

EFFECT OF DEXAMETHASONE INFILTRATION ON PERIODONTAL LIGAMENT AFTER EXPERIMENTAL MOLAR LUXATION IN RATS

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ABSTRACT

Aim of the study: Dexamethasone is a popular corticosteroid in dental practice. Submucosal infiltration of dexamethasone proved to be effective to relieve post-operative symptoms in different clinical and research application. This study was conducted to evaluate the effect of submucosal dexamethasone single injection on the healing of injured periodontal ligament fibers after molar luxation in rats via histological and gene expression studies.

Materials and methods: 24 Wistar rats were selected for the study. Luxation injuries were performed for all first mandibular molars of both mandibular sides, right & left. Immediately after luxation, the right sides of the mandibles received submucosal injections of dexamethasone sodium phosphate (0.4mg/ml), while left sides were not dexamethasone injected. Follow up periods were 1, 3 & 7 days. Mandibular specimens were studied by routine histology, and quantitative gene expression analysis was done for fibroblast growth factor (FGF2), caspase-3 & alkaline phosphatase (ALP).

Results: Histological features revealed superior results of the dexamethasone group with better regeneration and organization of fibers. Gene expression results revealed significant increase in the expression of both FGF2 and alkaline phosphatase (ALP) and a significant decrease in the expression of caspase-3 in the study side when compared to the control side.

Conclusion: histologically dexamethasone enhanced the regeneration of torn periodontal ligament fibers with noticeable reduction in inflammation and rise in cellular proliferation. Gene expression reflected the possible benefit of dexamethasone single local injection in the management of periodontal ligament injuries.

KEYWORDS: dexamethasone, rat, luxation, periodontal ligament, alkaline phosphatase, FGF2, caspase-3.

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INTRODUCTION

Corticosteroids in dentistry are usually used to alleviate post-operative symptoms in minor surgeries, endodontic periapical surgery² and mucogingival surgery³. Topical corticosteroids are also used in dentistry for their immuno-suppressive and anti-proliferative property⁴

Dexamethasone is one popular corticosteroid due to its market availability and reasonable cost. Clinical and experimental applications of dexamethasone reveal variable outcomes depending on its dosage, route as well as frequency of administration.⁴

Local injection of dexamethasone, in dental practice has been experimented for its efficacy in reduction of postoperative inflammatory symptoms since the middle of the last decade^{5, 6, 1}. Studies on submucosal dexamethasone injection demonstrated its efficacy in relieving postoperative pain, swelling and trismus when it was compared to intra-muscular route.⁷

In vitro experiments on periodontal ligament cells dealt with dexamethasone as an osteogenic inducer and as regeneration promoter in various researches.⁸

The effect of dexamethasone is always modified according to dose amount and frequency variation that makes it an osteoporotic drug⁹ upon repeated and prolonged administration. On the other hand, it may be an osteogenic and healing promoter on lower doses with shorter duration.^{10, 11}

Although dexamethasone is widely used in dentistry, clinical and experimental, yet its introduction with periodontal problems is still limited.⁴

Experimental studies on tooth movement are applied in dental research to evaluate different tissue changes and understand physiologic events and periodontal adaptation accompanying such movements¹². These researches are useful for periodontal, orthodontic and dental trauma clinical aspects. However, the traumatized supporting

structure is not fulfilled till the present concerning survival under treatment and clinical follow up. The present research is designed to study the effect of local dexamethasone injection on periodontal ligament after molar luxation in rats.

MATERIALS & METHODS

Animals: 24 Wistar rats were purchased for the study, 120-150gm weight & 12-16 weeks age, from the animal house of Faculty of Medicine-Cairo University. The animals were maintained in an air-conditioned animal house under controlled room temperature $25\pm 2^{\circ}\text{C}$ with 12/12 h light/dark cycle with free access to water and softened food. Rats were housed in standard polycarbonate cages as 3 animals per cage. General animal care was according to the guidelines of the institutional animal care and use committee of Cairo University.

Sample size was calculated using the (G power software). We found that 8 rats for each duration was appropriate sample size for the study with total sample size 24 rats (1, 3 and 7 days). The power is 80% and α error probability = 0.05. the effect size = 1.52 with each rat representing the control and the study groups.

Experimental procedure:

Animals were anesthetized by intraperitoneal injection of intraperitoneal injection of ketamine/xylazine (ketamine 40–100 mg/kg IP/ xylazine 5–13 mg/kg IP, *Trittau, Germany*) To induce periodontal injury each rat's first mandibular molar was gently luxated bucco-lingually as an incomplete extraction movement using a Mosquito hemostat. Luxation was considered done when the tooth is raised about 0.5 mm in its socket¹².

Then luxation was performed on both mandibular sides taking the right side as experimental and the left as control. On the right mandibular sides supra-periosteal infiltration of $50\mu\text{l}$ (0.4mg/ml), of dexamethasone sodium phosphate (*AMRIA pharmaceuticals, Egypt*) into the buccal alveolar mucosa

opposite to the mandibular 1st molar ¹³. Dexamethasone was diluted 1:10 with sterile saline to provide a maximum dose of 0.02 mg/injection. Injection was performed using a micro-syringe and the solution slowly injected upon touching the bone surface. The exact procedures were performed on the left sides except for dexamethasone. Molars with broken or damaged roots were excluded from the study.

Animals were euthanized 1, 3 & 7 days post-operatively, 8 animals/interval. The mandibles were sectioned each into two halves as the left is considered as control (untreated) and the right as experimental (treated). Then mandibles were prepared for histological examination and for quantitative analysis of gene expression of FGF, caspase 3 & Alkaline phosphatase by real time polymerase chain reaction (PCR)

Histological examination: dissected mandibles were decalcified in 10% ethylene diamine tetra-acetic acid (EDTA) for 3 weeks. Then dehydrated in alcohol, cleared in xylol and embedded in paraffin. Sections of 4-5 μ thickness were mounted on regular glass slides and stained by Hematoxylin and Eosin for routine examination according to the conventional method.

Quantitative analysis of gene expression of FGF 2, Caspase 3 and ALP by real time PCR:

Total RNA extraction

Total RNA was extracted according to manufacturer instructions using SV Total RNA isolation system. (Promega, Madison, WI, USA). The RNA concentrations and purity were measured with an ultraviolet spectrophotometer.

Complementary DNA (cDNA) synthesis

The cDNA was synthesized from 1 μ g RNA using SuperScript III First-Strand Synthesis System as described in the manufacturer's protocol (#K1621, Fermentas, Waltham, MA, USA).

Real-time quantitative PCR (RT-PCR)

RT-PCR amplification was done with Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction includes SYBR Green Master Mix and gene-specific primer pairs (Table 1). It was designed by Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences of the gene bank. Every set of primers exhibited 60° annealing temperature. RT-PCR was done in 25- μ l reaction volume formed of 2X SYBR Green PCR Master Mix, 900 nM of every primer plus 2 μ l of complementary DNA. Amplification was applied under specific time and temperature conditions of several cycles of denaturation and annealing/extension. v1.7 sequence detection software from PE Biosystems (Foster City, CA) was used to calculate the records of RT assays. Comparative Ct technique was used to determine the relative expression of mRNA gene. Data were standardized to beta actin which is the control housekeeping gene.

TABLE (1) Showing the primer sequence of the studied genes

Primer sequence	
FGF2	F - 5'GCGGCTCTACTGCAAGA-3', R - 5'-CGTCCATCTTCCTTCATAGC-3',
Caspase- 3	F: 5'-GTGGAAGTACGATGATATGGC-3' R: 5'-CGCAAAGTGACTGGATGAACC-3'
Alkaline phosphatase	F -5-CAGTGTCAGCCGTTAATTGAC-3 R - 5-AGAGTAGGCTCCCCGCCACG-3
Beta actin	F-5-GCGCTCGTCGTCGACAACGG-3 R-5-GATGGCTACGTACATGGCTG-3

Statistical analysis

Microstat7 for Windows statistical package was used for statistical analysis in this study. One-way ANOVA was used to evaluate effect of time on the selected parameters in each group. Paired "t" test was used to compare both groups in different intervals. Differences were considered statistically significant at $p \leq 0.05$ ¹⁴.

RESULTS

Histological results

Longitudinal jaw sections revealed the area of the luxated molars with variable changes along the different follow up periods.

One day post-operatively

Control untreated

The periodontal fibers were torn around the roots with irregular pattern and diffuse hemorrhagic areas and evidence fibroblast degeneration with pyknotic nuclei. Aggregations of inflammatory cells concentrated around the traumatized area with widening of the interstitial spaces. Occasional osteoclasts were distributed along the pdl alveolar bone margin (fig.1).

Dexamethasone treated

The histological presentation of this group was like that of the control with apparent fewer hemorrhage spots despite the dilated capillaries that exhibited obvious fenestrations. Lesser inflammatory infiltration was also noticed with dexamethasone. Few pyknotic nuclei were also detected within fibroblasts (fig.1).

3 days post-operatively

Control untreated

Inflammatory infiltration was a characteristic sign of the current interval. The inflammatory cells were overlapping collagen fibers that exhibited discrete areas of hyalinization. The fibroblasts appeared with pyknotic nuclei in different locations around the roots. Sporadic osteoclasts on the alveolar bone surface were also detectable. (fig.2)

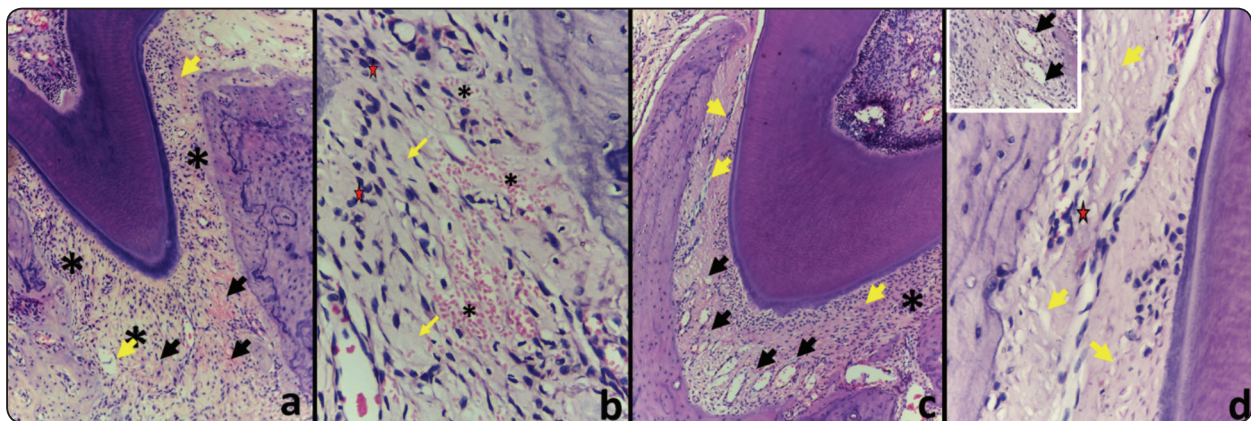


Fig. (1) Photomicrographs of the one-day interval showing

- Control (untreated) group showing; inflammatory infiltration (asterisks), areas of hemorrhage (black arrows) & torn collagen fibers (yellow arrows).
- Higher magnification of control group showing; torn collagen fibers (yellow arrows), hemorrhage (asterisks) & inflammatory cells (red stars).
- Dexamethasone treated group showing; inflammatory infiltration (asterisks), dilated interstitial spaces with lesser congestion (black arrows) & torn collagen fibers (yellow arrows).
- Higher magnification of the Dexamethasone treated group showing; torn collagen fibers (yellow arrows), inflammatory cells (red stars) & dilated interstitial space (inset). (H&E orig. mag. a & c, 100X, b & d, 400X)

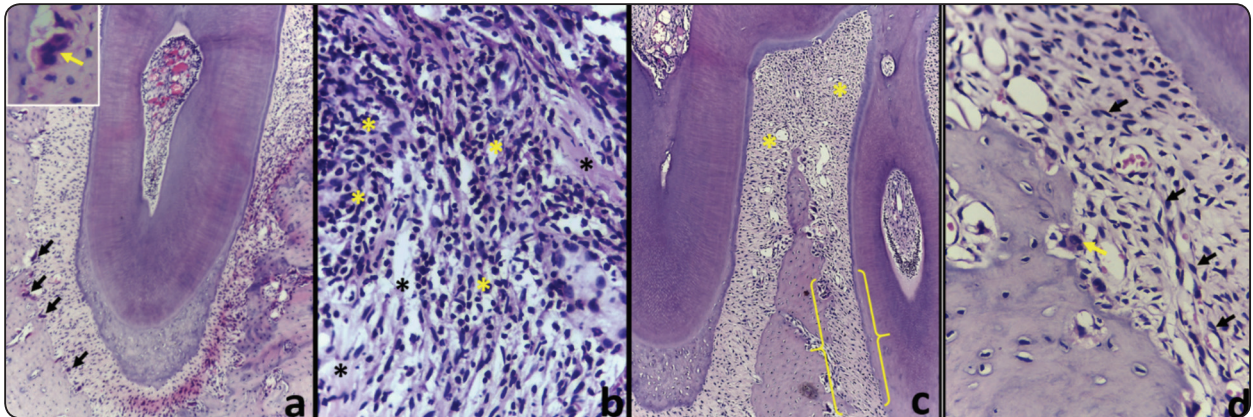


Fig. (2) Photomicrographs of the three-days interval showing

- Control group showing; inflamed periodontal ligament around the root with osteoclasts on bone surface (black arrows), osteoclast (inset).
- Higher magnification of control group showing; inflammatory infiltration (yellow asterisks) & hyalinized collagen fibers (black asterisks).
- Dexamethasone treated group showing; zones of proliferation and condensation (yellow asterisks) & organized oblique group of fibers (yellow double brace)
- Higher magnification of dexamethasone treated group showing; collagen fibers with spindle shaped fibroblasts (black arrows) & osteoclast (yellow arrow). (H&E orig. mag. a & c, 100X, b & d, 400X).

Dexamethasone treated

Apparently, the periodontal condition appeared characterized by increased proliferation of the fibroblasts with the characteristic spindle spindle-shaped nuclei and increased density of collagen fibers as well. The fibers started to regain its attachment with both tooth and bone surfaces with some aligned as definite oblique fibers. A shaped that approached the well-known configuration. Fewer osteoclasts were evident than that in the untreated group (fig.2)

7 days post-operatively

Control untreated

Periodontal changes in the present interval included fiber regeneration with a bizarre pattern, as there was no definite identified pattern of fiber grouping seen. The ligament included multiple voids interrupting the continuity of collagen fibers around

the root surface of the luxated molar. It was hard to identify interstitial spaces in the studied areas. (fig.3) some locations of the periodontal ligament appeared devoid of the nuclei of fibroblasts while other nuclei appeared pyknotic. Massive alveolar bone resorption was also a feature of the current group with evidence of osteoclasts. Multinucleated giant cells were detected within the periodontal ligament as well. (fig.3)

Dexamethasone treated

Periodontal healing in the current group showed more improvement when compared to the preceding group. More organized pattern of periodontal fibers with aligned fibroblasts with spindle nuclei. Pyknotic nuclei were less evident than the control group. The ligament appeared more intact than the untreated group except few locations where the fibers exhibited an irregular pattern. The adjacent alveolar bone exhibited a healthier appearance with multiple resting lines denoting its activity and osteoclasts were scarcely seen (fig.3)

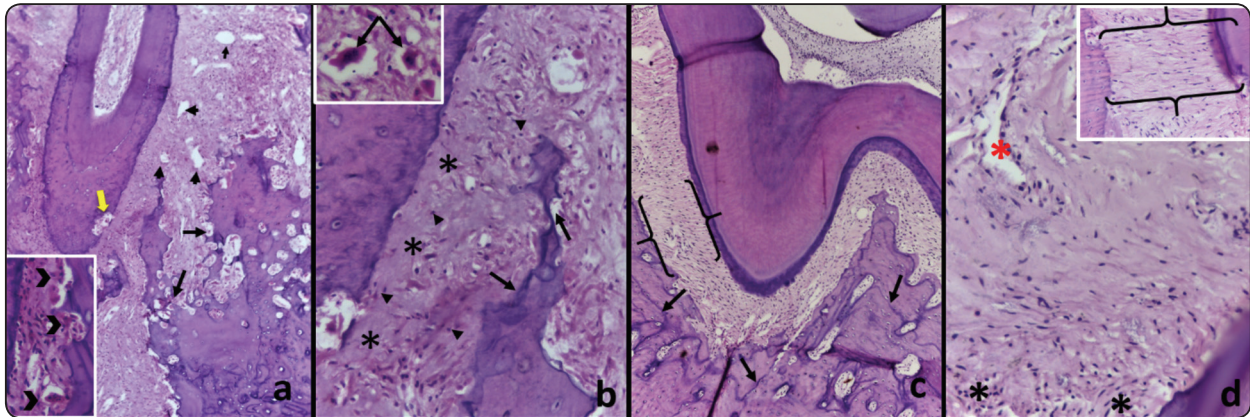


Fig. (3) Photomicrographs of the seven-days interval showing;

- Control group showing; multiple voids in the periodontal ligament (short black arrows), resorption of the alveolar bone (long black arrows), root resorption (yellow arrow) & osteoclasts (arrow heads-inset).
- Higher magnification of control group showing; alveolar bone resorption (arrows), homogenized areas of periodontal ligament without cellular existence (asterisks), nuclear pyknosis (arrow heads) and osteoclast (inset)
- Dexamethasone treated group showing; intact periodontal ligament with fewer voids with defined oblique group (double brace), resting bone lines (arrows).
- Higher magnification of dexamethasone treated group showing; cellular proliferation (black asterisks) fewer discontinuity (red asterisk) & organized horizontal fiber group (inset) (H&E orig. mag. a & c, 100X, b & d, 400X).

Gene expression results: Comparisons between the study groups & effect of time within the same group

FGF2: Comparing the two groups revealed that the highest mean value was recorded in treated group at 3 days, while the least value was recorded in untreated group at 1 day. The difference between groups was statistically significant (table 2). Within the same group, treated or untreated, the difference was not statistically significant (untreated: $p=0.362$), (treated: $p=0.083$)

In both groups there was no significant between three intervals

The study group showed statistically significantly higher values than control group in all intervals. (table 2, fig. 4)

Caspase-3: Comparing the two groups revealed that the highest mean value was recorded in untreated group at 1 days, while the least value was recorded in treated group at 7 days. The difference between groups was statistically significant (table 3). Within the treated group, the highest mean value

was recorded at day1, while the least value was recorded at day7 with no statistically significant difference.

In untreated group there was insignificant decrease after 3 days ($p=0.033$). There was no statistically significant difference between the three intervals ($p=0.582$). The treated group in general showed statistically significantly lower values than control group in all intervals (table 3, fig. 5)

ALP: Comparing the two groups confirmed that the highest mean value was recorded in treated group at 7 days, while the least value was recorded in control. Within the untreated group, the highest mean value was recorded at 3 days, while the least value was recorded at day1. The difference by time was not statistically significant ($p=0.868$). Within the treated group, the mean value gradually increased by time, with no statistically significant difference ($p=0.211$). In both groups there was no significant between three intervals. The study group showed statistically significantly higher values than control group in all intervals table (4, fig 6)

TABLE (2): One-Way ANOVA evaluating effect of time on FGF in both groups and independent student “t” test comparing both groups in each interval.

FGF	Control (untreated)		Study(treated)		“t”	Probability
	Mean	St Dev	Mean	St Dev		
Day 1	2.04	0.15	5.43	0.25	20.019	0.0000 *
Day 3	2.30	0.36	6.91	1.07	7.076	0.001 *
Day 7	2.33	0.21	6.47	0.35	17.536	0.0000 *
F ratio	1.211		3.885			
Probability	0.362 NS		0.083 NS			
LSD	0.511		1.709			

* = Significant

NS = Not significant

TABLE (3): One-Way ANOVA evaluating effect of time on Caspase3 in both groups and independent student “t” test comparing both groups in each interval.

Caspase3	Control(untreated)		Study(treated)		“t”	Probability
	Mean	St Dev	Mean	St Dev		
Day 1	4.50	0.89	1.74	0.31	5.085	0.004*
Day 3	3.34	0.29	1.53	0.15	9.429	0.0000*
Day 7	2.93	0.25	1.50	0.36	5.646	0.002*
F ratio	6.327		0.592			
Probability	0.033*		0.582 NS			
LSD	1.119		0.576			

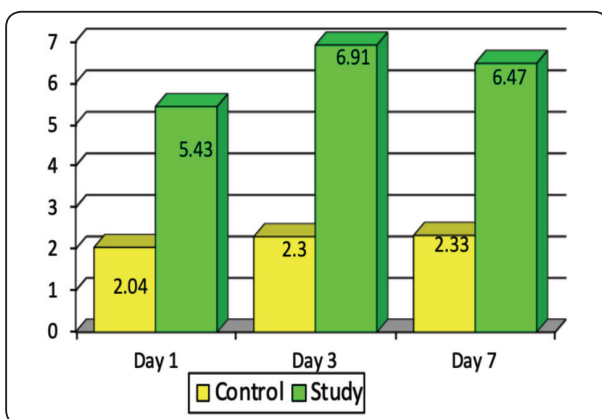
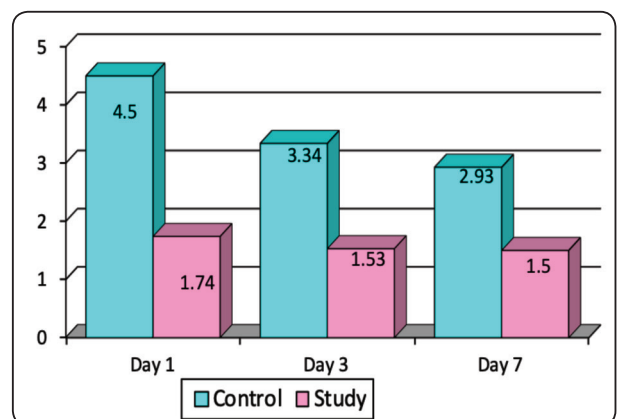
Fig. (4) Mean values of **FGF2** in both groups during follow up intervals. Control refers to the untreated & study refers to the dexamethasone treated.Fig. (5) Mean values of **Caspase3** in both groups during follow up intervals. Control refers to the untreated & study refers to the dexamethasone treated.

TABLE (4) One-Way ANOVA evaluating effect of time on Alkaline Phosphatase in both groups and independent student “t” test comparing both groups in each interval.

Alkaline Phosphatase	Control(untreated)		Study(treated)		“t”	Probability
	Mean	St Dev	Mean	St Dev		
Day 1	1.37	0.15	4.33	0.15	23.786	0.0000*
Day 3	1.48	0.39	4.53	0.38	9.679	0.0003*
Day 7	1.43	0.15	4.78	0.23	21.292	0.0000*
F ratio	0.145		2.042			
Probability	0.868 NS		0.211 NS			
LSD	0.518		0.538			

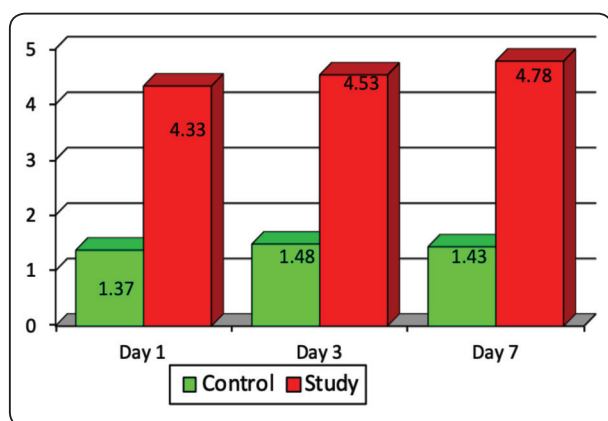


Fig. (6) Mean values of **Alkaline Phosphatase** in both groups during follow up intervals. Control refers to the untreated & study refers to the dexamethasone treated.

DISCUSSION

The present study was based on multiple trials dealing with the use of local injection of dexamethasone in dentistry as an anti-inflammatory and an anti-edematous agent^{1, 7, 3}.

Experimental work considers rat successful animal model is for being easily manipulated and a for obtaining similar tissues to the human body¹⁵ and different researches on dexamethasone effect used rats as the model of choice.^{2, 16}

The use of dexamethasone periodontal tissue was studied in different in vitro experiments¹⁷⁻¹⁸ and

in vivo available research work dealt with the drug action as repeated injectable doses or as a topical application.^{4, 19, 20}

Tooth luxation can be induced by trauma, infection, periodontitis, malocclusion or undue orthodontic force application. If the practitioner can overcome the hazards of tooth luxation by a simple technique that would be of a great value for the patient which developed the idea of the present research.

In the ongoing research mandibular first molar luxation was performed on both sides of the rat's mandible to induce periodontal ligament trauma. Submucosal dexamethasone was injected on the buccal alveolar mucosa on the right side opposite to the luxated tooth while the left side received no injection. Animals were euthanized at 1,3 and 7 days after procedure. The mandibles were dissected, and the area of interest was prepared for histological examination and for gene expression of fibroblast growth factor (FGF), caspase-3 and alkaline phosphatase activity.

The histological results of the current research revealed in general better reaction to dexamethasone injection over the follow up intervals. In the earlier follow up detection of hemorrhage and inflammation was seen in both treated and untreated groups with lesser capillary congestion and apparently less

inflammatory infiltration in the dexamethasone treated group which coincides with the drug effect in different research work^{3,21-23}. The detected shape of torn fibers and the hemorrhagic areas was also described by Nagayama et al, 2012¹⁵.

The current results are also in agreement with Harshita et al, 2018⁴ who explained that corticosteroids can induce the production of lipocortin, a polypeptide group, that block the action of vasodilators such as histamine and bradykinin which explains the decreased capillary congestion among the dexamethasone group.

Upon longer follow after three days the inflammation subsided in the treated group with dexamethasone better than that of the untreated group and this period was characterized by increased cellular proliferation and beginning of formation of new fibers which demonstrated the ability of dexamethasone to induce cellular proliferation. That coincided with other investigators who revealed the ability of dexamethasone to stimulate proliferation and regeneration of fibroblasts & osteoblasts in culture^{24, 8}. The noticed decrease in inflammatory infiltration in this interval (three days) is in accordance with Hakim et al, 2005²⁵ who detected a significant decrease in inflammatory markers at a similar interval, three days, when dexamethasone was injected into muscles after muscular injury.

Local injection of a single dose of dexamethasone after muscle injury was also helpful in restoring the contractile tension of the muscle after three days of injection which is further supported by the current results in the three days interval²⁵.

Regenerative effect of dexamethasone also extended to nerve tissue as I.M. drug injection helped in the regeneration of crushed sciatic nerve in rat. The drug increased nerve function and controlled Wallerian degeneration and enhanced the regeneration of myelinated nerve fibers²⁶

Moreover, Hakki et al, 2009²⁷ suggested that dexamethasone maybe of advantage in periodontal

regeneration as it inhibited the expression of matrix metalloproteinases MMP-1, 3 & 9 which inhibited degeneration of periodontal ligament cells in culture. which further coincides with the herein findings.

Furthermore, Kirakozova et al, 2009²⁸ studied the effect of intra-canal corticosteroids on periodontal healing in avulsed teeth in dogs. they concluded that canals filled with corticosteroids showed superior healing criteria when compared with gatta percha filled canals which supports the ongoing study findings.

On the other hand, the current findings were not in agreement with other investigators whose findings revealed that the administration of dexamethasone may inhibit soft tissue healing¹⁶ or delay extraction socket healing²⁹. other researchers demonstrated that dexamethasone suppressed osteoblasts both in vivo and in vitro^{9, 10}.

However, on reviewing the mentioned researches it was evident that they depended on repeated or prolonged administration of the drug which explains the different outcome in the present work that depended on single dose application. Harshita et al, 2018⁴ confirmed that the effect of corticosteroids on any tissue is usually a dose and concentration dependent effect which supports the mentioned assumption for the current work.

Reviewing the next follow up period, 7 days, noticeable changes were detected in both periodontal ligament and alveolar bone histology. healing of the periodontal ligament area was nearly established and approached its normal configuration which further supports the results in previous research work²⁵. In addition, the ongoing results are in accordance with Kirakozova et al, 2018 who revealed that dexamethasone treatment improved periodontal healing of replanted teeth in dogs. They suggested that corticosteroids in general could be effective in stimulation of periodontal ligament recovery²⁸. Moreover, the organized spindle shape of fibroblasts demonstrated in the current interval of the treated group was also recorded by other researchers' in vitro

studies^{27,31}. However, the herein results of the treated group were not in accordance with Choi et al, as they reported that dexamethasone treatment induced the production of DKK-1 gene by periodontal ligament stem cells in vitro which in turn inhibited the growth of human periodontal ligament stem cells¹⁷.

Massive alveolar bone resorption was detected in the control, untreated, group while in the dexamethasone-treated group bone activity was normal represented by resting and reversal lines mapping the bone surface. moreover, the osteoclasts were more detected in the untreated group. This demonstrates the ability of the drug to arrest bone resorption that may result from traumatic tooth movement which supported previous research work¹¹ that revealed that dexamethasone inhibited bone resorption during the early stage of orthodontic movement in rat.

Nuclear pyknosis was also shown in different locations of the periodontal ligament of the control group a finding that coincides with other investigators¹⁵.

Gene expression results supported the histological findings as well. The values of statistical analysis revealed statistically significant increase in the dexamethasone treated groups over the untreated groups regarding FGF2 and ALP expression means in general comparison between the two groups. However, within each group there was no statistically significant difference upon comparing the different follow up intervals. Additionally, the expression of Caspase 3 was noticed to be lower in the dexamethasone treated group than the untreated group with a statistically significant difference. Meanwhile, between each group intervals there was no statistical difference.

These results coincided with previous literature as periodontal ligament was proved to express FGF2 during healing of periodontal defects³². Furthermore, it was recorded by Hakki et al, 2009²⁷ that the addition of dexamethasone to FGF2 to cultured periodontal ligament cells would enhance the periodontal regeneration. While the sole addition of dexamethasone induced biomineralization and

osteogenic differentiation³¹. An observation that also explains the elevated means of alkaline phosphatase expression in the current study. Dexamethasone was demonstrated to improve periodontal healing in research work which is confirmed by the elevated level of FGF2 in the present study. Dexamethasone also resulted in increased levels of ALP in the treated group samples which was also demonstrated in previous research work³³. The elevated level of ALP may be one of the factors that prevented the massive bone resorption detected in the untreated group. On the other hand, dexamethasone was found to lessen the ALP activity in female rats after repeated, twice weekly, injection¹⁰, which is not in accordance with the present findings.

The levels of caspase 3 were significantly lower among the treated group coinciding with other investigators³⁴. However, other researches demonstrated that dexamethasone induced apoptosis in human periodontal ligament stem cells³⁴.

In summary the present study suggests that a single local injection of dexamethasone can have a regenerative effect on the damaged periodontal ligament which can be of a therapeutic value in cases with traumatized periodontal ligament in luxated or avulsed teeth. This was based on the histological findings that were further confirmed by the PCR results that coincided with most of the reviewed research work results. The difference in the drug effect can vary according to its frequency, dosage and route of administration. Further investigation is needed with varying doses and longer follow up intervals.

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