: mild inflammatory response to the group supplemented with omega (e-h); moderate inflammatory response to groups with water supplementation (E-H); At 30 days: mild to absent inflammatory response to the group supplemented with omega (i-l); moderate

to mild inflammatory response to groups with water supplementation (I-L). (Hematoxylin-eosin staining, x400).

IL-6 Inflammatory mediator expression

Representative images of the different specimens from all groups indicating the immunolabeling of IL-6 are shown in Figure 2. According to the statistical tests it can be observed a lower immunolabeling for IL-6 in the TO animals at 15 days ($p<0.001$) and 30 days ($p<0.001$) as compared with TW groups, regardless of the sealer used (Table 2). After the comparison among all TO groups, it can be seen statistical differences on the period of 5 days for Endofill and control group ($p=0.028$). Among all TW groups, it can be seen statistical differences on the period of 15 days for Endofill and AH Plus ($p<0.001$). The comparison for each group supplemented with omega 3 or water enable to observe differences in the Control group at 15 days ($p=0.038$) and 30 days ($p=0.038$), for the AH Plus at 5 days ($p=0.015$), 15 days ($p=0.010$) and 30 days ($p=0.010$), for the Endofill at 5 days ($p=0.007$) and for the Sealapex at 15 days ($p=0.010$) and 30 days ($p=0.010$) (Table 2). In these cases, and periods of analysis the animals that received omega 3 supplementation presented lower immunolabeling for IL-6 inflammatory mediator.

Table 2. Scores observed for the immunolabeling of Il-6 by endodontic sealers with and without the supplementation of omega 3.

Period of analysis	Score	IL-6 Staining for Groups								Statistical analysis
		Control	Control + Omega	AH Plus	AH Plus + Omega	Endofill	Endofill + Omega	Sealapex	Sealapex+ Omega	
5 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	0/8	0/8	2/8	0/8	3/8	0/8	0/8	
	3	3/8	6/8	3//8	6/8	3/8	5/8	8/8	8/8	
	4	5/8	2/8	5/8	0/8	5/8	0/8	0/8	0/8	
	Median**	4^a	3^a	4^a	3^b	4^a	3^b	3^a	3^a	
15 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	0/8	0/8	6/8	3/8	6/8	0/8	6/8	
	3	3/8	8/8	8/8	2/8	5/8	2/8	8/8	2/8	
	4	5/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	4^a	3^b	3^a	2^b	3^a	2^a	3^a	2^b	
30 days	1	0/8	0/8	0/8	0/8	0 /8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	3/8	0/8	6/8	3/8	6/8	0/8	6/8	
	3	5/8	5/8	8/8	2/8	5/8	2/8	8/8	2/8	
	4	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	3^a	3^b	3^a	2^b	3^a	2^a	3^a	2^b	

* Mann-Whitney test between all groups with omega supplementation versus all groups without omega supplementation

** Superscript lowercase letters for two groups analysis (Control versus Control + omega; AH Plus versus AH Plus + omega; Endofill versus Endofill + omega; Sealapex versus Sealapex + omega)

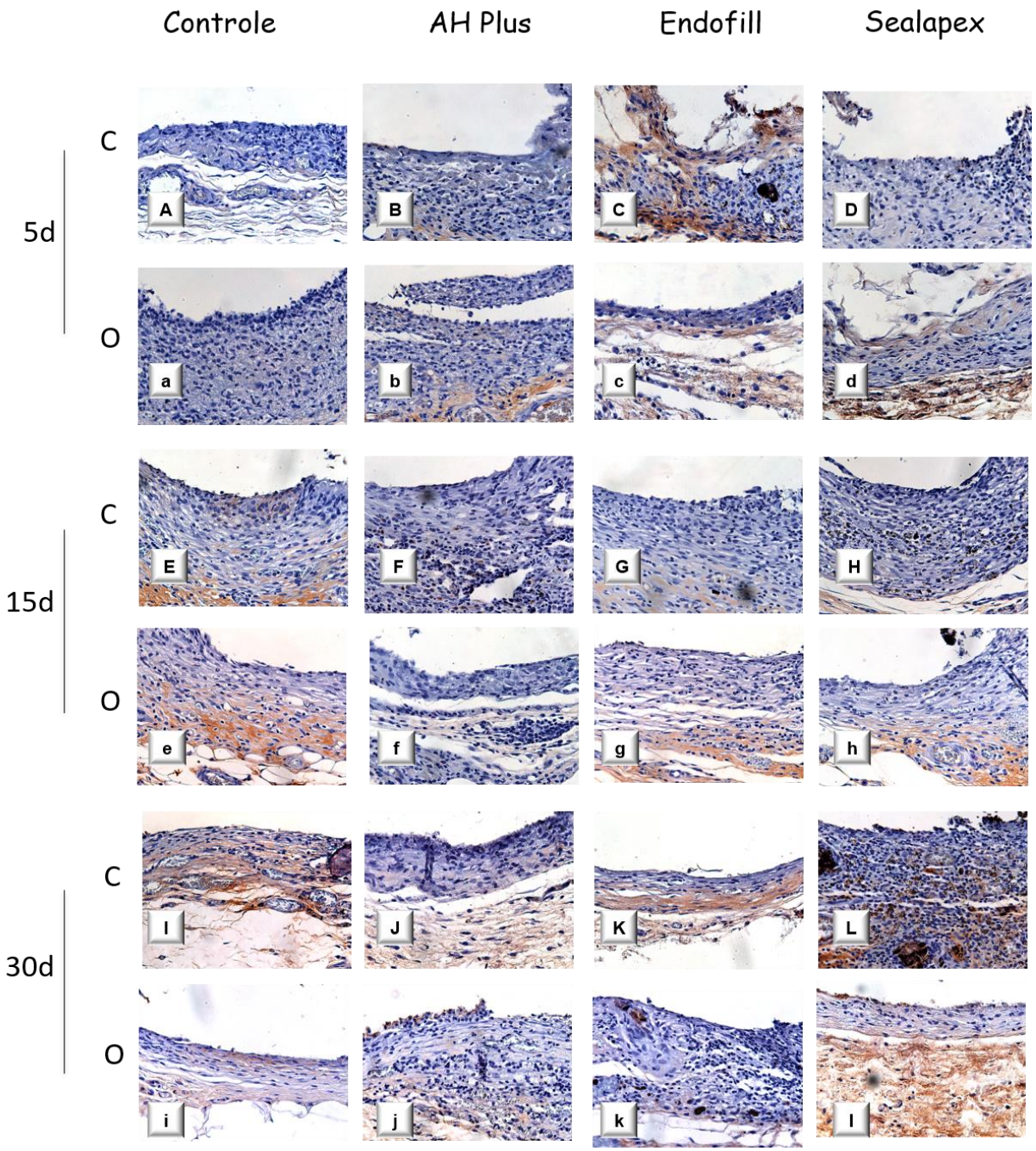

Figure 2. Immunohistochemistry images of in situ IL-6 expression for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

TNF-α Inflammatory mediator expression

Representative images of the different specimens from all groups indicating the immunolabeling of TNF-α are shown in Figure 3. According to the statistical tests it can be observed a lower immulabeling for TNF-α in the TO animals in all periods of the analysis (5 days ($p<0,001$), 15 days ($p<0,004$) and 30 days ($p<0,001$)] as compared with TW animals, regardless of the sealer used (Table 3). There was no statistical difference in the comparison between the TO groups among themselves in any periods of analysis ($p>0.05$). Likewise, there was no statistical difference in the comparison between the groups with TW among themselves in any period of analysis, considering the imunolabeling of the TNF-α ($p>0.05$). The comparison for each group with the supplementation with omega 3 or water enable to observe differences in the Control tube at 30 days ($p=0.038$), and for the sealers AH Plus at 5 days ($p=0.049$) and 30 days ($p=0.038$), Endofill at 5 days ($p=0.038$) and 15 days ($p=0.038$) and for Sealapex at 15 days ($p=0.038$). In these cases, the animals that received omega 3 supplementation presented lower immunolabeling for TNF-α inflammatory mediator (Table 3).

Table 3. Scores observed for the expression of TNF-α by endodontic sealers with and without the supplementation of omega 3.

Period of analysis	Score	TNF-α Staining for Groups								Statistical analysis
		Control	Control + Omega	AH Plus	AH Plus + Omega	Endofill	Endofill + Omega	Sealapex	Sealapex+ Omega	
5 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	2/8	0/8	2/8	0/8	3/8	0/8	0/8	
	3	5/8	6/8	6/8	6/8	5/8	5/8	8/8	8/8	
	4	3/8	0/8	2/8	0/8	3/8	0/8	0/8	0/8	
	Median**	3^a	3^a	3^a	3^b	3^a	3^b	3^a	3^a	
15 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p=0.004
	2	0/8	2/8	0/8	3/8	0/8	5/8	0/8	5/8	
	3	5/8	6/8	8/8	5/8	8/8	3/8	8/8	3/8	
	4	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	3^a	3^a	3^a	3^a	3^a	2^b	3^a	2^b	
30 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	3/8	0/8	5/8	2/8	5/8	8/8	8/8	
	3	5/8	5/8	8/8	3/8	6/8	3/8	0/8	0/8	
	4	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	3^a	3^b	3^a	2^b	3^a	2^a	2^a	2^a	

* Mann-Whitney test between all groups with omega supplementation versus all groups without omega supplementation

** Superscript lowercase letters for two groups analysis (Control versus Control + omega; AH Plus versus AH Plus + omega; Endofill versus Endofill + omega; Sealapex versus Sealapex + omega)

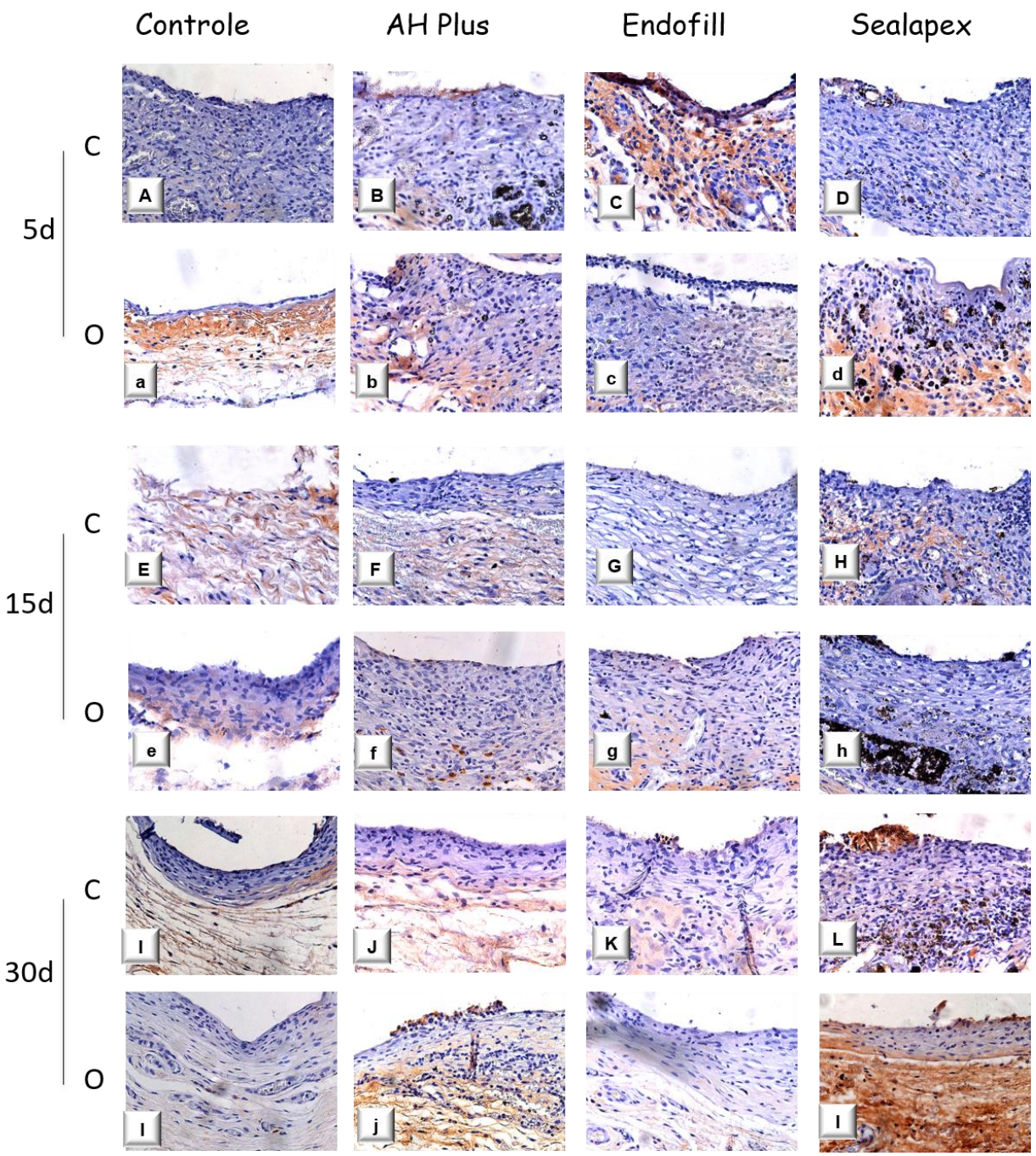

Figure 3. Immunohistochemistry images of in situ TNF-α expression for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

Collagen Maturity

Representative images from the Picrosirius red staining to identify the maturity of the collagen fibers for all groups are show in Figure 4. According to the Mann-Whitney statistical test, between all TO groups versus all TW groups, a higher percentage of mature fibers was observed in the groups without supplementation at 15 days (p=0.034) and 30 days (0=0.008). On the other hand, in the TO groups there was a higher percentage of immature fibers at 15 days (p=0.034) and 30 days (0=0.008). There was no statistical difference in the comparison between TO groups among themselves in any of the analysis periods (p>0.05). Among all TW groups, it can be seen statistical differences at 5 days between Sealapex and AH Plus group (p=0.013) and at 15 days between Control and Endofill group (p=0.016). The comparison for each group with supplementation or not of the omega 3 enable to observe differences only at 30 days in the Control tube (p=0.038) and in the Sealapex group (p=0.015), which presented higher amount of immature fibers in the presence of supplementation.

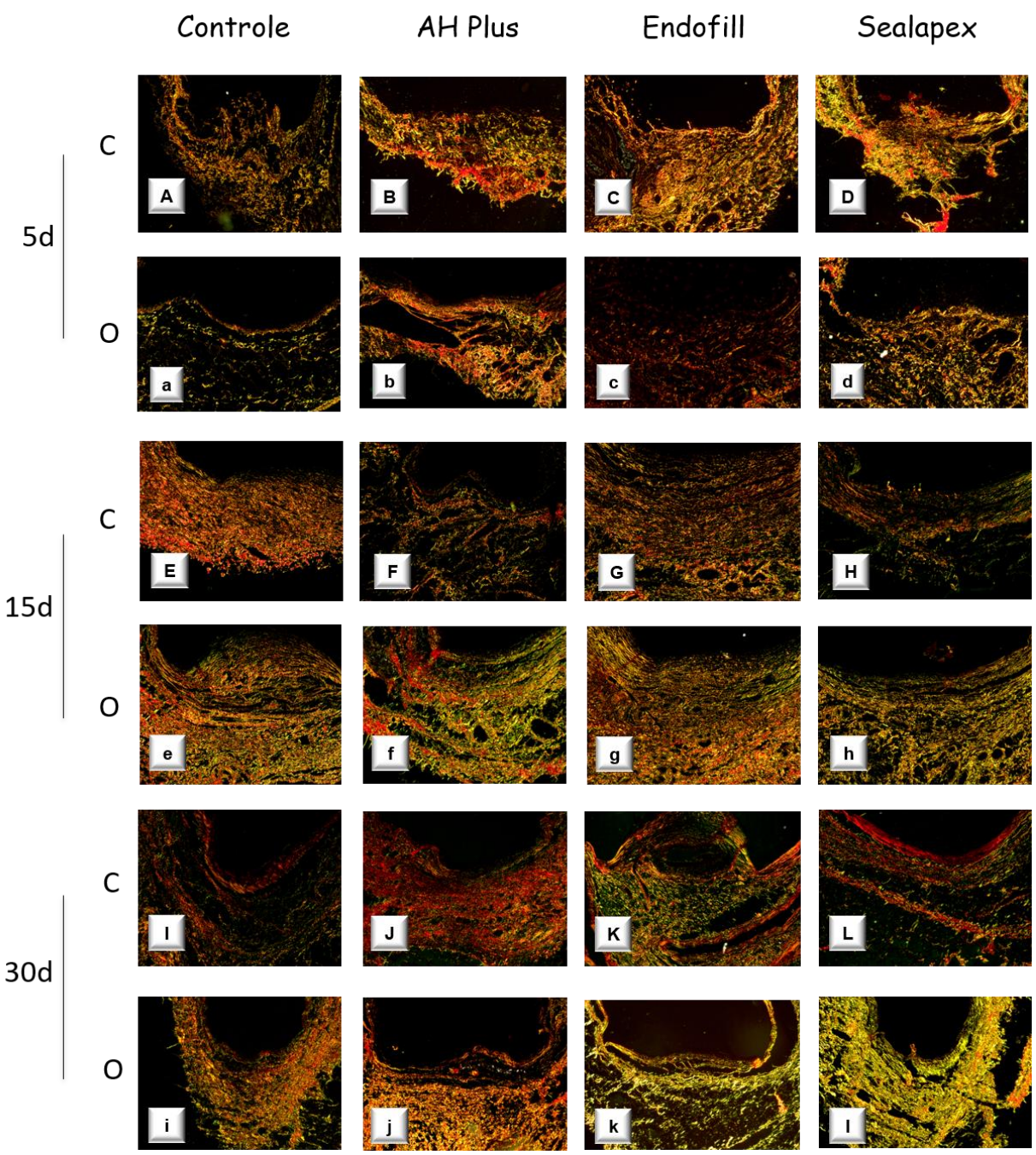

Figure 4 – Representative images from the Picrosirius red staining to identify the maturity of the collagen fibers for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,I), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (PSR staining, x100).

Mineralization

Representative images of von Kossa and Polarized light are shown in figure 5 and 6. It was possible to identify calcium deposition in both groups of supplementation at all periods only for Sealapex. In von Kossa staining it was observed calcium carbonate (Figure 5) and in polarized light, it was encountered biorrefringent crystals of calcium (Figure 6) near the tube opening. However, these structures were not observed in the other groups of sealers and in the control tube.

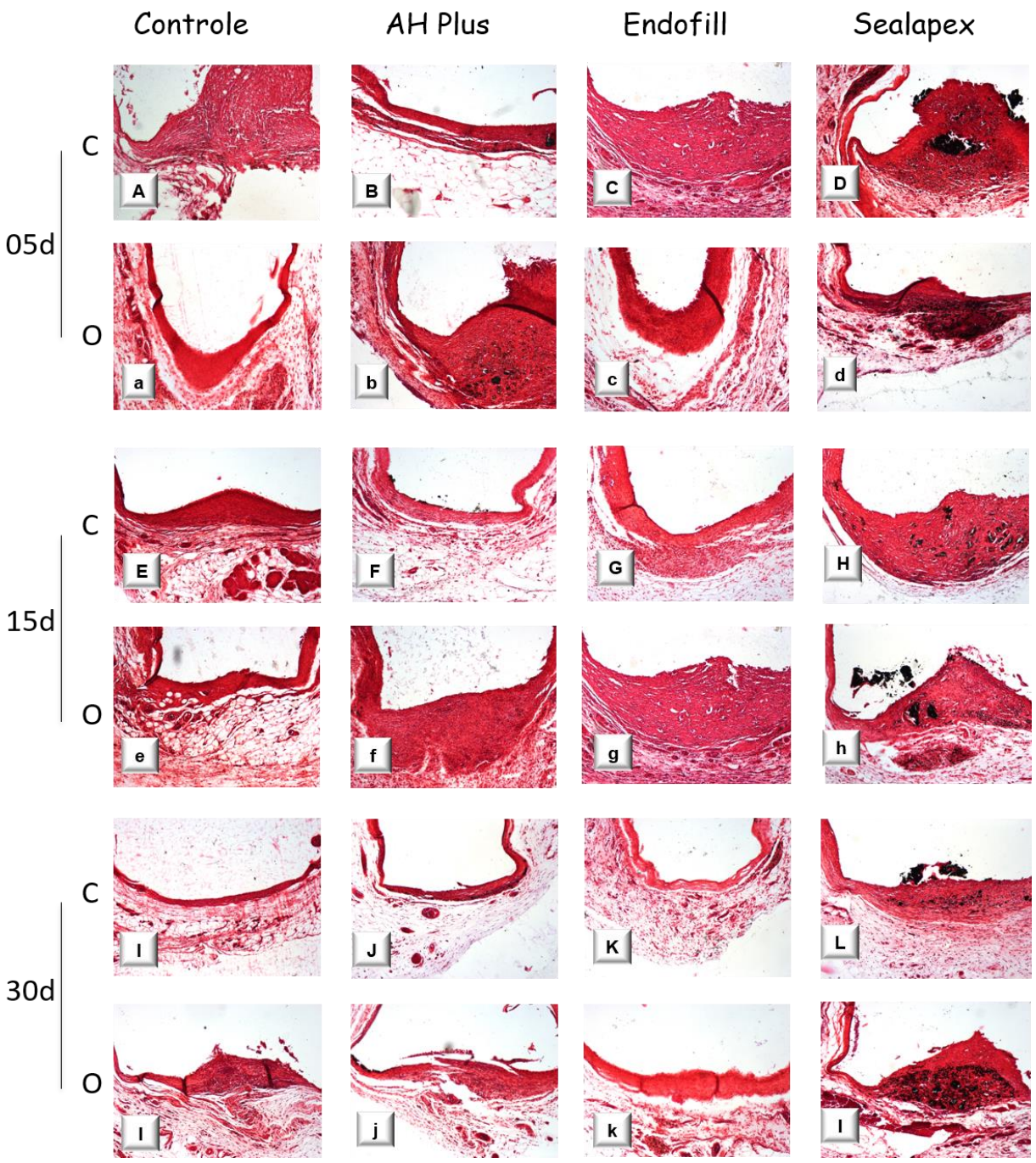

Figure 5. Representative images from Von Kossa staining for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis.. Structures positive for von Kossa staining can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation with omega 3 (D,d; H,h; L,l). (staining according to the von Kossa technique, x100).

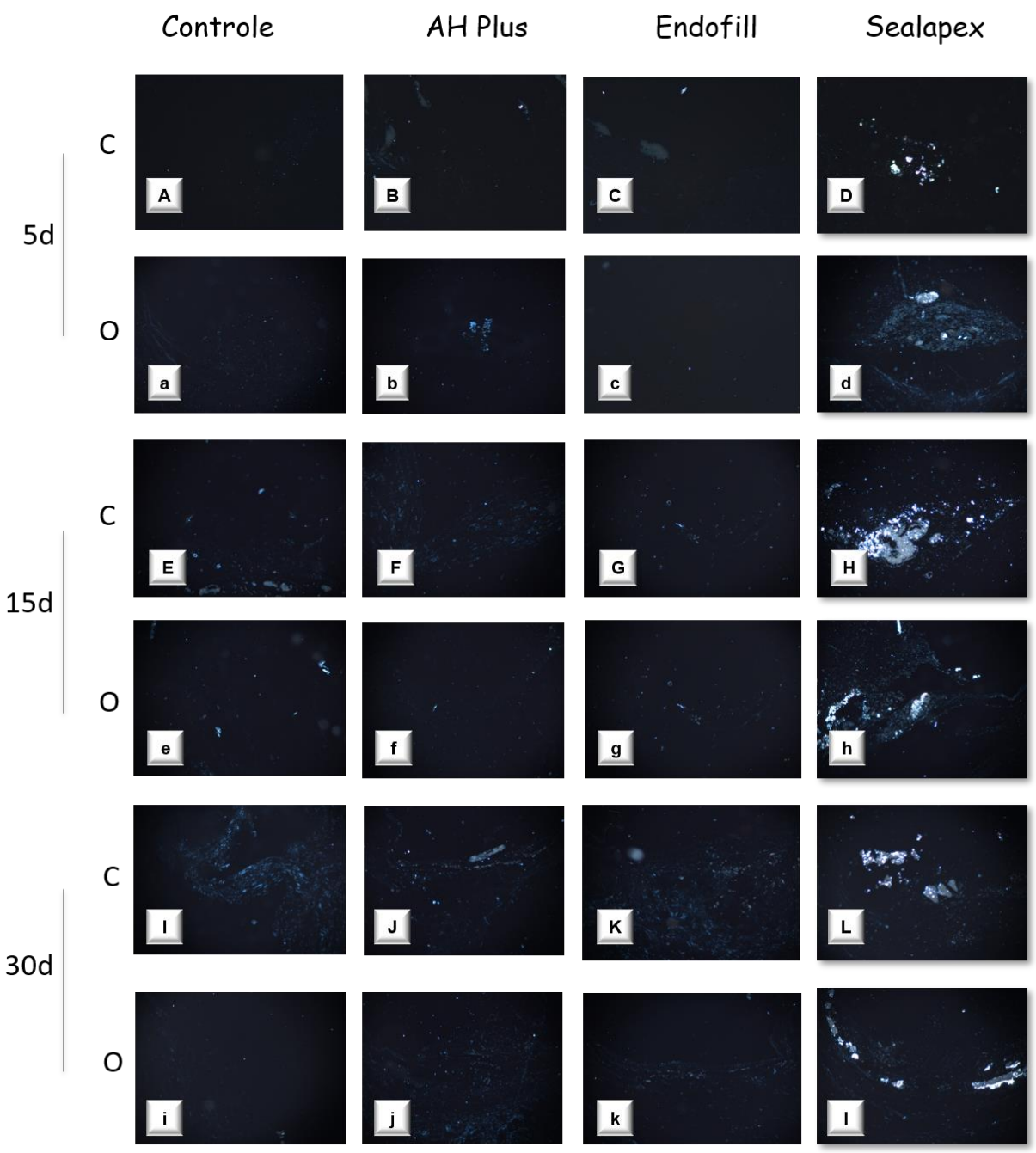

Figure 6. Representative images from Polarized light for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. The biregringent calcium crystals can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation with omega 3. (D,d; H,h; L,l). (polarized light visualization, x100x).

Table 4. Median of mature and immature fibers and mineralization capacity for each sealer in both experimental groups (Results on %)

Groups	Time	Material	Collagen Maturity (%)		Mineralization (%)	
			Mature Fibers	Imature Fibers	VK	POL
Control	05 days	Control	52.697	47.303	0	0
		AH Plus	66.158	33.842	0	0
		Endofill	61.065	38.935	0	0
		Sealapex	39.223	60.777	100	100
	15 days	Control	65.508	34.492	0	0
		AH Plus	64.982	35.018	0	0
		Endofill	47.279	52.721	0	0
		Sealapex	51.587	48.413	100	100
	30 days	Control	69.665[a]	30.335[a]	0	0
		AH Plus	56.26	43.74	0	0
		Endofill	61.686	38.314	0	0
		Sealapex	78.405[a]	21.595	100	100
Omega 3	05 days	Control	51.261	48.739	0	0
		AH Plus	57.801	42.199	0	0
		Endofill	69.384	30.616	0	0
		Sealapex	50.895	49.105	100	100
	15 days	Control	58.926	41.074	0	0
		AH Plus	52.386	47.614	0	0
		Endofill	51.368	48.632	0	0
		Sealapex	48.578	51.422	100	100
	30 days	Control	43.216[b]	56.784[b]	0	0
		AH Plus	63.669	36.301	0	0
		Endofill	49.433	50.567	0	0
		Sealapex	54.658[b]	45.342	100	100

* Lowercase letters overwritten for two groups in each analysis period by mature and immature fibers (control versus control + omega; AH Plus versus AH Plus + omega; Endofill versus Endofill + omega; Sealapex versus Sealapex + omega)

DISCUSSION

This study investigated the influence of omega 3 on sealer's tissues response. The results showed that the omega 3 can decrease the inflammatory infiltrate and it is able to stimulate the repair. Moreover, it did not interfere in the deposition of calcium particles, which rejects partially the initial hypothesis.

Some natural substances, such as the omega 3 have the potential of acting on the inflammatory process (Bittiner et al. 1988, Vardar et al 2004, Azuma et al. 2017, 2018) and it has been used as an adjuvant therapy for chronic inflammatory diseases, such as diabetes and cardiovascular problems, degenerative inflammatory conditions (Lee et al. 2012, Schunck 2018). Considering the fact that the endodontic sealers are able to promote inflammatory reaction of different intensity depending on the type of sealer used (Kim et al. 2010, Bueno et al. 2016); It would be reasonable to explore the omega 3 properties associated with the endodontic sealers tissue's response (Massaro M. et al. 2006).

At 30 days period, it was possible to observe a significant reduction on the inflammation promoted by the Sealapex sealer in the TO group when compared to TW group. This decrease can be explained by the fact that the omega 3 acts on the arachidonic cascade, regulating the immunolabeling of pro and pre inflammatory mediators that cause these reactions and it can strettghen cell's membrane, making them more resistant to the immunologic reactions (Vardar et al 2004, Azuma et al. 2017, 2018). The same result was observed for Endofill sealer, which can be considered remarkably interesting since Endofill can difficult the biological sealing as well as induce some postoperative symptoms, such as post-operative pain and edema that occurs specially because of the presence of zinc oxide and eugenol, which are very inflammative coumpounds (Zmener et al. 2012).

The immunohistochemistry is a quite common form of analyzing the inflammatory process because it assesses cellular mechanisms (Nibali et al. 2011). The Il-6 cytokine is secreted by the T-cells, fibroblasts, macrophages in response to the attack of antigens and other types of cytokines. Also, they are very easily found in acute reactions (Heinrich et al. 2003). Overall, the TO group allowed a lower immunolabeling of IL-6 when compared to the TW group and it was possible to see differences among the materials' response, having a decrease of this mediator in specific periods for each sealer, Specifically, it was possible to verify that its immunolabeling was lower for the Control tube at 15 and 30 days, for the AH Plus at

5,15 and 30, for the Endofill at 5 days and for Sealapex at 15 and 30 days. A previous research has shown that this interleukin is increased in the presence of root canal sealers (Silva et al. 2015). Consequently, the results observed in this study affirms the regulation of the omega 3 on the immunolabeling of such immune mediators and it also opens a lot of forms of exploring the action of the omega 3 and the benefits that the substance could bring to patients.

TNF-α is a protein that acts as a regulator of the inflammation over time. It can recruit cells, such as leukocytes, monocytes and they are even able to induce endothelial cells to secret adhesion molecules and chemokines (Pfeffer et al. 2003). When the supplementation of omega 3 occurred, some materials responded in a better way, with lower immunolabeling of TNF-α than their correspondents in the TW group. These results can be observed on the reaction of control tube at 30 days, AH Plus at 5 and 30 days, Endofill at 5 days and Sealapex at 15 days. This interpretation affirms the findings of the literature that indicate that this substance supplementation can regulate the production of TNF-α, by interrupintg the complete arachidonic inflammatory cascade (Körner et al. 2008).

Still, to better comprehend the influence of the omega 3 on the endodontics sealers response, the maturity of the collagen fibers was explored. After the inflammation process be controlled, the fibroblasts cells are proliferated, and they migrate to the area that suffered injury (Diegelmann et al. 2004). Then, the next step is the maturation process of the collagens fibers that were formed. The type of collagen can be observed by the Picrosirius red analysis. The mature fibers indicate injured areas that are in repair process for longer periods. For the immature fibers, they will be observed in areas that are in renovation collagen fibers process. However, because of the fibrosis process, they might as well be encountered in inflamed areas. In this study, the differences of collagen fibers could not be observed when the groups of sealers were isolated. But, we could see that when it was considered the kind of treatment, the TO group showed more immature fibers than the TW group. Considering the lower inflammatory response of the materials in the TO group, the presence of more immature collagen fibers can be a result of the remodeling collagen process. If it induced less fibrosis and accelerated the renovation of the tissue, it can be assumed that the inflammatory process is controlled or even decreased (Cintra et al. 2017), which it is expected, since being omega 3 able to reduce the inflammation, it facilitates tissue repair (Lee et al. 2012 e Jain et al, 2015, Azuma al. 2017, 2018).

At last, an interesting ability of a sealer, it is the capacity of forming calcium particles on tissues, being this ability analyzed by the von Kossa staining and the polarized light (Gomes-Filho et al. 2012, Cintra et al. 2017a). The omega 3 is reported as a bone metabolism regulator, not influencing negatively the biomineralization or deposition of calcium, being even mentioned as a substance that ameliorates the mineralization process. (Maggio et al. 2009, Azuma et al. 2017). However, the analysis showed that the omega did not interfere on sealer's ability of depositing calcium on tissues. Among the evaluated sealers in TW and TO only Sealapex was able of stimulating calcium particles formation. This result was already expected because of Sealapex's compounds, which contains calcium hydroxide, a molecule able to promote the calcification (Bueno et al. 2016).

Unfortunately, there are not many studies that can contribute to these results. Thus, some researches can explore more the influence of the omega 3 in the repair process induced by endodontic sealers. In summary, the omega 3 supplementation can control tissue reactions expressed by endodontics sealers. Therefore, it is of very importance in the scientific field to consider its use as an adjuvant treatment to enhance biomaterials response, facilitating the endodontic treatment.

CONCLUSION

It can be concluded that omega 3 supplementation influence on the inflammatory response of endodontic sealers, modulating the inflammatory process, immunolabeling of IL-6 and TNF-α, the repair process and, it does not interfere on biomineralization.

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The authors deny any conflicts of interest related to this study.

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Legends

Table 1. Scores observed for inflammatory response of endodontic sealers and oral supplementation.

Table 2. Scores observed for the immunolabeling of Il-6 by endodontic sealers with and without the supplementation of omega 3.

Table 3. Scores observed for the expression of TNF-α by endodontic sealers with and without the supplementation of omega 3.

Table 4. Median of mature and immature fibers and mineralization capacity for each sealer in both experimental groups (Results on %)

Figure 1. Representative images from the subcutaneous tissue reactions for groups with water supplementation (A-D. E-H; I-L) and groups with omega 3 supplementation (a-d. e-h, i-l) for each kind of sealer and the control tube used at 5, 15 and 30 days of analysis (A,a; E,e. I,I – Control; B,b; F,f;J,j – AH Plus; C,c; G,g;K,k – Endofill and D,d, H,h;L,l – Sealapex). At 5 days: intense inflammatory response for every sealers in both groups of analysis (A-D; a-d); At 15 days: mild inflammatory response to the group supplemented with omega (e-h); moderate inflammatory response to groups with water supplementation (E-H); At 30 days: mild to absent inflammatory response to the group supplemented with omega (i-l); moderate to mild inflammatory response to groups with water supplementation (I-L). (Hematoxylin-eosin staining, x400).

Figure 2. Immunohistochemistry images of in situ IL-6 expression for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

Figure 3. Immunohistochemistry images of in situ TNF-α expression for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

Figure 4 – Representative images from the Picrosirius red staining to identify the maturity of the collagen fibers for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j),

Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (PSR staining, x100).

Figure 5. Representative images from Von Kossa staining for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis.. Structures positive for von Kossa staining can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation with omega 3 (D,d; H,h; L,l). (staining according to the von Kossa technique, x100).

Figure 6. Representative images from Polarized light for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. The biregringent calcium crystals can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation with omega 3. (D,d; H,h; L,l). (polarized light visualization, x100x).

2. Artigo

Influence of supplement administration on the tissue response of endodontic sealers. Part 2: Supplementation with Melatonin

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Influence of supplement administration on the tissue response of endodontic sealers. Part 2: Supplementation with Melatonin

ABSTRACT

Introduction: The aim of this study was to evaluate the influence of melatonin supplementation on tissue's response of endodontic sealers. **Methods:** Forty-eight Wistar rats received subcutaneous implants of four polyethylene tubes, one was empty (control) and three filled with endodontic sealers (AH Plus, Endofill e Sealapexl). Half of the animals were treated with melatonin (TM) and the remaining animals received water (TW) for 15 days before the implantation until the euthanasia day. After 5, 15 and 30 days (n=8), the tubes were removed and processed for analysis of inflammation, immunolabeling for IL-6 and TNF-α, collagen matury and calcium deposition. The results were statistically analyzed ($p<0.05$). **Results:** The inflammatory infiltrate decreases over time and, at 30 days, the TM group showed lower inflammatory response than TW group ($p<0.05$). In the TW, it was observed that the Endofill evoked a more intense inflammatory infiltrate compared to the AH Plus group and control group in the 30-day period ($p<0.05$). Among TM groups, there was no difference among all tube's response in any period of analysis ($p>0.05$). Comparing each group as a function of the supplementation with melatonin, there are difference for Endofill ($p=0.05$) and Sealapex ($p<0.05$) sealer in the 30-day period, presenting lower inflammatory infiltrate in the TM animals. For IL-6 and TNF-α it was observed that the immunolabeling was lower in the TM animals in all periods, regardless of the sealer used. In addition, a higher percentage of mature fibers in TM groups at 5 days ($p<0.05$). Structures positive for von Kossa staining and birefringent to polarized light were observed only for Sealapex in all periods, regardless of melatonin supplementation. **Conclusion:** It can be concluded that melatonin influence on the inflammatory response of endodontic sealers, modulating the inflammatory process, immunolabeling of IL-6 and TNF-α, stimulating tissue repair and, it did not interfere on calcium particles deposition.

KEY WORDS

Endodontic sealers; tissue response; inflammation; mineralization; melatonin

A proper cleaning and sealing are the main goal of an endodontic treatment (Jianag Lei-Meng et al. 2012, Lacerda M et al. 2017). The filling of the root canals is done due endodontics sealers that along with the gutta percha allow the canal obturation (Ørstavik 2014). An ideal endodontic sealer must present specific biological and physicochemical characteristics, such as biocompatibility and dimensional stability (Guttuso 1963, Morse et al. 1984)

Different responses can be observed in tissues, depending on the sealer base compound (Guttuso 1963, Morse et al. 1984, Benetti et al. 2019). Consequently, these reactions can influence on periapical repair, inducing or even delaying it (Geurtsen et al. 2001, Kim et al. 2010). The ones based on oxide zinc, for example, can promote a more exacerbated inflammatory response, while others that contains different formulation induce lower inflammation, such as those based on calcium silicate, which also has the ability of inducing the mineralization process (Morgental et al. 2011, Zmener et al. 2012, Benetti et al. 2019).

The local inflammatory response can be modulated by different substances (Slominski et al. 2012, Azuma et al. 2017, Noori et al., 2019), one of them is the melatonin (N-acetil-5-metoxitriptamina), an endogenous indolamine, produced in the absence of light by the pineal gland and other areas of the body, such as the retina, salivary gland and even inflammatory cells (Reiter et al. 2009, Singh et al. 2014, Acuña-Castroviejo et al. 2014). This indolamine performs different functions, such as chronobiological, immunomodulatory, sedative, antitumor, analgesic, anti-inflammatory and antioxidant functions (Mauriz et al. 2013, Dong et al. 2016, Colares et al. 2016, Ostjen et al. 2019). As an anti-inflammatory agent, several studies have demonstrated that the melatonin can regulate the inflammatory process ceasing innumerable events within the cell cascade by the signaling of NF-k*β* (Nuclear transcription factor – Kappa *β)*, which is translocated to the nucleus of cells, binding itself to specific elements that modulate the transcription of pro-inflammatory genes (Baeuerie et al. 1996, Reiter et al. 2000, Cuzzocrea et al. 2001, Vriend et al. 2014, Favero et al. 2017). In addition, the melatonin can influence in the expression of pro-inflammatory and anti-inflammatory cytokines, and the recruitment of polymorphonuclear leukocytes to sites of inflammation in different pathophysiological situations (Leon et al. 2014, Habtemariam et al 2017).

Considering that endodontic sealers can develop local inflammatory reaction on connective tissues and that the melatonin can regulate the inflammation, makes it

possible to evaluate the influence of melatonin supplementation on the tissue's response of endodontic sealers. Null hypothesis: Melatonin would not interfere in the tissue's response of endodontic sealers.

MATERIAL AND METHODS

Experimental Animals

Following the protocols of previous studies (Cintra et al. 2017; Benetti et al. 2019), an amount of 48 male Wistar albino rats weighing 250-280g were utilized in this study. They were caged in a controlled temperature and light cycle environment (22 ± 1 ° C, 70% humidity at 12 hours light and 12 hours dark), received solid diet and ad libitum water, except for the first 24 hours after the intervention. The experimental procedures were approved and performed according to the guidelines of the ethical committee (process no. 00229-2017).

Melatonin supplementation

Half of the animals were supplemented with melatonin (Group treated with melatonin – TM) and the other half with water (Group treated with water – TW). The supplementation occurred 15 days before the implantation of the endodontic sealers and lasted until the sacrifice day, as a therapy supplementation. The dose of melatonin was of 10mg/kg/day dissolved in 1% of ethanol and 1ml of water (Santos et al. 2017, Li et al. 2015). The TW group received 1ml of water instead of melatonin.

Subcutaneous Implant

Endodontic sealers (Sealapex, AH Plus and Endofill) were prepared according to the manufacturer's recommendations and injected in polyethylene tubes (Abbot Labs of Brazil, São Paulo, Brazil) with 1.0-mm internal diameter, 1.6-mm external diameter, and 10.0-mm length (ISO 10993-6: 2007), sterilized in ethylene oxide (ISO 10993-7: 1995); One tube got empty and it was used as a control. The surgical procedure was performed following previous studies (Cintra et al. 2017). The rats were anesthetized and shaved in their dorsa, and a 2.0-cm incision was made in a head-to-tail orientation with a #15 Bard-Parker blade (BD, Franklin Lakes, NJ). The skin was reflected in the extremities, creating two pockets on the right and left side of the incision; Then, the tubes were allocated randomly to avoid the contact with only one type of tissue and the skin was sutured.

After 5, 15 and 30 days (n = 8 rats / period), the animals were euthanized by an over-dose of anesthetic solution. The implanted tubes were removed with the surrounding tissues and fixed in 1% formalin solution at a pH of 7.0. The specimens were imbibed in paraffin and sectioned into 5-μm cuts for staining with hematoxylin-eosin, picrosirius red (PSR), immunohistochemistry for IL-6 and TNF-α and 10-μm cuts for staining with von Kossa and not stained for polarized light technique (Gomes-Filho et al. 2012, Bueno CR et al. 2016, Benetti F et al. 2018).

Histological and immunohistochemical analysis

Histological and immunohistochemical analysis were performed by a single calibrated operator in a blinded manner under light microscopy (400x, DM 4000 B: Leica, Wetzlar, Germany). The inflammatory infiltrate was graded in scores from 1 to 4 (1 - no or few inflammatory cells and no reaction; 2 - less than 25 cells and little reaction; 3 - between 25 and 125 cells and moderate reaction; 4 - 125 or more cells and severe reaction). The scores for the immunolabeling of IL-6 and TNF-α were graded also from 1 to 4 (1 - Absence of immunostaining in the region of interest; 2 - Low immunolabeling pattern, about ¼ of labeling of cells and poor labeling in extracellular matrix; 3 - Moderate immunolabeling pattern, about ½ of the immunolabeled cells and moderate labeling in the extracellular matrix; 4 – High immunolabeling pattern, about ¾ of immunolabeled cells and high labeling in the extracellular matrix (Benetti et al. 2018).

The maturation of the collagen was analyzed by the Picrosirius Red staining technique, allowing the identification of greenish yellow fibers, which were considered immature and thin and yellowish red fibers that were considered mature and thick (Cintra et al. 2017a). The LASQWin program was used (400X magnification, Leica LASQWin V3, Leica Microsystems), the variation of colors was selected and automatically the marked area was calculated, indicating the percentage of each kind of fibers observed (Cintra et al. 2017a). The analysis of biomineralization was assessed by the evaluation of calcium deposits in von Kossa staining with deposition of calcium carbonate or in a non-stained technique, the Polarized light, detecting the presence of birefringent crystals (Cintra et al. 2017a).

Statistical Analysis

Statistical analysis was performed using the SigmaPlot 12.0™ program (Chicago, IL, USA). The normality of the data was verified for all the analyses. The Kruskal-Wallis

test or Mann Whitney test was performed for nonparametric data. The Tukey post hoc test was performed for parametric data. The level of significance attributed was 5%.

RESULTS

Inflammatory infiltrate

Representative images of the different specimens from all groups are shown in Figure 1. Overall, it was observed a reduction of the inflammatory infiltrate over time. At 5 days, it was observed an intense inflammatory response for all groups, except for AH Plus and Sealapex in TM that was considered moderate. At 15 days, control and Sealapex in the group supplemented with melatonin showed mild inflammatory response, whereas in all other groups it was predominantly moderate. At 30 days, in TM mild to absent inflammatory response for the control tube and Sealapex. In the TW, it was observed that the Endofill evoked a more intense inflammatory infiltrate compared to the AH Plus group and control group in the 30-day period ($p=0.010$). There was no difference among all tube's response in any period of analysis ($p>0.05$) in the TM. According to the Mann-Whitney statistical tests between all groups with melatonin supplementation versus all groups with water supplementation, it can be observed a lower inflammatory infiltrate in the melatonin supplemented group at 30 days ($p=0.022$) (Table 1). Comparing each group as a function of the supplementation with melatonin, there are differences for Endofill ($p=0.05$) and Sealapex ($p=0.038$) sealers in the 30-day period, presenting lower inflammatory infiltrate in the animals that had been treated receiving the melatonin supplementation (Table 1).

Table 1. Scores observed for inflammatory response of endodontic sealers and oral supplementation of Melatonin

Period of analysis	Score	H.E. Staining for Groups								Statistical analysis
		Control	Control + Melatonin	AH Plus	AH Plus + Melatonin	Endofill	Endofill + Melatonin	Sealapex	Sealapex+ Melatonin	
5 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p= 0.707
	2	2/8	2/8	1/8	3/8	1/8	0/8	2/8	1/8	
	3	3/8	3/8	3/8	3/8	3/8	4/8	3/8	4/8	
	4	3/8	3/8	4/8	2/8	4/8	4/8	3/8	3/8	
	Median**	3,5[a]	3,5[a]	4[a]	2,5[a]	4[a]	3,5[a]	3,5[a]	3[a]	
15 days	1	1/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p= 0.624
	2	1/8	4/8	2/8	3/8	1/8	2/8	2/8	4/8	
	3	4/8	2/8	5/8	4/8	4/8	2/8	6/8	2/8	
	4	2/8	2/8	1/8	1/8	3/8	4/8	0/8	2/8	
	Median**	3[a]	2[a]	3[a]	3[a]	3[a]	4[a]	3[a]	2,5[a]	
30 days	1	3/8	3/8	2/8	2/8	0 /8	1/8	0/8	4/8	*Mann-Whitney p= 0.022
	2	3/8	4/8	3/8	3/8	1/8	5/8	4/8	3/8	
	3	2/8	1/8	3/8	3/8	5/8	1/8	4/8	1/8	
	4	0/8	0/8	0/8	0/8	2/8	1/8	0/8	0/8	
	Median**	1,5[a]	2[a]	2,5[a]	2,5[a]	3[a]	2[b]	2,5[a]	1[b]	

* Mann-Whitney test between all groups with Melatonin supplementation versus all groups with water supplementation

** Superscript lowercase letters for two groups analysis (Control versus Control + Melatonin; AH Plus versus AH Plus + Melatonin; Endofill versus Endofill + Melatonin; Sealapex versus Sealapex + Melatonin)

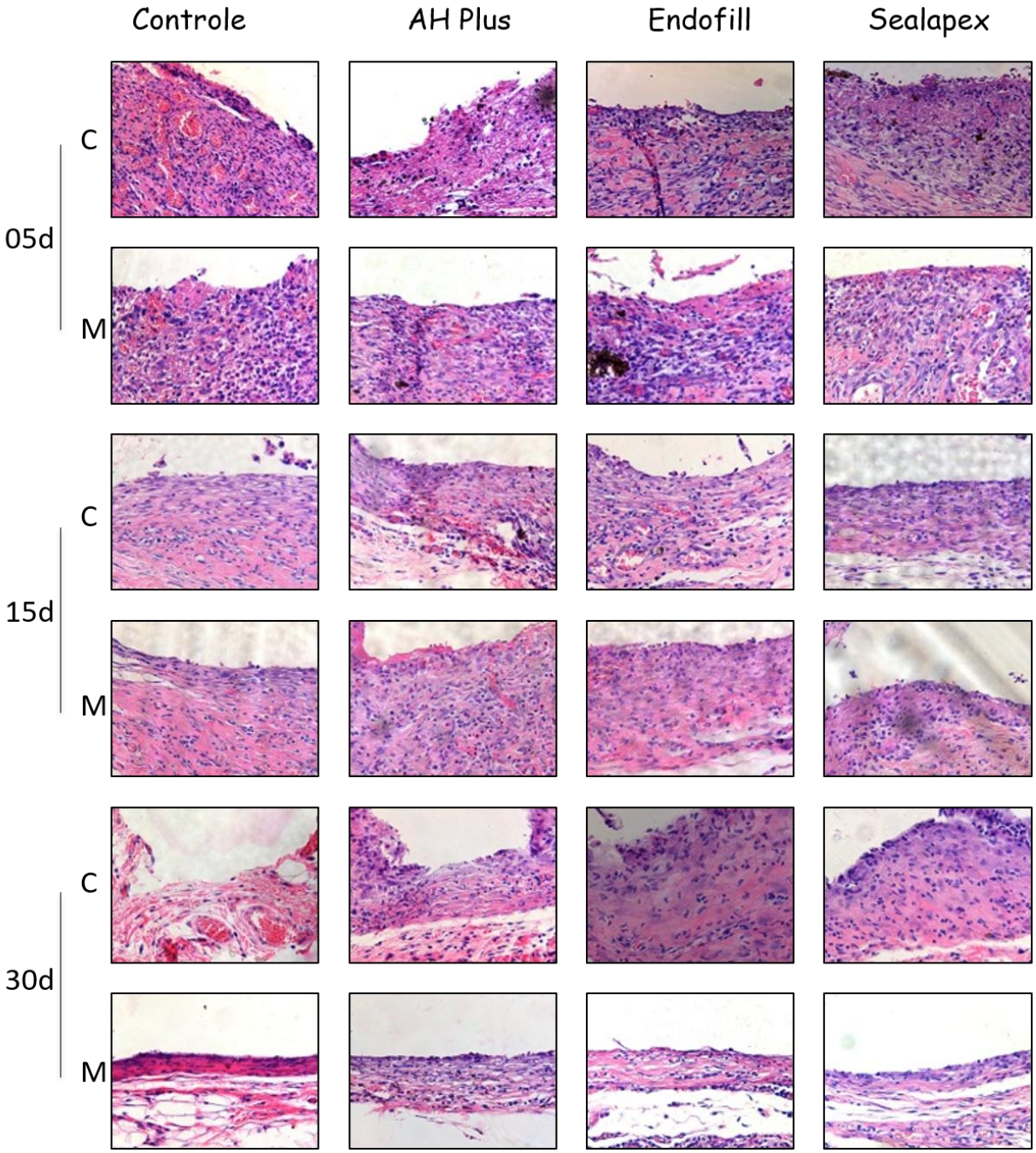

Figure 1. Representative images from the subcutaneous tissue reactions for groups with water supplementation (A-D. E-H; I-L) and groups with melatonin supplementation (a-d. e-h, i-l) for each kind of sealer and the control tube used at 5, 15 and 30 days of analysis (A,a; E,e. I,I – Control; B,b; F,f;J,j – AH Plus; C,c; G,g;K,k – Endofill and D,d, H,h;L,l – Sealapex).

Inflammatory Mediators

IL-6

Representative images showing the immunolabeling of IL-6 in the different specimens of all groups in Figure 2. According to statistics it can be affirmed that the immunolabeling of IL-6 was lower in the animals that were supplemented with melatonin at 5 ($p<0.001$); 15 ($p=0.005$) and 30 days ($p<0.001$) compared with animals that received water, regardless of the sealer used (Table 2).

Comparing the immunolabeling of IL-6 in the TW groups, it can be observed a statistical difference between the control tube and Endofill at 15 days ($p<0.001$), with more immunolabeling of the inflammatory mediator for Endofill. No statistical difference was observed among the TM groups in any period ($p>0.05$). On the other hand, comparing each tube's response considering the supplementation of melatonin or water, it was observed a lower immunolabeling of IL-6 in the control group at 5 ($p=0.015$) and 15 days ($p=0.021$), for the AH Plus sealer at 5 ($p=0.038$), 15 days ($p=0.010$) and 30 days ($p=0.010$), and for the Sealapex sealer at 30 days ($p=0.010$) when the melatonin was used (Tabela 2).

Table 2. Scores observed for the expression of IL-6 by endodontic sealers with and without the supplementation of melatonin.

Period of analysis	Score	IL-6 Staining for Groups								Statistical analysis
		Control	Control + Melatonin	AH Plus	AH Plus + Melatonin	Endofill	Endofill + Melatonin	Sealapex	Sealapex+ Melatonin	
5 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	2/8	0/8	5/8	0/8	0/8	0/8	2/8	
	3	3/8	6/8	3//8	1/8	3/8	6/8	8/8	6/8	
	4	5/8	0/8	5/8	2/8	5/8	2/8	0/8	0/8	
	Median**	4^a	3^a	4^a	2^b	4^a	3^b	3^a	3^a	
15 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p=0.005
	2	0/8	1/8	0/8	6/8	3/8	2/8	0/8	1/8	
	3	3/8	7/8	8/8	2/8	5/8	6/8	8/8	7/8	
	4	5/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	4^a	3^a	3^a	2^a	3^a	3^b	3^a	3^b	
30 days	1	0/8	0/8	0/8	0/8	0 /8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	0/8	0/8	6/8	3/8	6/8	0/8	6/8	
	3	5/8	8/8	8/8	2/8	5/8	2/8	8/8	2/8	
	4	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	3^a	3^b	3^a	2^b	3^a	2^a	3^a	2^a	

* Mann-Whitney test between all groups with melatonin supplementation versus all groups with water supplementation

** Superscript lowercase letters for two groups analysis (Control versus Control + melatonin; AH Plus versus AH Plus +melatonin; Endofill versus Endofill + melatonin; Sealapex versus Sealapex + melatonin)

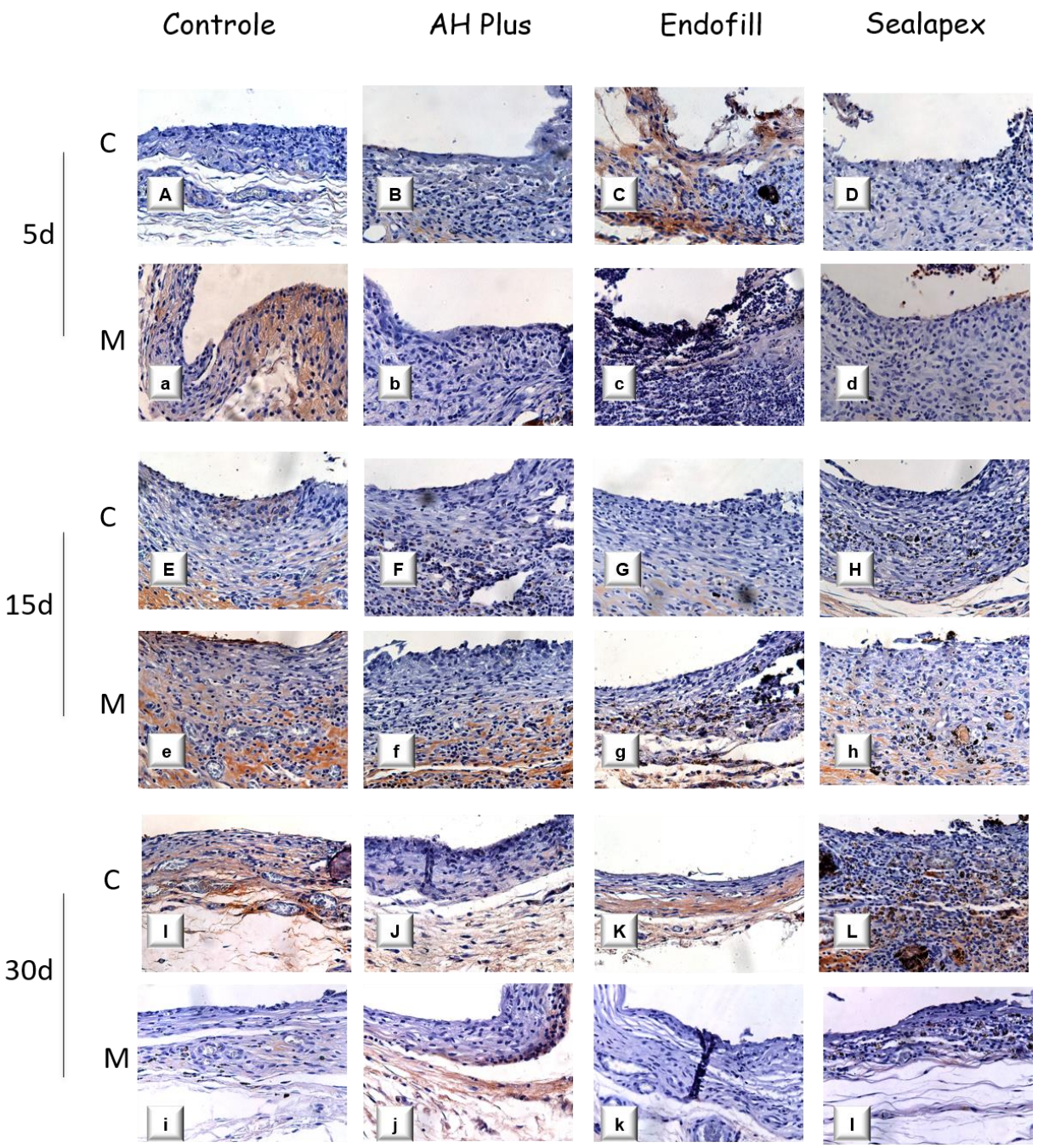

Figure 2. Immunohistochemistry images of in situ IL-6 expression for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

TNF- α

Representative images showing the immunolabeling of TNF-α in the different specimens of all groups are shown in Figure 3. According to statistics, the immunolabeling of TNF-α was lower in the TM groups at 5 ($p<0.002$), 15 ($p<0.001$) and 30 days ($p=0.004$) as compared with animals that received water, regardless of the sealer used (Table 3).

In the TW group, it was observed a statical difference between the response of Control and Sealapex ($p<0.001$), and AH Plus and Sealapex at 30 days ($p<0.001$). For the TM groups, it was observed a difference between Endofill and AH Plus ($p<0.001$), and Sealapex and AH Plus at 5 days ($p<0.001$) and Control and Sealapex at 30 days ($p=0.001$). Considering each tube's response according to the supplementation with melatonin or water, it was observed a lower immunolabeling in the AH Plus group at 5 ($p=0.038$), 15 ($p=0.002$) and 30 days ($p=0.002$) when the melatonin was used (Tabela 3).

Table 3. Scores observed for the immunolabeling of TNF-α by endodontic sealers with and without the supplementation of melatonin.

Period of analysis	Score	TNF-α Staining for Groups								Statistical analysis
		Control	Control + Melatonin	AH Plus	AH Plus + Melatonin	Endofill	Endofill + Melatonin	Sealapex	Sealapex+ Melatonin	
5 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.002
	2	0/8	2/8	0/8	7/8	0/8	0/8	0/8	0/8	
	3	5/8	6/8	6/8	1/8	5/8	8/8	8/8	8/8	
	4	3/8	0/8	2/8	0/8	3/8	0/8	0/8	0/8	
	Median**	3^a	3^a	3^a	2^b	3^a	3^a	3^a	3^a	
15 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.001
	2	0/8	2/8	0/8	7/8	0/8	2/8	0/8	2/8	
	3	5/8	6/8	8/8	1/8	8/8	6/8	8/8	6/8	
	4	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	3^a	3^b	3^a	2^b	3^a	3^a	3^a	2^a	
30 days	1	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	*Mann-Whitney p<0.004
	2	0/8	1/8	0/8	7/8	2/8	5/8	8/8	8/8	
	3	5/8	7/8	8/8	1/8	6/8	3/8	0/8	0/8	
	4	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	
	Median**	3^a	3^b	3^a	2^b	3^a	2^a	2^a	2^a	

* Mann-Whitney test between all groups with melatonin supplementation versus all groups with water supplementation

** Superscript lowercase letters for two groups analysis (Control versus Control + melatonin; AH Plus versus AH Plus +melatonin; Endofill versus Endofill + melatonin; Sealapex versus Sealapex + melatonin)

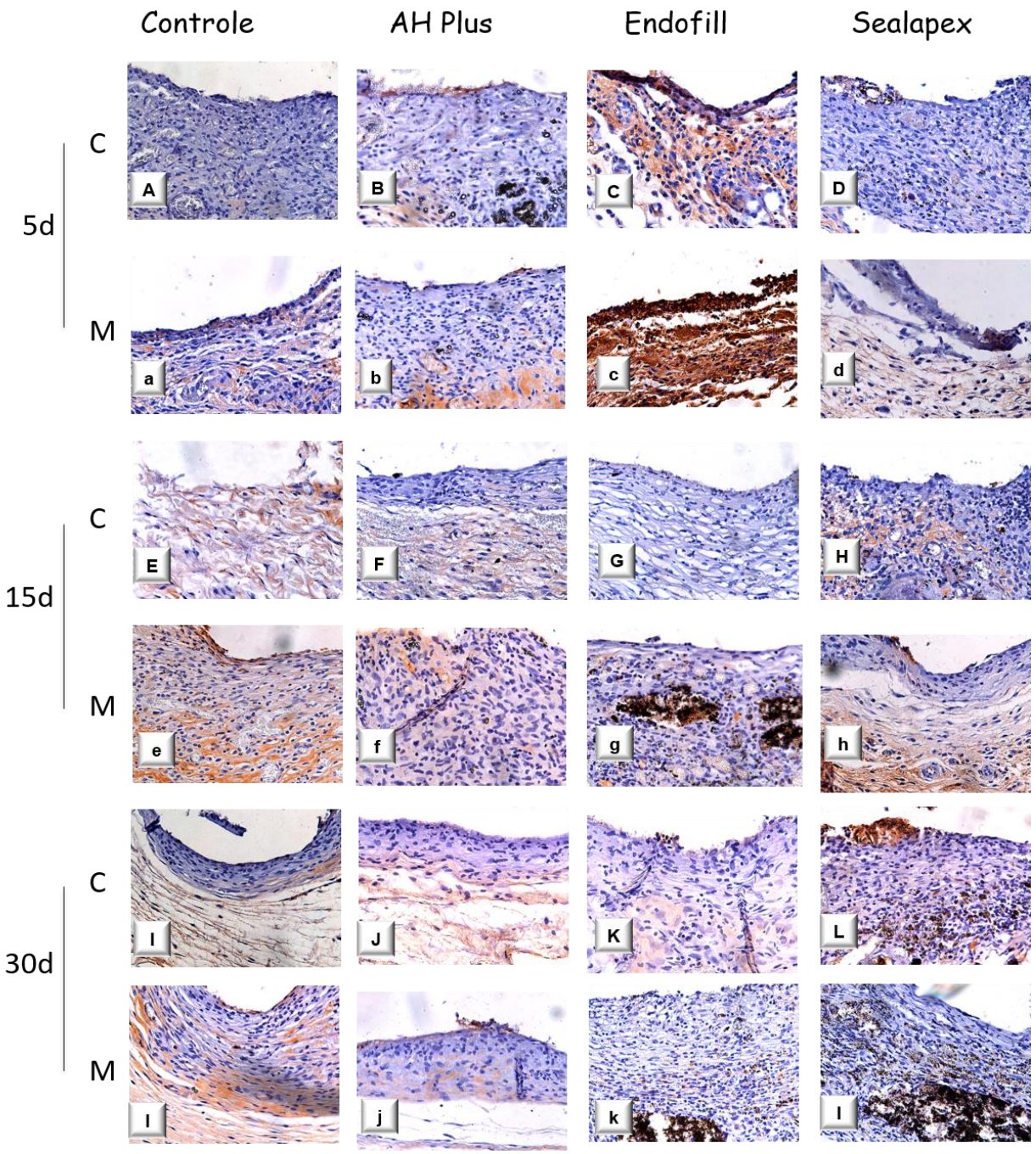

Figure 3. Immunohistochemistry images of in situ TNF-α expression for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

Collagen Maturity

Representative images from the Picrosirius red staining to identify the maturity of collagen fibers for all groups are shown in Figure 4. According to the Mann-Whitney statistical tests between all groups with melatonin supplementation versus all groups with water supplementation, it can be observed a higher percentage of mature fibers in the groups supplemented with melatonin at 5 days ($p=0.024$). On the other hand, in the TW groups the percentage of immature was higher 5 days ($p=0.024$). The comparative analysis among all TW groups at 5 days, the AH plus group showed more mature fibers compared to Sealapex ($p=0.036$) and, at 15 days, the Control group showed more mature fibers compared to Endofill ($p=0.029$). The comparative analysis among all TM groups at 5 days, revealed that control and Endofill tubes had higher percentage of mature fibers than Sealapex and AH Plus ($p<0.001$). However, there was no statistical differences between them ($p>0.05$). At 30 days there was more mature fibers in the AH Plus and Endofill when compared to the control tube ($p=0.005$), also with no statistical difference between them. The comparison for each group with supplementation of melatonin or water enable to observe differences in the Control group at 5 days ($p<0.001$), in the AH Plus group ($p=0.011$) at 30 days, and in the Endofill group at 5 days ($p=0.021$), which presented higher amount of mature fibers in the presence of melatonin. On the other hand, the control group, at 15 days, showed higher immature fibers in the presence of melatonin ($p=0.015$).

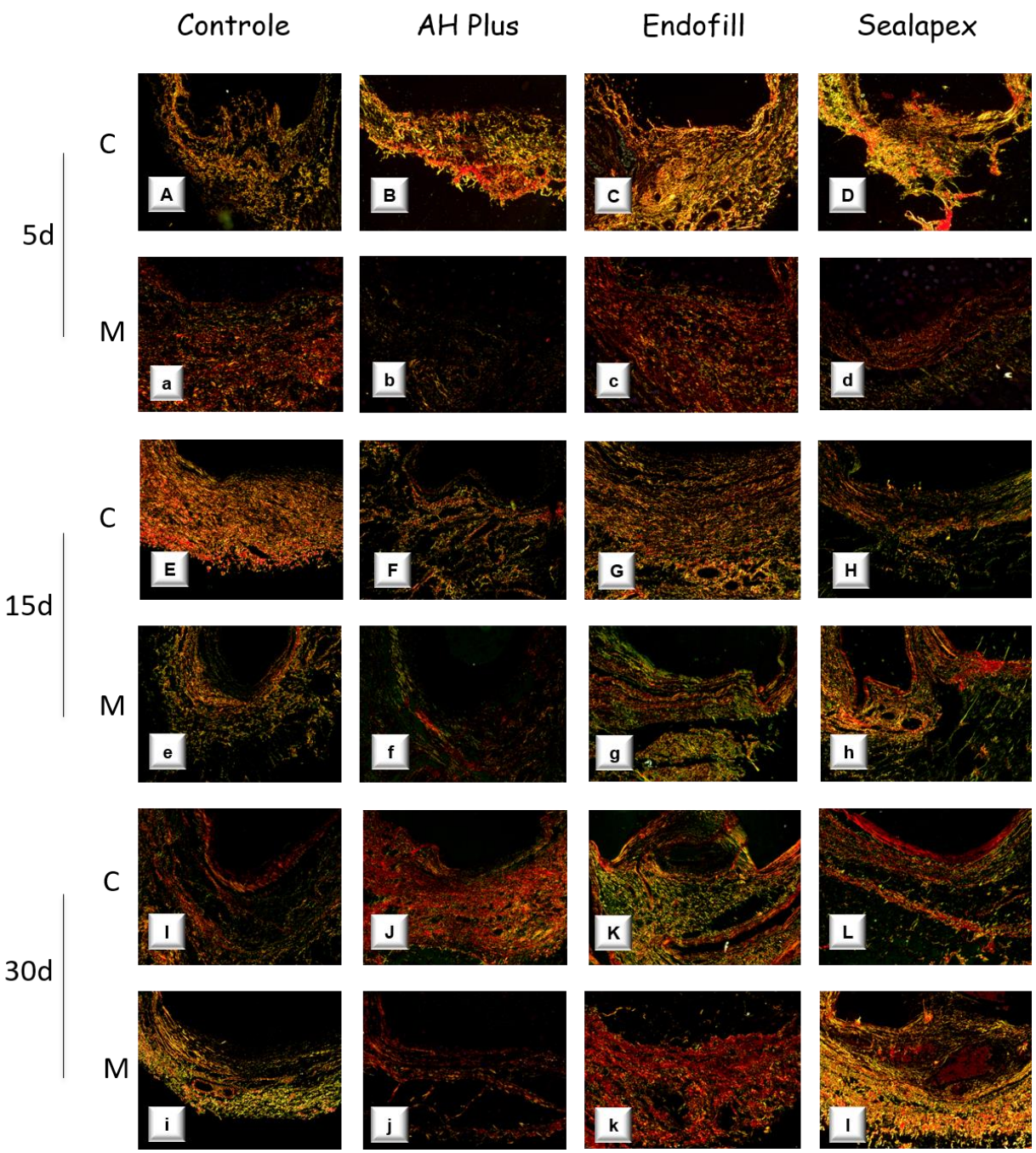

Figure 4 – Representative images from the Picrosirius red staining to identify the maturity of the collagen fibers for groups supplemented with water (A-D, E-H, I-L) and with melatonin. (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (PSR staining, x100).

Mineralization

Representative images of Von Kossa staining and Polarized light to identify calcium positive structures for all groups are shown in the Figures 5 and 6.

In the figures it is possible to observe for Sealapex the presence of structures positive for von Kossa staining (Figure 5) and birefringent crystals in polarized light at all periods of analysis, regardless of supplementation of melatonin (Figure 6). For AH Plus, Endofill, as well as in the control tube it was not identified any of these structures, neither in TW groups nor in TM groups.

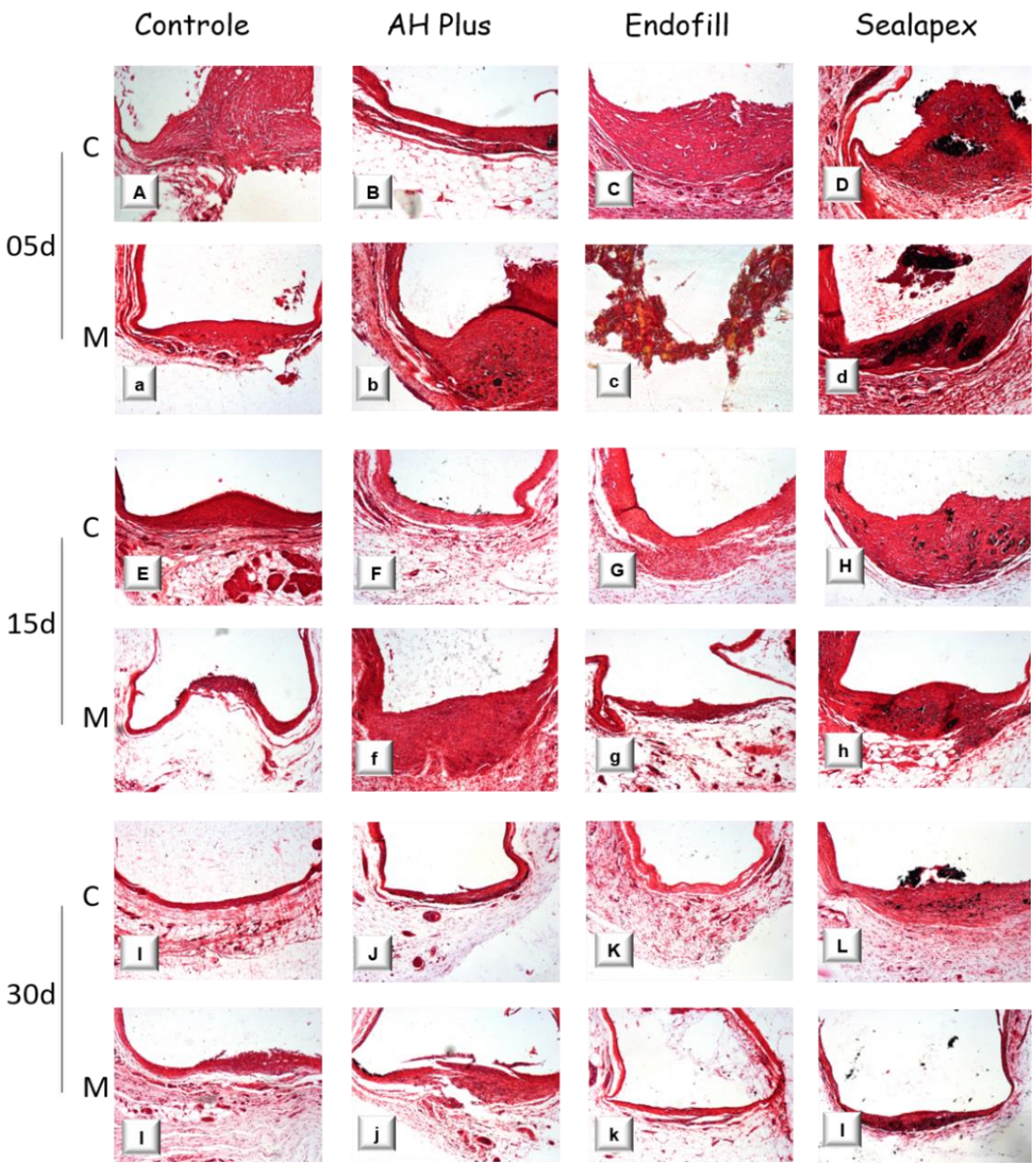

Figure 5. Representative images from Von Kossa staining for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. Structures positive for von Kossa staining can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation of melatonin (D,d; H,h; L,l). (staining according to the von Kossa technique, x100).

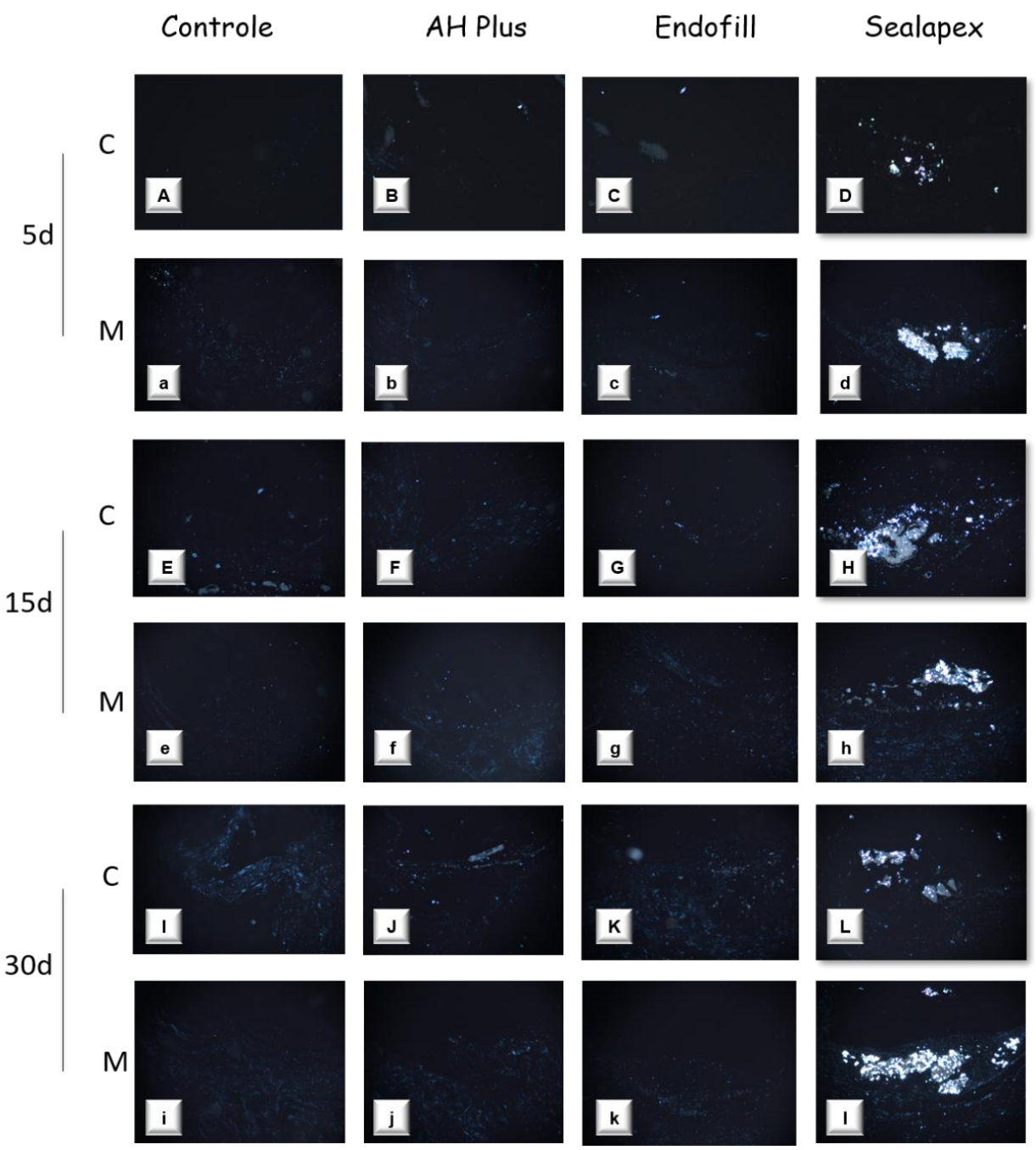

Figure 6. Representative images from Polarized light for groups supplemented with water (A-D, E-H, I-L) and with omega 3 (a-d, e-h, i-l) for control tube (A,a; E,e; I,l), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. The biregringent calcium crystals can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation with melatonin. (D,d; H,h; L,l). (polarized light visualization, x100x).

Table 4. Median of mature and immature fibers and mineralization capacity for each sealer in both experimental groups (Results on %)

Groups	Time	Material	Collagen Maturity (%)		Mineralization (%)	
			Mature Fibers	Imature Fibers	VK	POL
Control	05 days	Control	52.697[a]	47.303	0	0
		AH Plus	66.158[A]	33.842	0	0
		Endofill	61.065[a]	38.935	0	0
		Sealapex	39.223[B]	60.777	100	100
	15 days	Control	65.508[A]	34.492[a]	0	0
		AH Plus	64.982	35.018	0	0
		Endofill	47.279[B]	52.721	0	0
		Sealapex	51.587	48.413	100	100
	30 days	Control	69.665	30.335	0	0
		AH Plus	56.260[a]	43.740	0	0
		Endofill	61.686	38.314	0	0
		Sealapex	78.405	21.595	100	100
Melatonin	05 days	Control	80.029[Ab]	19.971	0	0
		AH Plus	55.311[B]	44.681	0	0
		Endofill	81.923[Ab]	18.077	0	0
		Sealapex	50.441[B]	49.559	100	100
	15 days	Control	52.810	47.190 [b]	0	0
		AH Plus	59.995	40.005	0	0
		Endofill	45.185	54.815	0	0
		Sealapex	55.357	44.643	100	100
	30 days	Control	48.862[A]	51.138	0	0
		AH Plus	84.011[Bb]	15.989	0	0
		Endofill	74.249[B]	25.751	0	0
		Sealapex	66.823	33.177	100	100

* Superscript capital letters represent statistical diferences among the melatonin supplemented groups or water supplemented groups in each period (analysis among 4 groups)

* Superscript lowercase letters for two groups in each period of analysis by Mature and Imature Fibers (Control versus Control + melatonin; AH Plus versus AH Plus + melatonin; Endofill versus Endofill + melatonin; Sealapex versus Sealapex + melatonin)

DISCUSSION

After being cleaned and shaped, the root canal must be filled with specific materials, the gutta percha and endodontic sealers (Ørstavik 2014, Sadr et al. 2015). However, these biomaterials when in contact with periapical tissues can induce inflammatory conditions that can even interfere negatively the periapical repair (Zmener et al. 2012, Benetti et al. 2019). This study evaluated the influence of melatonin supplementation on tissues response of endodontic sealers. The results showed that the melatonin was able to decrease the inflammatory infiltrate and to stimulate the repair process and did not unable calcium deposition in tissues, which rejects partially our null hypothesis.

The melatonin is a hormone produced specially in the pineal gland. Although its main action is to regulate the circadian rhythm (Cajochen et al. 2013), this molecule can modulate the inflammatory process, besides regulating the oxidative stress and the proliferation of tumoral cells (Mauriz JL et al. 2013, Dong Y et al. 2016). These actions are due its ability of penetrating the cells, acting on their DNA and cell's structures, such as the plasma membrane (Tan et al. 2011, Melchiorii et al. 1995). In that context and knowing that the endodontic sealers can irritate tissues (Benetti et al. 2019), the melatonin was a potential reducer of the inflammatory process induced by the endodontic sealers. Also, in this study to potentiate the anti-inflammatory actions of the melatonin, a prophylactic supplementation dose was administered to increase the concentrations of melatonin's molecule in the rat's organisms, enhancing the actions of this substance (Renn et al. 2018)

The assess of tissues responses induced by biomaterials can be observed by the implantation of standardized polyethylene tubes filled with specific materials in the subcutaneous tissue of rats. These reactions can be compared, because both tissues, the subcutaneous, as well as the periapical one has the same origin, being both connective tissues (ISO 10993-6; ISO 10993-7, Cintra et al. 2017). The staining of hematoxylin and eosin can show the expression of inflammatory cells indicating the inflammatory process (Bueno et al. 2016, Cintra et al. 2017, Benetti et al. 2019). Three different periods of analysis were considered in this project, becoming possible to see a reduction of the inflammatory response of sealers over time, which is referenced in the literature as a natural process promoted by endodontic sealers (Benetti et al. 2019). Nevertheless, when it was made a comparison between the groups that had the supplementation of melatonin and the group that was supplemented with the vehicle (water), a notable difference was observed, mainly at 30 days, having a lower

inflammation on Endoffill and Selapex groups. Endofiil is based on oxide zinc and because of this matter its capacity of inducing the inflammation is extremely high (Zmmer et al. 2012), which could be observed in TW group, having the Endofill expressing a very intense inflammatory infiltrate. On the other hand, in TM any difference was observed in the inflammatory infiltrate among the different groups of sealers and the control tube, which shows the reduction of the inflammation. In addition, to comprehend even more the inflammatory response of the sealers associated with the supplementation of a potential anti-inflammatory substance, the immunohistochemistry was assessed (Niballi et al. 2011). The evaluation of the immunolabeling of IL-6 and TNF-α was done; These inflammatory mediators are associated with the regulation of the inflammation, inducing, and modulating it. The IL-6 is produced by several cells, specially by the T-cells and it also can be very easily found in acute responses and it has been reported as an interleukin that increases in high levels when tissues get in contact with endodontic sealers (Henrich et al. 2003, Silva et al. 2015) For the TNF-α, it is a protein able to recruit inflammatory cells and induce the production of pro and pre inflammatory mediators, being present in every step of the inflammatory process (Pfeffer et al. 2003). Consequently, knowing all this information and associating it with its relation in endodontic sealer tissue's response, the assessment of the immunolabeling of these mediators is considered a particularly important aspect. (Chang et al 2015, Silva et al. 2015).

According to the findings of the immunohistochemistry analysis there was a decrease of these mediators over time, which is expected, since the inflammatory infiltrate is diminished (Cintra et al. 2017, Silva et al. 2015). However, when the melatonin was supplemented, the immunolabeling of TNF-α and IL-6 were even lower. In addition, in the groups that received melatonin no statistical difference was observed among them for IL-6. The results prove that melatonin has the potential of decreasing the inflammatory process by the regulation of the expression of this type of cytokine (Henrich et al. 2003, Pfeffer et al. 2003, Silva et al. 2015). Still, to investigate even more these associations, more studies must be done to understand the interrelation of these pro inflammatory mediators and the response promoted by endodontic sealers.

The type of collagen fibers expressed in the tissues can contribute to the understanding of a biomaterial tissue's response. It is known that after the inflammation be controlled, there are a proliferation of fibroblasts cells that migrate to

the area injured; These cells produce collagen that can be classified in mature, typically found in areas that suffered irritation for longer periods and immature fibers, associated with tissues that are in the beginning of the repair process (Diegelmann et al. 2004). The Picrosirus red is a technique that enable to detect which type of collagen fibers are being expressed in the analyzed area (Cintra et al. 2017). After the staining, the detection of mature fibers is seen in a variation of red color and the immature fibers in a variation of green, both in polarized light microscopy (Cintra et al. 2017). In this study it was observed more expression of mature fibers over time in the groups that had the supplementation of the melatonin when compared to the groups that was supplemented with water. Therefore, associating to the fact that the melatonin has the ability of decreasing the inflammation in tissues in a variation of mechanisms (Mauriz et al. 2013, Dong et al. 2016) and the results of this experiment, it is possible to say that the expression of more mature fibers was due the maturity of the inflammatory process, which shows tissues renewal indicating its domination by the rat's immunologic system (Mauriz et al. 2013, Dong et al. 2016). Although these results can contribute to the affirmation of the decrease of the inflammation and the influence of the melatonin on the repair process induced by endodontic sealers, specific studies should be done to explore the specific type of collagen and understand exactly which step of the repair process can be observed at this periods.

Some endodontic sealers, especially those that contain calcium hydroxide in their composition has the capacity of allowing calcium deposition in tissues, which can be interpreted as the beginning of a mineralization process (Bueno et al. 2016). These characteristics could also be evaluated in this study by the von Kossa staining, with the expression of calcium carbonate and Polarized light, with the identification of areas with birefringent calcium crystals (Cintra et al. 2017). Sealapex was the only sealer that had in its composition the calcium hydroxide and the other materials were not based on calcium inducers; Thus, as expected, the only sealer that promoted depositis of calcium particles in all periods was Sealapex, reveling that melatonin did not interfere on calcium deposistion (Bueno et al. 2016).

These data affirm that melatonin is a substance able to modulate the inflammatory process, decreasing the inflammatory infiltrate, the immunolabeling of cytokines, such as TNF-α and IL-6; Also, it probably accelerates the repair process and do not influence in the deposition of calcium induced by sealers. Such results are

remarkably interesting and should be more explored in dentistry, enabling the amelioration of treatment and the knowledge of clinicians and researches.

CONCLUSION

It can be concluded that melatonin modulated the inflammatory process, decreasing inflammation and the immunolabeling of IL-6 and TNF, stimulating tissue repair and did not influence the evaluation of calcium deposits.

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The authors deny any conflicts of interest related to this study.

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Legends

Table 1. Scores observed for inflammatory response of endodontic sealers and oral supplementation.

Table 2. Scores observed for the immunolabeling of Il-6 by endodontic sealers with and without the supplementation of melatonin.

Table 3. Scores observed for the expression of TNF-α by endodontic sealers with and without the supplementation of melatonin.

Table 4. Median of mature and immature fibers and mineralization capacity for each sealer in both experimental groups (Results on %)

Figure 1. Representative images from the subcutaneous tissue reactions for groups with water supplementation (A-D. E-H; I-L) and groups with melatonin supplementation (a-d. e-h, i-l) for each kind of sealer and the control tube used at 5, 15 and 30 days of analysis (A,a; E,e. I,I – Control; B,b; F,f;J,j – AH Plus; C,c; G,g;K,k – Endofill and D,d, H,h;L,l – Sealapex). At 5 days: intense inflammatory response for every sealers in both groups of analysis (A-D; a-d); At 15 days: mild inflammatory response to the group supplemented with melatonin (e-h); moderate inflammatory response to groups with water supplementation (E-H); At 30 days: mild to absent inflammatory response to the group supplemented with melatonin (i-l); moderate to mild inflammatory response to groups with water supplementation (I-L). (Hematoxylin-eosin staining, x400).

Figure 2. Immunohistochemistry images of in situ IL-6 expression for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,I), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

Figure 3. Immunohistochemistry images of in situ TNF-α expression for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,I), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (hematoxylin-eosin staining, x400).

Figure 4 – Representative images from the Picrosirius red staining to identify the maturity of the collagen fibers for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,I), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. (PSR staining, x100).

Figure 5. Representative images from Von Kossa staining for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,I), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. Structures positive for von Kossa staining can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation with omega 3 (D,d; H,h; L,l). (staining according to the von Kossa technique, x100).

Figure 6. Representative images from Polarized light for groups supplemented with water (A-D, E-H, I-L) and with melatonin (a-d, e-h, i-l) for control tube (A,a; E,e; I,I), AH Plus (B,b; F,f; J,j), Endofill (C,c; G,g; K,k) and Sealapex (D,d, H,h; L,l) at 5 days (A-D, a-d), 15 days (E-H, e-h) and 30 days (I-L, i-l) of analysis. The biregringent calcium crystals can be seen in the tissue reaction induced by Sealapex at 5, 15 and 30 days regardless of supplementation with melatonin. (D,d; H,h; L,l). (polarized light visualization, x100x).

Conclusão Geral

Conclusão geral

Considerando as limitações do presente estudo, pode-se afirmar que a suplementação com ômega-3 e a melatonina modularam a inflamação, diminuindo o infiltrado inflamatório, a imunomarcação de IL-6 e TNF- α, estimularam o reparo tecidual e não influenciaram no processo de mineralização.

Anexos

unesp **UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"**

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Influência de suplementações sistêmicas sobre a resposta tecidual de cimentos endodônticos"**, Processo FOA nº 00433-2018, sob responsabilidade de Luciano Tavares Angelo Cintra apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 14 de Agosto de 2018.

VALIDADE DESTE CERTIFICADO: 01 de Maio de 2020.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 01 de Junho de 2020.

CERTIFICATE

We certify that the study entitled **"Influence of the systemic supplementation on the influence of endodontics sealers"**, Protocol FOA nº 00433-2018, under the supervision of Luciano Tavares Angelo Cintra presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on August 14, 2018.

VALIDITY OF THIS CERTIFICATE: May 01, 2020.
DATE OF SUBMISSION OF THE FINAL REPORT: June 01, 2020.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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