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Evaluation of the effects of melatonin and vitamin D on wound healing in immunosuppressive rats following tooth extraction

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SUMMARY

Background. The aim of this study was to evaluate the effects of melatonin (MLT) and paricalcitol (PRC) on the healing of the extraction socket following tooth extraction in rats with cyclosporine-A (CsA).

Material and methods. 76 male Wistar rats were divided into five different groups, one of which was a control (C) group. All groups other than the control group were applied CsA (10 mg/kg/daily) intraperitoneally 7 days before the tooth extraction. The left upper incisors were extracted on day 8 and CsA injections were continued in all groups until sacrification. Starting from the day of tooth extraction, in group 1 (CsA) were given CsA, in group 2 (CsA+MLT) were applied intraperitoneal MLT injection in a dose of 4 mg/kg, in group 3 (CsA+PRC) were applied intraperitoneal PRC injection in a dose of 200 ng/kg, and in group 4 (CsA+MLT+PRC) were applied intraperitoneal PRC injection and intraperitoneal MLT until the day of sacrification of all groups. The subjects were sacrificed on day 7 and 14 following tooth extraction. For histopathological examination, the subjects stained Hemotoxylin-Eosin and evaluated histologically under the light microscope. The intensity of inflammation in extraction socket was scored based on a four-grade system.

Results. The level of inflammation was found to be lower in group C on day 7, while the inflammation value was found to be higher in the group 1 on day 14 ($p>0.05$). Statistically significant differences were found in the ossification values on day 7 between the groups($p<0.05$). The percentage of ossification on day 7 was significantly lower in the group 1 than in the C and group 3, and significantly lower in the group 2 than in the group 3. The percentage of ossification on day 14 in the group 1 was significantly lower than in group C ($p<0.05$).

Conclusions. CsA has a negative effect on bone healing. The application of MLT and PRC following the toxicity produced by CsA was found to positively affect the healing of the socket that develops after tooth extraction.

Keywords: cyclosporine, melatonin, paricalcitol, rat, tooth extraction.

INTRODUCTION

The toxicity associated with cyclosporine-A (CsA) has been shown in some studies to be decreased by the use of antioxidative substances (1-3). There have also been studies on the application of various antioxidative substances with the aim of stimulating bone healing (4, 5). Antioxidants have recently been suggested to limit the oxidative damage caused by the reactive oxygen radicals released secondary to CsA use. For this purpose, the natural immune systems of the organism or pharmacological agents with antioxidative properties have been used to strengthen the endogenous immune system. Melatonin (MLT) is among the agents classified as exogenous immune systems (6).

MLT is soluble in both liquid and lipid phases, and since there is no known morphophysiological barrier against MLT, it has easy access to all intracellular components, thus efficiently protecting

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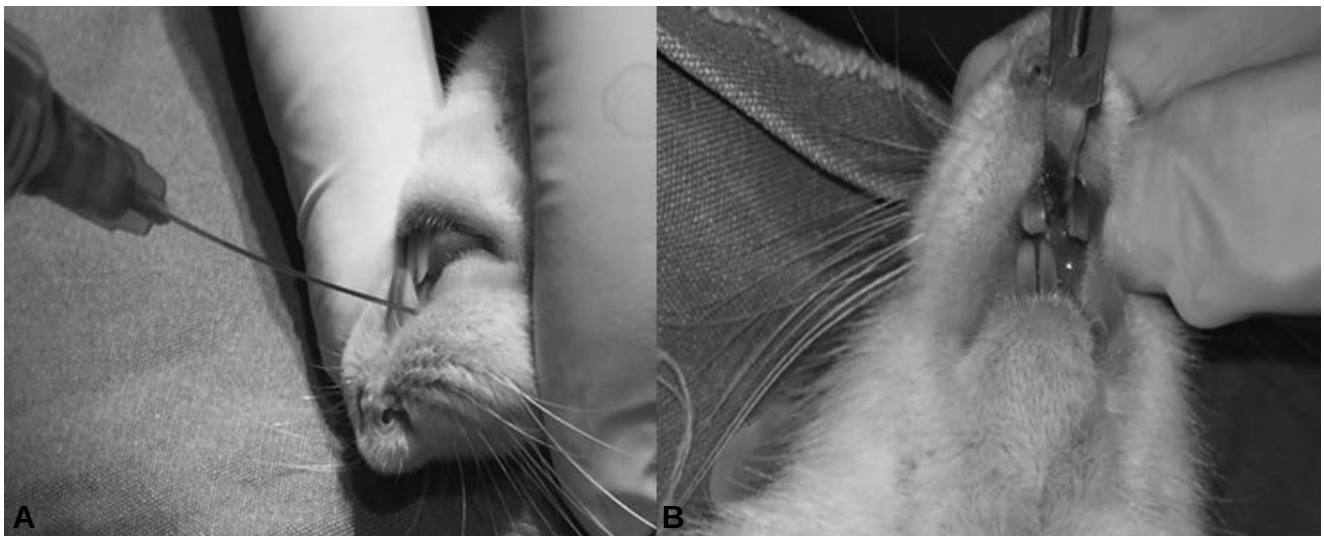

Fig. 1. A – dental anesthesia application; B – tooth extraction.

the cell membrane, organelles and nucleus from free radical damage. MLT attaches to the external surface of the phospholipid layer when it comes into contact with the cell membrane, and thus comes into contact with free radicals prior to the membrane, detoxifies them and protects the membrane. An advantage of MLT is its ability to reach the cell nucleus and thus protect DNA from oxidative damage (7), and additionally, no toxic effect of MLT has been reported, even in very high doses and long-term use (300 mg/day) (8).

Positive results have been obtained from studies investigating the possible direct anticancerogenic effects of MLT, and from the studies of the possible effects of the supportive properties of MLT on the immune system and its anticancerogenic effects (9).

In addition to the classical effects of calcitriol (1, 25 dihydroxy vitamin D3), the active metabolite of Vitamin D on the calcium metabolism, its positive effects on the endothelial functions, and its immunomodulator and anti-tumor efficacy have been proven in many studies (10, 11). All these properties, although not the same in all vitamin D analogs, are similar, and this effect of vitamin D on the immune system may explain the association between autoimmune disease and vitamin D deficiency (12).

Paricalcitol (PRC) is a substance that exerts vitamin D-like effects without causing hypercalcemia. It stimulates the vitamin D receptors found in all organs and exerts pleiotropic and antioxidant effects (13). In the study of Ari *et al.*, the protective effect of PRK on rats with contrast nephropathy (CIN) was demonstrated (14).

PRC has been used as an MLT and vitamin D analog in studies into the removal of free radicals produced in the organism (15).

The present study evaluates the possible protective effects of MLT and PRC, have been reported to have antioxidant properties, on the healing of extraction sockets in CsA applied rats.

MATERIALS AND METHODS

Experimental Model

A total of 76 Wistar male rats weighing 270–410 gr were acquired after ethics board approval for the study was obtained. The rats were divided into five different groups, one of which was a control group. A tooth extraction of the rats was performed on the start day of the study in group C, and on Day 8 after the application of CsA for 1 week in the test groups.

CsA (Sandimmun, Novartis) was applied as an intraperitoneal injection in the rats in the experimental groups starting on day 1 of the study in a dose of 10 mg/kg/day. After the tooth extraction on day 8 (Fig. 1), CsA applications were continued until the day of sacrification.

CsA application was continued until the day of sacrification at a dose of 10 mg/kg/day following tooth extraction in test group 1 (CsA).

The rats in the test group 2 (CsA+MLT) were applied an intraperitoneal MLT injection in a dose of 4mg/kg starting from the day of tooth extraction until the day of sacrification. A solution was prepared following the solubilization of MLT in pure alcohol by adding distilled water at a ratio of 1/10 after making sure that no particles remained. The rats in this group were kept in a dark environment during the day and in a luminous environment at night. All injections were performed at the same time each day (between 14:00–16:00 hours).

The rats in the test group 3 (CsA+PRC) were applied PRC intraperitoneally in a dose of 200 mg/kg starting on the day of tooth extraction until the day of sacrification.

The rats in the test group 4 (CsA+MLT+PRC) were applied PRC intraperitoneally in a dose of 200 ng/kg, and MLT intraperitoneally in a dose of 4 mg/kg starting on the day of tooth extraction until the day of sacrification.

The rats were sacrificed on days 7 and 14. The maxillary bones were resected anterior to the zygomatic arch using a diamond separated disk. The samples were subsequently trimmed to include the extraction socket, the newly formed bone tissue and the adjacent central tooth.

Histological Evaluation

The samples were decalcified for approximately 1 week in 10% formic (neutral phosphate-buffered formalin) acid. They were then passaged from low degree alcohols to high degree alcohols (from 70% to 100%) and embedded in paraffin blocks following a routine tissue follow-up procedure. Approximately 4- to 5-μm thick sections were obtained from the tissues that were prepared on adhesive slides (Surgipath, X-tra Adhesive Microslides, Illinois, USA) for staining with hematoxylin-eosin.

The presence of necrotic bone, osteblastic/osteoclastic activity, microorganisms, abscess and inflammatory cell infiltration in the hematoxylin-eosin stained sections of the samples from the anterior part of the maxilla were evaluated. Each extraction socket was evaluated in terms of the percentage of new bone filling and the intensity of inflammation in the hematoxylin-eosin stained sections. The intensity of the inflammation in the area of the defect (tooth extraction socket) was scored using a four-grade system (16).

Statistical Evaluation

The data obtained in the study were analyzed using IBM SPSS Statistics (Version 20.0. Armonk, NY: IBM Corp.). Chi-Square analysis was applied when analyzing the associations between nominal variables; a Fisher's Exact Test was used when the expected values in the cells in 2×2 tables had insufficient volume; and a Pearson Chi-Square analysis was used for RxC tables with the help of a Monte Carlo Simulation. A Chi-Square analysis with the help of a Monte Carlo Simulation was carried out since 20% of the expected values in the cells were less than 5.

A Kappa Test was used for the analysis of consistency between the ratios in dependent groups. The level of significance was accepted as 0.05, and $p<0.05$ was determined as presence of significant difference, while $p>0.05$ reflected no significant difference.

RESULTS

The study was conducted with 76 Wistar rats: 12 rats in the control group, 16 in the CsA group, 16 in the CsA+MLT group, 16 in the CsA+PRC group, and 16 in the CsA+MLT+PRC group. Five of the rats were lost to surgery and seven to feeding problems. The study was thus completed with 64 subjects.

Histological Findings

Group C (Control Group)

The general inflammation score in the group was determined to be 2.00 (++) on day 7. An osteoid formation was observed to fill the cavity in percentages in the range of 5–25% starting from the 1/3 base of the socket in all the samples (Fig. 2, A).

On day 14, the entire extraction socket was found to be filled with a fibrovascular connective tissue, and a mononuclear inflammatory cell infiltration (MNCI) was noted in all rats at a minimal level. An osteoid formation was observing to fill the cavity in percentages in the 20–70% range, starting from the 1/3 base of the socket in all the samples (Fig. 2, B).

Group 1 (CsA)

The general inflammation score in the group was determined to be 3.00 (+++) on day 7. Resorption and osteoclasts were observed in the host bone of the extraction socket in all samples. No new bone formation was seen in any of the samples (Fig. 2c).

Resorption and osteoclasts were identified in the host bone in the ceiling of the extraction sockets in the general group on day 14. Actinomyces clusters were observed in sample 1. Less than 10% osteoid formation was observed around the host bone and the base of the cavity in general in the group (Fig. 2, D).

Group 2 (CsA + PRC)

An inflammation score of 3.00 (+++) was recorded on day 7 in general in the group. The bone walls forming the socket were vital in general in the group. Osteoid formation was observed in rates ranging from 5 to 40% starting from the 1/3 base of the socket and around the wound opening, and also around the host bone in general in the group (Fig. 2, E).

An inflammation score of 2.00 (++) was recorded on day 14 in general in the group. Osteoid formation in rates varying from 5 to 40% was observed starting

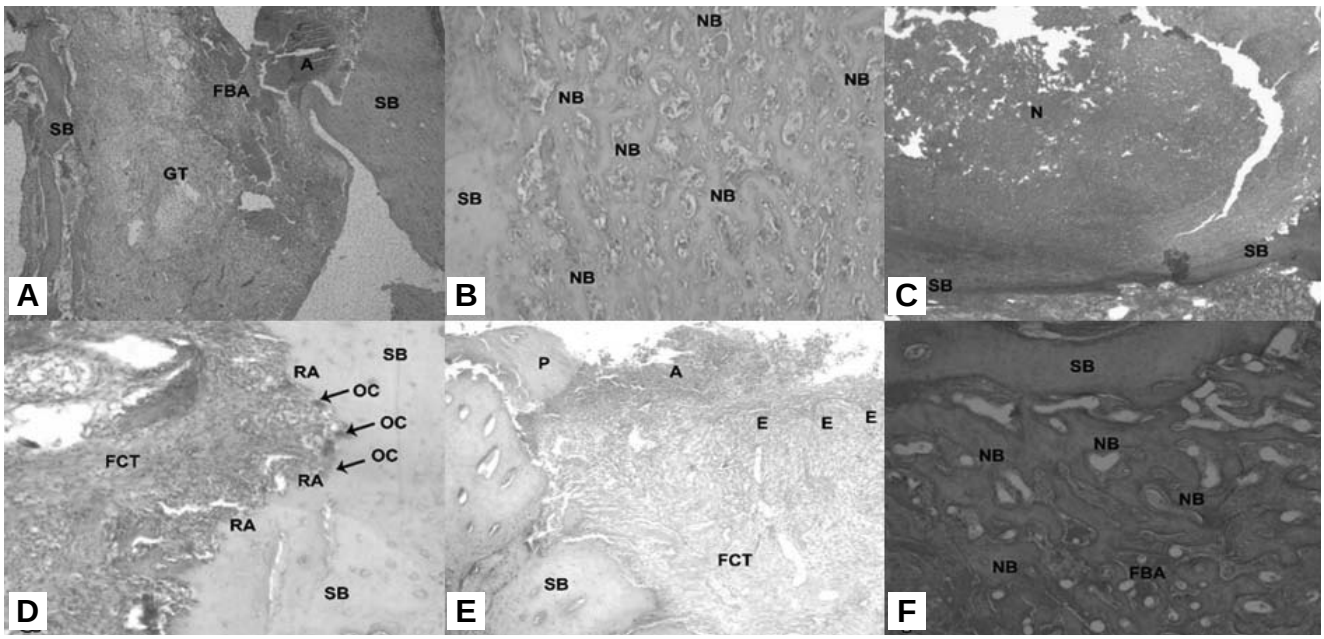

Fig. 2. A – group C on day 7 (H&E, ×100); B – group C on day 14 (H&E, ×200); C – group 1 on day 7 (H&E, ×100); D – group 1 on day 14 (H&E, ×200); E – group 2 on day 7 (H&E, ×200); F – group 2 on day 14 (H&E, ×200). (SB – socket bone; GT – granulation tissue; FBA – free bleeding area; A – abscess; NB – new bone; N – necrosis; FCT – fibeocellular connecting tissue; RA – resorbtion area; OC – osteoclast; P – plaque; E – epithelium).

from the 1/3 base of the socket and around the host bone in general in the group (Fig. 2, F).

Group 3 (CsA + MLT)

Mononuclear cell infiltration was observed in variable degrees in half of the group on day 7. An inflammation score of 3.00 (+++) was recorded in general in the group. Marked areas of free hemorrhage and stasis were seen starting from the cavity base up to half of the cavity. Resorption was seen in the host bone to varying degrees, starting from the mouth of the extraction cavity down to the deepest 1/3, and osteoclasts were identified in these areas in all of the samples. Osteoid formation was seen in a single sample in 15% (Fig. 3, A).

On day 14, slight resorption and osteoclasts in an area covering 1/3 of the single side of the socket mouth bone was noted in most samples in the group. An inflammation score of 2.00 (++) was recorded on day 2 in general in the group. The osteoid formation identified in the mouth of the cavity was observed to fill the socket together with fibrovascular connective tissue to varying degrees, starting from the base. An osteoid formation filling the cavity in ranges of 30–70%, starting from the 1/3 base of the socket, was observed in all samples (Fig. 3, B).

Group 4 (CsA+MLT+PRC)

Diffuse multi nuclear giant cells in the connective tissue were observed on day 7 in general in the group. Osteoid formations in rates of 3–15% were observed starting from the 1/3 base of the socket and around the host bone in general in the group (Fig. 3, C).

Table 1. New bone formation results of Kruskal Wallis Test

Groups		n	Mean	Median	Min	Max	Ss	Kruskal Wallis H Test		
								Average	H	P
New Bone Formation (Day 7)	Group C	5	9.4	5	5	25	8.76	21.2	20.883	0.001
	Group 1	8	0	0	0	0	0	6.5		
	Group 2	7	17.14	10	5	40	13.49	24.86		
	Group 3	6	2.5	0	0	15	6.12	9.83		
	Group 4	6	6.33	5	3	15	4.32	18.58		
	Total	32	7.66	5	0	40	11.23		2-1 2-4 3-4	
New Bone Formation (Day 14)	Group C	6	45.83	45	20	70	19.6	25.08	16.646	0.41
	Group 1	6	6	5	3	10	2.53	6.33		
	Group 2	7	18.57	10	6	40	13.71	15.43		
	Group 3	7	29.14	30	3	70	23.04	17.71		
	Group 4	7	36.43	15	5	80	32.11	20.07		
	Total	33	26.82	20	3	80	23.99		2-1	

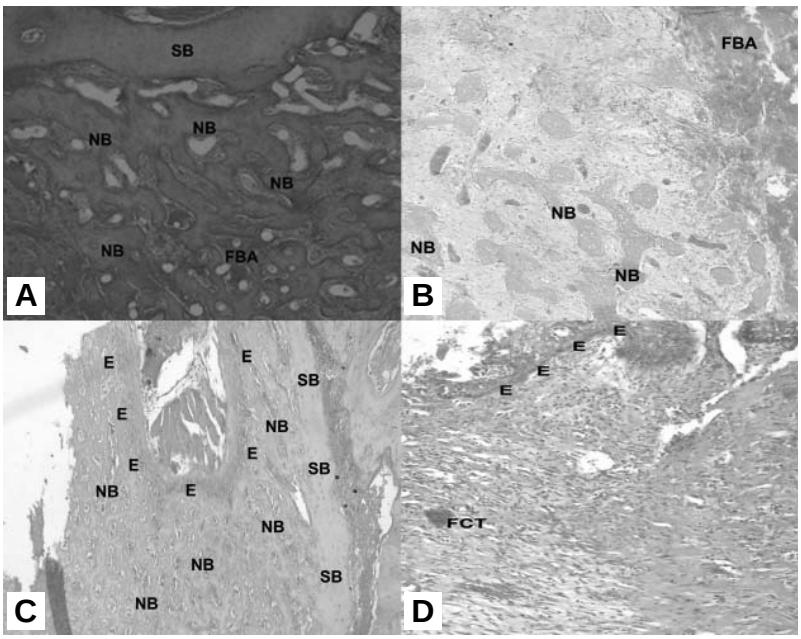

Fig. 3. A – group 3 on day 7 (H&E, ×100); B – group 3 on day 14 (H&E, ×100); C – group 4 on day 7 (H&E, ×200); D – group 4 on day 14 (H&E, ×100). (SB – socket bone; VS – vascular structure; FCT – fibeocellular connecting tissue; FBA – free bleeding area; NB – new bone; E – epithelium).

The mouth of the extraction cavity was found to be covered by stratified squamous epithelium on day 14 in general in the group, and osteoid formation at variable rates of 5–80% was observed starting from the 1/3 base of the socket and around the host bone in general in the group (Fig. 3, D).

Statistical Evaluation of New Bone Formation

A statistically significant difference was found between the groups in terms of the percentage of new bone formation on day 7 ($p<0.05$). The ossification value on day 7 was found to be significantly lower in the group 1 than in the group C and group 2, and significantly lower in the group 3 than in the group 2.

A statistically significant difference was noted in the percentage of new bone formation between the groups on day 14 ($p<0.05$). The ossification value was found to be significantly lower in the group 1 than in the group C on day 14 (Table 1, 2).

Statistical Evaluation of Inflammation Findings

No statistically significant difference was noted between the day 7 inflammation parameters of the groups ($p>0.05$), while the inflammation parameters were found to be lower than in the group C on day 7, though not to a statistically significant degree (Table 2). No statistically significant difference was noted in the inflammation parameters at different time points ($p>0.05$).

DISCUSSION

CsA first started to be used in 1976 as a calcineurin inhibitor and was known for its dose-dependent side effects. The use of CsA has been limited as a result of its immunosuppressive qualities and side effects, although it is known to increase the duration and quality of life of transplantation patients. The most serious side effects of CsA are nephrotoxicity, hypertension, and malignancy and infection due to immunosuppression (17).

Jayasheela and Mehta evaluated the effects on periodontal tissue of subcutaneous CsA in a 10 mg/kg dose in their study of rats, and reported alveolar bone loss and gingival growth in the experiment group (18) . Bolzani *et al.* administered subcutaneous CsA in a dose of 10 mg/kg in rats, and reported that matrix metalloproteinases 1 and 3 to be inhibited by CsA in a gingival fibroblast culture (19).

Compatible with the previously published studies, CsA was found to have a negative effect on the healing of the extraction socket in the group to which

Table 2. New bone formation results of Wilcoxon Test (Fig. 4)

Groups		N	Mean	Median	Min	Max	Ss	Wilcoxon Testi		
								Rank Mean	z	P
Group C	Day 7	5	9.4	5	5	25	8.76	0	-2.023	0.043
	Day 14	6	45.83	45	20	70	19.6	3		
Group 1	Day 7	8	0	0	0	0	0	0	-2.226	0.026
	Day 14	6	6	5	3	10	2.53	3.5		
Group 2	Day 7	7	20.71	15	5	40	15.66	4.5	-0.682	0.495
	Day 14	7	16.43	10	5	35	12.15	3.33		
Group 3	Day 7	6	2.5	0	0	15	6.12	0	-2.207	0.027
	Day 14	7	29.14	30	3	70	23.04	3.5		
Group 4	Day 7	6	6.33	5	3	15	4.32	0	-1.826	0.068
	Day 14	7	36.43	15	5	80	32.11	2.5		

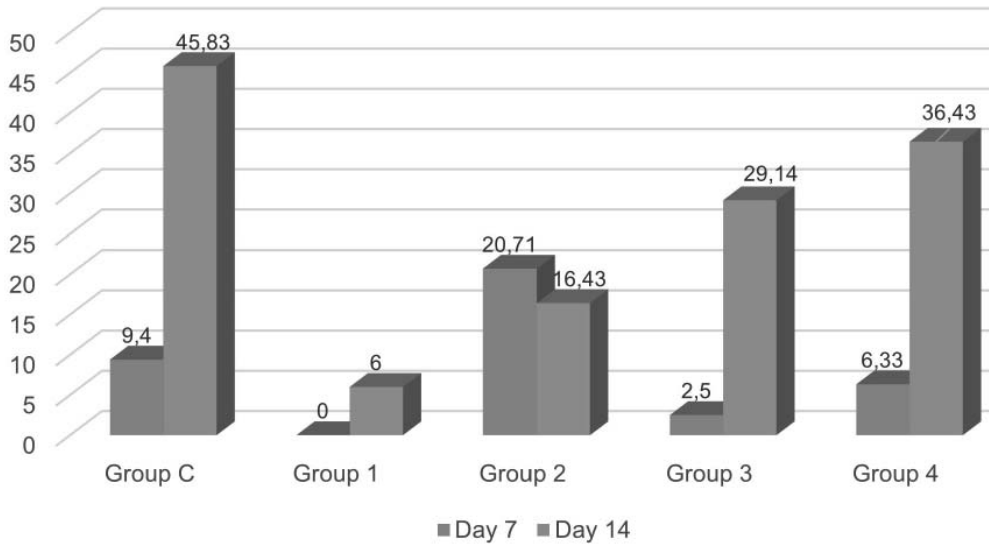

Fig. 1. Scatterplot of new bone formation percentages on day 7 and day 14.

it was applied, to delay epithelization in extraction sockets, to increase inflammation and to significantly decrease new bone formation when compared to the control group in the present study.

Many studies suggest that the toxicity induced by CsA may be linked to oxidative stress, and so using antioxidant agents, it has been suggested, may aid in in decreasing the rate of side effects induced by CsA. A review of literature revealed many studies evaluating the effects of CsA on gingival cell metabolism, although those focusing on hard tissues, teeth and the surrounding bone tissue are scarce (20). The effects of the addition of antioxidants to treatment protocols on the healing of the socket and the surrounding bone tissue are evaluated in the present study.

Rezzani *et al.* applied subcutaneous CsA in a dose of 15 mg/kg/day and intraperitoneal MLT in a dose of 1 mg/kg/day for 21 days in their study evaluating the antiapoptotic effects of MLT on protection of the heart from the cardiotoxicity produced by CsA in rats (21). They reported that MLT decreased substantially the cardiotoxicity of CsA, and was also effective on apoptotic processes. They further concluded that MLT decreased the cardiotoxicity caused by CsA, due primarily to its antioxidant effects.

Reiter *et al.* in their study reported that both in vivo and in vitro MLT applications in doses of 1-10 mg decreased lipid peroxidation and oxidative damage (22). Li *et al.*, on the other hand, demonstrated that intraperitoneal application of 10 mg/kg MLT in rats with experimental pulpitis had a protective effect on pulpal inflammation (23). The MLT dose determined for the present study was 4 mg/kg, as in many other rat studies.

MLT plays an active role in many stages of new bone formation. Similar to the findings in literature, both the systemic and local administration of MLT supported new bone formation, and the differentiation, function and maturation of osteoblasts in the present study. The findings of the present study concur with those of earlier studies in literature in terms of the antioxidant properties exerted by MLT through its direct stimulating effects on antioxidant enzymes (24).

The present study further concurs with literature on the prevention and decrease in the cytotoxic effects of CsA on osteoblasts and bone tissue by MLT. The parameters related to new bone formation were found to be significantly high in the long term in the CSA+MLT group when compared to the CSA group, which supports the idea that MLT can induce bone formation by facilitating osteoblast function in cases of immunosuppression.

Piao *et al.* evaluated the protective effects of PRC on renal damage to CsA in an experimental CsA nephrotoxicity model in which PRC (50 ng/kg/day and 200 ng/kg/day) was applied to rats in addition to CsA (15 mg/kg/day) for 28 days (25). The use of PRC in renal damage induced by CsA has been reported to have antiinflammatory and antifibrotic benefits. PRC, a Vitamin D analog, was used in the present study intraperitoneally in a dose of 200 ng/kg/day, and the protocol used in the present study was compatible with that applied in the study by Piao *et al.* (25).

Boyce *et al.* in their study applied Vitamin D in daily injections and reported an increase in bone resorption in the first two days, and in bone formation after 14 days (26). They thus concluded the effect of vitamin D on osteoclasts to be short acting, while its effect on osteoblasts was more persistent. In the present study, vital weight loss from the time of extraction until the sacrification on day 14 was found to be lower in the CsA +MLT + PRC group than in group C, and the observed vital weight loss in the CsA group from the time of extraction until the sacrification was higher than in group C.

Pinto *et al.*, in their study evaluating tooth extraction sockets, reported that the extraction socket was had filled completely with a blood clot composed primarily of macrophages (27). The socket was seen to be filled with newly formed connective tissue including fibroblasts in a large number on day 7, new

bone trabeculae started to be seen on day 15, and the socket was found to be filled with well-demarcated trabecular bone on day 28.

Boyce *et al.* (26) and Kawakami *et al.*, (28), supporting their previously reported studies, claimed that the addition of vitamin D supported a greater amount of new bone formation and decreased inflammation, which suggests to us that PRC may be advantageous in clinical applications.

The results of this present study support many earlier studies in the literature. A statistically significant difference was noted in the day 7 percentage ossification values between the groups ($p<0.05$). The percentage of ossification on day 7 was found to be significantly lower in the CsA group than in the C and vitamin D groups, and significantly lower in the MLT group than in the vitamin D group. The ossification value on day 7 was higher in the vitamin D group, although not to a statistically significant degree.

The contribution of free radicals to the increased damage mediated by CsA has been shown in rat tissues in many studies performed to date (20, 29). MLT has been suggested to decrease oxidative stress and the associated damage both by a direct route, in a sweeping effect, and by an indirect route, increasing the antioxidant enzyme level (30).

In this present study, CsA was demonstrated to inhibit both bone healing and new bone formation, and in turn, osteoblast function in the extraction socket. The results of the present study support the application of MLT and PRC due to the decrease in inflammation and augmentation of the physiologic bone production process, and the increases the amount of newly formed bone.

CONCLUSION

Vitamin D and MLT agents are herein shown to have positive effects on tooth extraction socket healing and in the production of protective effects against oxidative stress damage during healing in rats in an immunosuppression model performed involving the application of CsA. Further clinical and experimental studies are required to clarify the complete mechanism of the effects of the agents in order to widen the use of antioxidant agents following oral surgical procedures.

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Conflict of interest

The authors state no conflict of interest.

Ethics

This research was approved by the the Gulhane Military Medical Academy Animal Experiments Local Ethics Committee (Acceptence Date 10.04.2015 and Acceptence No. 15/43).

Author contributions

Dilber CELIK wrote the main manuscript text and Sibel Elif GULTEKIN prepared and analyzed figures 2-3. All authors reviewed the manuscript.

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