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Morphometric parameters of dental pulp in immature teeth in a sheep model after mechanical pulp exposure and restoration with reinforced zinc oxide-eugenol

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ABSTRACT

Background: The aim of the study was to investigate the morphometric parameters of dental pulp in open apices immature teeth in a sheep model after mechanical pulp exposure and restoration with reinforced zinc oxide-eugenol.

Materials and Methods: In this experimental study, a total of 12 immature mandibular central incisors from six adult male sheep, weighing 30–40 kg and with the age of 1 year old with Merino race were examined. After anesthesia, the pulps of the teeth in the case group were mechanically exposed and then were restored with reinforced zinc oxide-eugenol and amalgam. In the control group, the teeth remained intact. The animals were sacrificed at intervals of 2, 4, 6, and 8 weeks (E2, E4, E6, and E8) in the case and 2 and 8 weeks (C2 and C8) in the control groups. Then, their teeth were removed with the surrounding supporting tissues and alveolar bones. Tissue processing and staining were done, and the sections were examined under a light microscope. The Kruskal–Wallis and Mann–Whitney *U* tests were used to analyze the data and compare the changes between the two groups. $P < 0.05$ was considered statistically significant.

Results: In response to mechanical exposure, reparative or tertiary dentin was formed, and its thickness increased during the time of the study. The thickness of the odontoblastic layer in the E4 group was the highest amount. The pulp chamber diameter in the C2 group was significantly larger than the other groups, and the diameter of the apical foramen in the E8 was decreased significantly compared to the controls ($P < 0.05$).

Conclusion: In response to mechanical exposure and restoration with reinforced zinc oxide-eugenol, some morphometric parameters of the dental pulp changed significantly in the sheep model compared to the controls.

Key Words: Dental pulp, dental pulp exposure, root canal filling materials, sheep, tooth apex, zinc oxide-eugenol cement

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INTRODUCTION

There are three causes of vital pulp exposure: caries, trauma, and mechanical sources. Mechanical exposure occurs during the preparation of a cavity without caries. Mechanical exposure of the pulp is typically due to a misadventure during tooth preparation.[1] Pulp exposure initially leads to reversible changes, irreversible, and partial necrosis of the tissue and eventually completes necrosis in the tissue. Tissue necrosis is accompanied by structural changes in the pulp and periapical tissues. These changes include decreasing the number of odontoblast cells and eventually destroying them in different areas of the canal walls.[2-4] Odontoblast cells are responsible for making dentin. If the root dentin is destroyed and odontoblast cells are destroyed, Hertwig's Epithelial Root Sheath (HERS) sends signals to the dental papilla, and under these signals, dental pulp the stem cells differentiate into odontoblast-like cells, and then they start to build up a tertiary dentin at the site of the decay.[5-8] Generally, the rate of restorative dentin formation depends on the amount of primary dentin degradation. The immature tooth is characterized by an open apex and thin dentin walls. Embryonic tooth formation is a result of the interaction between the ectodermal coating and the underlying mesodermal tissues. During tooth growth, various molecules, including FGF, TGF-β, BMP4, and BMP2 send signals to the ectodermal and mesodermal tissues.[9,10] This interaction at different evolutionary stages results in the formation of the tooth and its retaining tissues. Root formation is the last stage of tooth formation that starts from the cervical arch. The cervical arch is where the internal and external enamel epithelium collide. Cells in this region multiply in the shape of a bilayer epithelial sheath termed HERS that acts as a template for root formation.[11,12] Inducement and regulation of root formation are among the different functions attributed to these cells, including the size, shape, and number of roots. If the HERS disappears during infection, inflammation, or necrosis, it may lead to a nonevoluted root in future. The HERS is in full contact with pulp cells in healthy teeth. However, with more severe inflammation and necrosis, the integrity between the apical papilla and the HERS is compromised, and the HERS is discontinuously seen around the pulp. As this process continues, the HERS is destroyed, and as a result, root apical evolution does not occur.[2] Suzuki *et al*. studied the function of the HERS cells during root formation. Their results showed that the formation of HERS is due to the invasion of dental follicle cells. Differentiation of papilla dental cells into odontoblast cells occurs following their attachment to the HERS.[13]

Ricucci *et al*. in their study on the teeth with irreversible pulpitis found that there was severe inflammation with small pieces of necrosis in the pulp tissue of the tooth. They showed that large parts of the canal walls lacked odontoblast cells, and the apical papilla was significantly reduced; in addition, the HERS was either disrupted or completely obliterated.[2]

Given that the treatment of immature teeth with open apex is a problem, on the other hand, defects in the composition of dentin and the thin walls of the roots have made immature teeth very sensitive to breakage. Therefore, immature apex has caused problems in the endodontic treatment of these teeth.[14,15] The description of pathological changes in the pulp and immature apical structures as a consequence of caries in immature teeth is not available in the articles. The answer to the question of what changes occurs in the HERS and tooth apical region as caries progresses is debatable. Therefore, this study aimed to evaluate the morphological changes that occurred in the dental pulp after mechanical exposure and restoration with reinforced zinc oxide-eugenol in immature teeth in the sheep, as an animal model.

MATERIALS AND METHODS

In this experimental study, six male adult sheep with immature teeth weighing between 30 and 40 kg with the age of 1 year old and Merino race were used in this study. Of these, four sheep were used as the experimental group, and two sheep remained intact as controls (to avoid killing more animals only two intervals were chosen).

Two central mandibular incisors with immature teeth (total number: 12) were used from each sheep. After anesthesia with the muscular injection of 0.01 mg/kg xylazine, lidocaine hydrochloride 2% with 1:80000 epinephrine (Persocaine, Darou Paksh, Iran) for local anesthesia. The isolation was performed using the split dam so that the incisor teeth were outside of the rubber dam. Teeth disinfected with 0.2% chlorhexidine. An access cavity was created at the lingual surface of the tooth, using a high-speed diamond fissure bur (Tizcavan, Tehran, Iran) with copious water irrigation. The pulp was mechanically

exposed. The size of pulp exposure in all teeth was similar and was 1.1.1 mm.

Initial hemorrhaging was controlled by placing sterile cotton pellets moisturized with saline over the pulp stump using slight pressure and waiting 5 min for hemostasis. Then, the pulp was covered with reinforced zinc oxide eugenol cement (Kemdent, United Kingdom). Cement was prepared according to the manufacturer's instructions and then inserted into the cavity. Using a dry sterile cotton pellet, the cement was gently adapted on the blood clot-free pulpal wound and dentinal walls, and the coronal portion of the cavity was permanently filled with amalgam (Cinalux plus, Iran). Central mandibular incisors from the control group remained intact.

Inclusion criteria

Male sheep had not received any medicine or antibiotics in the past 2 weeks; the permanent teeth of the mandibular incisors had just erupted, and the access cavity preparation site was located above the gums, the apex of the mandibular incisor was open in the radiograph, and the diameter of the apical region of the canal was about 2–3 mm in all samples.

Exclusion criteria

Preparation of the access cavity with any accident, failure to stop pulp bleeding after 5 min of packing with cotton soaked in normal saline, teeth restoration defects during the study period, crown fracture in the studied teeth, and occurrence of systemic diseases in animals during the study period.

The ARRIVE recommendations were considered for conducting this animal research (https://arriveguidelines.org/arrive-guidelines). This study was approved by the Ethics Committee of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1397.054).

In the experimental group, animals were sacrificed in the 2nd, 4th, 6th, and 8th weeks (named: E2, E4, E6, and E8, respectively). Immediately after sacrificing the animals, central mandibular incisors were resected along with the surrounding supporting tissues and alveolar bone. In the control group, the teeth were removed in the interval of 2 and 8 weeks (named: C2 and C8).

The samples were placed in 10% formalin saline for at least 14 days for tissue fixation. Then, they were cleaned and embedded in 10% formic acid, 9.2% citric acid, and 8.1% trisodium citrate solution for decalcification for about 21 days at the room temperature. To verify full decalcification, teeth radiography was done. Then, the samples were dehydrated in a graded series of ethanol, cleared in xylene, and impregnated in paraffin wax using a Leica TP 1010 tissue processor (Leica, Germany). Paraffin tissue blokes were prepared as the samples were placed longitudinally along the long axis of the tooth. Then, sectioning was done using the fully automated Leica RM2255 instrument (Leica, Germany). 4 μm serial sections were prepared from each specimen, and the samples were mounted on glass slides.

Sections were stained with routine H and E staining, and histological and morphometric studies were done.

To calculate the morphometric parameters, the images of the relevant slides were transferred to the computer using the camera transfer (CME ×50, Euromex), and by considering the magnifications of the images, the morphometric calculations for each sample were examined in five areas, comprising the apical foramen region and compared with the parallel slides from the control group using the image analyzing software of the photomicroscope (Leica, Germany).

In addition, each sample was observed under the microscope for the presence or absence of tertiary dentin in the root canal.

The present study focused on the histologic qualitative interpretation of changes in the root canal at different time durations. Histology of apical and periapical area, thickness of odontoblast layer, presence or absence of tertiary dentin and its thickness, and HERS changes such as size and shape of the inner enamel epithelium (IEE) and outer enamel epithelium (OEE) cells were studied.

Statistical analysis

The Kruskal–Wallis and Mann–Whitney *U* tests were used to analyze the data and compare the changes at different times between the experimental and control groups. $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the morphometric information and the changes between the case and control groups. The changes in the histologic status of the periapical tissue in the case groups showed that the connective tissue was denser and its fibers became thicker than the control group. Furthermore, in the experimental

group, from the 2nd week to the 8th week, there was a trend of progressive increase in density and thickness of connective tissue fibers. However, bone tissue in this area appears to be gradually diminishing.

A comparison of changes in the odontoblast cells in the experimental and control groups is shown in Figure 1. There was a significant difference in the thickness of the odontoblast cell layer between the experimental and control groups. The highest thickness of the odontoblast layer was observed in the E4 group and the lowest thickness was observed in the C2 group. In the other groups, the thickness of the odontoblast layer was similar.

The results of Figure 2 showed that there is a significant difference in the tertiary dentin diameter between the experimental and the control groups. No tertiary dentin was formed in the control group. However, in the experimental groups, the rate of tertiary dentin formation had increased from the E2 to the E8, so the highest amount of tertiary dentin was seen in the E8 group.

The apical foramen diameter was compared in the experimental and the control groups. It is shown in Figure 3. According to our results, the diameter of the apical foramen was greatest in the C2 group and smallest in the E8 group. After the C2 and C8 groups, the E2 group showed the largest apical foramen diameter between all the groups.

In our study, there are no significant differences in the thickness of predentin between experimental and control groups.

Changes in HERS in experimental and control groups are shown in Figure 4. There were no significant differences in the height of IEE between the experimental and control groups.

There was a significant difference in the height of the OEE between the experimental and control groups. Our study results showed that there was the highest OEE in the E6 group and the lowest was observed in the control groups.

We also observed that in the E6 group, the cells of the HERS were found only in a small area near the cervical loop and in other areas of the root were destroyed. While in the C8 group, these cells were found throughout the primary apical foramen.

The apical papilla tissue in the control group was somewhat denser, had narrower arteries, and was less

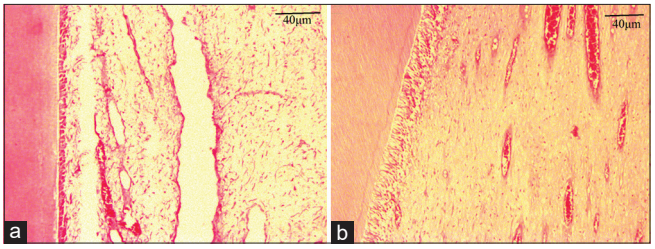

Figure 1: Thickness reduction of the odontoblastic layer cells in the second week of the control group (a) compared to 4th week (b) of the study after mechanical pulp exposure in the experimental group (arrows indicate odontoblastic layer cells); (hematoxylin-eosin stained sections).

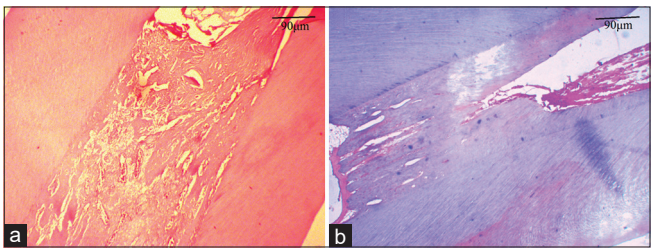

Figure 2: Tertiary dentin formation (formation of dentin bridge in below the exposure area) in the second (a) and eighth (b) weeks of the study after mechanical pulp exposure in the experimental group (arrow).

Table 1: Comparison of morphometric parameters of dental pulp with mechanical exposure compared with the control group

Value	Groups						*P*
	Control		Case				
	Second week	Eighth week	Second week	Fourth week	Sixth week	Eighth week	
Thickness of odontoblastic layer (µm)	11±0.00	17.6±3.207	19.80±3.73	31.90±3.64	22.00±3.47	22.00±5.76	0.013
Tertiary dentin diameter (mm²)	-	-	32.00±1.38	37.60±1.38	78.13±1.162	94.40±1.38	<0.0001
Apical foramen diameter (µm)	2440±7.07	2000±70.71	1880±7.07	1260±7.07	1720±7.07	660±7.07	<0.0001
Predentin thickness (µm)	20.68±1.46	23.16±0.78	18.52±1.85	17.80±0.98	26.74±2.76	16.00±1.60	0.074
Height of cells IEE (µm)	20.56±5.46	37.70±5.92	23.86±1.34	-	20.00±3.77	-	0.240
Height of cells OEE (µm)	5.83±0.76	3.80±0.300	6.13±0.49	-	10.60±0.51	-	0.026

Values are given as mean±SEM; the significant difference between groups (P<0.05). IEE: Inner enamel epithelium; OEE: Outer enamel epithelium; SEM: Standard error of mean

dilated relative to the higher areas. In all experimental groups, the connective tissue view of this area was similar to the control groups [Figure 5].

DISCUSSION

The present study showed that in response to mechanical exposure, the thickness of the odontoblastic layer in the C2 group was the lowest and in the E4 group was the highest amount. In the case groups, tertiary dentin was formed and its thickness increased during the time of the study. The diameter of the apical foramen in the E8 was decreased significantly compared to control groups. Our study results showed that there was the highest OEE in the E6 group and the lowest was observed in the control groups.

The results of this study showed that pulp stimulation causes the formation of connective tissue with denser and thicker fibers in the apical region. This is one of the differences between the stimulated and the unstimulated pulp in the experimental and control groups. In addition, the bones of the periapical area degenerate over time. Ricucci *et al*. showed that even with moderate inflammation in the pulp, secretion of proinflammatory mediators and cytokines and their entry into the apical region causes periapical bone resorption. They also have shown that in cases of irreversible pulpitis, the number of cells in the apical area is drastically reduced, and in some areas, there are no cells. Furthermore, the pulp tissue showed an abundance of dilated vessels and nerves, and in some areas, the pulp exhibited fewer cells and an abundance of fibers, this tissue showed an abundance of dense collagen bundles. All of these findings can indicate the inflammatory mechanism of pulp.[2]

Our results showed that in the control group, the cells of the odontoblast layer in the coronal region were higher with more specific odontoblastic appendages than in the apical region. In the experimental groups, the odontoblastic layer was similar to the control groups, except for the exposed areas of the pulp, where no odontoblasts were cells due to cavity formation. Odontoblast cells form the outermost layer of the dental pulp and they are like a barrier between the dentin and the vital tissues of the tooth. Odontoblast cells protect the dentin and living pulp against mild stimuli by reactive dentin deposition. When bacteria invade deep dentin and dentin tubules, odontoblast cells are the first pulp cells to encounter

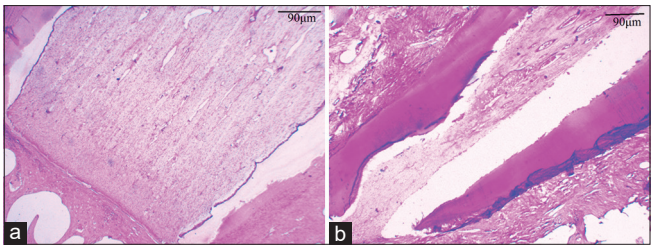

Figure 3: Larger apical foramen diameter in the 2nd week of the control group (a) compared to 8th week of the study (b) after mechanical pulp exposure in the experimental group (black arrows indicate apical foramen); in image B: red arrow indicates intermediate dilatation and blue arrow indicate minor foramen.

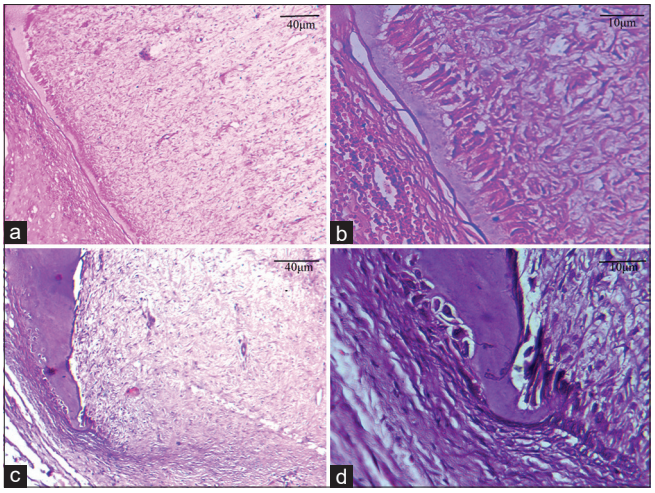

Figure 4: Hertwig's epithelial root sheath changes in the 8th week of the control group (a and b) and 6th week of the study after mechanical pulp exposure in the experimental group (c and d) (arrows); (hematoxylin-eosin-stained sections at different magnifications).

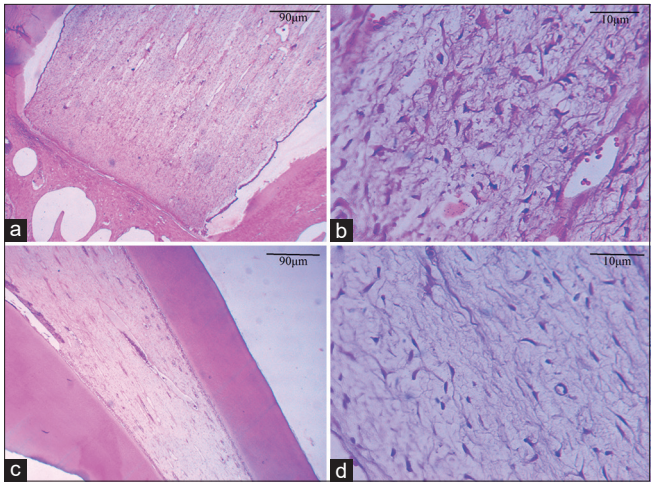

Figure 5: Apical papilla tissue changes in the control group (a and b) and the 4th week of the study after mechanical pulp exposure in the experimental group (c and d) (arrows indicate apical papilla tissue); (hematoxylin-eosin stained sections at different magnifications.

them. In very severe and long-lasting inflammations, odontoblast cells break down the pulp by producing pro-inflammatory mediators such as cytokines. Furthermore, immune cells that present in the pulp produce proteases, such as metalloproteinase, and various enzymes to combat invading pathogens. However, these molecules can also cause damage to dental pulp cells and lead to host cell death or reduce their number.[16,17]

We found that the level of tertiary dentin in the case group was significantly higher than the control group. Because no stimulation was performed on the teeth in the control group, no tertiary dentin was formed in this group. However, in the case group, tertiary dentin formed at the site of mechanical pulp exposure and its amount increased over time. So that the lowest amount of tertiary dentin was seen in the E2 group and then with an upward trend in other groups, the highest amount of tertiary dentin was seen in the E8 group. In our study, the tertiary dentin formed at the apex of the pulp chamber; under the exposed area and then progressed toward the canal walls. The amount of tertiary dentine produced is proportionally equal to the amount of dentin destroyed previously.[7,18] Our study is similar to the results of Zhang *et al*. they found a large strip of tertiary dentin beneath the area involved in the decay. They also showed that the number of odontoblast cells in the vicinity of tertiary dentin had decreased and showed that in response to pulp exposure, stem cells were differentiated into odontoblast cells and formed tertiary dentin.[19]

The results of this study also showed that by increasing tertiary dentin deposition and by continuing to make dentin, the diameter of the root canal in the experimental groups was significantly reduced compared to the control groups, so that, the lowest diameter of the root channel was observed in the E8 week of the case group.

It has been shown that the formation of hard tissue begins immediately after pulp exposure and is a pulp protective mechanism against local stimuli. It also protects the pulp from reinfection. Although the formation of hard tissue in an uncontrolled manner is a complication of this defense mechanism, the subsequent closure and narrowing of the apical foramen can also be part of the pulp defense mechanisms in the experimental group compared to the controls.[20,21] Although, we did not find any studies that show what changes occur in the condition of apical foramen inflammation. However, the present study showed that mechanical exposure accelerated the closure of the apical foramen.

The results of this study showed that the predentin layer in the experimental group was thinner than the control group. However, these differences were not statistically significant. The highest thickness of predentin was observed in the E6.

The results of our study showed that the apical papilla in the experimental group was histologically similar to the control group. In this area, the connective tissue was denser, and the blood vessels were narrower than in other areas of the papilla. These results are similar to the results of Ricucci *et al*.[2] In the advanced stages of root development, the volume of the dental papilla is significantly reduced. This reduction is observed in all parts of the dental papilla except the apical part of the root. This part of the root is called the apical papilla. Stem cells are located in the apical region of the papilla, which differentiate into odontoblast cells and form the dentin of the root region. Therefore, the dental papilla is the part of the dental pulp that helps teeth form.[22,23]

The present results also showed that the cells of the HERS became smaller in the experimental group, especially in the E6 week, and their intercellular space decreased. In addition, it seems like these cells become denser and more atrophic. In the Ricucci *et al*.'s study, the HERS was observed discontinuous and in some cases did not exist at all.[2] Luan *et al*. studied the evolution of the HERS in rat molar teeth. They believed that the HERS continued to grow in the early stages of root development and that this growth was due to the proliferation of HERS cells. In the later stages of root development, the distance between the HERS cells and the root surface increases. Eventually, with the final evolution of the root, the proliferation of HERS cells decreases and they undergo apoptosis. Furthermore, some of these cells migrate from the root surface to form the epithelial sheath of Malassez.[24] In another study, Wentz *et al*. showed that as the primary dentin formed, at the same time, the HERS cells were fragmented and then removed from the root surface to form the epithelial remnants of Malassez.[25]

After differentiating odontoblast cells from dental papilla cells, these cells begin to secrete predentin. With the onset of predentin formation, the HERS begin to fragment. These cells then attach to the surface of the root and could be recognized as Malassez

epithelial cells. If the integrity of HERS is disrupted prematurely, differentiation of root odontoblasts and the formation of predentin is impaired. Anyway any disturbance in the formation of the HERS leads to malformations affecting root shape, number, length, structure, and other features.[26]

CONCLUSION

The use of reinforced zinc oxide-eugenol for mechanical exposure of the pulp can lead to severe and progressive morphometric damage. These changes can include an increment of the density of connective tissue and destruction of odontoblast cells that then may lead to tertiary dentin formation and shrinkage and atrophy of HERS cells. Understanding the histology and pathology of the dental pulp can be helpful in the timely diagnosis and treatment of pulp injuries. Future studies will help us gain a better understanding of the premature damage to the root of the tooth and the resulting changes.

Acknowledgments

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Ethics approval

This study was approved by the Ethics Committee of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1397.054).

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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