

*Nathália Evelyn da Silva Machado*

**Supplementation with Omega-3 reduces the inflammatory and resorptive process, and improves the repair after delayed dental replantation in rats**

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*Nathália Evelyn da Silva Machado*

**Supplementation with Omega-3 reduces the inflammatory and resorptive process, and improves the repair after delayed dental replantation in rats**

Tese apresentada a Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para obtenção do título de Doutora em Ciências, com área de concentração em Endodontia.

Orientador:  
Profº Drº Luciano Tavares Ângelo Cintra

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*Charles Chaplin*

Machado N.E.D.S. **A suplementação com ômega-3 reduz o processo inflamatório e reabsortivo e melhora o reparo após reimplante dentário tardio em ratos.** 2023. 20f. Tese (Doutorado em Ciências, área de Endodontia) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2023.

## RESUMO

**Objetivo:** Avaliar a influência da suplementação com ácidos graxos ômega-3 no processo inflamatório, reabsortivo e reparador após reimplante dentário tardio em ratos.

**Metodologia:** Vinte animais foram suplementados com placebo para controle (C) ou ômega-3 (O). Os animais tiveram seus incisivos superiores direitos extraídos e tratados de acordo com o protocolo de reimplante tardio da Associação Internacional de Traumatologia Dentária. As suplementações foram realizadas por gavagem por 15 dias antes do reimplante até a eutanásia. Após 45 dias do reimplante, os animais foram sacrificados e as maxilas removidas e processadas para análise microtomográfica, histológica (H.E. e picrosirius red stain) e imuno-histoquímica (IL-6, TNF- $\alpha$ , RANK-L, OPG, TRAP e OCN). Os resultados foram analisados estatisticamente ( $p < 0,05$ ).

**Resultados:** No grupo O, há um infiltrado inflamatório de leve a moderada intensidade e menor percentual de reabsorção inflamatória, enquanto no grupo C a inflamação foi de moderada a severa com maior percentual de reabsorção inflamatória, a análise microCt também mostrou dentina remanescente mais espessa no grupo O ( $p < 0,05$ ). Além disso, o grupo O apresentou melhor organização do ligamento periodontal com fibras colágenas mais maduras e menor imunomarcagem para IL-6, TNF- $\alpha$  e TRAP, mais imunomarcagem para RANK-L, OPG e OCN em relação ao grupo C ( $p < 0,05$ ).

**Conclusões:** concluiu-se que a suplementação com ômega-3 reduziu o processo inflamatório e reabsortivo e melhorou o processo reparativo após reimplantação tardia de dentes em ratos.

**Palavras-chave:** ácidos graxos ômega-3, reimplante dentário, avulsão dentária.

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## **ABSTRACT**

**Aim:** To evaluate the influence of omega-3 fatty acid supplementation on the inflammatory, resorptive and reparative process after delayed tooth replantation in rats.

**Methodology:** Twenty animals were supplemented with placebo for control (C) or omega-3 (O). The animals had their right maxillary incisors extracted and treated according to the International Association of Dental Traumatology delayed replantation protocol. Supplementations were performed by gavage for 15 days before replantation until euthanasia. After 45 days from replantation, the animals were killed and the jaws removed and processed for microtomographic, histological (H.E. and picosirius red stain) and immunohistochemical (IL-6, TNF- $\alpha$ , RANK-L, OPG, TRAP and OCN) analysis. Results were statistically analyzed ( $p < .05$ ).

**Results:** In group O, there is an inflammatory infiltrate of mild to moderate intensity and a lower percentage of inflammatory resorption, while in group C the inflammation was moderately to severe with a higher percentage of inflammatory resorption, the microCt analysis also showed thicker remaining dentin in group O ( $p < 0.05$ ). Furthermore, group O showed better organization of the periodontal ligament with more mature collagen fibers and lower immunostaining to IL-6, TNF- $\alpha$  and TRAP, more immunostaining to RANK-L, OPG and OCN compared to group C ( $p < 0.05$ ).

**Conclusions:** It was concluded that omega-3 supplementation reduced the inflammatory and resorptive process and improved the reparative process after delayed replanted teeth in rats.

**Keywords:** omega-3 fatty acids, tooth replantation, tooth avulsion.

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## **Supplementation with Omega-3 reduces the inflammatory and resorptive process, and improves the repair after delayed dental replantation in rats**

### **INTRODUCTION**

Tooth avulsion is characterized by the complete displacement of the tooth from its socket (Roskamp et al., 2010; Mori et al., 2010, 2013; Najeeb et al., 2017). This displacement occurs due to the rupture of the fibres of the periodontal ligament (PDL) and the vascular-nervous bundle that nourishes the pulp (Najeeb et al., 2017). It usually happens due to an impact on the oral region caused by falls, accidents, fights, sports, among others (Mori et al., 2007, 2009; Glendor, 2008). In these cases, other oral tissues may be involved, such as bone, gum and lip (Roskamp et al., 2010). The highest occurrence involves people aged 7 to 14 years, in a ratio of 3:1 for males, with the upper incisors being the most affected dental group (Trope, 2011; Bergman et al., 2017). In addition, it is a type of dental trauma that occurs with great frequency, representing about 16 to 25% of the total trauma to permanent teeth (Martins et al., 2016).

When tooth avulsion occurs, the reposition of the tooth inside the socket is necessary, whose ultimate goal is to restore the patient's health and the physical and social well-being (Mori et al., 2010; Andersson et al., 2012). After replantation, healing of the PDL can occur by reattachment of its fibres to the root surface, when cell viability is maintained (Gullineri, 2008). PDL loss is the main cause of a local inflammatory reaction and dentoalveolar ankylosis (Campbell et al., 2005; Fuss et al., 2003). Extra-alveolar time is critical to determining cell survival (Mori et al., 2010; Roskamp et al., 2010; Andersson et al., 2012). Thus, long extra-alveolar periods eliminate any possibility of preserving PDL cell vitality (Andreasen, 1981; Pohl et al., 2005), which

makes the reattachment of PDL fibres to the root surface unfeasible. In situations where the extra-alveolar time is longer than 60 minutes, the PDL will not be expected to heal, and its removal is recommended (Andreasen 1981, Pohl et al., 2005).

Tooth resorption is the most frequent cause of replantation failure, as it leads to the progressive destruction of cement and dentin. The etiological factors of resorptions usually involve trauma, pulpal infection, and orthodontic treatment (Heboyan et al., 2022). After replantation, the alveolar portion of the PDL is separated from the cemental portion by a blood clot. The blood clot can organize as a granulation tissue and regenerate under ideal conditions of immediate replantation, or the granulation tissue may be replaced by bone tissue and start a resorption process (Andreasen, 1981; Pohl et al., 2005)

Systemic factors have been studied aiming at a better prognosis in different oral health treatments (Holland et al., 2016; Segura-Egea et al., 2022). Natural substances such as omega-3 have been used in the medical field due to their numerous properties and, in particular, modulating effect on the systemic and local inflammatory processes (de Oliveira et al., 2022). The positive effects observed with omega-3 are related to its ability to inhibit the synthesis of inflammatory mediators, alter cellular functions of polymorphonuclear leukocytes and modulate lymphocyte proliferation and cytokine production (Azuma et al., 2021). The supplementation with omega-3 fatty acid has been studied as an adjuvant therapy during treatments of oral diseases that involve inflammatory processes and bone resorption (Azuma et al., 2022).

Previous studies have demonstrated positive effects after dietary supplementation with omega-3 fatty acid on the development of endodontic infection (Azuma et al., 2017, 2018a). It was observed that supplementation not only inhibited

the action of osteoclasts leading to less bone loss but also promoted an increase in the osteoblasts counting and allowing the bone remodeling (Azuma et al., 2017). Furthermore, it was able to decrease the expression of pro-inflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, and increase the expression of anti-inflammatory mediators such as IL-10 in periapical tissues (Azuma et al., 2018b).

The pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ) are cytokines involved in the inflammatory and resorptive process of periodontal and periapical tissues (Marçal et al., 2010; Teixeira-Salum et al., 2010). Furthermore, the ratio RANK/RANK-L/OPG is close related to the osteoclast activity and consequently the bone resorption (Cosme-Silva et al., 2020). Tartrate-resistant acid phosphatase (TRAP) is a bone marker of osteoclasts, and osteocalcin (OCN) is a marker of bone formation, that is, it is responsible for the activities of osteoblasts. Thus, in order to better understand the mechanisms involved in the inflammatory process and bone turnover resulting from tooth replantation, it is important to study the behavior of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$ , as well as, RANK, OPG, TRAP and OCN (Justo et al., 2022).

Thus, the aim of the study is to evaluate the effects of omega-3 on the inflammatory response of the PDL after delayed tooth replantation, including organization of the PDL, inflammatory resorption, and dental ankylosis. The null hypothesis was that omega-3 supplementation does not reduce the inflammatory and resorptive process, and does not interfere with the reparative process after delayed replantation.

## **MATERIALS AND METHODS**

The research protocol and all experimental procedures were approved by the Animal Research Ethics Committee on Conduct and Ethics of the School of Dentistry of Araçatuba (CEUA – FOA/UNESP – Univ Estadual Paulista, Brazil; Protocol N° 00387-2020).

### **Experimental design and Distribution in groups**

Twenty male Wistar rats (*Rattus albinus*, Wistar), weighing approximately 250g were used. The animals were kept in mini-isolators (Alesco-Monte Mor, São Paulo, Brazil) with a temperature between 22 and 24°C with a controlled light cycle (12 hours of light and 12 hours of dark), they were fed throughout the experimental period with a solid diet and water “ad libitum”, except for the preoperative 12 h and the 7 days after the tooth avulsion procedure and reimplantation, when the animals were fed with crushed chow. The 20 rats were arranged into two experimental groups, containing 10 animals each group: C group - control rats supplemented with placebo; O group - rats supplemented with omega-3 fatty acids.

The rats in the O group received, by means of gavage, omega-3 at the rate of 40mg/kg (60% eicosapentaenoic acid (EPA) + 40% docosahexaenoic acid (DHA)) for 15 days before and 45 days after tooth replantation (Azuma et al., 2018b). The rats in the C group received water as placebo in the same manner.

### **Tooth avulsion simulation and Tooth replantation**

The animals were anesthetized using a sedative based on 2% xylazine (10 mg/kg, Dopaser; Laboratório Calier do Brasil Ltda. Osasco, SP, Brazil) and an anaesthetic based on 10% ketamine hydrochloride (80 mg/kg, Dopalen; AgriBrands

do Brasil Ltda. Paulínia, SP, Brazil). After anaesthesia, asepsis of the anterior portion of the maxilla was performed with 2% chlorhexidine (FGM Dental Group, Joinville, SC, Brazil), extraorally and 0.12%, intraorally (Colgate-Palmolive, Sao Paulo, SP, Brazil). Then, with the aid of adapted surgical instruments, syndesmotomy was performed, with the objective of helping to detach the periodontal fibres; dislocation to facilitate movement of the tooth in the alveolus, and finally extraction of the upper right incisor of each animal, simulating tooth avulsion.

After 60 minutes of extra-alveolar time in a dry environment, the dental papilla was removed. An access cavity was prepared via retrograde and the root canal was instrumented using pre-curved Flexofile #15, 20, and 25 (Dentsply Siron, Charlotte, North Carolina, USA) files. Intracanal irrigation was performed with 1% sodium hypochlorite (Prolink Industria Quimica, Guapiaçu, Sao Paulo, SP, Brazil). During treatment of the root surface, sterile gauze moistened with saline solution (Eurofarma Laboratorios Ltda, Sao Paulo, SP, Brazil) was used and the teeth were immersed in 2% sodium fluoride at pH 5.5 (Drogaderma, Presidente Prudente, Sao Paulo, SP, Brazil) for 20 minutes. Subsequently, the root canals were irrigated with the saline solution, dried with nº 25 sterile absorbent paper points (Dentsply Siron, Charlotte, North Carolina, USA) and filled with a 1g/mL calcium hydroxide powder (Biodinamica, Ibipora, PR, Brazil) and propylene glycol (Drogaderma, Presidente Prudente, Sao Paulo, SP, Brazil).

The tooth replantation procedure was based on previous study (Machado et al., 2020). The teeth were replanted in their respective dental socket after irrigation with the saline solution (Eurofarma Laboratorios Ltda, Sao Paulo, SP, Brazil). After reimplantation, each animal received a single dose of the antibiotic Gentamicin Sulfate (Gentatec® Agro-Veterinaria Ltda – Sao Paulo, SP, Brazil – 3.5 mg/Kg)

intramuscularly. The analgesic Dipyrone (Medley® Farmaceutica Ltda – Campinas, SP, Brazil – 150mg/kg) was administered intramuscularly every 12 hours for 3 days.

### **Obtaining and processing samples and form of analysis**

After 45 days of the replantation, the rats were euthanized with Sodium Thiopental (Thiopentax® Cristália – Produtos Químicos Farmacêuticos Ltda – Itapira, SP, Brazil- 150mg/kg) and anesthetic Lidocaine Hydrochloride 20mg/ml (Novafarma Indústria Farmacêutica Ltda – Anápolis, GO, Brazil - 10mg/Kg), both intraperitoneally. The specimens obtained were fixed in 4% neutral formaldehyde (Merck, Sao Paulo, SP, Brazil) for 24 hours and washed for 12 hours in running water. After fixation, the hemimaxillae were scanned for microtomographic analysis to quantify the extent of root resorption. The samples were scanned with the following settings: 70 kV, 167 µA, with a rotation step of 0.5° and exposure of 2100 milliseconds.

After scanning each sample, reconstruction was performed with the NRecon software (Skyscan, Belgium) and with the DATA VIEWER v. 1.5.1.2 Plans have been adjusted to better understand the area to be learned. For the three-dimensional (3D) analysis, the CTAn v.1.5.0 software (Skyscan) was used. The regions of interest (ROI) with the presence of inflammatory and/or replacement resorption were selected and quantified. The size of the region with the presence of inflammatory and/or replacement resorption was expressed in square millimeters.

Subsequently, the specimens were demineralized in a 10% EDTA solution and embedded in paraffin to obtain 5 µm thick semi-serial sections using a microtome (Leica - RM 2045). They were stained with hematoxylin and eosin and analyzed microscopically observing the following aspects: quantification of cells present per field to describe the scores regarding the present inflammatory infiltrate, organization of the

PDL, extent and quantification of inflammatory resorption and by replacement and ankylosis dental.

Analyzes were performed at 400x magnification under light microscopy (DM 4000 B, Leica, Wetzlar, Germany) by a single, calibrated, and blinded operator. The inflammatory reaction was scored according to the average number of inflammatory cells present in the middle third of root of the teeth, being: score 0, no or few cells (normal); score 1, <25 cells (mild); score 2, 25-125 cells (moderate); score 3, >125 cells (severe) (Cintra et al., 2013).

For the PDL organization analysis, the percentage of organization was considered, where: score 0, organization greater than 75%, score 1, organization greater than 50% and less than 75%; score 2, greater than 25% and less than 50%; and score 3, organization of less than 25% (Panzarini et al., 2012; Gulinelli et al., 2008; Negri et al., 2008 and collaborators).

The collagen fibers analysis was performed on PSR-stained sections under polarized light microscopy. Palatal, mesial and distal faces were analysed using QWin software (400x magnification; Leica QWin V3; Leica Microsystems), which allows selection of the corresponding colors for each type of collagen fiber. After this selection, the program automatically calculates the marked area of each type of collagen, and the yellow-green fibers are considered immature and thin, while the red-yellow fibers are considered mature and thick (Junqueira et al., 1982).

Inflammatory and/or replacement resorption and dental ankylosis were considered according to their intensity and extent, where: score 0, absent, without the presence of the mentioned events; score 1, change in an area less than 25%; score 2, change in an area greater than 25% and less than 50%; score 3, change in an area

greater than 50% (Panzarini et al., 2012; Gulinelli et al., 2008; Negri et al., 2008 and collaborators).

Immunohistochemical markers IL-6, TNF- $\alpha$ , RANK-L, OPG and OCN were evaluated according to the immunostaining pattern for recognition of scores as described by Cintra et al., 2016, where: the scores were assigned as follows: 0, complete absence of immune-reactive cells; 1 (low IR), a few immunoreactive cells and weak labeling of the extracellular matrix (approximately one quarter of the immuno-reactive cells); 2 (moderate IR), a moderate number of immunoreactive cells and moderate labeling of the extracellular matrix (approximately one half of the immunoreactive cells); and 3 (high IR), a large number of immunoreactive cells and strong labeling of the extracellular matrix (approximately three quarters of the immuno-reactive cells) (Cintra et al., 2016).

The immunohistochemical marker TRAP was evaluated considering the number of TRAP-positive cells per mm. The number of TRAP-positive cells in the perimeter of the area where resorption was present was quantified and the results were expressed in mm (Azuma et al., 2017).

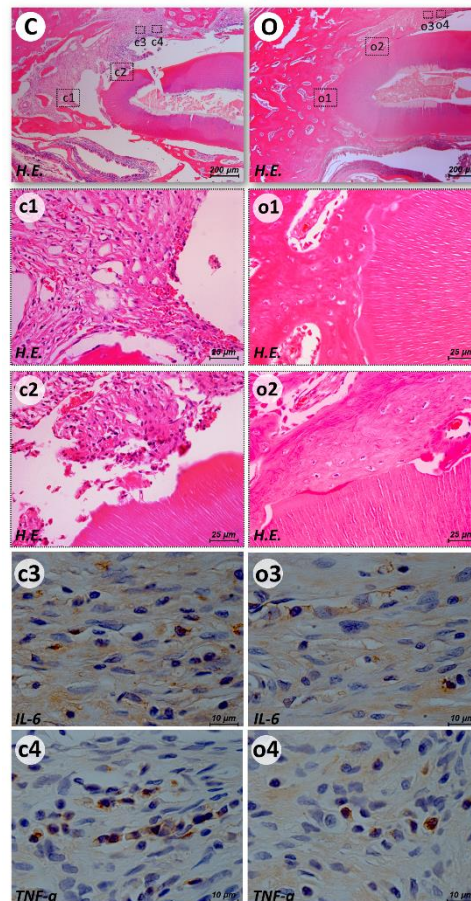
### **Statistical Analysis**

The sample size was established based on a previous study involving omega-3 supplementation (Azuma et al., 2018). We obtained a standard deviation estimate of 0.5163, the minimum detectable difference in median scores, considering an alpha error of 5% and power of 95%, obtaining a number of 9 animals per group. One more animal was included for safety in relation to the invasive surgical procedure and monitored reimplantation, totaling 10 animals per group.

Statistical analysis was performed using SigmaPlot 12.0™ software (Chicago, IL, USA). After the Shapiro–Wilk test of normality, the Mann–Whitney test was performed for nonparametric data and Student’s t-test was performed for parametric data. The level of significance was 5%.

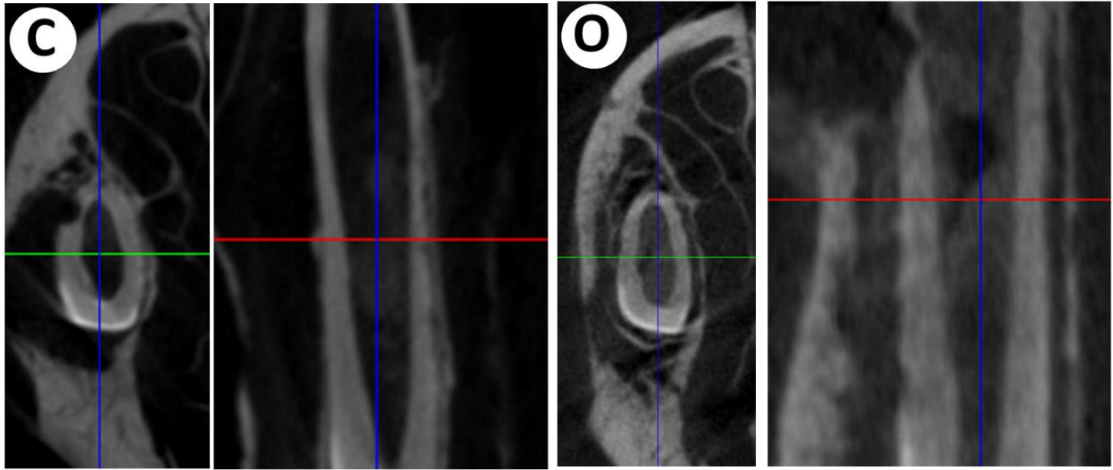
## RESULTS

Representative histological images stained in H.E. in the different experimental groups are shown in Figure 1.



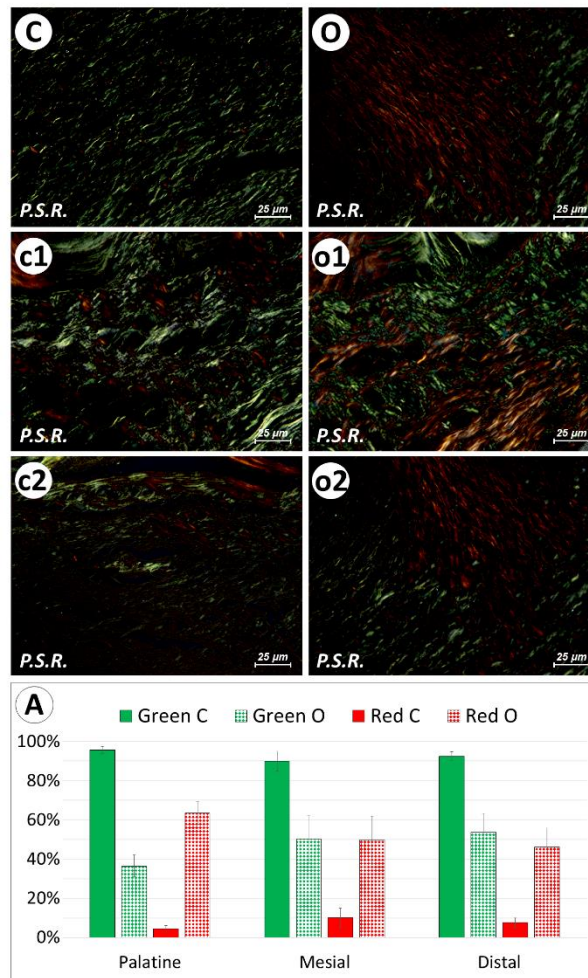
**Figure 1** - Representative images of H.E. at magnifications of 50x in the C group (C) and the O group (O), and 400x in the C group (c1, c2) and the O group (o1, o2); Immunostaining for IL-6 in the C group (c3) and the O group (o3). Immunostaining for TNF-α in the C group (c4) and the O group (o4). (x1000).

Representative MicroCT images in the different experimental groups are shown in Figure 2.



**Figure 2** - Computed microtomography ( $\mu$ CT) images of tooth resorption from each group. The C group (C) exhibited disintegration of bone structure in the upper right central incisor. And the O group (O) presents integrity of the bone structure in the upper right central incisor, where the adopted reimplantation took place.

Representative histological images stained in P.S.R. in the different experimental groups are shown in Figure 3.



**Figure 3** - Collagen fibers are shown as birefringent structures in the periodontal ligament. Yellow-green fibers indicate immature collagen and red-yellow fibers indicate mature collagen. Representative PSR staining images of groups C (C-c2) and O (O-o2) (x400x); Graph of the percentage of green and red fibers (A).

It can be seen that there were less inflammatory cells in the O group, with a prevalence of score 1, while the C group presented a prevalence of score 3 ( $p < 0.05$ ) (Fig. 1 and Table 1). The same can be observed in the quantification of inflammatory cytokines, where the immunohistochemical markers IL-6 and TNF- $\alpha$  were diminished in O group compared to C group ( $p < 0.05$ ). For IL-6, O group presented predominantly mild immunostaining pattern (score 1), while the immunostaining pattern was between scores 2 and 3 in the C group ( $p < 0.05$ ) (Fig. 1 and Table 1). For TNF- $\alpha$ , the

O group showed a mild pattern of immunostaining, with a prevalence of score 1, and the C group showed a mild pattern, characterized as score 2 ( $p < 0.05$ ) (Fig. 1 and Table 1).

In the analysis of inflammatory reabsorption, the O group had less areas of inflammatory reabsorption, characterized as a score of 1, compared to the C group that had a score of 3 ( $p < 0.05$ ) (Fig. 1 and Table 1). When observing the microtomographic analysis, it was possible to verify that the O group had a thicker remaining dentin when compared to the C group ( $p < 0.05$ ) (Fig. 2 and Table 2).

With regard to resorption by replacement and dental ankylosis, it was found that there was no significant difference between the groups ( $p > 0.05$ ). When reabsorption by substitution was observed, it was noted that it was absent in most of the specimens analyzed. In the analysis of dental ankylosis, it was possible to observe the same in C group, in O group it was possible to verify that there was half of the analyzed group with the presence of dental ankylosis, however, in low concentration, being classified as score 1, that is, less than 25% ( $p > 0.05$ ) (Fig. 1 and Table 1).

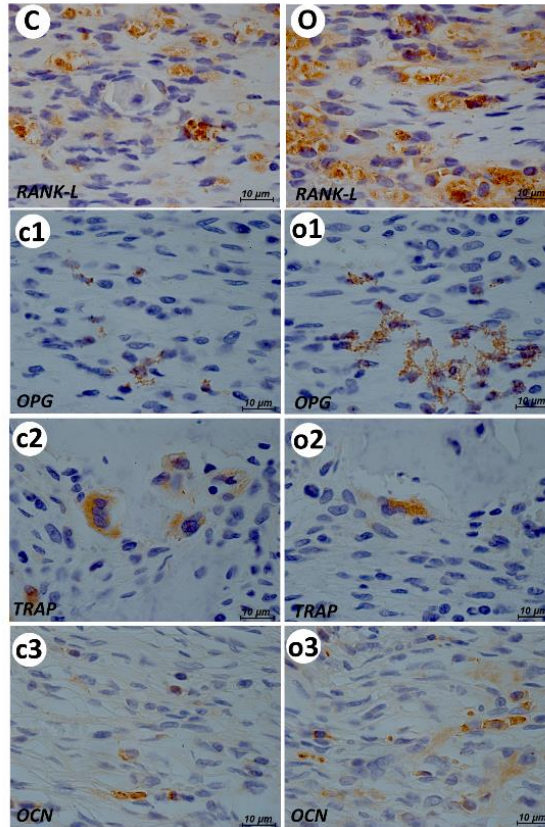
When analyzing the organization of the PDL, the O group presented more organization, characterized as score 1, while in group C, it was observed less organization, characterized as score 3 ( $p < 0.05$ ) (Fig. 1 and Table 1). The same can be observed when analyzing the percentage of collagen fibers present, where O group had a higher percentage of collagen fibers characterized as mature when compared to C group, independently of the analysed face ( $p < 0.05$ ) (Fig. 3 and Table 1).

**Table 1.** Scores, median, mean, standard deviation (SD) and p values for the inflammatory and reparative process criteria in groups C and O

Analysis Parameters			Groups		p Value
			C	O	
Inflammatory Infiltrate	0 - Absent		0/10	0/10	0.003
	1 - Mild		1/10	7/10	
	2 - Moderate		4/10	3/10	
	3 - Severe		5/10	0/10	
	Median*		2.5 <sup>a</sup>	1 <sup>b</sup>	
IL-6	0 - Absent		0/10	0/10	< 0.001
	1 - Mild		0/10	8/10	
	2 - Moderate		6/10	2/10	
	3 - Severe		4/10	0/10	
	Median*		2 <sup>a</sup>	1 <sup>b</sup>	
TNF-a	0 - Absent		0/10	2/10	0.008
	1 - Mild		2/10	6/10	
	2 - Moderate		7/10	2/10	
	3 - Severe		1/10	0/10	
	Median*		2 <sup>a</sup>	1 <sup>b</sup>	
Inflammatory resorption	0 - Absent		0/10	1/10	0.006
	1 - <25%		2/10	6/10	
	2 - >25% and <50%		2/10	3/10	
	3 - >50%		6/10	0/10	
	Median*		3 <sup>a</sup>	1 <sup>b</sup>	
MicroCt Analysis	(mean ± SD)		6.875 ± 0.606 <sup>a</sup>	8.438 ± 0.886 <sup>b</sup>	< 0.001
Replacement resorption	0 - Absent		5/10	6/10	0.769
	1 - <25%		3/10	2/10	
	2 - >25% and <50%		2/10	2/10	
	3 - >50%		0/10	0/10	
	Median*		0.5 <sup>a</sup>	0 <sup>a</sup>	
Ankylosis	0 - Absent		7/10	4/10	0.269
	1 - <25%		2/10	5/10	
	2 - >25% and <50%		1/10	1/10	
	3 - >50%		0/10	0/10	
	Median*		0 <sup>a</sup>	1 <sup>a</sup>	
Periodontal Ligament Organization	0 - >75%		0/10	0/10	0.001
	1 - >50% and <75%		1/10	8/10	
	2 - >25% and <50%		4/10	2/10	
	3 - <25%		5/10	0/10	
	Median*		2.5 <sup>a</sup>	1 <sup>b</sup>	
Collagen maturation (%± SD)	Mature fibres	Palatine	4.42 ± 1.72 <sup>a</sup>	63.53 ± 5.64 <sup>b</sup>	< 0.001
		Mesial	10.15 ± 4.95 <sup>a</sup>	49.84 ± 12.17 <sup>b</sup>	< 0.001
		Distal	7.60 ± 2.34 <sup>a</sup>	46.23 ± 9.59 <sup>b</sup>	< 0.001
	Immature fibres	Palatine	95.58 ± 1.72 <sup>a</sup>	36.47 ± 5.64 <sup>b</sup>	< 0.001
		Mesial	89.84 ± 4.95 <sup>a</sup>	50.15 ± 12.17 <sup>b</sup>	< 0.001
		Distal	92.39 ± 2.34 <sup>a</sup>	53.77 ± 9.59 <sup>b</sup>	< 0.001

\*Different superscript letters in rows indicate statistically significant difference (p &lt; .05).

Representative immunohistochemistry images for RANK-L, OPG, TRAP and OCN in the different experimental groups are shown in Figure 4.



**Figure 4** - Representative images of immunostaining for RANK-L in C group (C) and O group (O). Representative images of immunostaining for OPG in C group (c1) and O group (o1) (x1000). Immunoreactivity of multinucleated TRAP-positive cells in C group (c2) and O group (o2), and OCN-positive cells in C group (c3) and O group (o3) on the alveolar bone surface after reimplantation (x1000).

In the analysis of immunostaining for RANK-L and OPG, it was observed that there was a greater amount of them in group O when compared to group C ( $p < 0.05$ ). For RANK-L, the O group presented a predominantly severe immunostaining pattern, score 3 and the C group presented a predominantly moderate immunostaining pattern, score 2 ( $p < 0.05$ ) (Fig. 4 and Table 2).

In addition, O group had a higher density of immunostaining for OCN compared to O group, with a higher mean number of cells present, and lower for TRAP compared to C group, which had fewer labelled cells ( $p < 0.05$ ) (Fig. 4 and Table 2).

**Table 2.** Scores, median, mean, standard deviation (SD) and p values for the resorptive process criteria in groups C and O

Analysis Parameters	Groups		p Value	
	C	O		
RANKL	0 - Absent	0/10	0/10	0.031
	1 - Mild	2/10	1/10	
	2 - Moderate	6/10	3/10	
	3 - Severe	2/10	5/10	
	Median*	2 <sup>a</sup>	3 <sup>b</sup>	
OPG	0 - Absent	4/10	1/10	0.012
	1 - Mild	3/10	3/10	
	2 - Moderate	2/10	4/10	
	3 - Severe	1/10	2/10	
	Median*	0 <sup>a</sup>	2 <sup>b</sup>	
TRAP (mean ± SD)	2.35 ± 0.72 <sup>a</sup>	0.69 ± 0.63 <sup>b</sup>	< 0.001	
OCN (mean ± SD)	0/10	0/10	0.015	
	5/10	2/10		
	3/10	5/10		
	2/10	3/10		
	1 <sup>a</sup>	2 <sup>b</sup>		

\*Different superscript letters in rows indicate statistically significant difference ( $p < .05$ ).

## DISCUSSION

This study investigated the influence of omega-3 fatty acid supplementation on the repair process after delayed tooth replantation. The results of the present study identified the positive influence of omega-3 fatty acid supplementation on the tissue repair process after delayed tooth replantation, since the animals supplemented with omega-3 had less inflammatory infiltrate, greater organization of the PDL and less superficial inflammatory resorption when compared to control group rats ( $p < 0.05$ ). This way the null hypothesis was rejected.

Inflammation is a coherent and orderly progression of events initiated and controlled by endogenous chemical mediators. That is, it is a response to infection or tissue injury that occurs to eradicate microorganisms or irritating agents and to enhance tissue repair.

Omega-3 has been accepted as an adjunctive therapy in the treatment of oral diseases such as gingivitis, periodontitis and stomatitis recurrent aphthous disease (Azuma et al., 2018), since its polyunsaturated fatty acids play an important role in the inflammatory response, reducing pro-inflammatory mediators such as arachidonic acid (Azuma et al., 2021). Considering that delayed tooth replantation can lead to an inflammatory process, resulting in root resorption and ankylosis, it was proposed to evaluate the anti-inflammatory potential of omega-3 in the repair process after delayed replantation.

Regarding the inflammatory infiltrate, at the end of 45 days from the replantation, it was possible to observe that the group supplemented with omega-3 presented, for the most part, a mild infiltrate, while the control group presented a severe infiltrate, demonstrating a significant reduction in inflammation. This can be explained by the modulator effect of omega-3, since it inhibits the production of arachidonic acid metabolites through the cyclooxygenase 2 and lipoxygenase pathways, thus reducing the inflammatory response (Patterson and Georgel, 2004).

Regarding the organization of the PDL, the group supplemented with omega-3 showed better organization when compared to the control, which can be explained by the increased expression of MMPs-1 and TIMP- by the therapeutic diet with omega-3 fatty acids (Nicolai et al., 2017). MMPs are molecules that act on extracellular matrix degradation and are present in periodontal diseases. An imbalance between MMPs and TIMPs can lead to disorganization of periodontal tissues (Azuma et al., 2020), and this has a link when looking at supplementation in cases of delayed tooth replantation, as the processes of bone resorption are interconnected.

In the analysis of IL-6, O group presented lower scores when analyzing this marker in relation to C group ( $p < 0.05$ ). This can be explained because IL-6 is a

cytokine produced in response to infection and tissue injury, eager for host defense by stimulated acute phase responses, hematopoiesis and immune reactions (Azuma et al., 2014). And an ongoing unregulated synthesis of IL-6 exerts an exacerbating effect on chronic inflammation (Tanaka et al., 2014). Regarding TNF- $\alpha$ , it was possible to verify that it has lowered scored in the O group compared to the C group ( $p < 0.05$ ). In the periapical lesions, there is a considerable increase in TNF- $\alpha$  in the tissues surrounding the tooth, and it can be assumed that this cytokine can induce local osteoclastic activity (Colić et al., 2009).

Regarding inflammatory resorption, group O had a higher percentage (score 1) than group C (score 3) ( $p < 0.05$ ). Through micro-CT, it was also possible to verify less bone damage in group O ( $p < 0.05$ ). Inflammatory resorption is a complex event, initiated by inflammation that leads to progressive destruction of cementum and subsequently dentin, increasing osteoclast activity, leading to resorption (Heboyan et al., 2022). Omega-3 supplementation has been shown to inhibit osteoclast action and increase osteoblast activity, leading to bone remodeling (Azuma et al., 2017), which may explain the lower percentage of inflammation resorption in the O.

The parameters reabsorption by substitution and ankylosis did not differ between groups ( $p > 0.05$ ). In the analysis of resorption by substitution, it was noted that it was absent in most cases. As for dental ankylosis, it was possible to observe the same result when observing C group, however, in O group it was possible to verify that there was half of the group analyzed with the presence of dental ankylosis in low concentration. Resorption by replacement consists of replacing the tooth structure with bone tissue, that is, the bone and tooth will be reabsorbed and neoformed, without the presence of cementum and dentin, which will also be replaced by bone. Dental anemia, on the other hand, is considered as the late result of replacement resorption, as it

consists of integrating the tooth in the bone remodeling process. It usually occurs due to the loss or deficiency of the PDL that promotes the disappearance of the epithelial remains of Mallassez, with this, the neighboring bone cells together with the fibroblasts and cementoblasts carry out a periodontal reconstruction, which, without the epithelial remains of Mallassez, generated a new bone formation in the PDL space and its union with the tooth (Consolaro, 2010). This can be explained by the modulatory effect and broad organization of PDL in the group supplemented with omega-3 (Heboyan et al., 2022).

In the analysis of immunostaining for RANK-L and OPG, it was observed that there was a greater amount of them in O group when compared to C group ( $p < 0.05$ ). The RANK-L/RANK/OPG system is triggered when the RANK-L ligand binds to RANK or OPG. Its binding to RANK in the cell wall of osteoclasts leads to activation of these cells and consequently triggers bone resorption. When an OPG binds to RANK-L, consequently, osteoclasts are not activated and bone resorption is inhibited.

In the present study, the number of cells TRAP positive was smaller in the group supplemented with omega-3, which may be related to lower osteoclastic activities in the O group. TRAP is an osteoclast marker, and the same is present in bone resorption events, where its main function is to perform bone matrix degradation (Dal-Fabbro et al., 2021). On the other hand, OCN is a marker of bone formation involved in the control of the process of regulating bone identity and responsible for stimulating the activity of osteoblasts, which are cells related to bone formation. In this study, the number of cells OCN positive were smaller in the O group, which corroborates the results found in previous analyses, favoring the prophylactic and therapeutic use of omega-3.

## CONCLUSION

Therefore, we can conclude that omega-3 fatty acid supplementation can influence the repair process after delayed tooth replantation, reducing the inflammatory infiltrate, increasing the organization of the PDL, reducing inflammatory resorption and increasing the level of bone repair in the region. This type of evidence contributes to the use of this substance in dentistry, in cases of delayed tooth replantation.

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## ANEXO A



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 "**Suplementação com ácido graxo ômega-3 influencia no processo de reparo após replante dentário tardio**", Processo FOA nº 00387-2020, 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 02 de Outubro de 2020.

**VALIDADE DESTE CERTIFICADO:** 30 de Julho de 2021.

**DATA DA SUBMISSÃO DO RELATÓRIO FINAL:** até 30 de Agosto de 2021.

## CERTIFICATE

We certify that the study entitled "**Supplementation with omega-3 fatty acid influences the repair process after late dental replantation**", Protocol FOA nº 00387-2020, 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 October 02, 2020.

**VALIDITY OF THIS CERTIFICATE:** July 30, 2021.

**DATE OF SUBMISSION OF THE FINAL REPORT:** August 30, 2021.

Prof. Associado Guilherme de Paula Nogueira  
Coordenador da CEUA  
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais  
Faculdade de Odontologia de Araçatuba  
Faculdade de Medicina Veterinária de Araçatuba  
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP  
Fone (18) 3636-3234 Email CEUA: ceua.foa@unesp.br

## ANEXO B

### 1. Submission and Peer Review Process

Once the submission materials have been prepared in accordance with the Author Guidelines, manuscripts should be submitted online at <https://wiley.atyponrex.com/journal/IEJ>.

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  - data availability statement
  - funding statement
  - conflict of interest disclosure
  - ethics approval statement and document
  - patient consent statement
  - relevant reporting guidelines paperwork (see 5.2 Reporting Guidelines below)
  - permission to reproduce material from other sources
  - clinical trial registration
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- Remove the keywords from the abstract they should be on the title page only;
- Put a page break between the abstract and the introduction;
- Remove the numbering of the sections in the main document;

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**The title page should contain:**

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- ii. A short running title of less than 30 characters;
- iii. The full names of the authors;
- iv. The author's institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;
- v. Acknowledgements.

**Important: the journal operates a double-blind peer review policy. Please anonymise your manuscript and prepare a separate title page containing author details.**

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Manuscripts can be uploaded either as a single document (containing the main text, tables and figures), or with figures and tables provided as separate files. Should your manuscript reach revision stage, figures and tables must be provided as separate files. The main manuscript file can be submitted in Microsoft Word (.doc or .docx) or LaTeX (.tex) format.

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It is the policy of the journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

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For all articles, the journal mandates the CRediT (Contribution Roles Taxonomy)—more information is available on our [Author Services](#) site.

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These must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original Articles must conform to the highest international standards in the field.

**Review Articles (systematic, scoping and narrative):**

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**Clinical Commentaries:**

These can describe significant improvements in clinical practice such as the report of a novel technique, a breakthrough in technology or practical approaches to recognised clinical challenges. They should conform to the highest scientific and clinical practice standards.

**Case Reports:**

These can illustrate unusual and clinically relevant and new observations, but they must be of sufficiently high quality to be considered worthy of publication in the journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered. Illustrative material must be of the highest quality and healing outcomes, if appropriate, should be demonstrated after an extended period – normally four years.

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The *International Endodontic Journal* encourages submission of adjuncts to printed papers via the supporting information website (see submission of supporting information below). Authors wishing to describe novel procedures or illustrate cases more fully with figures and/or video may be encouraged to utilise this facility.

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### **Systematic, Scoping and Narrative Review Articles**

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Structured: Background, Objectives, Method, Results, Conclusion, Funding, Conflict of interest, Registration

No more than 350 words.

### **Case Reports and Clinical Commentaries**

Main text includes: Introduction, report, discussion and conclusion

Structured: Aim, summary, key learning points

Supporting information, such as data sets or supplemental figures or tables, that will not be published in the print edition of the journal, but which will be viewable via the online edition, can be submitted. It should be clearly stated at the time of submission that the supporting information is intended to be made available through the online edition.

### **Supporting Information**

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## **2.1 Reporting Guidelines**

### **2.1.1 Case reports/case series**

Case reports should be written to comply with the Preferred Reporting Items for Case reports in Endodontics (PRICE) 2020 guidelines (Nagendrababu et al. 2020, doi:10.1111/iej.13285).

When submitting manuscripts that have been written using the PRICE 2020 guidelines, authors should include the following statement in the beginning of "Report" section: "This case report has been written according to Preferred Reporting Items for Case reports in Endodontics (PRICE) 2020 guidelines.

A PRICE checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material. The PRICE [2020 checklist](#) and [flowchart](#) can be viewed by clicking the appropriate link. PRICE guidelines can be found [here](#), and PRAISE checklist link [here](#).

## 2.1 Reporting Guidelines

### 2.1.1 Case reports/case series

Case reports should be written to comply with the Preferred Reporting Items for Case reports in Endodontics (PRICE) 2020 guidelines (Nagendrababu et al. 2020, doi:10.1111/iej.13285).

When submitting manuscripts that have been written using the PRICE 2020 guidelines, authors should include the following statement in the beginning of "Report" section: "This case report has been written according to Preferred Reporting Items for Case reports in Endodontics (PRICE) 2020 guidelines.

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It is recommended that authors consult the following papers when writing case reports, which explains the rationale for the PRICE 2020 guidelines and their importance:

Nagendrababu V, Chong BS, McCabe P, Shah PK, Priya E, Jayaraman J, Pulikkotil SJ, Setzer FC, Sunde PT, Dummer PMH (2020) PRICE 2020 guidelines for reporting case reports in Endodontics: a consensus-based development. *International Endodontic Journal* 53, 619-26.

(<https://www.ncbi.nlm.nih.gov/pubmed/32090342>)

Nagendrababu V, Chong BS, McCabe P, Shah PK, Priya E, Jayaraman J, Pulikkotil SJ, Dummer PMH (2020) PRICE 2020 guidelines for reporting case reports in Endodontics: Explanation and elaboration. *International Endodontic Journal* 53, 922-47.

(<https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13300>)

### 2.2.2. Randomised clinical trials

Randomised clinical trials should be reported to comply with the Preferred Reporting Items for Randomised Trials in Endodontics (PRIRATE) 2020 guidelines (Nagendrababu et al. 2020, doi: 10.1111/iej.13294).

When submitting manuscripts that have been written using the PRIRATE 2020 guidelines, authors should include the following statement in the beginning of "Materials and Methods" section: "This randomised clinical trial has been written according to Preferred Reporting Items for RAndomised Trials in Endodontics (PRIRATE) 2020 guidelines.

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Nagendrababu V, Duncan HF, Bjørndal L, Kvist T, Priya E, Jayaraman J, Pulikkotil SJ, Pigg M, Rechenberg DK, Vaeth M, Dummer PMH (2020) PRIRATE 2020 guidelines for reporting randomised trials in Endodontics: a consensus-based development. *International Endodontic Journal* 53, 764-73.

(<https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13294>)

Nagendrababu V, Duncan HF, Bjørndal L, Kvist T, Priya E, Jayaraman J, Pulikkotil SJ, Dummer PMH (2020) PRIRATE 2020 guidelines for reporting trials in Endodontics: Explanation and elaboration. *International Endodontic Journal* 53, 774-03.

(<https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13304>)

## Clinical Trials

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### 2.2.3. Epidemiological observational trials

Observational studies should be written using the STrengthening the Reporting of OBServational studies in Epidemiology' (STROBE) guidelines. Compliance with this should be detailed in the "Materials and Methods" section. ([www.strobe-statement.org](http://www.strobe-statement.org)). A STROBE checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material.

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Von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP, Strobe Initiative (2014) The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies. *International Journal of Surgery* 12, 1495-9.

Vandenbroucke JP, von Elm E, Altman DG, Altman DG, Gøtzsche PC, Mulrow CD, Pocock SJ, Poole C, Schlesselman JJ, Egger M, STROBE Initiative. (2014) Strengthening the reporting of observational studies in epidemiology (STROBE): explanation and elaboration. *International Journal of Surgery* 12, 1500–24

### 2.2.4. Diagnostic accuracy studies

Diagnostic accuracy studies should be written using the Standards for Reporting of Diagnostic Accuracy Studies (STARD) 2015 guidelines. Compliance with this should be detailed in the "Materials and Methods" section. A STARD checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material. The STARD checklist and flowchart can be downloaded from: <https://www.equator-network.org/reporting-guidelines/stard/>

It is recommended that authors consult the following papers when writing manuscripts, which explain the rationale for the STARD guidelines and their importance:

Cohen JF, Korevaar DA, Altman DG, Bruns DE, Gatsonis CA, Hooft L, Irwig L, Levine D, Reitsma JB, de Vet HCW, Bossuyt PMM. (2016) STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. *BMJ Open* 6, e012799

<http://bmjopen.bmj.com/content/6/11/e012799.abstract>

### 2.2.5. Animal studies

Animal studies should be written using the Preferred Reporting Items for Animal Studies in Endodontology (PRIASE) 2021 guidelines (Nagendrababu et al. 2021, doi: 10.1111/iej.13477).

When submitting manuscripts that have been written using the PRIASE 2021 guidelines, authors should include the following statement in the beginning of "Materials and Methods" section: "The manuscript of this animal study has been written according to Preferred Reporting Items for Animal studies in Endodontology (PRIASE) 2021 guidelines.

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Nagendrababu V, Kishen A, Murray PE, Nekoofar MH, de Figueiredo JA, Priya E, Jayaraman J, Pulikkotil SJ, Camilleri J, RM S, Dummer PMH (2021) PRIASE 2021 guidelines for reporting animal studies in Endodontology: a consensus-based development. *International Endodontic Journal* 54, 848-57. (<https://onlinelibrary.wiley.com/doi/10.1111/iej.13477>)

Nagendrababu V, Kishen A, Murray PE, Nekoofar MH, de Figueiredo JA, Priya E, Jayaraman J, Pulikkotil SJ, Jakovljevic A, Dummer PMH (2021) PRIASE 2021 guidelines for reporting animal studies in Endodontology: Explanation and Elaboration. *International Endodontic Journal* 54, 858-86. (<https://onlinelibrary.wiley.com/doi/10.1111/iej.13481>)

### 2.2.6. Laboratory studies

Laboratory studies should be reported using the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al. 2021, doi: 10.1111/iej.13542).

When submitting manuscripts that have been written using the PRILE 2021 guidelines, authors should include the following statement in the beginning of “Materials and Methods” section: “The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines.

A PRILE checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material. The PRILE 2021 checklist and flowchart can be downloaded from: <http://pride-endodonticguidelines.org/prile/>

It is recommended that authors consult the following papers when writing manuscripts, which explain the rationale for the PRILE 2021 guidelines and their importance:

Nagendrababu V, Murray PE, Ordinola-Zapata R, OA Peters, IN Rôças, JF Siqueira Jr, E Priya, J Jayaraman, SJ Pulikkotil, J Camilleri, C Boutsoukis, G Rossi-Fedele, PMH Dummer (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontics: a consensus-based development. *International Endodontic Journal* May 3. doi: 10.1111/iej.13542. (<https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13542>)

Nagendrababu V, Murray PE, Ordinola-Zapata R, OA Peters, IN Rôças, JF Siqueira Jr, E Priya, J Jayaraman, SJ Pulikkotil, N Suresh, PMH Dummer (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontics: Explanation and elaboration. *International Endodontic Journal* (<https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13565>)

### 2.2.7 Systematic reviews

The abstract and main body of the systematic review should be reported using the PRISMA for Abstract and PRISMA guidelines respectively (<http://www.prisma-statement.org/>). Authors submitting a systematic review must register the protocol in one of the readily-accessible sources/databases at the time of project inception and not retrospectively (e.g. PROSPERO database, OSF registries). The protocol registration number, name of the database or journal reference should be provided at the submission stage in the “Registration” section in the abstract and ‘Methods’ section in the main body of the text.

A PRISMA checklist and flow diagram as a Figure (to be included in the manuscript for readers) should also be included in the submission material. Source of funding (grant number, if available) should be added in the ‘Acknowledgements’ section.

It is recommended that authors consult the following papers, which help in the production of high quality reviews:

Moher D, Liberati A, Tetzlaff J, Altman DG, PRISMA Group (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLOS Medicine* 6, e1000097.

Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D (2009) The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *Journal of Clinical Epidemiology* 62, e1-34.

Nagendrababu V, Duncan HF, Tsesis I, Sathorn C, Pulikkotil SJ, Dharmarajan L, Dummer PMH (2019) PRISMA for abstracts: best practice for reporting abstracts of systematic reviews in Endodontology. *International Endodontic Journal* 52, 1096-1107. (<https://onlinelibrary.wiley.com/doi/10.1111/iej.13118>)

Nagendrababu V, Dilokthornsakul P, Jinatongthai P, Veettil SK, Pulikkotil SJ, Duncan HF, Dummer PMH (2020) Glossary for systematic reviews and meta-analyses. *International Endodontic Journal* 53, 232-249. (<https://onlinelibrary.wiley.com/doi/full/10.1111/iej.13217>)

### 2.2.8 Scoping reviews

Reviews should be reported using the PRISMA guidelines. A checklist for scoping reviews should also be included in the submission material - see: <http://www.prisma-statement.org/Extensions/ScopingReviews>.

### 2.2.9 Guidelines for reporting of microarray and next-generation sequencing data

Submission will be assessed according to MIAME and MINSEQE standards. The complete current guidelines are available at

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### Appendix

#### Abbreviations:

The International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

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### Examples of correct forms of reference

#### Standard journal article

Jakovljevic, A., Duncan, H.F., Nagendrababu, V., Jacimovic, J., Milasin, J. & Dummer, P.M.H. (2020) Association between cardiovascular diseases and apical periodontitis: an umbrella review. *International Endodontic Journal*, 53, 1374–1386.

Selman, P. (2016) The global decline of intercountry adoption: what lies ahead? *Social Policy and Society*, 11(3), 381–397.

#### Corporate author

British Endodontic Society (1983) Guidelines for root canal treatment. *International Endodontic Journal* 16, 192-5.

Department of Health. (2009) Living well with dementia: a national dementia strategy.

#### Journal supplement

Frumin AM, Nussbaum J, Esposito M (1979) Functional asplenia: demonstration of splenic activity by bone marrow scan (Abstract). *Blood* 54 (Suppl. 1), 26a.

Holding, M.Y., Saulino, M.F., Overton, E.A., Kornbluth, I.D. & Freedman, M.K. (2008) Interventions in chronic pain management. 1. Update on important definitions in pain management. *Archives of Physical Medicine and Rehabilitation*, 89 (3, Supplement 1), S38–S40.

#### Books and other monographs

##### Personal author(s)

Gutmann J, Harrison JW (1991) *Surgical Endodontics*, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Barnes, R. (1995) *Successful study for degrees*, 2nd edition, London: Routledge.

**Chapter in a book**

Wesselink P (1990) Conventional root-canal therapy III: root filling. In: Harty FJ, ed. *Endodontics in Clinical Practice*, 3rd edn; pp. 186-223. London, UK: Butterworth.

Partridge, H. & Hallam, G. (2007) Evidence-based practice and information literacy. In: Lipu, S., Williamson, K. & Lloyd, A. (Eds.) *Exploring methods in information literacy research*. Wagga Wagga, Australia: Centre for Information Studies, pp. 149–170.

**Published proceedings paper**

DuPont B (1974) Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. *Proceedings of the Third Annual Meeting of the International Society for Experimental Rematology*; pp. 44-46. Houston, TX, USA: International Society for Experimental Hematology.

Wittke, M. (2006) Design, construction, supervision and long-term behaviour of tunnels in swelling rock. In: Van Cotthem, A., Charlier, R., Thimus, J.-F. and Tshibangu, J.-P. (Eds.) *Eurock 2006: multiphysics coupling and long term behaviour in rock mechanics: proceedings of the international symposium of the international society for rock mechanics, EUROCK 2006, 9–12 May 2006, Liège, Belgium*. London: Taylor & Francis, pp. 211–216.

**Agency publication**

Ranofsky AL (1978) *Surgical Operations in Short-Stay Hospitals: United States-1975*. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8.

Wittke, M. (2006) Design, construction, supervision and long-term behaviour of tunnels in swelling rock. In: Van Cotthem, A., Charlier, R., Thimus, J.-F. and Tshibangu, J.-P. (Eds.) *Eurock 2006: multiphysics coupling and long term behaviour in rock mechanics: proceedings of the international symposium of the international society for rock mechanics, EUROCK 2006, 9–12 May 2006, Liège, Belgium*. London: Taylor & Francis, pp. 211–216.

**Dissertation or thesis**

Saunders EM (1988) *In vitro and in vivo investigations into root-canal obturation using thermally softened gutta-percha techniques* (PhD Thesis). Dundee, UK: University of Dundee.

The University Encyclopedia (1985) London: Roydon.

**URLs**

Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text.

Smith A (1999) Select committee report into social care in the community [WWW document]. URL <http://www.dhss.gov.uk/reports/report015285.html> [accessed on 7 November 2003].

European Space Agency. (2015) Rosetta: rendezvous with a comet. Available at: <http://rosetta.esa.int> [Accessed 15th June 2015].