Regenerative potential of simvastatin on stem cells of different sources in dog's teeth: a histologic and radiographic study

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Dedication

This work is dedicated to my parents, my wife and my little kids for the countless sacrifices they have made throughout the years. Without their love and support, none of my accomplishments, however small, would have been possible.

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4.	MSCs	mesenchymal stem cells	2
5.	Ca(OH)2	calcium hydroxide	4
6.	NaOCl	Sodium Hypochlorite	7
7.	PBSC	penicillin, bacitracin, streptomycin and caprylate sodium	7
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1. Introduction

Treatment of immature non vital teeth represents a big challenge during root canal treatment procedures. It is difficult to perform chemo mechanical debridement and create an effective apical seal by using conventional endodontic methods. Also immature roots are week, short, and more susceptible to fracture due to thin fragile dentinal walls [1].

Historically, apexification using calcium hydroxide was the treatment of choice but it had several disadvantages including increased root dentin brittleness, and increased risk of root fracture because of long-term presence of calcium hydroxide inside the root canal space [2]. Alternatively, Apical barrier technique was introduced as a replacement for specification [3] by placing a barrier material such as Mineral Trioxide Aggregate (MTA) at the apex to facilitate root canal obturation. Although clinical studies have shown high success rates of this method but it neither promote root development nor able to obturate root canals with blunderbuss apex [4].

Another treatment option used was surgical endodontics in an attempt to seal tubular or blunderbuss apical opening. Unfortunately, Surgical treatment is an invasive treatment gained the possibility of surgical complications and might lead to a compromised crown root ratio in a tooth already weakened as a result of immature root development [5].

Recently, a biologically based treatment called regenerative endodontics was introduced as an innovative treatment protocol that allow for introduction of vascularity of root canal system with deposition of a calcific material at the apex as well as on the lateral dentinal walls resulting in complete root maturation [6]. To complete this procedure successfully, three components including stem cells, scaffolds and growth factors should be involved in such protocol [7, 8]. With

advancement of regenerative protocols, it is now possible to use stem cells for dental tissue engineering either from dental or non-dental origin [9,10].

Alternatively, different pharmacologic agents have been introduced to allow for enhancing this regenerative protocol. Recently, Simvastatin (SIM), a widely accepted drug used primarily to lower the cholesterol levels for the treatment of hypercholesterolemia has revealed increase in bone mineral density with long term systemic administration in humans [11]. Furthermore, simvastatin-treated dental pulp stem cells exhibited enhanced odontogenic differentiation and accelerated mineralized tissue formation [12,13,14]. This observation led to the possibility of utilizing Dental Pulp Stem Cells (DPSCs) to regenerate pulp and dentin for clinical applications [15].

It is also not known whether mesenchymal stem cells (MSCs) from different sources are comparable in their differentiation potential in vivo or whether their capabilities are influenced by the niche of their origin [16].

From the histological point of view, the nature of the tissue formed in the canal space in human revascularized immature permanent teeth with apical periodontitis is speculative because no histologic studies are available[17]. Therefore, animal models may shed some light to address this issue [18].

The combination of simvastatin with stem cells from different sources to restore a functional dentin-pulp complex is not fully investigated in dental literature.

2. Review of literature

Section outline:

Treatment of the immature non vital teeth with apical pathosis presents several treatment challenges. The mechanical cleaning and shaping of a tooth with blunderbuss canal are difficult, if not impossible. The thin, fragile lateral dentinal walls can fracture during mechanical filing, and the large volume of necrotic debris contained in a wide root canal is difficult to completely disinfect. Also obturation of wide canal systems requires precise fabrication of a customized gutta-percha cone, and there is danger of splitting of the root during lateral condensation. Many blunderbuss canals with flaring walls cannot be obturated and sealed by orthograde methods and might require apical surgery and retrograde sealing of the canal[19].

2.1. Management of teeth with open apex

Although acceptable endodontic results have been achieved through apexification procedures using a long-term calcium hydroxide Ca(OH)2 but this technique might alter the mechanical properties of dentin. In a retrospective study by **Cvek**, the risk of cervical root fractures has been found to be markedly higher in immature teeth treated with Ca(OH)2 apexification and gutta percha, and its frequency was related to the stage of root development [20].

Recent treatment strategies include the creation of an artificial apical barrier using MTA with or without an apical matrix followed by compaction of obturating material and placement of a coronal restoration [21]. Unfortunately, even after treatment, these teeth have an elevated risk for fracture [20].

An alternative approach is to provide treatment under conditions where continued dentin formation is promoted. Several reports document that under conditions where at least some pulp tissue appears vital, a pulp cap treatment permits continued dentin formation, described as either continued root development (maturogenesis) or apical closure (apexogenesis)[22].

Thus, there is continued need to develop a biologically based treatment that offer the potential for continued hard tissue formation of teeth with a necrotic root canal system and an incompletely developed root.

Prior to the development of organized regenerative procedure in the early 2000, trials at pulp regeneration and apical repair (closure) were being done. The beginning of the regenerative endodontics can be traced back to the land mark paper in **1961** by **Nygaard ostby** [23] titled the "role of blood clot in endodontic therapy" an experimental histological study". In this study the pulp was removed from the canal, bleeding was induced inside the apical part of the canal, finally obturation of the coronal part of the canal was done using gutta percha coated with kloroperka paste. The aim of the study was to evaluate the histological changes in the apical area and the results showed soft tissue formation inside the root canals of all the roots with variable extensions. It was suggested that this method could be used as an alternative variant in case of immature teeth.

One of the most interesting articles was published by **John Ham et al.** [24] in **1972** before the era of regeneration titled "Induced apical closure of immature pulp-less teeth in monkeys". The study used a radiographic and histological evaluation of two approaches in non-vital immature teeth by calcium hydroxide apexification and induced root continuation using a blood clot. The results indicated that both periapical healing and calcified tissue formation can occur after either of the treatments. They concluded that no new dentin was formed and the apical closure was due to calcification through the apical foramen or along the canal walls and the Hertwig's root sheath had no role in the process of apical closure. They also concerned that infection control inside the canal was very important regarding the results that showed more apical closure in teeth with short term infection.

In a series of a three consecutive articles**, Alan J. Nevins et al.**[25,26,27] used collagen-calcium phosphate gel contain potassium iodide to induce physiologic

closure in implanted polyethylene tubes , pulp less immature teeth of monkeys, and traumatized immature maxillary central incisor of a child. The results showed physiologic closure of the tube by scars of mineralized connective tissue and continued root development in both animals and the child.

2.2. Root canal infection

In **1890,** research done by **Miller** [28], was the first to demonstrate the presence of bacteria in necrotic human pulp tissue. However, the cause and effect relationship between pulp necrosis and bacterial contamination was according to **Kakehashi et al.** [29] They observed the pathologic changes resulting from untreated experimental pulp exposures in germ-free rats as compared with conventional rats with normal micro flora. This study is considered a classic reference, which altered out understanding of endodontic microbiology because the results of this study indicates that the presence or absence of microbial flora is the major determinant in the healing of exposed rodent pulps.

Then, **Bergenholtz** [30] demonstrated the presence of bacteria in the traumatized teeth. Despite the fact that pulp chambers were not exposed, bacterial growth was observed and he found numerous types of anaerobic microorganisms including Bacteroides, Corynebacterium, Peptostreptococcus, and Fusobacterium.

In **1982**s, **Fabricius et al.** [31] investigated the Influence of combinations of oral bacteria on periapical tissues of monkeys, the combinations of oral bacteria showed the greatest capacity' of inducing apical periodontitis, as revealed by radiography, and the most pronounced type was the obligate anaerobic species.

In **1987**s, **Nair** [32]. studied the structure of the endodontic flora, its relationship to the dentinal wall and microbial interactions using correlated light and transmission electron microscopes. This study showed that the root canals of all periapically-affected teeth contain bacteria. The endodontic flora consists of a mixture of cocci, rods, filamentous forms, and spirochetes.

2.7. **Root canal disinfection**

The creation of a bacteria-free environment inside the root canal space through mechanical preparation and chemical disinfection is one of the essential elements for a successful endodontic regeneration protocol [33].

Disinfecting agents used as irrigants and intracanal dressings between appointments with a wide variety of medicaments. However due to complex root canal anatomy that harbour more bacteria, mechanical procedures and irrigation with sodium hypochlorite (NaOCL) has been proven that it is not enough to eradicate root canal infection [34,35,36].

A study was done by **George Stewart** [37] in **1955** titled "The importance of chemo mechanical preparation of the root canal" to evaluate the presence of bacteria inside the root canal after mechanical preparation. He cultured specimens immediately afterward and obtained negative cultures in 94% of the specimens. The percentage dropped to 76% when specimens were cultured during the next visit. The conclusion was that, irrigation with NaOCL may improve the adequate reduction of intra canal bacteria.

Antibiotics was discovered in 1928 and was labelled as (Antibiotics) in the 1940s [38] and was not available for civilian. The first reported use in Endodontics was by **Groomsman** [39] in **1948**. Within the next decade Grossman published another two articles outlining the use of antibiotics in different concentrations and combinations [40]. In his article, he used a combination of antibiotics including penicillin, bacitracin, streptomycin and caprylate sodium (PBSC) aiming to reduce the time of the treatment and this was the first usage of poly antibiotic in endodontics which was a precursor to modern triple antibiotic paste. The results showed reduction in the treatment time by one half. The conclusion was that "this combination is designed to destroy any type of organism likely to be encountered during treatment of infected root canals".

Between 1970 and 1990 the research was scant about the effect of antibiotics in the management of root canal infection. Calcium hydroxide was used for root canal disinfection [41,42,43.,44,45 ,46,47].

In **1990 molander et al.** [48] used a single antibiotic (clindamycin) as intracanal medicament. Their results, with regard to root canal disinfection, clindamycin has no more advantages over the conventional dressings such as calcium hydroxide. Their conclusion was that "clindamycin cannot be recommended for routine endodontic therapy".

After that, group of Japanese researchers including **Satto** and **Hoshino** did a series of research papers on the effect of different antibiotic combinations on dentin caries. Their research has led the way in the dental community in the field of topical antibiotics for the disinfection of carious dentin, infected root canals and periapical lesions.

In 1993 **Satto et al.** [49] published a paper in which the disinfection capacity of a mixture of ciprofloxacin, metronidazole, plus a third antibiotic amoxicillin, cefaclor, cefroxadine, fosfomycin or rokitamycin was evaluated with regards to carious dentin and infected pulpal tissues. The results showed that no bacteria were recovered in the presence of any antibiotic combination. The conclusion was that the antibiotic combination inhibits the growth of bacteria in the samples.

Furthermore, in another pioneering study done by **Satto et al.** in **1996** [50] to examine the ability of triple antibiotic paste to penetrate root canal dentin and eradicate bacteria. Triple antibiotic paste consisting of a mixture of ciprofloxacin, metronidazole and minocycline (TAP) was evaluated. The results and the conclusion were that "A mixture of ciprofloxacin, metronidazole and minocycline is useful for sterilization of infected root dentine, and that the drug mixture can be applied to root canals".

Following the establishment of a protocol [19] for Regenerative Endodontics many researchers have used different protocols to disinfect the root canals.

In an interesting study published in 2005 by **Windley et al**. [51] done to evaluate the affectivity of TAP to disinfect root canals of dog teeth associated with apical periodontitis. The canals were sampled before (S1) and after (S2) irrigation with 1.25% NaOCl and after dressing with TAP (S3). The results showed that at S1, 0% of the samples cultured were bacteria free, at S2 10% of the samples cultured were bacteria free and at S3 70% of the samples cultured were bacteria free. These results indicate the effectiveness of a triple antibiotic paste in the disinfection of immature teeth with apical periodontitis.

In another study done by **Sabrah et al.** [52], TAP was compared with double antibiotic paste (DAP) consisting of (metronidazole and ciprofloxacin), and calcium hydroxide against a biofilm formed of Enterococcus faecalis and Porphyromonas gingivalis. The results showed that both TAP and DAP were more effective than $Ca(OH)_2$ against E. faecalis and P. gingivalis bacteria. The conclusion was that "DAP can be considered an effective and comparable antibacterial substitute to TAP without the discoloration associated with the latter medicament.

In another study done by **Sabrah et. Al** [53], to evaluate the effect of different dilutions of TAP and DAP with regard to root canal disinfection and survival of dental pulp stem cells (DPSCs). The results showed that all dilutions of TAP and DAP were effective against the bacteria however all antibiotic dilutions except 0.125 mg/ml significantly reduced the viability of DPSCs. The conclusion was that "0.125 mg/ml of DAP and TAP showed a significant antibacterial effect with no cytotoxic effects on DPSCs".

In summery from all the above-mentioned studies it becomes evident that root canal infection can hinder any efforts for successful regeneration. Secondly mechanical preparation is not enough to completely eradicate root canal infection.

2.4. Tissue engineering

Tissue engineering is an emerging multidisciplinary field that applies the principles of engineering and life sciences for the development of biological substitutes that can restore, maintain, or improve tissue function. The tissues of interest in regenerative endodontics include dentin, pulp, cementum and periodontal tissues [(6)].

Regenerative medicine offers an exciting opportunity to replace or restore tissues of the body after disease and trauma. It aims to fabricate new replacement body tissues, and such approaches commonly involve seeding of cells at various stages of differentiation within scaffolds, which can then be implanted [(54)].

Regenerative Endodontic Therapy (RET) includes treatments such as revascularization via blood clotting technique, postnatal stem cell therapy, pulp and scaffold implantation, injectable scaffold delivery, three-dimensional cell printing, and gene delivery[(55)]. However, the use of advanced tissue engineering techniques for dental tissue regeneration, although promising, is still only at a laboratory and animal trial stage [(56)].

The three key elements of tissue engineering are stem cells, morphogens or growth factors, and an extracellular matrix scaffold.[(57)]

2.4.1. Triad of regeneration

2.4.1.1. Stem cells

2.4.1.2. Scaffolds

2.4.1.3. Signaling molecules (Morphogenic Factors)

2.4.1.1. Stem cells:

Cell-based therapies are the most common approaches in regenerative medicine. Challenges in applying this approach clinically are to acquire the appropriate source of cells, to identify methodologies to induce cell proliferation and differentiation, to maintain cell survival, and to remove unwanted cells.

The term "stem cell" generally refers to a cell possessing the ability to self-replicate and give rise to daughter cells which undergo an irreversible, terminal differentiation process. They are present in many vertebrate regenerative tissues including the hematopoietic system, nervous system, gut, gonads, skin, olfactory epithelium, and teeth [58].

A classic stem cell should possess two properties namely **self-renewal** and **potency**. A self-renewal is the capacity of the cell to undergo numerous cycles of cell division maintaining the undifferentiated state while Potency means the differentiation capacity of the stem cell [59].

Stem cell potency describes the potential of the cell to divide and express different cell phenotypes. **Totipotent** stem cells are able to divide and produce all the cells in an individual, including extraembryonic tissues. **Pluripotent** stem cells have not completely divided and can become many cells. They are able to differentiate into any of the three germ layers: endoderm, mesoderm or ectoderm, where the progeny have multiple distinct phenotypes, whilst **multipotent** stem cells can differentiate into cells from multiple, but a limited number of lineages. [60]

Based on their origin, there are two types of stem cells: embryonic and postnatal. Embryonic stem cells are pluripotent cells capable of differentiating into any cell type as well as maintaining an undifferentiated state. The immunological

and ethical problems of allogenic embryonic stem cells may limit their use to some extent [61].

Postnatal stem cells are multipotent and can be classified according to their **origin**, i.e. haematopoietic or mesenchymal (MSC), and **differentiation potential**. They are less potent and more limited in their differentiation potential than embryonic cells with a concise lifespan, but still fulfil the criteria of stem cells.

Use of stem cells, either of embryonic or postnatal derivation, for tissue engineering is attractive because it offers greater scope for cell fate to try and mimic physiologic tissue architecture. The discovery of stem cells in the pulp of permanent teeth [14] raised the possibility of using dental pulp stem cells for tissue engineering [62,63]. The dental pulp stem cells have been shown to be capable of self-renewal and multilineage differentiation [64].

The concept of using stem cells for dental tissue engineering was explored by **Sharpe** and **Young** [9,10]. They and others demonstrated that it is possible to engineer murine teeth by using adult stem cells of non-dental or dental origin.

The first successful attempt to engineer complex whole tooth structures used single-cell suspensions dissociated from third molar tooth buds and suggested the existence of dental pulp stem cells in this tissue [65].

It is speculated that stem cells from each tissue are somewhat **‘‘primed’’** to regenerate that same tissue, and, therefore, it is likely that the best stem cells for dental pulp tissue engineering are pulp stem cells. However, it is rather unclear at this time what the relative potential of each one of these oral stem cells for dental pulp tissue engineering is [66].

The stem cell population in the pulp is very small; approximately 1% of the total cells and the effect of aging reduces the cell pool available to participate in regeneration which reflects the better healing outcomes seen in younger patients [67].

A postnatal population of human DPSCs has been identified and isolated which show a higher proliferation capacity compared with osteogenic cells, have the ability to differentiate into odontoblast-like cells which express the early odontoblast cell marker, dentine sialophosphoprotein, and can form a dentine–pulp complex when transplanted in vivo [68].

Almushayt *et al* [69] have confirmed the expression of odontoblast specific markers and presence of collagenous matrix and calcified deposits in DPSCs to show that these cells differentiate into odontoblast and form dentin material.

In **2000 Gronthos et al.** concluded that in vitro DPSCs have been shown to produce sporadic but densely calcified nodules. In addition, he found that these cells are capable of forming ectopic mineralized tissue, similar to dentin, but only when grafted in vivo or when placed on the surface of human dentin in vivo [70] and when exposed to tooth germ conditioned medium [71] or similar differentiation factors [72].

DPSCs can be cryopreserved and revived whenever; they are needed for future regenerative therapies [73]. Some of the diseases are being cured by DPSCs include type 1 diabetes, neurological diseases, Immunodeficiency diseases and diseases of bone and cartilages [14,74].

As DPSCs have comparable therapeutic potential similar to bone marrow stem cells (BMSCs), DPSCs is another alternative noninvasive source to be used for future regenerative therapies. The most striking feature of DPSCs is their ability to regenerate a dentin-pulp-like complex that is composed of mineralized matrix with tubules lined with odontoblasts, and fibrous tissue containing blood vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth [75].

The isolation of stem cells is not restricted to the permanent dentition. Stem cells from Human Exfoliated Deciduous teeth (SHED) have been identified as a population of highly proliferative, colony-forming cells able to differentiate into more specialized cell lines, and capable of producing bone and dentine when transplanted in vivo. SHED are distinct from DPSCs in their greater proliferation rate and increased population doublings [76].

Another dental stem cell source is Stem cells from the apical papilla (SCAP) which are a population of multipotent stem cells isolated from the root apical papilla of human teeth [77]. SCAP might be the source of primary odontoblasts involved in the development of root dentine, in contrast to DPSCs, which are most likely involved in reparative dentine formation [78].

In year **2012**, **Shinya Yamanaka** and **John Gurdon** have won Noble prize award for their excellent work on induced pluripotent stem cells (iPSCs) derived from adult somatic cells. This work has resulted into development of innovated technology to make an iPSCs from individual patient who needs treatment for specific disease. It is proposed that dental pulp stem cells (DPSCs) can develop iPSCs which can be used for therapies of various diseases [79].

In conclusion, there are several studies that used animal models have confirmed that DPSCs implantation in the root canal could lead to pulp regeneration [80,81,82].

Other sources of stem cells

Bone Marrow

Bone marrow stem cells (BMSCs) can be harvested from sternum or iliac crest. It is composed of both hematopoietic stem cells and MSCs. The majority of oro-maxillofacial oral structures are formed from mesenchymal cells. The advantage of bone marrow is that it has a larger volume of stem cells and can be differentiated

into a wide variety of cells. Isolation of BMSCs can be carried out only under general anesthesia.

Adipose Tissue

They can be harvested from the lipectomy or liposuction aspirate. Adipose-derived stem cells (ADSCs) contain a group of pluripotent MSCs that exhibit multilineage differentiation. Advantage of adipose tissue is that it is easily accessible and abundant in many individuals [(83)].

Another type of non-dental adult stem cells is Adipose-Derived Stem Cells (ASCs) which have several advantages including ease of isolation by local excision or suction-assisted liposuction, relative abundance, rapidity of expansion, and multipotency that is independent upon serum source and quality. They also have high cell yield and rapid in vitro expansion [(84)].They have demonstrated broad multipotency with differentiation into number of cells lineages including adipo-, osteo-, chondro-cytic lineages.

However, the easy and repeatable access to subcutaneous adipose tissue and the approximately 500-fold greater number of fresh mesenchymal cells derived from equivalent numbers of other types like bone marrow provide a clear advantage. One gram of human adipose tissue could yield over 70,000 ASCs within 24 h of culture. These cells could be further expanded in vitro for over 2 weeks.[(85)] Also isolated adipose tissue could be easily cryopreserved. These favorable features of ASCs make them more applicable for clinical treatments. [(86)] ASCs have neither ethical nor immune-reactive consideration and might be an alternative cell source for pulp tissue regeneration.[(87)]

Hicok et al. [(88)] found that ASCs produced more osteoid when being seeded on hydroxyl appetite tricalcium phosphate (HA/TCP) than cells that were cultured

on collagen/HA–TCP composite, indicating that the osteogenic capacity of ASCs could be heavily influenced by the scaffold on which cells were seeded.

It is also not known whether MSCs from different sources are comparable in their differentiation potential in vivo or whether their capabilities are influenced by the niche of their origin [82].

The role of seeding cells in tissue regeneration is controversy. Some groups reported that scaffold alone or scaffold modified with growth factors is sufficient for bone regeneration [89,90], while other studies showed that no functional repair could be achieved without seeding cells by tissue engineering approach [91,92].

It is becoming apparent that revascularization alone without cell transplantation in necrotic teeth is accompanied by the resolution of periapical lesion and partial apical closure, but it does not enable the generation of a fully functional dental pulp tissue throughout the full length of the root canal [93,94]. On the other hand, transplantation of human stem cells generates a dental pulp throughout the entire length of human premolars transplanted in the subcutaneous space of immunodeficient mice. [95]

The anatomy of the dental root is a major limiting factor regarding access to vascularization, considering that all blood vessels have to come through a system of narrow foramina located exclusively in 1 end of the tooth. In young, immature teeth, the apical opening of the root is relatively wide. However, in people aged 21 years or older, the dimensions of apical foramen are very narrow [96] and tend to decrease progressively over time. It appears that necrotic immature teeth with open apices are the prime candidates for dental pulp tissue engineering at this stage of development of the technique.

However, the translation of stem cell–based dental pulp tissue regeneration into routine clinical use faces significant **challenges**. For example, it is still unclear what the ideal source of multipotent stem cells for pulp regeneration is. We do not know if dental pulp stem cells are necessarily better than gingival stem cells or bone marrow–derived mesenchymal stem cells in regenerative endodontics. An additional challenge is that one would have to establish cell handling protocols that follow good manufacturing practice standards, defined by the Food and Drug Administration as ex vivo manipulation of clinical-grade cells that are safe for the patient while being effective therapeutically, in dental clinics and supporting laboratories [(97)].

3.2. Scaffolds:

On the other hand, the potential role of the scaffold as a delivery vehicle for cells has become increasingly important in a wide variety of tissues and organs in cell therapy for local repair. Scaffolds are three dimensional structures that can support cell organization and vascularization [(98)]. A scaffold is necessary to aid the ingrowth of new tissue into the canal space. They share common features such as allowing cell attachment, diffusion of nutrients and oxygen, being biodegradable, and having physical properties aligned with those of the tissue/organ to be regenerated [(99,100)].

Scaffolds can be classified according to the **origin** into ***natural*** or ***synthetic***, and according to the **fate** into ***biodegradable*** or ***permanent***.[(101)] The synthetic scaffolds include polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL), poly(dioxanone), poly(methyl methacrylate) (PMMA), and poly(glycerol-sebacate) which are all common polyester materials that degrade within the human body [(102,103,104)].

Natural scaffolds are either blood clot or different derivatives of the extracellular matrix. Several materials, such as collagen, fibrin, alginate, agarose,

hyaluronic acid derivatives, chitosan, have been suggested for use as scaffolds [105,106,107].

Platelet rich plasma (PRP) [7,108,109], and platelet rich fibrin (PRF) [110,111,112] are examples of natural scaffolds which have been used as a scaffold instead of a blood clot because they are rich in growth factors, which might help improve regeneration of pulp-dentin complex.

Polymers, such as polyglycolic acid (PGA), have been previously found to act as suitable matrices for seeding of dental pulp fibroblasts, allowing their proliferation and development of a tissue with similar cellularity to normal pulp. PGA was a more conducive scaffold for dental pulp cell proliferation than a hydrogel and an alginate [104].

Also, scaffolds can be divided into (1) **casted** (i.e., fairly rigid and custom-made for specific purposes) and (2) **injectable** (i.e., low viscosity gels that can be delivered and ‘‘molded’’ at the site that requires tissue regeneration).

Various approaches have been considered in tissue engineering and regenerative medicine, but currently the most common is to use a biodegradable scaffold in the shape of the new tissue that is seeded with either stem cells or autologous cells [113].

Key considerations in development of a strategy for tissue engineering is the choice of scaffold and cells, which show potentiality to differentiate into the various cell populations, especially the dentin-secreting odontoblasts and the cells of the vasculature to provide vitality to the tissue. The scaffold provides an environment that allows the implanted cells to proliferate, differentiate, and form the desired tissue or organ. [114]

The role of a scaffold is to provide support for delivering cells and growth factors to the proposed site of tissue regeneration. There are important features to

consider in scaffold selection, including the physical and mechanical aspects of the material, its biocompatibility, and its degradation timeline. Hydrogels are polymeric structures that are cross-linked and swell in water. For a hydrogel, important aspects include swelling behavior and diffusivity of the hydrogel should be examined[115].

A beneficial clinical feature for dental pulp regeneration would be if the scaffold is injectable, as are some of the natural scaffold materials and hydrogels. In these cases, the gelation time would need to be taken into consideration when seeding cells in a scaffold for implantation into a host[116]. Also the degradation process of the scaffold is important, and should closely follow the rate of tissue regeneration [117].

It is becoming increasingly evident that the ideal scaffold for dental pulp tissue engineering will be injectable, not casted. This is because of the narrow spaces within the root canal and the complexity of its anatomy, particularly in the apical region. In addition, there are concerns related to the use of solvents (e.g., chloroform, dichloromethane, and acetone) that are typically used to solubilize casted scaffold. For example, solvent-casted poly (lactic-co-glycolic acid) requires more than 2 days for significant volatilization, and residual levels of solvents may be toxic to cells. On the other hand, hydrogels can be injectable and therefore penetrate throughout the root canal system [118].

2.4.1.3. Signaling molecules (growth Factors)

The observation that dentin is a reservoir of bioactive morphogenic signals that can be recruited on demand [119] constitutes a major discovery in the field of dental pulp tissue regeneration. This discovery represents a true paradigm shift in the field because it elevated the dentin to the status of a morphogenic source that enables and guides regenerative processes and tissue repair, rather than being simply an inert and passive tissue.

Growth factors are polypeptides produced by immuno-inflammatory and tissue cells and bound to extracellular matrix. They can bind to receptors on the cell and act as signals to induce cellular proliferation and/or differentiation. They regulate many aspect of cellular function, including survival, proliferation, migration and differentiation [120]. Growth factors typically have a short half-life and are rapidly eliminated.

Several growth factors can have one target cell and one growth factor can have several target cells. Growth factors determine the fate of stem/progenitor cells and are often immobilized in scaffold to help promote tissue regeneration in tissue engineering [121].

Among the growth factors released from dentine matrix, Transforming growth factor (TGF -β1), fibroblast growth factors 2 (FGF2) and platelet derived growth factors (PDGF) enhance cell migration; PDGF and vascular endothelial growth factors (VEGF) control angiogenesis; TGF -β1, FGF2, VEGF and insulin - like growth factors stimulate cell proliferation; Bone morphogenetic proteins and FGF2 promote dentinogenesis. Non-collagenous proteins such as dentine matrix protein and dentine phosphoprotein, and glycosaminoglycan such as chondroitin sulfate and dermatan sulphate also promote dentinogenesis. [122]

Disinfection irrigants and medicaments used in regeneration can influence growth factor release from dentine [123]. It has been shown that varieties of biological molecules are embedded in dentine matrix and can be released when demineralization occurs [124].During regeneration, dentine conditioning agent is used to liberate the entrapped biological elements from dentine matrix before apical bleeding is evoked. These dentine matrix molecules include growth factors, non - collagenous proteins and glycosaminoglycan [125]. These biological molecules can direct the behaviors of cells mobilized into root canals toward pulp regeneration.

The discovery that dentin-derived proteins are sufficient to induce full differentiation of dental pulp stem cells into odontoblasts has an important implication for pulp tissue engineering. It indicates that one does not need to provide additional morphogenic signals to achieve odontoblastic differentiation of stem cells transplanted or recruited into the root canal. The focus could be simply to protect these dentin-derived factors from degradation (e.g., avoid exposure to sodium hypochlorite) and to enhance their mobilization by treating the dentin surface with mild organic acids (e.g., Ethelyne Diamine Tetra Acetic acid (EDTA) [126].

Some investigators have shown that dentin-derived proteins are sufficient for odontoblastic differentiation [127]. Therefore, intentional degradation of dentin-derived proteins with sodium hypochlorite eliminated its inductive potential [128]. This finding raised the possibility that sodium hypochlorite might not be the ideal solution for root canal irrigation in regenerative endodontics [129].

Another important dentin-derived morphogenic signal is transforming growth factor beta 1 (TGF-b1) [127] that present in sound dentin and can be released by the acidic activity of cariogenic bacteria [130] or when EDTA is applied over sound dentin [131].

In addition to the dentin, there are several other sources of morphogenic signals that include, but are not limited to, resident pulp cells (e.g., fibroblasts, neural cells, and endothelial cells), circulating cells (e.g., circulating progenitor cells and inflammatory cells), and the pulp extracellular matrix itself. The understanding of the role of these morphogenic signals in the maintenance of dental pulp homeostasis and in the processes that lead to pulp regeneration is emerging[132].

3. Statins and regeneration

Over the past two decades, a variety of pharmacological agents have been studied for their possible roles in the management of disease and regeneration of different structures.

Statins are a class of drugs which is used to lower the cholesterol levels. They mainly act by inhibiting the enzyme (HMG-CoA) 3-hydroxy-3-methylglutaryl Coenzyme A reductase, which plays a central role in the production of cholesterol in the liver, and contributes to produce about 70 % of total body cholesterol. This group of drugs was primarily introduced as cholesterol-reducing drugs but have been found to increase bone mass [(133)].

Statins have been shown to have certain ***pleiotropic effects***, including the prevention of inflammation, induction of angiogenesis, and improvement in vascular endothelial function [(134)].

The potential anti-inflammatory of statins when used in systemic and local application can enhance osteoblastic differentiation and bone formation. It has also been suggested that statin use was associated with decreased tooth loss in chronic periodontitis patients [(135)].

Amongst the various pleiotropic effects of statins, their anabolic effect on bone metabolism, reported for the first time in 1994, has recently been receiving a great attention. Although the exact molecular mechanism of increase in bone formation remains elusive[(136)], the collected in vitro and in vivo evidence supports the notion that stimulation of vascular endothelial growth factor (VEGF) and bone morphogenic protein-2 (BMP-2) is responsible for the anabolic effects of statins on bone metabolism [(137,138)].

The statin family reportedly increased bone mineral density in humans and decreases the risk of fractures in osteoporotic and elderly patients. **Horiuchi** and

Maidea [139] pointed out that statins may be useful for treating periodontal disease in patients with osteoporosis. Furthermore, systemic administration of simvastatin (SIM) is found to be associated with a reduced risk of tooth loss in patients diagnosed with chronic periodontitis.

On searching for alternatives to the application of exogenous genetically engineered proteins, some authors have suggested the topical use of drug compounds aimed at upregulating intrinsic bone growth factors such as bisphosphonates or statins to upregulate bone growth through distinct and complex biochemical pathways [140].

As statins can improve osteoblasts function and suppress function of osteoclast leading to enhanced bone formation [141], Therefore, they might improve odontoblastic function resulting in improved dentin formation [12]. Statins are also thought to induce angiogenesis and increase neuronal cell. Consequently, they might have a role in pulp regeneration along with dentin regeneration [13].

Classification of statins:

The statins differ with respect to their ring structure and these differences in structure affect the pharmacological properties of the statins, such as the affinity for the active site, rates of entry into hepatic and non-hepatic tissues, availability in the systemic circulation for uptake into non-hepatic tissues and routes and modes of metabolic transformation and elimination [142].

Statins include Lovastatin, Provastatin, Aterovastatin and Simvastatin. Simvastatin, a lipophilic Statin, was selected because it has a higher bone growth stimulation capability compared with the more hydrophilic Statins[143].

Simvastatin:

Simvastatin (SIM) is a hypolipidemic drug used to control elevated levels of cholesterol and has recently been found to have potent anti-inflammatory action. By

targeting inflammation itself, simvastatin was found to reduce the damage done by the body and thus giving way to healing [144]. Organogels are capable of delivering a variety of lipophilic and hydrophobic drugs like simvastatin. They are also easy to prepare, require fewer ingredients, are good permeation enhancers and can have a sustained release effect [145].

Regarding the anti-inflammatory effect, **Libby et al** [146] stated that statins can cause an improvement in inflammatory pathways including, vascular reactivity and coagulation. Recent evidence suggests that these anti-inflammatory properties may be of some clinical relevance.

Also Simvastatin seems to play an important role in bone regeneration by participating directly in osteoblast activation (increasing Bone Morphogenic Protein expression) and in osteoclast inhibition [147] and also indirectly, by stimulating neovascularization increasing the secretion of Vascular Endothelial Growth Factor [148].

Mundy et al [149] reported that simvastatin stimulated new bone formation in vitro as well as in vivo. In addition, in vitro studies have shown that statins promote osteoblastic differentiation in mouse osteoblastic cells [150], human osteosarcoma cells [151], murine embryonic stem cells [137], and human periodontal ligament cells [152], all of which can result in bone formation.

Regarding pulp-dentin complex regeneration, few investigations have assessed the effects of SIM-loaded biomaterials. **Miyazawa et al** [153] developed a gelatin hydrogel polylactic acid micelles loaded with low concentrations of SIM. These authors determined that this biomaterial enhanced alkaline phosphatase ALP activity, calcium deposition, and BMP-2 secretion by DPSCs seeded on its structure. After subcutaneous implantation into mice, the constructs containing SIM exhibited increased dental pulp cell, DSP expression and calcium deposition.

Stein et al. [154] showed that topically administrated Simvastatin resulted in dose-dependent bone formation. **Min et al** [13] showed that SIM promotes odontogenesis in human dental pulp cells.

Wu et al [155] studied the different local concentrations of simvastatin to promote bone formation in vivo to find an appropriate delivery system. There are a number of advantages to an appropriate carrier, including localization and retention of the molecule to the site of application thus reducing the loading dose and providing a matrix for mesenchymal cell infiltration and a substrate for cell growth and differentiation. The carrier may also help to define the shape of resulting new bone and the optimal carrier has a degradation rate that does not inhibit bone growth and prevent fibrous tissue formation or fibrous encapsulation of the carrier.

Previous studies have shown that SIM stimulates the expression of bone morphogenetic protein (BMP)-2 [156], thereby promoting bone formation by bone marrow stem cells. Similarly, it has been also shown to stimulate the mineralizing phenotype in dental pulp stem cells (DPSCs).

Okamoto et al [12] reported that simvastatin-treated dental pulp stem cells exhibited enhanced odontogenic differentiation as well as accelerated mineralized tissue formation. Moreover, simvastatin markedly diminished the severity of induced rat periapical lesions.

Also, **Emani et al** in an in vitro study found a potent antimicrobial activity for simvastatin against major pathogen microorganisms in periodontitis [157].

Another study found that **3Mixtatin** (a combination of metronidazole, minocycline, and ciprofloxacin) and statin, showed toxicity to tissues to some degree, which was concentration-dependent and the higher the concentration of medicaments, the lower the cell survival and cell viability. As a result, cytotoxicity

of 0.1 mg/mL 3Mixtatin was medium and 3Mixtatin at 1 mg/mL concentration was severely cytotoxic. These findings revealed no significant difference in cytotoxicity of 0.05 mg/mL 3Mixtatin and that of MTA group and thus, 0.05 mg/mL concentration of 3Mxtatin was found to be non-toxic and there is no significant harmful effect to the cells up to 0.05 mg/ml of 3Mixtatin [158].

Paradeep et al. have been investigated the use of topical simvastatin to decrease inflammation around natural teeth [159]. In periodontal disease, tissue destruction results from the interaction of the host's immune responses with microorganisms in dental plaque. Statins has been suggested to have several anti-inflammatory effects which may also be important in treating periodontal disease.

In **Nyan's** experiment, he applied different doses of simvastatin combined with osteoconductive Tri Calcium Phosphate (TCP) particles. The results showed that Simvastatin doses of 0.25 and 0.5mg caused inflammation of the soft tissue at the graft site whereas 0.1mg simvastatin is the optimal dose for stimulation of the maximum bone regeneration in rat calvarial defects without inducing inflammation and it could be applied as an effective bone graft material [160].

In a previous study, **Aminabadi et al** [161] applied SIM at concentrations of 1 µml/L, 5 µml/L, and 10 µml/L, along with highly viscous sodium carboxyl methylcellulose as a carrier, to exposed pulp in human primary molars. Those authors observed that the higher SIM concentrations led to dental pulp inflammation and that a small amount of this substance stimulated dentin bridge formation at the pulp exposure site. SIM at 1 mmol/L induced odontoblast like cell differentiation from host DPCs followed by deposition of mineralized tissue at the pulp-dentin border; however, pulp inflammation was still detected. These data may be correlated with the toxic effect of SIM at high concentrations.

3. Aim of the study

The current study was directed to evaluate the regenerative potential of simvastatin on postnatal stem cells of different sources in non-vital immature dogs' teeth both radiographically and histopathologically. The null hypothesis was stated as there is no significant difference among the experimental groups.

4. Materials and Methods

Section outline:

Materials and Methods

This study was approved by the Ethical Committee at Faculty of Dentistry, Al Azhar University based on the guidelines and regulations. The procedures were carried out in the Department of Veterinary Surgery, General veterinary hospital in Alabbasyya, Cairo, Egypt. Every effort was done to minimize the discomfort of the dogs involved in this study with reduction of the number of used animals as possible.

Out of 10 examined dogs, the present study was carried out on 6 adult healthy male mongrel dogs aged from 4 to 6 months and their weight ranged from 12 to 17 kg. The dogs were examined by the staff member of Veterinary Surgery to rule out the presence of any disease. The animals were kept under clinical observation for two weeks preoperatively and fed cooked meat, liver, bread, milk and water. The animals were housed in separate cages, supplied with food and water tanks and allowed to live in optimal conditions according to the hospital housing protocol and under the supervision of the staff members of the Veterinary Hospital.

The cages were sprayed with 6/1000 Neocidal Diazinone and the dogs were bathed in 1/1000 Neocidal Diazinone. The dogs were injected with Ivomac 0.1mg/Kg of body weight subcutaneously to guard against ecto-endoparasitic infections. Furthermore, dogs were vaccinated by the triple vaccine against rabies, distemper, leptospirosis and hepatitis viruses. This was to eliminate the presence of parasites such as Ankylistoma, Tania solium and Tania saginata, which was determined by urine and fecal analysis, to exclude diseased dogs.

4.1. Selection of dogs' teeth (Animal model)

Three premolars (two rooted) in each quadrant from 6 healthy mongrel dogs were included in this study summing up the total number of teeth to 72 (12 premolars x 6 dogs). Under dog's sedation, clinical and radiographic examination was done to confirm shedding of the primary teeth with eruption of their permanent successors

with incomplete root formation **(Figures 1 and 2)**. All the selected teeth were intact and free from dental caries.

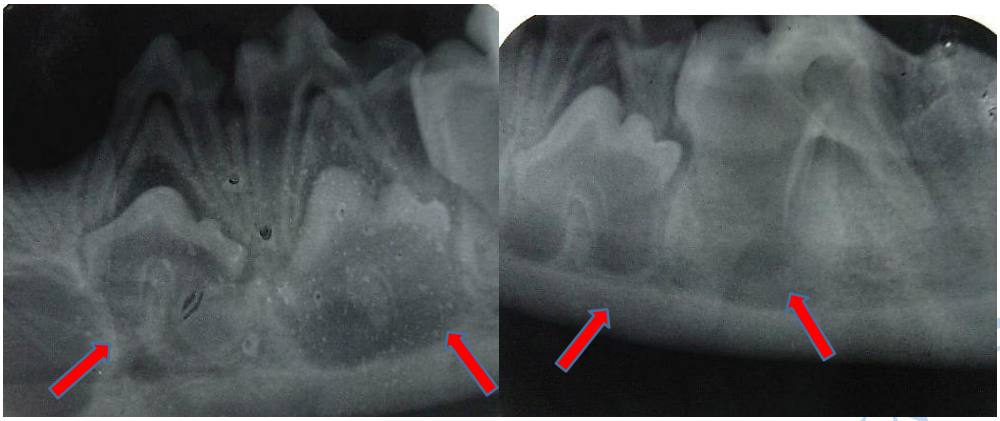


Fig. (1): showing radiographs of deciduous teeth before shedding

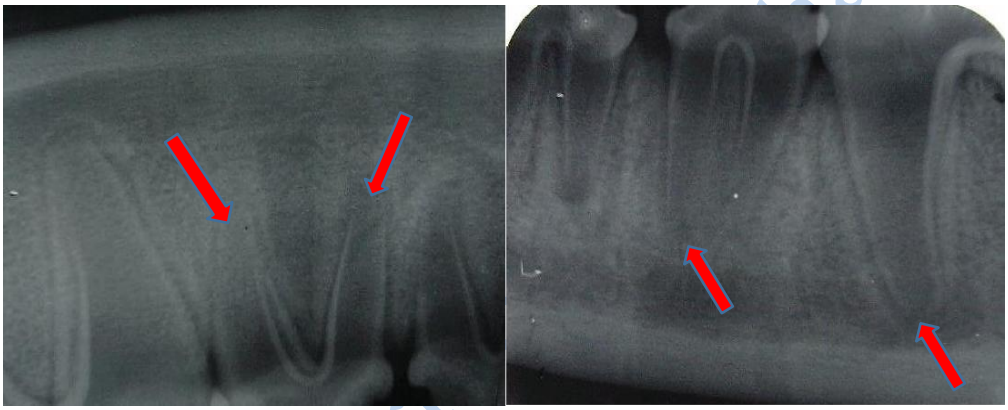


Fig. (2): showing teeth with incompletely formed roots with open apices with thin dentinal walls

Radiographs were exposed using an X-ray machine (Philips Medical Systems, Shelton, Connecticut) using the following exposure factors: 7.5 mA, 65 kVp, and 0.66 seconds.

4.2. Anesthetization of the dogs

Food and water were kept away from the dogs 12 hours prior to the operation to prepare them for anesthesia. Dogs were weighted to calculate the dose of the drugs used and to get a preliminary record for the general health of the dogs. All dogs were

pre-medicated with subcutaneous injection of atropine sulphate 0.05 mg/kg body weight (Atropine Sulphate; Misr Co., Cairo, Egypt) and intramuscular Xylazine HCl 1.1 mg/kg body weight (Xylaject; ADWIA Co., Cairo, Egypt), Diazepam 0.5mg/kg (Diazepam Bayer, Leverkusen, Germany) and Amoxicillin and flucsacillin (flumox 1000mg vial E.I.P.I.Co 10th of Ramadan Egypt) as a prophylactic antibiotic which injected intramuscularly, 20 minutes, before induction of general anesthesia.

A cannula, 18-20 gauges, was fixed in the radial vein for injection of drugs. The anesthesia was induced by intravenous Ketamine HCl 5mg/1kg body weight (EIMC. Pharmaceuticals co., Egypt). Then the anesthesia was maintained by 25mg/1kg intravenous incremental doses of 2.5% solution of Thiopental sodium (EIPICO, Cairo, Egypt). One third of the estimated dose was injected within 15 seconds, the remainder was administered slowly until loss of pedal and corneal reflexes, constriction of the pupils and development of shallow regular respiration **(Figure 3)**. Each animal was placed on the operating table in supine position with tilted head to provide unblocked air-way. Special mouth gag was used to keep the mouth opened. Between visits, intra appointment analgesia were given by Torbugesic (Butorphanol Tartrate, Fort Dodge Animal Health, Fort Dodge, Iowa) 0.2 mg/kg post-operatively after all subsequent operative procedures for analgesia.

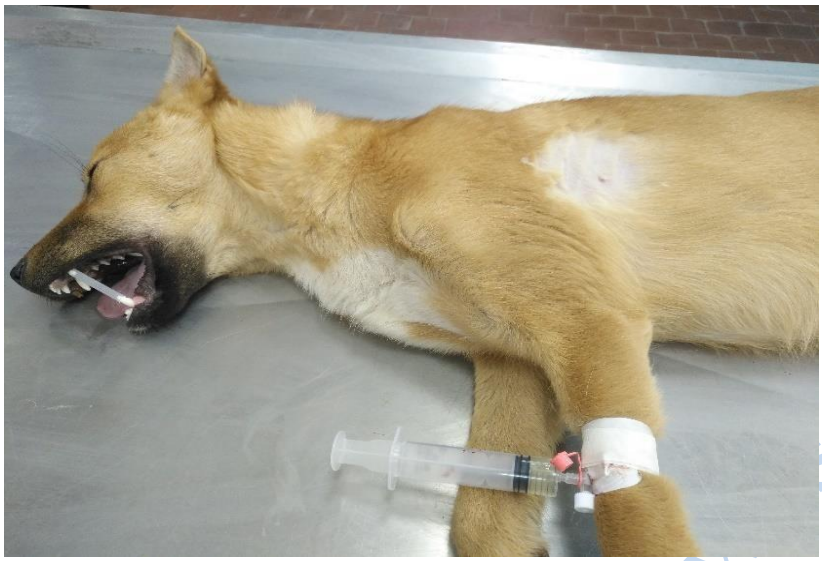


Fig. (3): a photograph showing intravenous anesthesia

The respiratory airway was kept patent by applying an endotracheal tube if needed**.** Then the operative site was painted with betadine mouthwash (Nile Company for Pharmaceuticals and Chemical Industries, Cairo, Egypt) and the site of the operation was isolated while the dog was draped in the regular surgical manner.

4.3. Induction of periapical radiolucency

In the first visit, endodontic access cavities were prepared in all experimental teeth in the buccal surface and the vital pulps of teeth were exposed. This procedure was performed with high-speed hand piece (NSK, Tokyo, Japan) under constant coolant with a size no. 2 sterile carbide bur (Brassler USA, Savannah, Georgia). The depth of penetration was equal to the bur diameter so that furcal perforation would be avoided. Then a size 25 sterile k-file (Mani, Inc., Tochigi, Japan) was used to disrupt the pulp tissue in the canals followed by placement of a piece of cotton into the entrance of each canal **(figures 4,5 & 6)**.

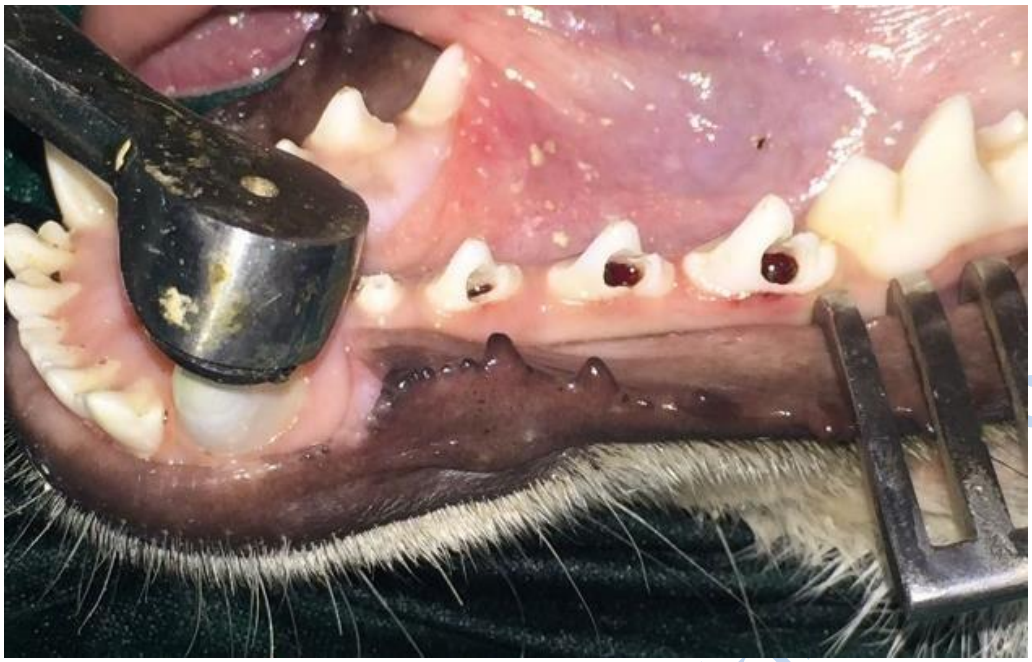


Fig. (4): a photograph showing access cavities of the premolars

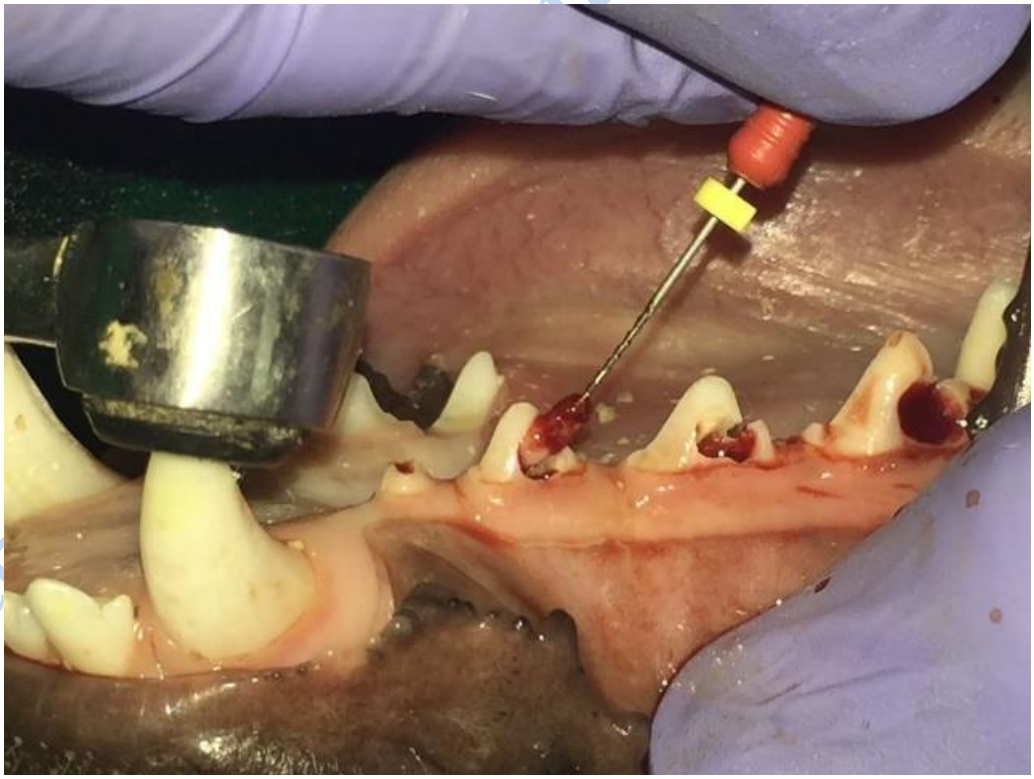


Fig. (5): a photograph showing disruption of pulp tissues

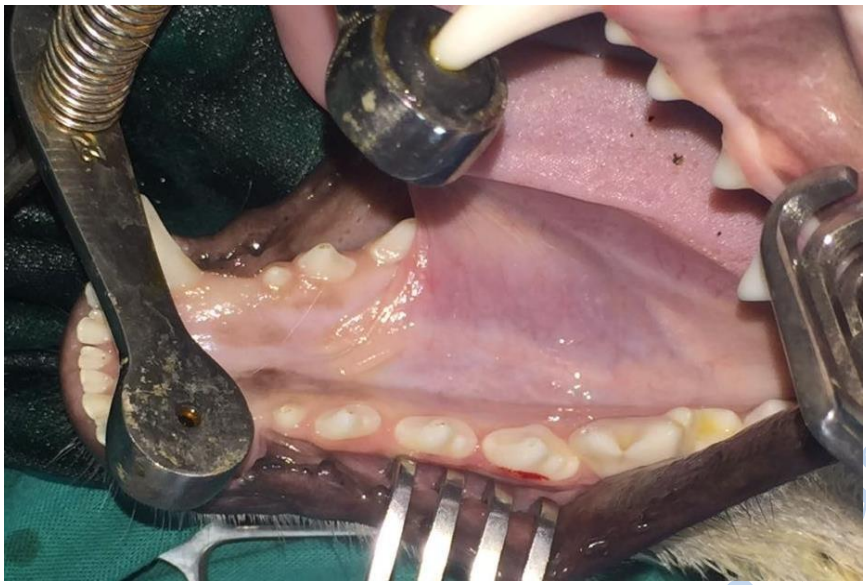


Fig. (6): a photograph showing cotton pellets inside access opening

Then the access cavity was left open till the radiographic evidence of periapical radiolucencies was appeared that considered as the preoperative radiograph for future comparison. Once apical periodontitis (radiolucent area related to the root apex with interruption of lamina dura) was verified radiographically **(figure 7)**, the dogs were placed under general anesthesia for a disinfection protocol of the infected pulp spaces.

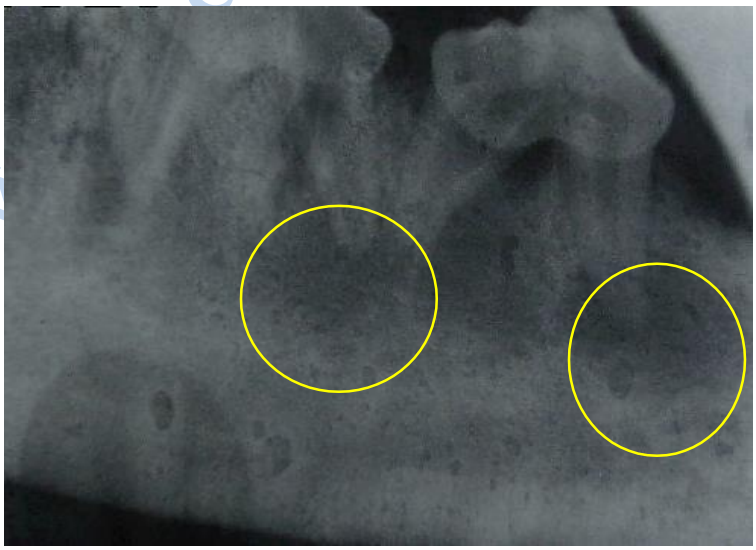


Fig. (7): a photograph confirming apical radiolucencies

4.4. Disinfection protocol

In the second visit, rubber dam isolation of the selected teeth was done followed by removal of previously placed cotton from their access cavities. The root canals were irrigated using 10 mL of 1.5 % sodium hypochlorite (Regular household Bleach, Clorox, Egypt), flushed with 10 ml of sterile saline (0.9% sodium chloride, Novartis, Egypt) and dried with sterile paper points (Dentsply Maillefer, Tulsa, Oklahoma). **(figures 8,9)**

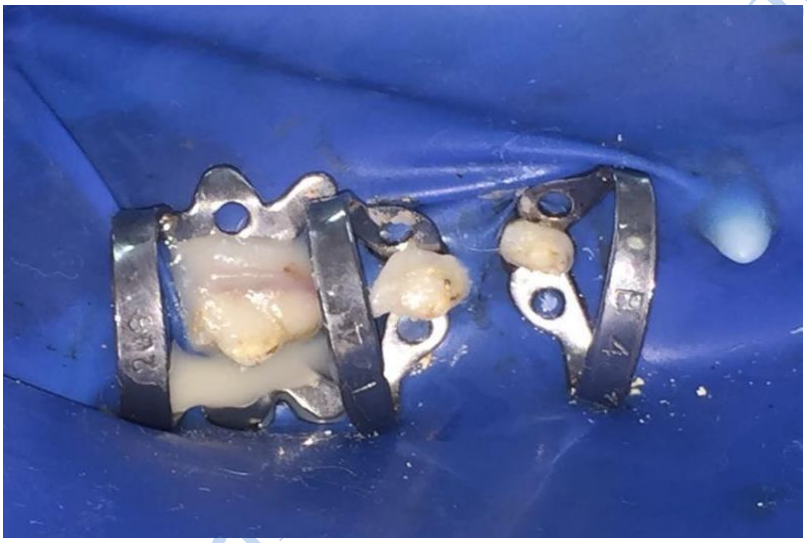


Fig. (8): a photograph showing rubber dam application

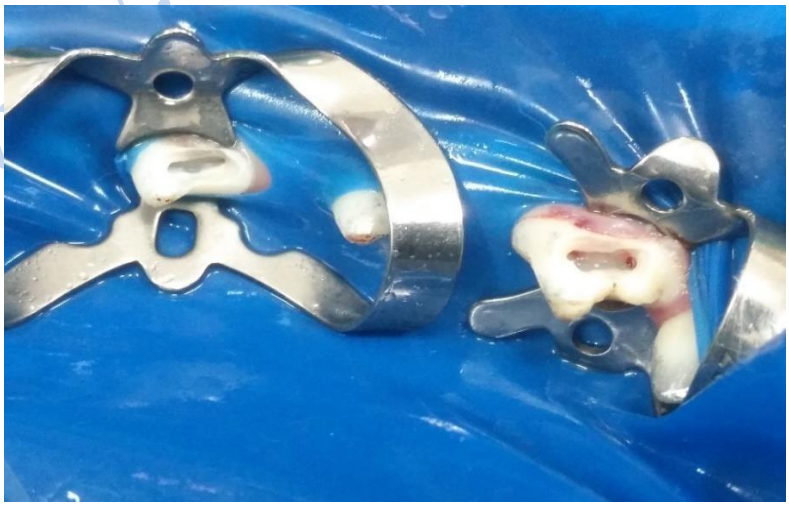


Fig. (9): a photograph showing access cavities after irrigation and dryness

The triple antibiotic paste (TAP) was prepared using metronidazole 500 mg tablets (Flagyl 500 mg, Aventis, Cairo, Egypt), ciprofloxacin 250 mg tablets (Ciprocin 250 mg, EPICO, Cairo, Egypt) and doxycycline 100 mg capsules (Vibramycin, Pfizer, Cairo, Egypt) in equal portions of each antibiotic by concentration mixed with 3-4 drops of sterile saline to a paste like consistency **(Figure 10)**. TAP was injected into the canal using a 16-gauge needle attached to a 10 ml sterile plastic needle placed 2 mm shorter than the working length after that the syringe was drawn back coronally to back fill the canal to the level of the Cemento Enamel Junction (CEJ). Then the access cavity was sealed with a resin reinforced glass ionomer (Ketacfill, 3MESPE, USA) for a period of 3 weeks (disinfection period).

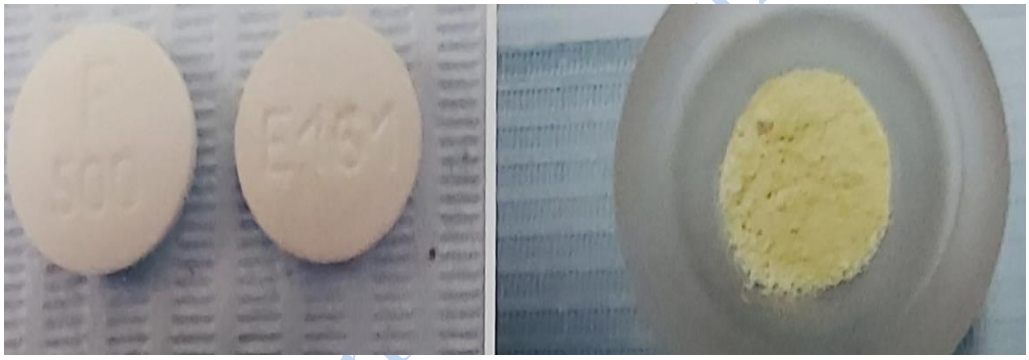


Fig. (10): showing preparation of TAP

4.5. Grouping of the teeth

The teeth were re-entered under the same anesthesia and aseptic conditions and the antibiotic paste was removed by copious irrigation using 10 mL of 1.5 % sodium hypochlorite then flushed with normal saline using a 27-gauge side vented closed end endodontic irrigating needle. A 17% EDTA liquid was used as final rinse then the root canals were dried using paper points. According to sample size calculation, the number of selected double rooted premolars teeth included within the study was set to be 72 teeth included in 6 dogs. Teeth were divided into four

equal groups according to different treatment protocols used for each experimental group as follows:

Group 1: treated with dental Pulp Stem Cells (DPSCs) added to simvastatin (SIM) hydrogel **(18 teeth)**

Group 2: treated with Adipose-derived Stem Cells (ASCs) added to simvastatin (SIM) hydrogel **(18 teeth)**

Group 3: treated with Dental Pulp Stem Cells (DPSCs) **(18 teeth)**

Group 4: treated with Adipose-derived Stem Cells (ASCs) **(18 teeth)**

Then each main group was subdivided into 3 subgroups according to post treatment evaluation periods as follows:

Group 1: evaluation period after 1 week of treatment **(24 teeth)**

Group 2: evaluation period after 1 month of treatment **(24 teeth)**

Group 3: evaluation period after 3 months of treatment **(24 teeth)**

Randomization of the groups:

Each dog was taken a number from 1 to six for preoperative randomization using research randomizer software (www.randomizer.com) to be blindly selected for each group. Furthermore, within each dog another randomization was done for eah quadrant. **(Figure11)**

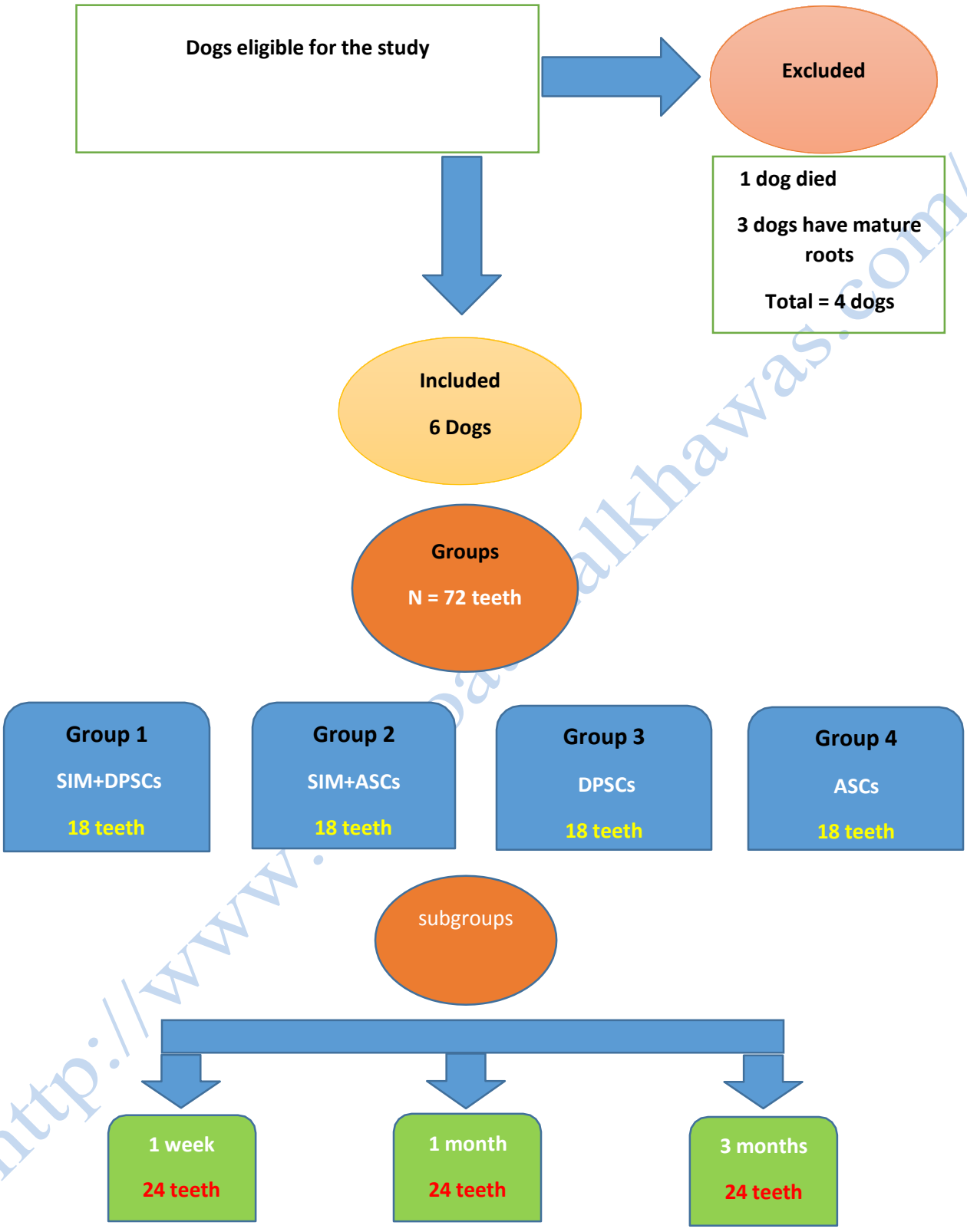


Fig. (11): Schematic drawing represents grouping of dogs teeth

4.6. Tissue harvesting and isolation of stem cells

4.6.1. Harvesting dental pulp tissue

Extraction of maxillary lateral incisor with vital pulp was done from each dog to isolate dental pulp stem cells **(Figure 12)**. Following extraction, each tooth was immersed in saline solution containing mixture of antibiotics (penicillin, streptomicin amphotericin B (Sigma Aldrich, St. Louis, MO, USA) for disinfection of external root surfaces. A longitudinal inciso-apical groove was done using (round bur size 1) under water coolant to preserve cell viability of the pulp. A vertical split was done using a pair of sterile extraction forceps while the pulp was picked up using toothed forceps. The harvested pulp was placed in a minimum essential medium (MEM) transporting media (Sigma Aldrich, St. Louis, MO, USA) in a validated, temperature-controlled 2°C transport box fitted with a frozen cold pack and shipped to the stem cells laboratory for processing **(Figure 13)**.

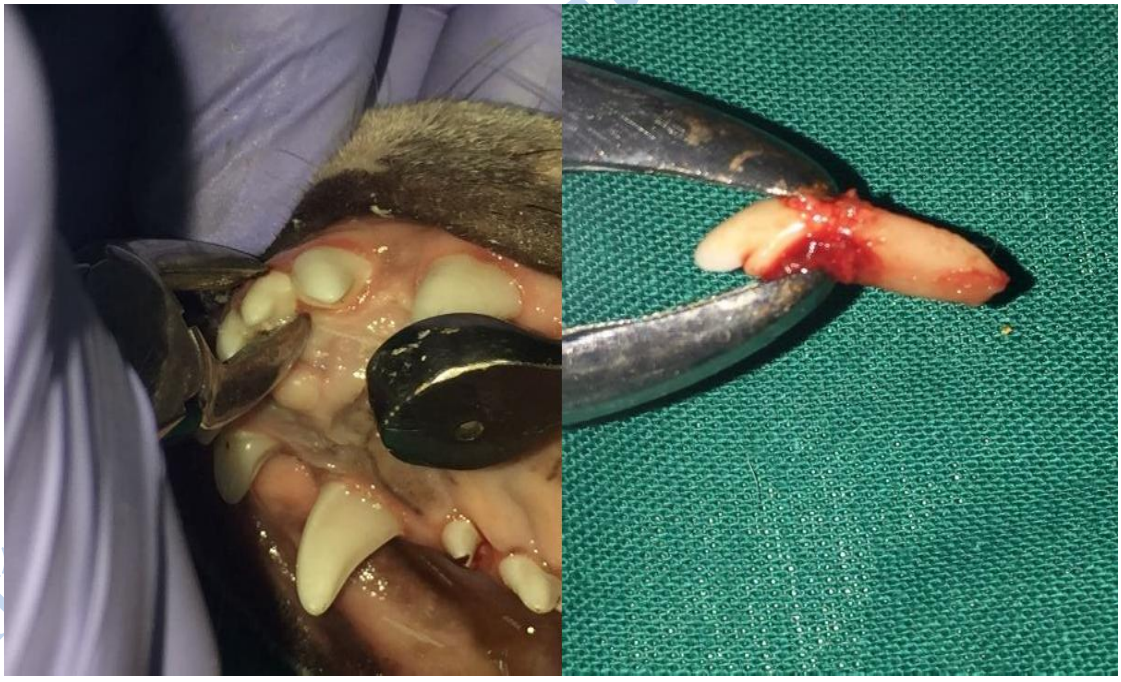


Fig. (12): photographs showing extraction of maxillary lateral incisor

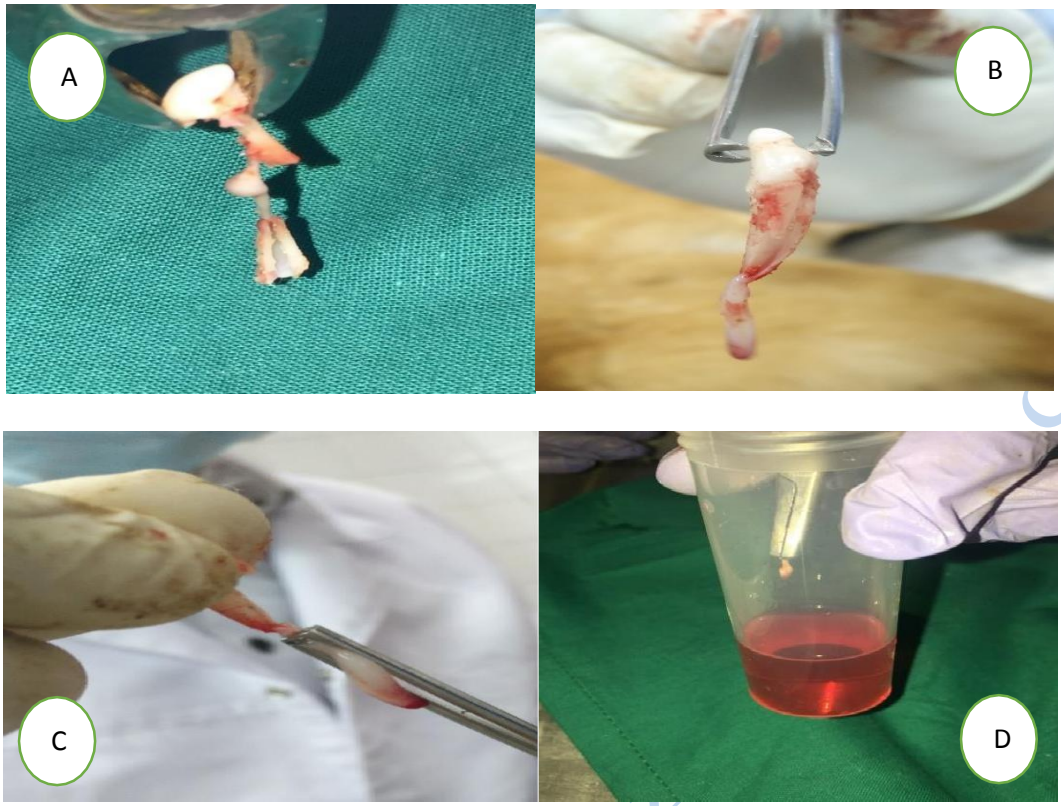


Fig. (13): Photographs showing pulp tissue harvesting procedures

a) Root splitting, b) Exposure of pulp tissue, c) Pulp tissue separation d) Placing pulp tissue into transporting media

4.6.2. Harvesting adipose tissue

Autologous adipose tissue was harvested from the pad of fat in the abdominal region of each dog. Following hair shaving and disinfection of the surgical site using betadine, an elliptical incision through the skin was done till reaching the fatty layer. Injection of 1:50:000 epinephrine into the adipose compartment was made to minimize blood loss and to decrease blood flow at the surgical site. Then adipose tissue was resected using size 15 surgical scalpel. Thereafter, the harvested adipose tissue was placed in a minimum essential medium (MEM) transporting media and shipped to the stem cells laboratory for processing in the same way of pulp tissue. . **(figure 14)**

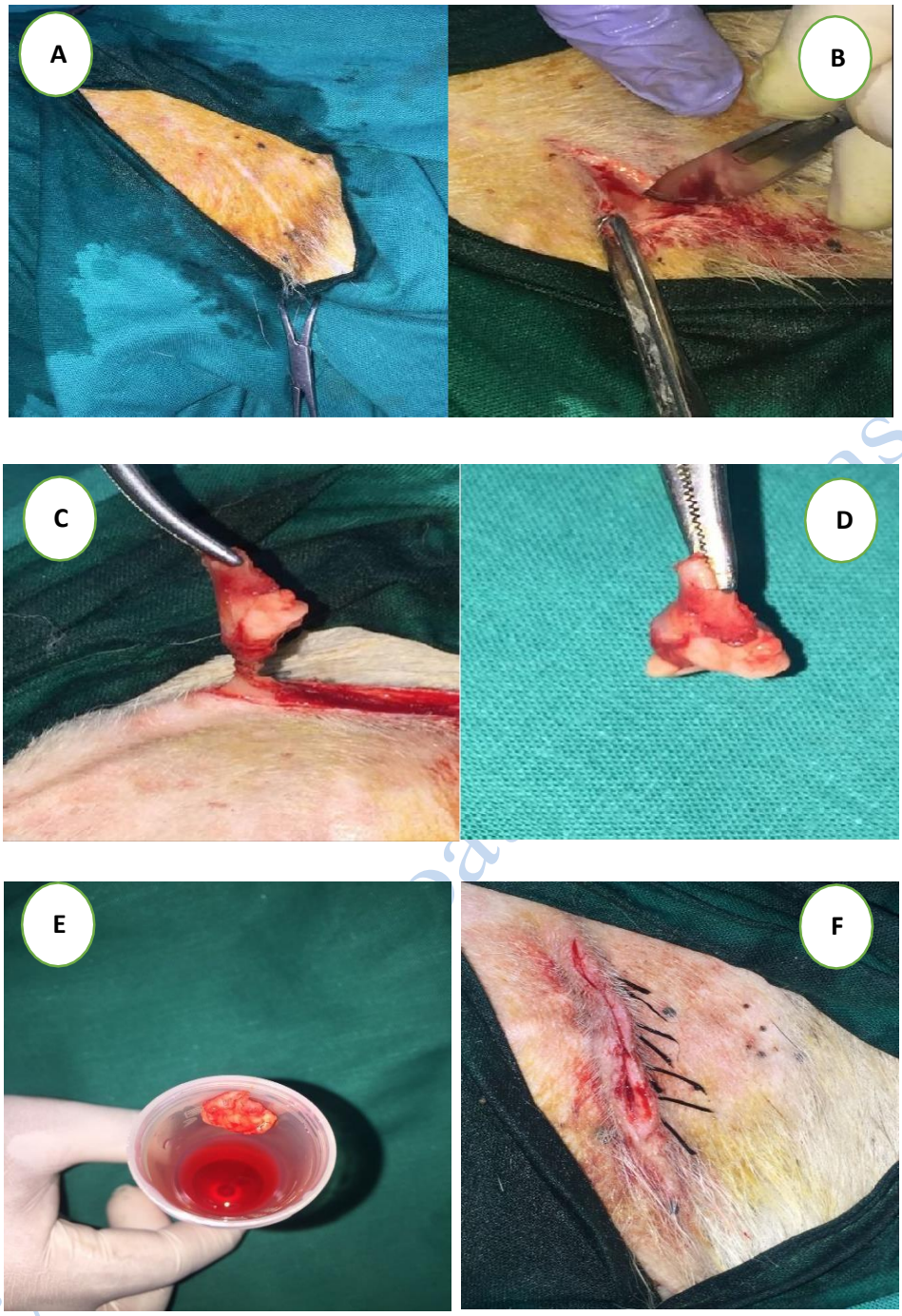


figure 14: photographs showing Adipose tissue harvesting procedures

(A) Disinfection of the surgical site (B) elliptical incision deep to the fatty layer (C) Adipose tissue attached to the fatty layer (D) Adipose tissue separation (E) Transport media containing adipose tissue (F) surgical wound closure

4.6.3. Isolation of stem cells:

The harvested tissue (both of dental pulp and adipose tissue) was removed from the transport media, washed in Phosphate buffered Saline (PBS) to ensure removal of any exogenous debris, placed in a sterile petri dishes and cut into small pieces using a sterile scalpel blade. The dissected tissue was transferred to a polycabrolactone tubes containing MEM then centrifuged at 1000 rpm for 10 minutes.

Enzymatic digestion of tissue was done as described by **Gronthos et al** [14] where 3 mg/ml collagenase type I were used as digesting solution. The harvested tissues were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for the time necessary to allow cells to slip down from the explants. After incubation, the digestive reaction was stopped by the addition of fresh basic medium which was added to neutralize collagenase. The basic fresh culture medium consists of minimum essential medium (MEM) (Sigma Aldrich, St. Louis, MO, USA), inactivated **fetal bovine serum** (FBS) and antibiotics (200 U/ml **penicillin,** 200 µg/ml **streptomicin** and 2.5 µg/ml **amphotericin B** (Sigma). The tubes were then centrifuged for five minutes at room temperature to concentrate cells at the bottom of the tubes. A sterile serological pipette was used to transfer the medium containing cells into T-25 cell culture flask (Falcon, Franklin Lakes, NJ) with filter top cap. The flask was labeled by the date and then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for the time necessary for adherence.

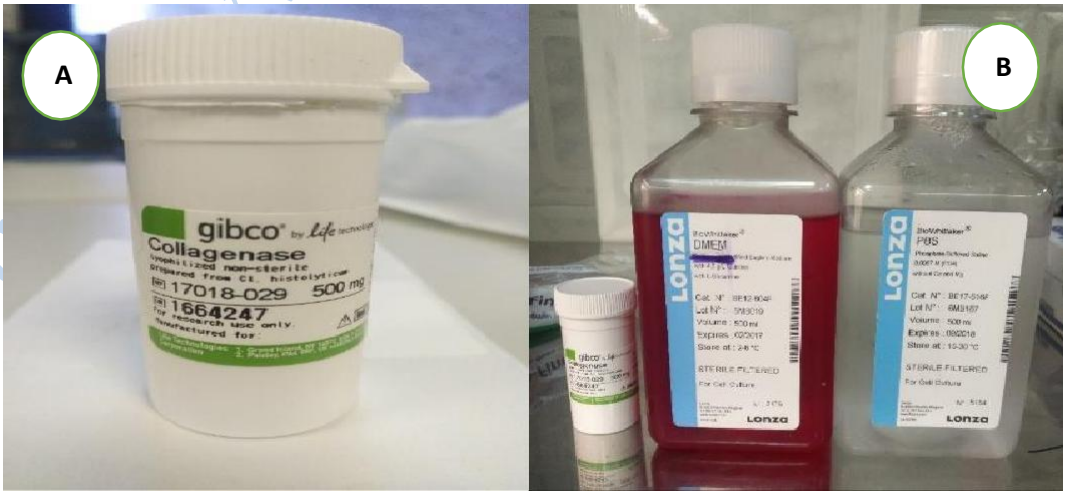
Expanding the cell culture:

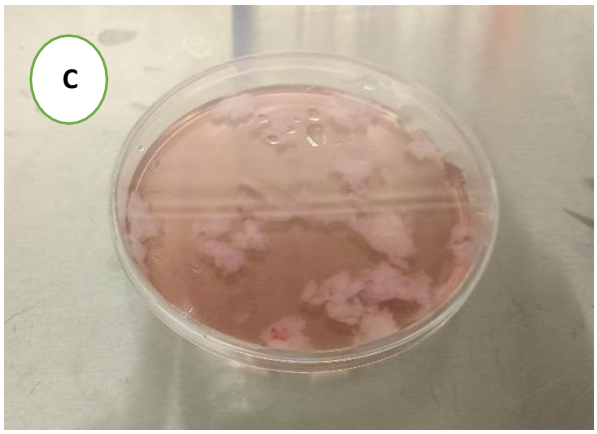
After washing the cells with Hanks Balanced Salt Solution (HBSS), 1 ml of **Trypsin** was added to the flasks in non- cell side to detach the cells from the flask floor (trypsinization). Then 5 ml of medium was added to the flask and

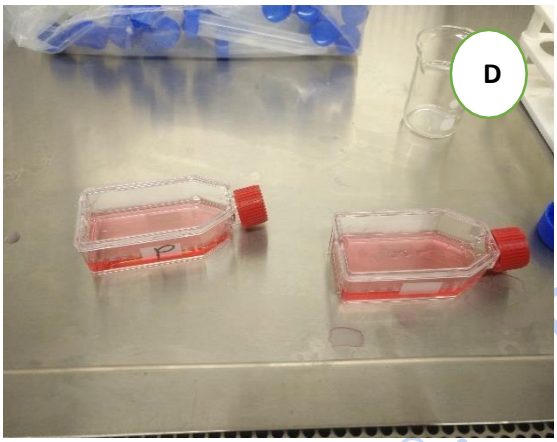
trypsin/medium/cell suspension had been collected in 15 ml test tube for centrifuging at 600 rpm for 6 minutes. Suspension was vacuumed out to get out as close as possible to the pellet without touching the cells. Then splitting of the cell suspension was done equally in T 25 flasks and the flasks were put on their sides in the incubator with loose caps. After 7 days, the cells were observed under microscope and non-adherent cells were discarded. The basic fresh medium was changed twice a week till the adherent cells reached confluence. In these experimental conditions the cells reached confluence within 15-20 days of culturing through three passages**.** All the previous steps were done under aseptic conditions in an air laminar flow safety cabinet using sterile instruments. **(figures15)**

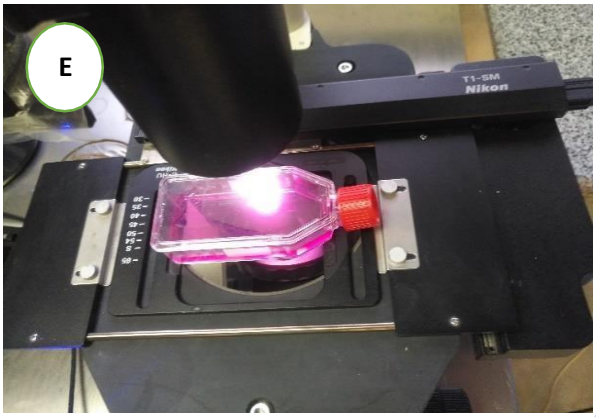
Counting the cells:

Counting of the cells was done using hemocytometer and Trypan blue stain was used to detect viable cell count. This method depends on the principle that viable cells do not able to take up certain dyes. With the aid of inverted phase contrast light microscope, all the viable (big, rounded) cells were counted from the cell suspension.









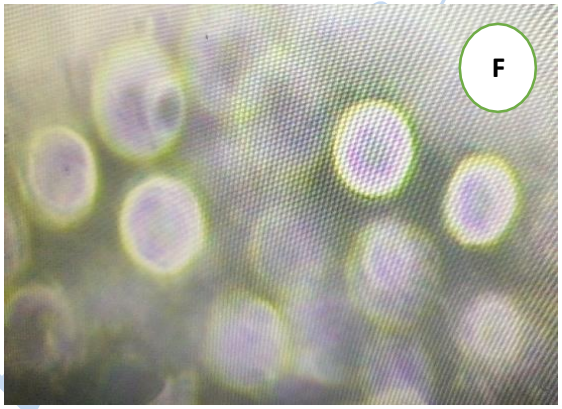


Fig. (15): (A&B) showing components of complete media from left to right; collagenase, Dulbecco's modified Eagle's medium DMEM and phosphate-buffered saline PBS (C&D) harvested tissue in petri dish and Falcon flasks (E&F) exmination of stem cells under microscope (G) centrifuge (H) incubation of cells in the incubator (I): air laminar flow safety cabinet

4.7. Preparation of Simvastatin hydrogel [162]

Simvastatin (Sigma-Aldrich, St Louis, MO) was prepared 1 day before the surgical procedures. Simvastatin is a white to off-white, non-hygroscopic, crystalline powder that is practically insoluble in water, and freely soluble in chloroform, methanol and ethanol. Therefore, Simvastatin was dissolved in absolute ethanol (50 mg: 1 ml) and mixed with a magnetic stirrer then stored at 4°C for future use. Solutions of the different concentrations (0.1 mg/50 µl, 0.5 mg/50 µl, 1.5 mg/50 µl, and 2.2 mg/50 µl) were prepared and examined to reach the appropriate concentration to be less toxic on stem cells using minimum inhibitory dose test. The selected dose was 0.1µl/L of simvastatin. Methylcellulose gel (MC) (Guangzhou Qiyun Biotechnology, China) served as the simvastatin delivery system in which 4.0% (w/v) methylcellulose was dissolved in hot triple-distilled water to produce

methylcellulose gel, which was used as the simvastatin carrier after it cooled down. All drug and carrier manipulations were performed in aseptic conditions.

Transplantation

DPSCs or ASCs were transplanted as previously described by (**Krebsbach *et al.*, 1997**) [163]. Stem cells were seeded in 24-well culture plates at a density of 2 x 10^4 cells per well and preincubated in a growth medium for 24 hours. Then, the cells were exposed to 0.1 µml/L simvastatin hydrogel [164].

4.8. Postoperative care

When the operative procedures were completed, the animals were received Zyleject, 3ml intramuscularly every 12 hours for 3 days to control pain. Animal were injected with flumox 1000 mg every 24 hours for seven post-operative days. Dogs were kept on soft diet composed of milk, rice, meat, liver, and bread for the first post- operative week. On the second postoperative week, dogs were able to eat the usual diet. All the animals were evaluated clinically for assessments of the general health until euthanasia. Also daily examination was carried out for the presence of signs of infection as redness, hotness, inability of the mouth opening, eating, and inability of swallowing.

4.9. Radiographic evaluation

post-operative radiographic evaluation of each root was done in compared to pre-operative radiograph to evaluate the following criteria

1. **Radiolucency:**

Score 0: no healing

Sore 1: decrease in size or partial resolvement

Score 2: complete disappearance of radiolucency

2. **Apical closure:**

Score 0: open apex

Score 1: closure with apparent canal at the apex

Score 2: complete obliteration of canal

3. **Canal calcification:**

Score 0: absence of calcification

Score 1: presence of calcification

Periapical radiographs were taken after induction of the periapical lesion and compared with follow-up radiographs taken according to each group, at 1 week, 1 month and 3 months. Periapical radiographs were digitized using a transparency scanner (HP Scanjet G3110, Hewlett-Packard Development Company, Palo Alto, CA, USA).

4.10. Euthanasia of the dogs:

After each evaluation period, the dogs were sacrificed under general anesthesia provided by pentobarbital (Socumb, Butler Company, Columbus, Ohio) at 30 mg/kg IV. The carotid arteries were exposed and cannulated then the dogs were euthanized with additional pentobarbital at a dose of 90 mg/kg IV. The animals were perfused with 10% buffered formalin (Fisher Scientific, Fair Lawn, New Jersey).

4.11. Histologic preparation and evaluation:

After euthanasia of the dogs, the maxillary and mandibular jaws were surgically removed and divided at the midline into two halves right and left one using a bone saw **(figure 16).**

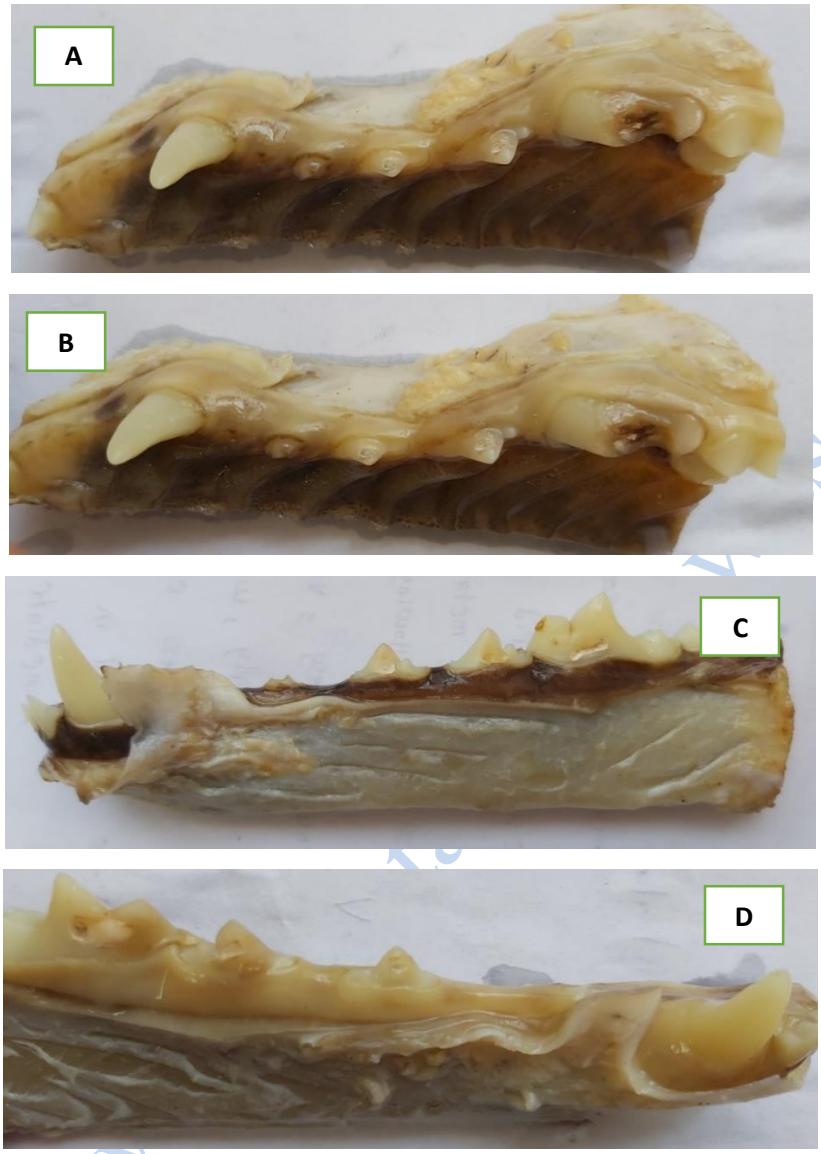


Fig. (16): photographs of upper and lower jaws after separation

A, B) upper right and left segments

C, D) lower right and left segments

The jaws with the involved teeth were placed immediately in formaldehyde (Fisher Scientific, Fair Lawn, New Jersey). After removal of all soft tissue and excess hard tissue from the specimens, they were next placed in Formical (Decal Chemical Corporation, Congers, New York) for decalcification for six days, including one change of the solution. The specimens were subsequently decalcified in Immunocal (Decal Chemical Corporation, Tallman, New York) for two months,

undergoing four changes of the solution over that time. Upon removal from the decalcification solution, the specimens were placed under a running tap water wash for 20 minutes followed by immersion in 70% ethyl alcohol. The specimens were then dehydrated through ascending gradations of ethanol and processed on a Leica TP 1020 dip n' dunk processor (Leica, Wetzlar, Germany) at 45 minutes per station in the following manner: one cycle of 70% ethanol, two cycles of 80% ethanol, two cycles of 95% ethanol, two cycles of 100% ethanol, two cycles of xylene and two cycles of Paraplast paraffin (Kendall, Mansfield, Massachusetts) at 58°C. The tissues were then removed from the storage cassettes and embedded in paraffin, and were sectioned on a Leica Jung RM 2045 microtome (Wetzlar, Germany). Sections were made longitudinally every 5 µm through the apical foramen of the roots and placed on probe on plus slides. Tissues were stained with Hematoxylin and Eosin (H&E) and evaluated under light microscopy at up to 400 X magnification for the presence or absence of the newly formed tissues.

Hematoxylin and Eosin

The dried sections were stained with 0.1% Mayer's hematoxylin solution for 10 minutes then rinsed in cool running double-distilled water for 5 minutes, dipped in 0.5 eosin 12 times, dipped in distilled water, and dehydrated in ascending concentrations of ethanol. The sections were dipped in xylene several times, mounted on slides, and covered with a coverslip with Cytoseal (Thermo Fisher Scientific, Waltham, MA) and examined under a light microscope.

Each individual root was taken as a unit of measurement/assessment and graded for the following parameters:

1. **Scoring system related to the inflammatory changes:**

Sore 0: no inflammatory changes

Score 1: mild inflammatory changes

Score 2: severe inflammatory changes

2. **Scoring system related to the intracanal tissue changes (based on odontoblastic differentiation and distribution):**

Score 0: no cellular changes

Score 1: non organized cellular changes

Score 2: well organized cellular changes

3. **Scoring system related to hard tissue formation:**

Score 0: absence of hard tissue formation

Score 1: immature hard tissue formation

Score 2: mature hard tissue formation

4. **Scoring system related to apical closure:**

Score 0: no apical closure

Score 1: immature apical closure

Score 2: mature apical closure

Radiographic and histologic evaluation was made for each root within each tooth. The final score was the mean of both roots. Summing up and correlating radiographic and histopathologic assessment was done for obtaining comprehensive conclusions regarding the outcome of different treatment modalities.

4.12. Statistical analysis of the data:

Statistical analysis was performed with IBM® SPSS® Statistics Version (Statistical Packages for the Social Sciences 20, IBM, Armonk, NY, USA). Data were represented by total number and percentage for each method used in the study. Descriptive statistics was done using one way ANOVA and Pearson correlation (r) tests for comparing the relation between different groups. Significance was set at P value ≤ 0.05.

5. Results

Section outline:

5.1. Evaluation of the histological findings:

5.1.1. Histological comparison among the tested groups after different evaluation periods

Four criteria were compared at different periods, all data and comparisons were represented at **table (1,2,3 & 4), figures (17,18,19,20).**

- **Inflammatory changes**

For all cells (DPSCS+SIM, ASCS+SIM, DPSCS and ASCS), there was a statistically significant inflammatory changes between different periods one weak, one month and 3 monthss as p= 0.003, 0.003, 0.028 and 0.003 respectively. While there was no any difference between one week and 3 months as p=0.834.

For one weak, one month and 3 months, there was no statistically significant difference between the 4 cells as p= 1, 0.226 and 0.856 respectively.

- **Intracanal tissue changes**

For all cells (DPSCS+SIM, ASCS+SIM, DPSCS and ASCS) , there was no statistically significant changes between different periods one weak, one month and 3 months as p= 0.521, 1, 0.277 and 0.827 respectively.

For one weak, one month and 3 months, there was a statistically significant difference between the 4 cells as p= 0.019, 0.007 and 0.003 respectively. DPSCs+SIM recorded the better change followed by ASCs+SIM then DPSCs while ASCs as the least one .

- **Hard tissue changes**

For all cells (DPSCS+SIM, ASCS+SIM, DPSCS), there was a statistically significant changes between different periods one weak, one month and 3 monthss as p= 0.00, 0.001 and 0.00 respectively. There was no any difference beween one month and 3 monthes as p = 0.45, 0.32 and 0.11 respectively.

While for ASCs there was no statistically significant changes between different periods one weak, one month and 3 monthss as p=0.14.

For one month and 3 months, there was a statistically significant difference between the 4 cells as p= 0.032and 0.02 respectively.

For one month, there was no a statistically significant diference between (DPSCS+SIM, and ASCs+SIM) as p=0.34. there was no difference between (ASCs and DPCs) as p = 0.57.

For 3 months, there was no a statistically significant diference between (DPSCS+SIM, ASCS+SIM, DPSCS) as p=0.34 while there was a difference beteen them and ASCs repectively as p = 0.00 , 0.001and 0.0023 respectivly.

- **Apical closure**

For DPSCs+Sim, there was a statistically significant difference between one weak, month and three months as p=0.00. better apical closure was recorded at 3 months followed by one month.

For ASCs+Sim, there was a statistically significant difference between one weak, month and three months as p=0.00. better apical closure was recorded at 3 months followed by one month.

For DPSCs, there was a statistically significant difference between one weak, month and three months as p=0.01. better apical closure was recorded at 3 months followed by one month.

For ASCs, there was no statistically significant difference between one weak, month and three months as p=0.152. better apical closure was recorded at 3 months followed by one month.

For one month, there was no statistically significant difference between 4 cells as p=0.226.

For 3 months, there was a statistically significant difference between 4 cells as p=0.0.019. DPSCs+SIm showed the better apical closure followed by ASCs+SIM then DPSC and ASCs.

Table (1): The mean inflammatory changes values and standard deviation (SD) when comparing each group after different evaluation periods

			1 week	1 month	3 months	P value
Inflammatory changes	DPSCs+SIM	Mean	1.80^{A}	0.80^{B}	0.40^{C}	0.003 *
		SD	0.52	0.63	0.52	
	ASCs+SIM	Mean	1.80^{A}	0.80^{B}	0.40^{C}	0.003 *
		SD	0.52	0.63	0.52	
	DPSCs	Mean	1.80^{A}	0.80^{B}	0.40^{C}	0.003 *
		SD	0.52	0.63	0.52	
	ASCs	Mean	1.80^{A}	0.80^{B}	0.40^{C}	0.003 *
		SD	0.52	0.63	0.52	
		P value	1	0.926	0.956	

*** Significance,**

(Small letters) for significance between the same column of the same color only,

(Capital letters) for significance between the same row of the same color only, $p \leq 0,05$.

Table (2): The mean Intracanal tissue changes values and standard deviation(SD) when comparing each group after different evaluation periods

			1 week	1 month	3 months	P value
Intracanal tissue changes	DPSCs+SIM	Mean	1.50[a]	1.67[a]	1.83[a]	0.521
		SD	0.55	0.52	0.41	
	ASCs+SIM	Mean	1.33[b]	1.44[b]	1.55[b]	1.000
		SD	0.52	0.52	0.52	
	DPSCs	Mean	0.83[c]	1.00[c]	1.33[c]	0.277
		SD	0.41	0.52	0.63	
	ASCs	Mean	0.67[d]	0.69[d]	0.70[d]	0.827
		SD	0.52	0.55	0.55	
		P value	0.019*	0.007*	0.003*	

*** Significance,**

(Small letters) for significance between the same column of the same color only,

(Capital letters) for significance between the same row of the same color only, $p \leq 0,05$.

Table (3): The mean hard tissue formation changes values and standard deviation(SD) when comparing each group after different evaluation periods

			1 week	1 month	3 months	P value
Hard tissue formation	DPSCs+SIM	Mean	0.00^{A}	1.50^{Ba}	1.67^{Ba}	0.000^{*}
		SD	0.00	0.55	0.52	
	ASCs+SIM	Mean	0.00^{A}	1.00^{Ba}	1.33^{Ba}	0.001*
		SD	0.00	0.63	0.52	
	DPSCs	Mean	0.00^{A}	0.67^{Bb}	1.17^{Ba}	0.000*
		SD	0.00	0.41	0.52	
	ASCs	Mean	0.00^{A}	0.50^{Bb}	0.67^{Bb}	0.141
		SD	0.00	0.55	0.82	
		P value		0.032*	0.020^{*}	

*** significance,**

(small letters) for significance between the same column of the same color only,

(large letters) for significance between the same row of the same color only, $p \leq 0,05$.

Table (4): The mean Apical closure changes values and standard deviation(SD) when comparing each group after different evaluation periods

			1 week	1 month	3 months	P value
Apical closure	DPSCs+SIM	Mean	0.00^{A}	1.00^{Ba}	1.83^{Ca}	0.000*
		SD	0.00	0.63	0.41	
	ASCs+SIM	Mean	0.00^{A}	0.67^{Bb}	1.50^{Cb}	0.000*
		SD	0.00	0.52	0.55	
	DPSCs	Mean	0.00^{A}	0.50^{Bc}	1.00^{Cc}	0.010*
		SD	0.00	0.55	0.63	
	ASCs	Mean	0.00^{A}	0.33^{Bd}	0.67^{Cd}	0.152*
		SD	0.00	0.52	0.82	
		P value		0.226	0.019*	

*** Significance,**

(Small letters) for significance between the same column of the same color only,

(Capital letters) for significance between the same row of the same color only, $p \leq 0,05$.

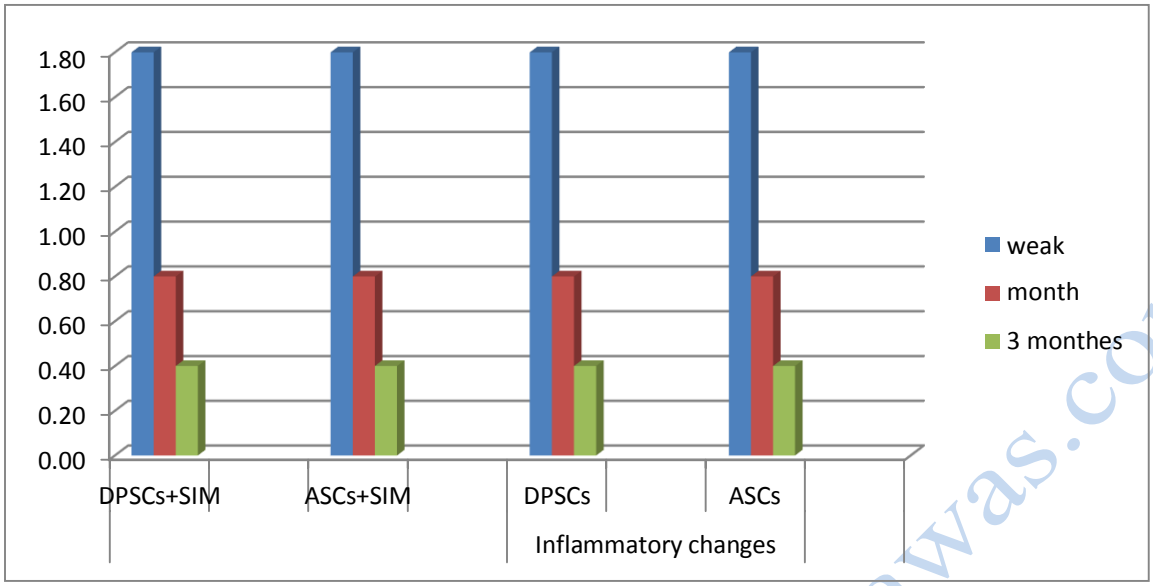


Figure 17: Bar chart representing the mean inflammatory changes of the four groups

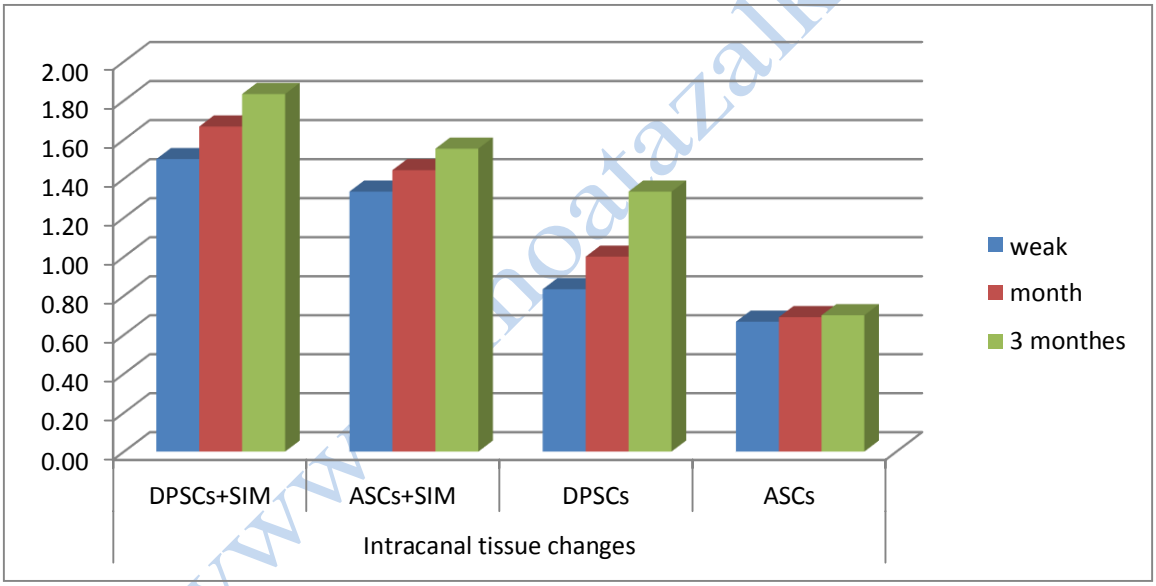


Figure 18: Bar chart representing the mean intracanal tissue changes of the four groups

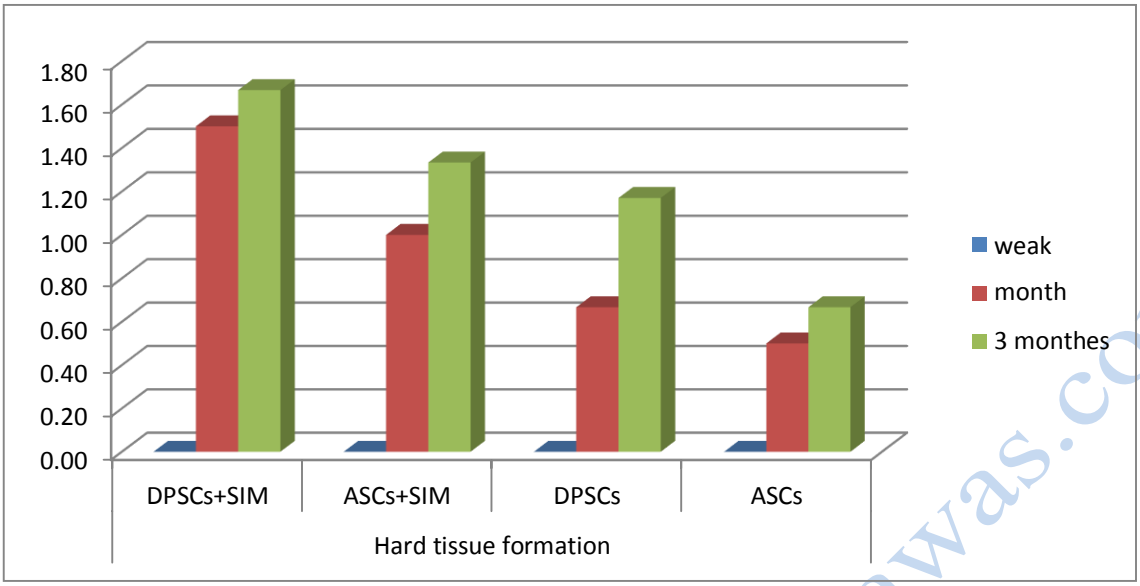


Figure 19: Bar chart representing the mean hard tissue formation changes of the four groups

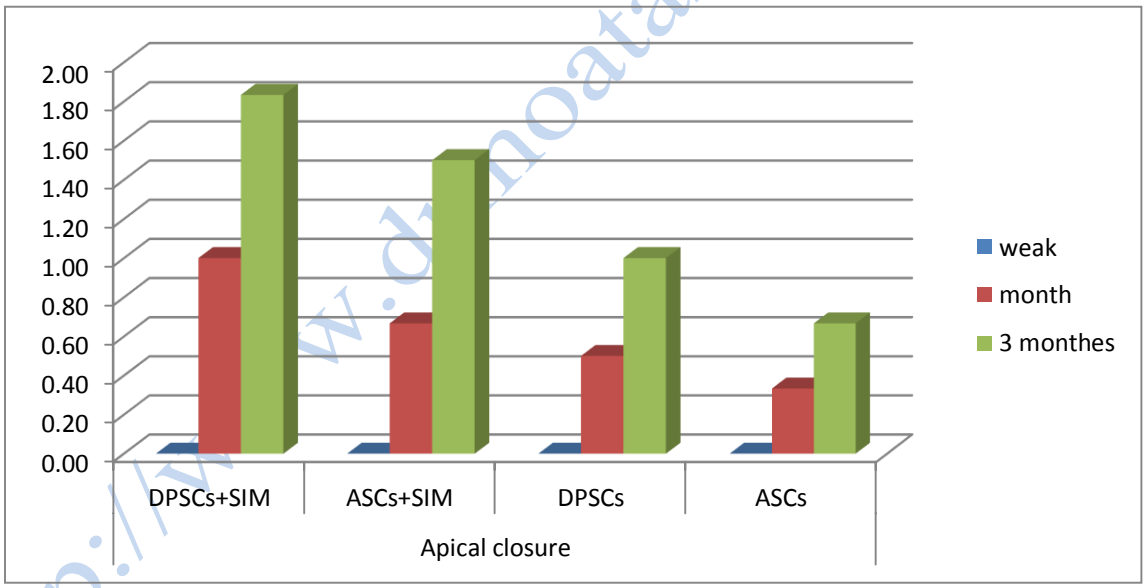


Figure 20: Bar chart representing the mean apical closure changes of the four groups

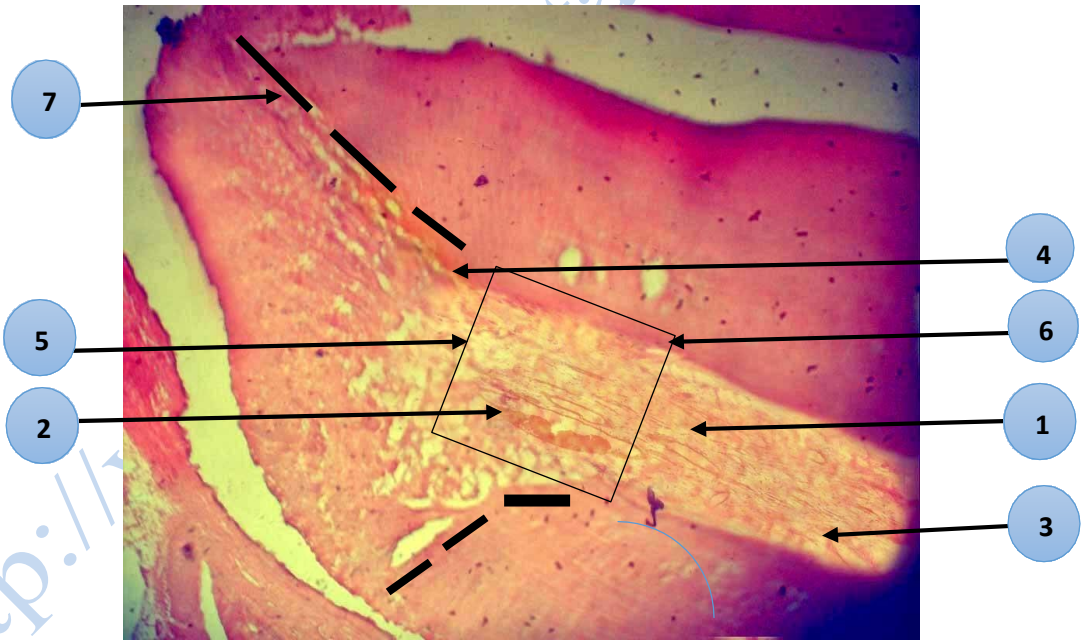
Decalcified sections of teeth were examined for evaluation of the effect of the four tested groups considering the histological structure of the tissues formed.

Group 1:

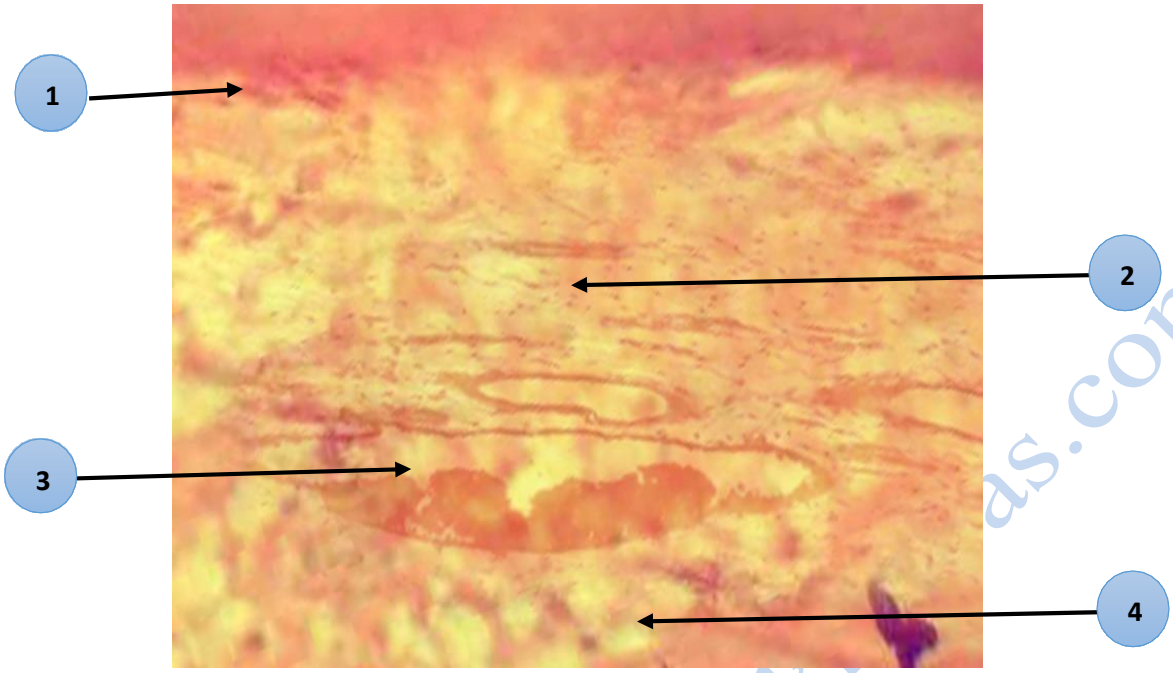
Teeth treated with dental pulp stem cells (DPSCs) added to simvastatin (SIM) hydrogels:

***After one-week evaluation period*:**

The histologic section stained with H&E revealed that the radicular formed tissues had a variable degrees of regeneration that was observed through appearance of dilated blood vessels (BVs) with coagulated red blood cells (RBCs), thrombus formation and there was a presence of one of the most important characteristic features through disorientation of preodontoblast like cells on one side with very short distance extending from apex toward cervical line **(figure 21 A&B)**.



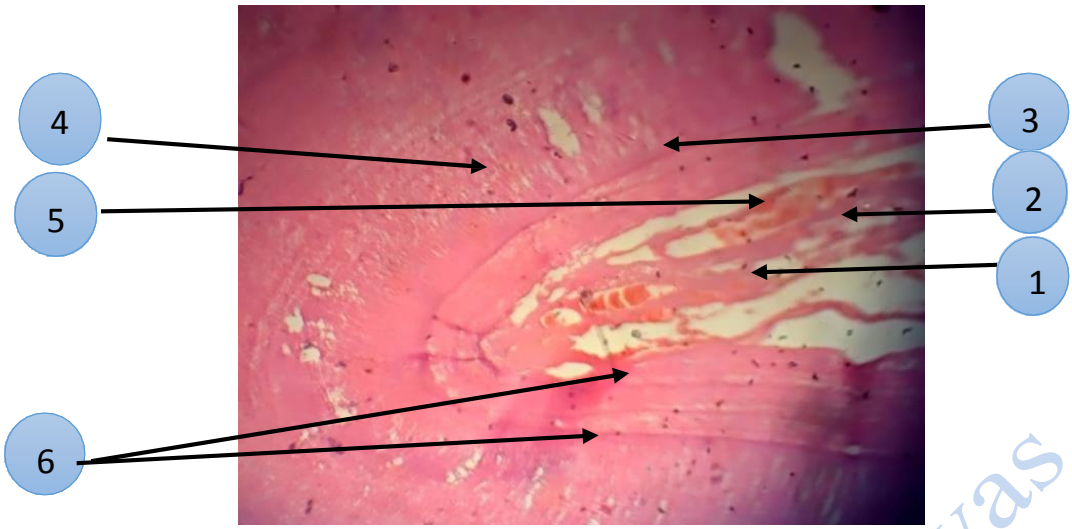
(fig. 21 A): Photomicrograph of group 1 animals at one-week interval treated with (DPSCs) + SIM showing, 1) inflammatory cell infiltrate, 2) coagulated blood vessels, 3) intracellular edema, 4) preodontoblastic like cells and 5) dilated BVs 6) magnified area 7) intermittent lines demarcate the boundries of open apex(H&E100 x).



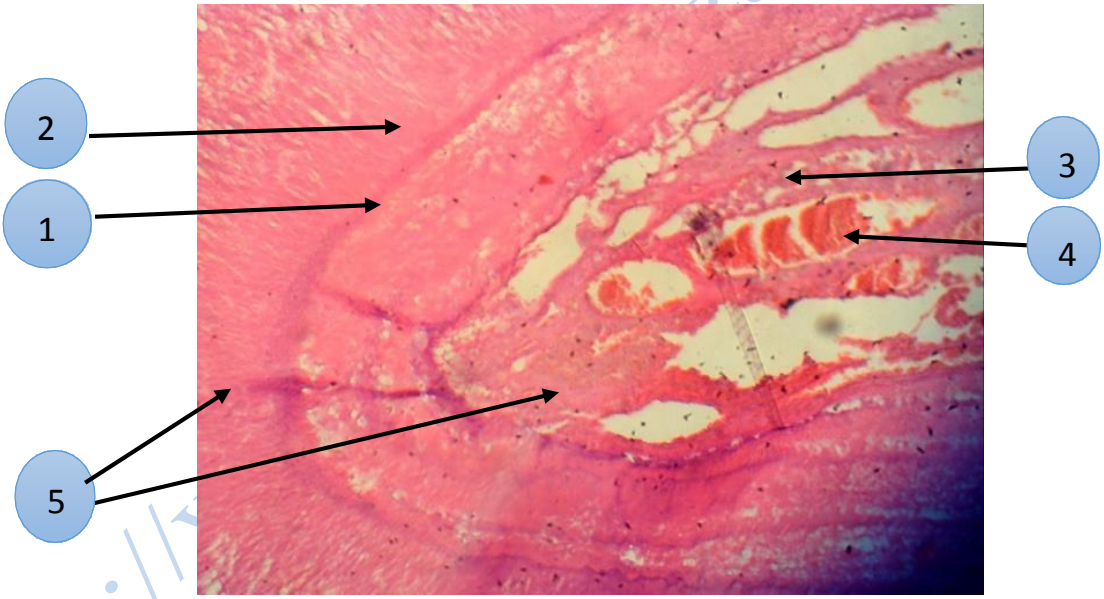
(fig. 21 B): Photomicrograph with higher magnification of the previous figure showing 1) preodontoblast like cells, 2) inflammatory cells, 3) coagulated BVs and 4) intracellular edema (H&E400 x).

After one month evaluation period:

The histologic section stained with H&E revealed organized granulation tissue, intracellular edema with variable degree, odontoblast like cells, dentinal tubules, pre dentine, coagulated BVs and appearance of islands of internal cementum like tissue (ectopic cementum) that can be described as internal ankyloses (appear as intermittent islands extending for short distance apico-cervically). However, cementum like tissue clearly appear on the external part of the root that subsequently lead to nearly apical closure. **(figure 22 A&B).**



(figure: 22 A) Photomicrograph of group 1 animals at 1-month interval treated with (DPSCs) + SIM showing: -, 1) organized granulation tissue, 2) intracellular oedema, 3) odontoblast like cells, 4) dentinal tubules, 5) coagulated BVs and 6) cementum like tissue. (H&E100 x).



(figure: 22 B) Photomicrograph showing higher magnification of the previous figure showing 1) odontobalsts, 2) predentin, 3) organized granulation tissue, 4) coagulated BVs and nearly closed apex, 5) cementum like tissue. (H&E400 x).

After three months' evaluation period,

The histologic section stained with H&E revealed that the radicular pulp tissues showing marked degree of regeneration of the pulp tissue with huge intercellular edema and large dilated blood vessels with thrombus formation. One of the most important dramatic signs for regeneration was aggregation of odontoblast like cells with variable thickness not only extending along the remaining whole length of the root but also under the MTA portion that forming predentin along the root length (cervico-apically) and dentin bridges like tissue extending (mesiodisally) under MTA portion. However, proper apical closure was appeared but not was with the same natural apical configuration. **(figure: 23 A&B)**

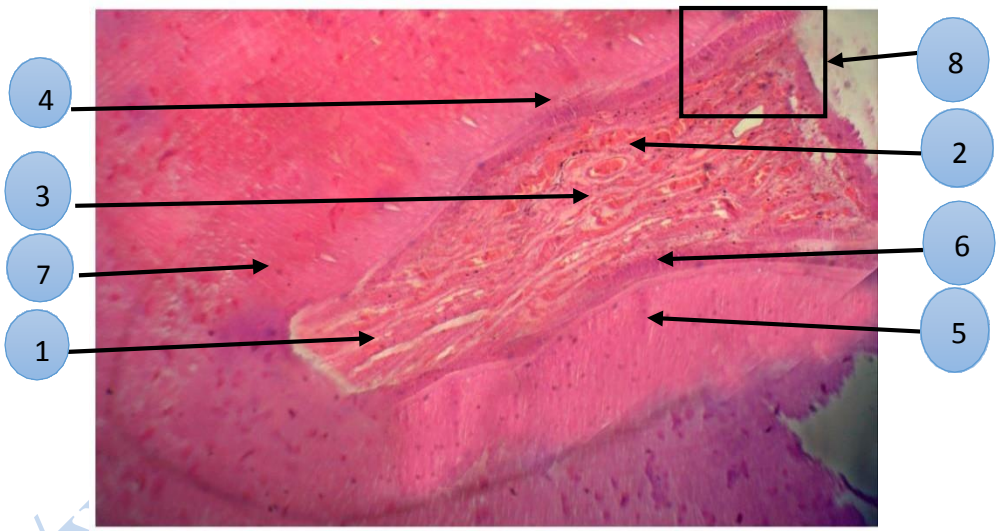


figure: 23 A: Photomicrograph of group 1 animals *After 3 months interval* treated with DPCSs+SIM showing, 1) well organized granulation tissue, 2) dilated blood vessels, 3) intracellular oedema , 4) bilateral well-arranged odontoblast like cells, 5) dentinal tubules, 6) predentin, dentine like tissue, 7) cementum like tissue and dentin like tissue bridge. 8) magnified area (H&E100 x).

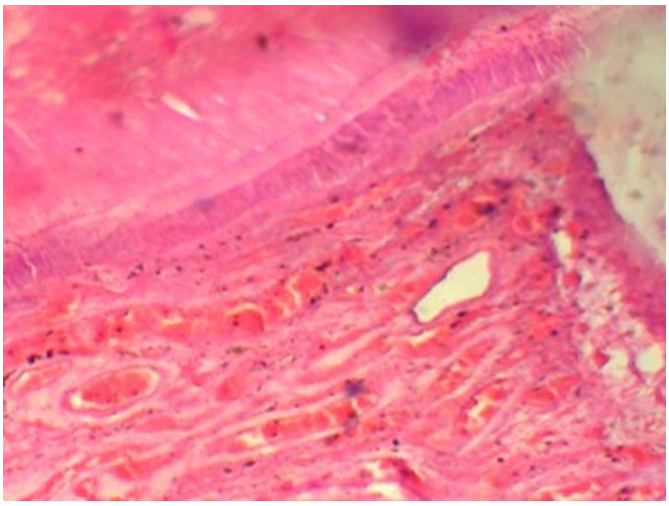


figure: 23 B: Photomicrograph showing higher magnification of the previous figure showing: - odontoblast like cells, predentin, dentin like tissue well organized granulation tissue, intraclleular cementum like tissue. (H&E400 x).

Group 2:

Teeth treated with Adipose-derived stem cells (ASCs) added to simvastatin (SIM) hydrogels:

After one week evaluation period:

The histologic section stained with H&E revealed that the intra canal changes nearly as present in group treated with dental pulp stem cells and simvastatin at the same period. These changes showed in the presence of intercellular edema, large dilated blood vessels with thrombus formation, inflammatory cells infiltrate and open apex (**Figure: 24 A&B).**

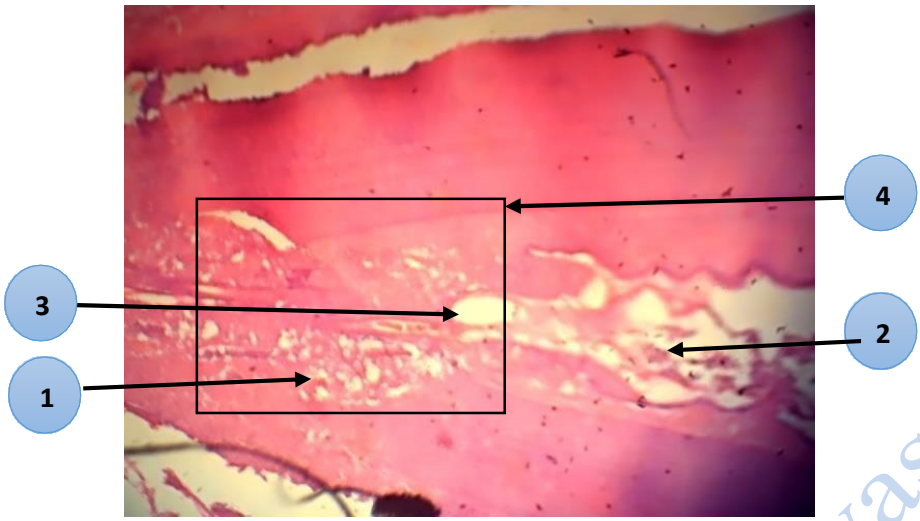


Figure: 24 A: Photomicrograph of group 2 animals After 1 week period treated with (ASCs) + SIM showing, 1) inflammatory cell infiltrate,2) coagulative dilated blood vessels, 3) intracellular edema 4) magnified area (H&E100 x).

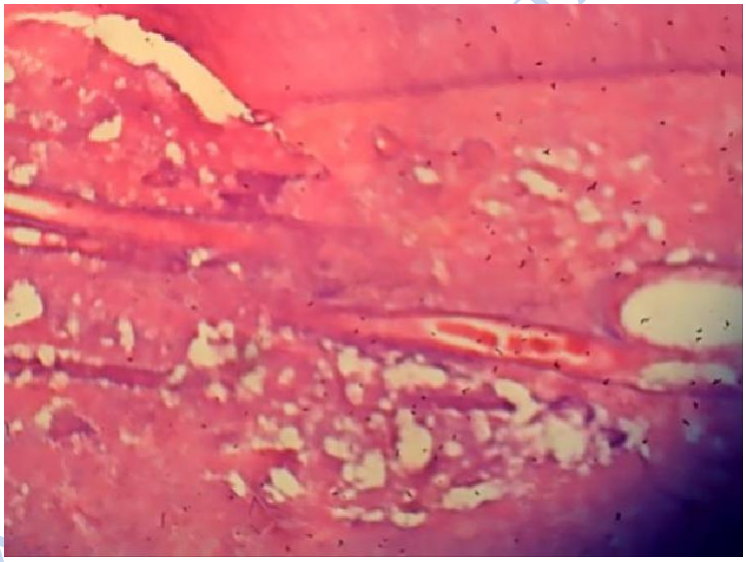


Figure: 24 B: Photomicrograph showing higher magnification of the previous figure showing, inflammatory cells, granulation tissue, coagulated BVs, and intracellular edema (H&E400 x).

After one month evaluation period:

The histologic section stained with H&E revealed that the radicular portion of the pulp showing regeneration through marked narrowing of the root canal through internal formation of islands of hard tissue like with variable thickness that extending apically. As well as the appearance of external cementum like tissue (orthotropic cementum) formation on the root surface leading to narrowing of the root apex. However, intercellular edema, **(Figure 25 A&B).**

After 1 month:

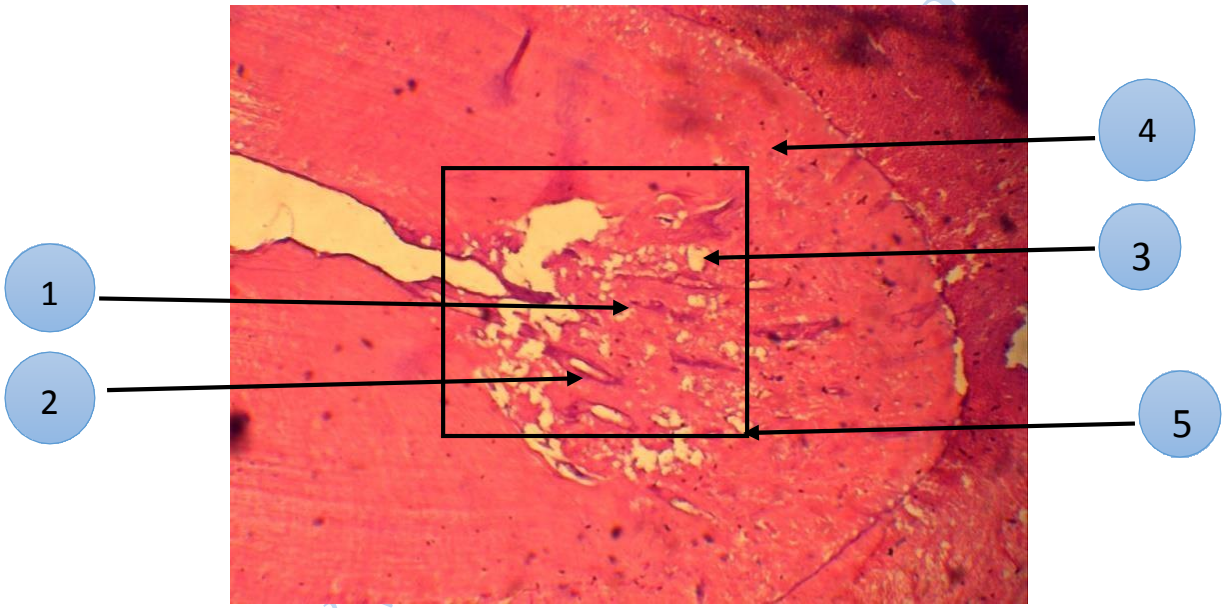


Figure 25 A: Photomicrograph of group 2 animals After 1 month interval treated with (ASCs) + SIM showing, 1) mild inflammatory cell infiltrate, 2) coagulated blood vessels, 3) intracellular edema,4) orthotropic cementum 5) magnified area (H&E100 x)

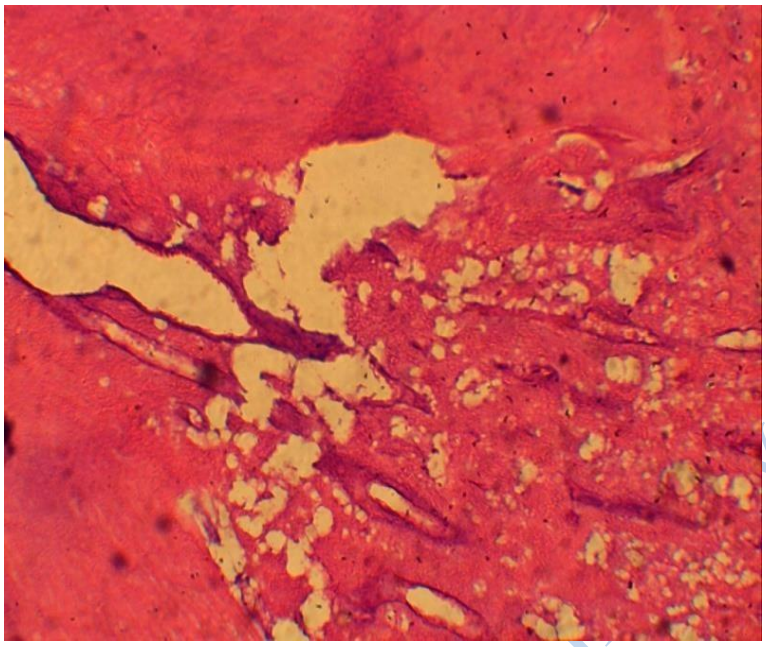


Figure 25 B: Photomicrograph showing higher magnification of the previous figure showing, mild inflammatory cells, coagulated BVs and intracellular edema(H&E400).

After three months' evaluation period:

The histologic section stained with H&E revealed that the radicular pulp space starting the symphony of regeneration through the appearance of predentin with marked appearance of apical closure architecture but still open. **(Figure 26: A&B)**

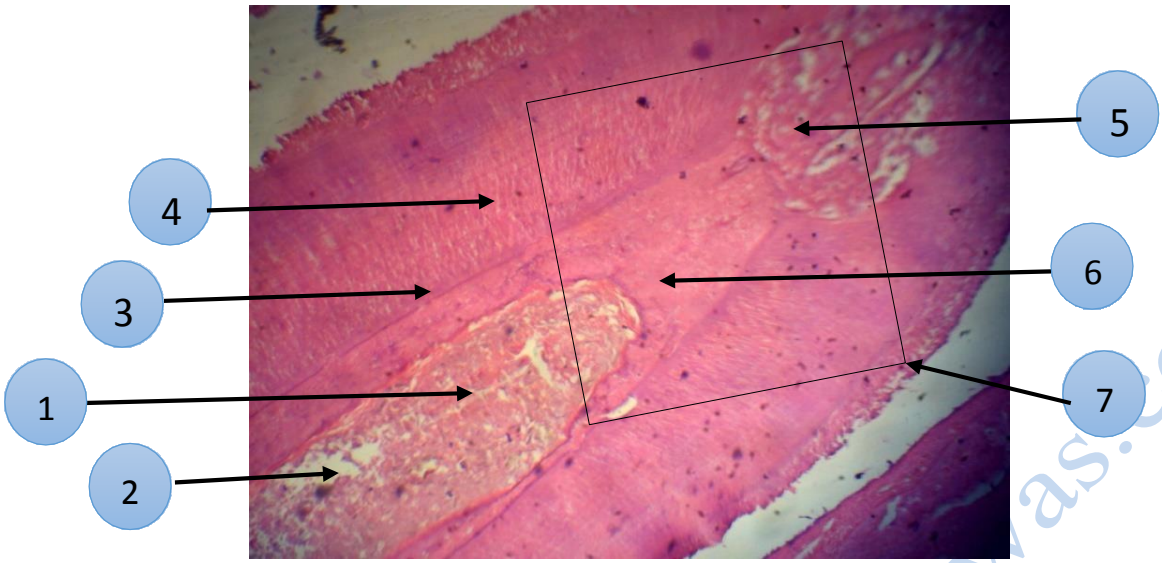


Figure: 26 A: Photomicrograph of group 2 animals After 3 months interval treated with (ASCs) + SIM showing, 1) well organized granulation tissue, 2) intracellular edema, 3) odontoblast like cells, 4) dentinal tubules, 5) coagulated BVs and 6) cementum like tissue, 7) magnified area (H&E100 x).

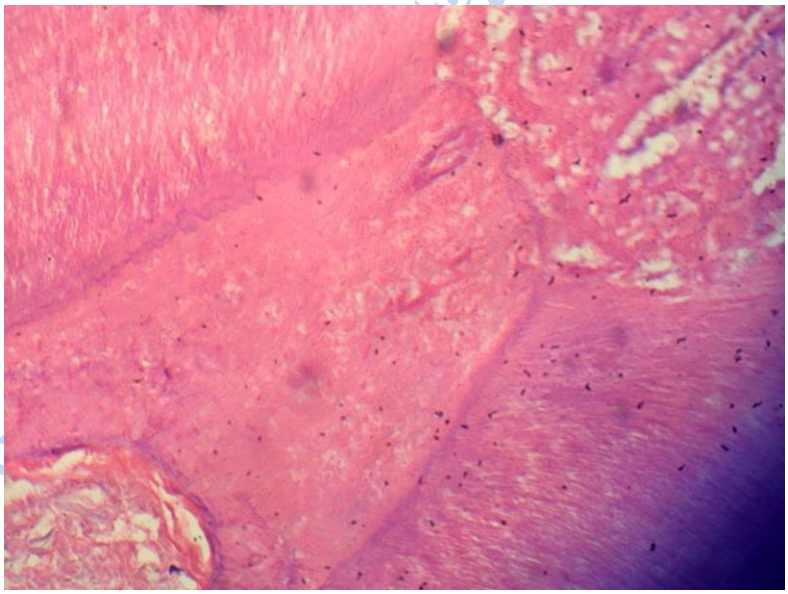


Figure: 26 B: Photomicrograph showing higher magnification of the previous figure showing odontobalst, predentin, well organized granulation tissue, coagulated BVs, and periapical intracellular edema nearly closed apex, cementum like tissue. (H&E400 x).

Group 3:

Teeth treated with dental pulp stem cells (DPSCs)

After one week evaluation period:

The histologic section stained with H&E revealed that the intra canal immediate reactions were with low intensity compared to combination groups(group 1&2) that containing simvastatin at the same periods. These reactions showed in the presence of intercellular edema, large dilated blood vessels (angiogenesis) with thrombus formation, inflammatory cells infiltrate and marked open apex **(Figure 27 A&B).**

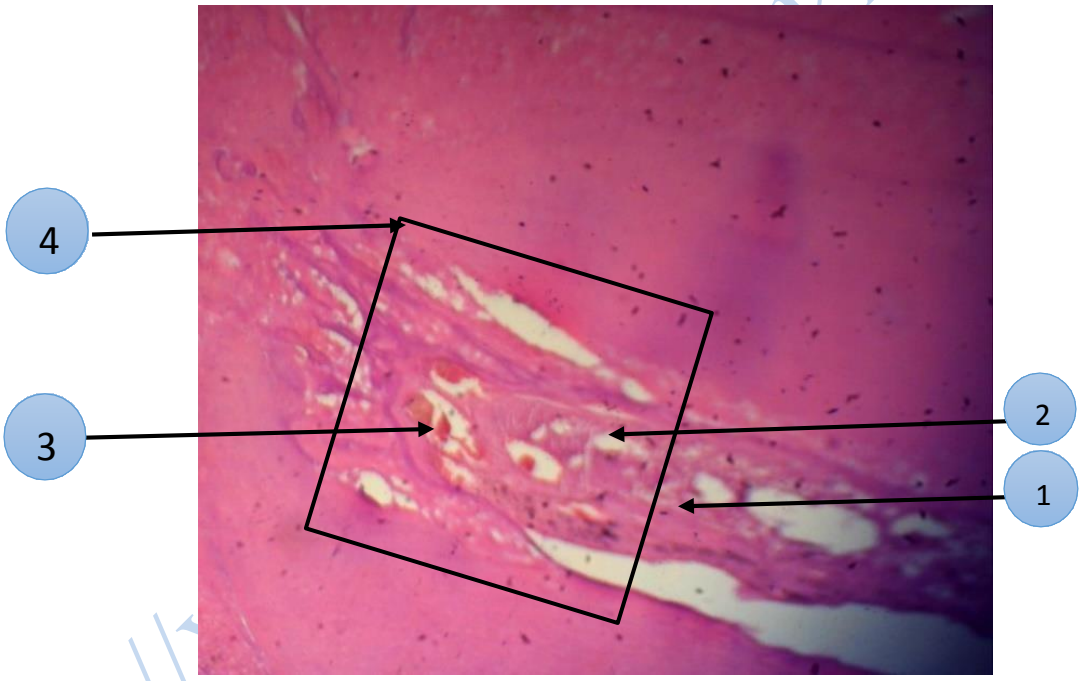


Figure: 27 A: Photomicrograph of group 3 animals After 1 week treated with (DPSCs) showing, 1) inflammatory cell infiltrate, 2) coagulated blood vessels, 3) intracellular oedema, and dilated BVs 4) magnified area (H&E100 x).

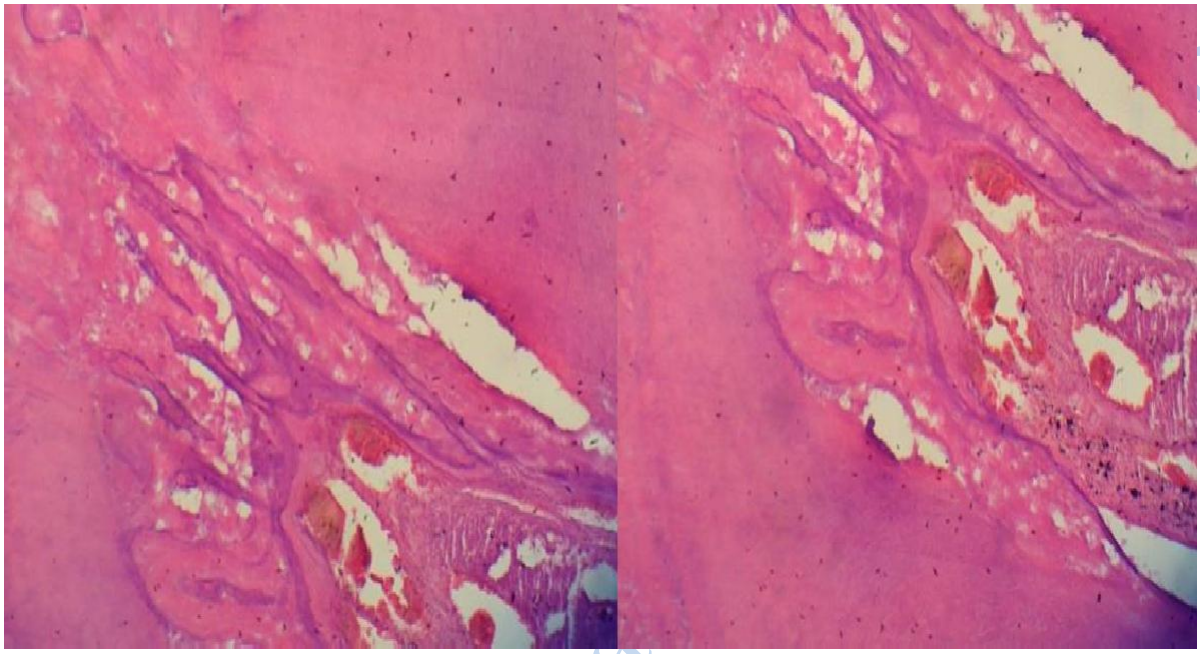


Figure: 27 B: Photomicrograph showing higher magnification of the previous figure showing, inflammatory cells, coagulated BVs and intracellular edema (H&E400 x).

After one month evaluation period:

The histologic section stained with H&E revealed organized granulation tissue, intracellular edema with variable degree as well as periapical edema, odontoblast like cells, pre dentine, coagulated BVs, internal cementum like tissue (ectopic cementum) but still open apex with a little degree. **(figure 28A&B).**

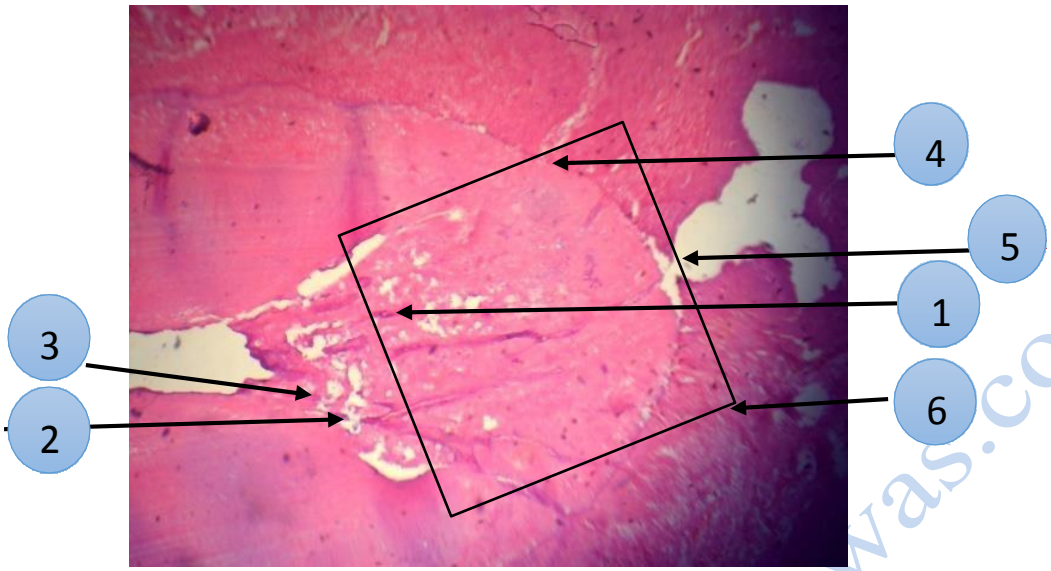


figure 28 A: Photomicrograph of group 3 animals After 1 month treated with (DPSCs) showing, 1) inflammatory cell infiltrate, 2) intracellular oedema, 3) cementoid tissue, 4), excomntosis like tissue, 5) periapical edema and open apex. 6) magnified area (H&E100 x)

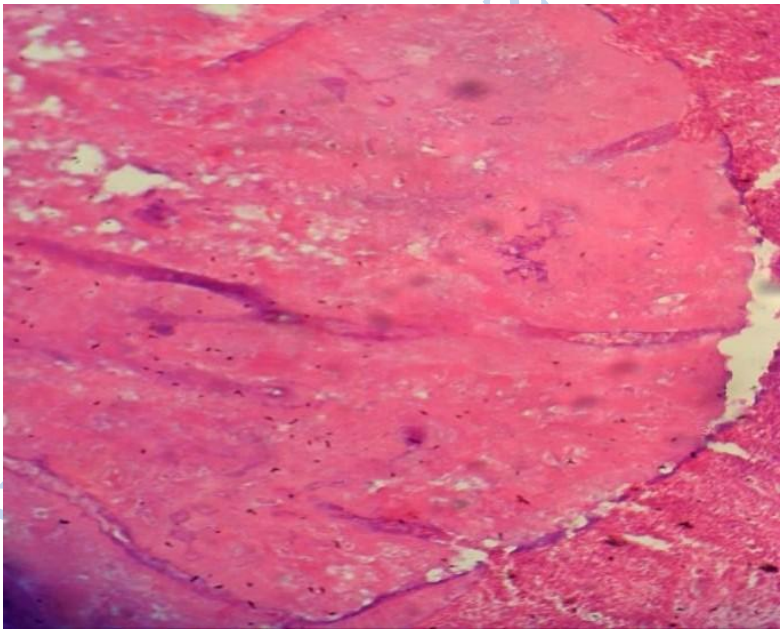


figure 28 B: Photomicrograph showing higher magnification of the previous figure showing, well organized granulation tissue. cementum like tissue nearly closed irregular apex. (H&E400 x)

After three months evaluation period:

The histologic section stained with H&E revealed that the radicular pulp space showing some degree of regeneration of the canal by internal hard tissue formation with proper apical closure appear at the apical third of the root. However, islands of hard tissue present for short distance at the middle third of the root that reflect internal ankyloses. Also Intercellular edema, Dilated blood vessels with thrombus formation and odontoblast like cells with variable thickness were found. **(figure 29 A&B).**

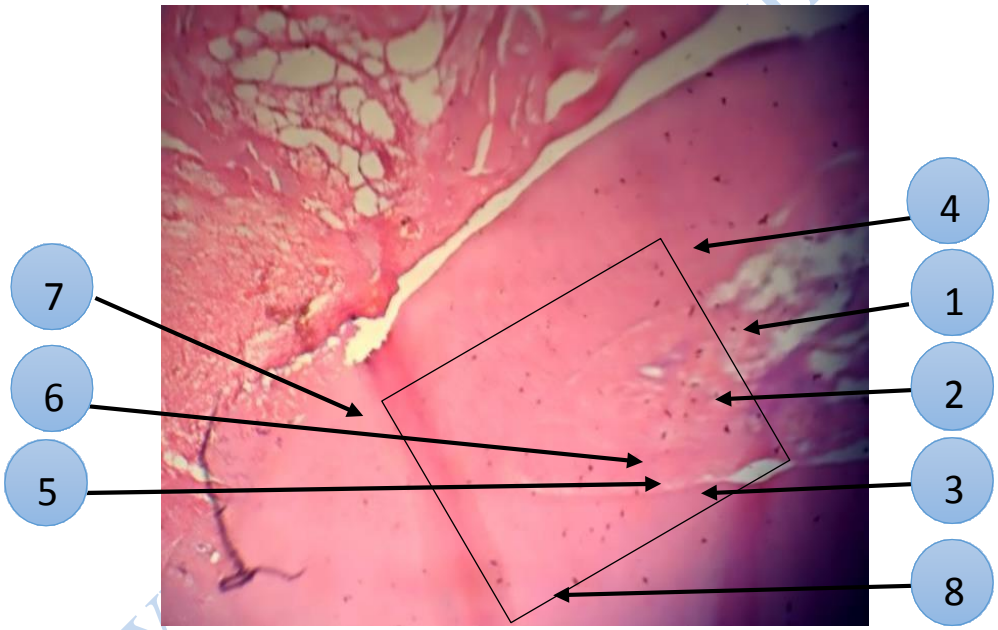


figure 29 A: Photomicrograph of group 3 animals After 3 months treated with (DPSCs) showing, well organized granulation tissue, 1) dilated blood vessels, 2) intracellular oedema , 3) bilateral well-arranged odontoblast like cells, 4) dentinal tubules, 5) predentin, 6) dentine like tissue, 7) cementum like tissue 8) magnified area (H&E100 x).

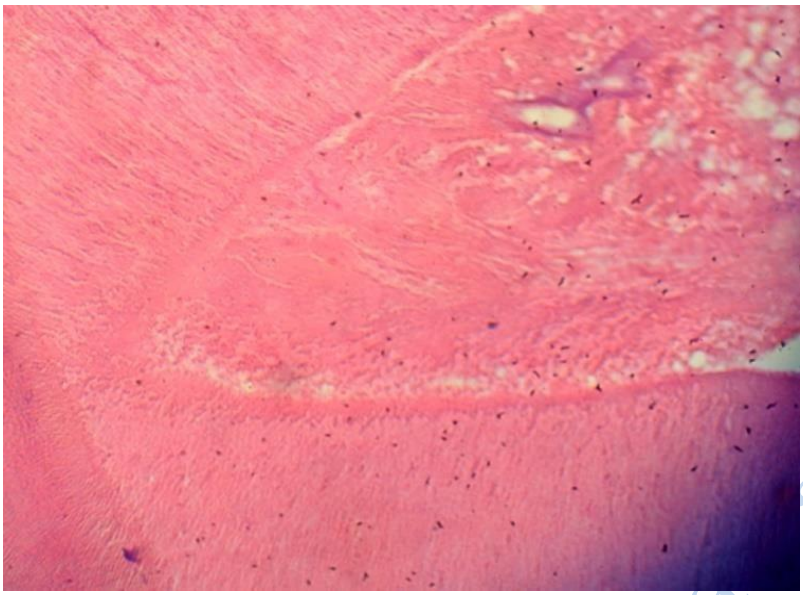


figure 29 B: Photomicrograph showing higher magnification of the previous figure showing: - odontoblast like cells, predentin, dentin like tissue well organized granulation tissue, intraclleular cementum like tissue. (H&E400 x).

Group 4:

Teeth treated with Adipose-derived stem cells (ASCs)

After one week evaluation period:

The histologic section stained with H&E revealed that the radicular pulp tissue showing, moderate inflammatory cells, Intercellular edema, large dilated blood vessels with thrombus formation, irregularities of odontoblastic layer and predentine and dentinal tubules. **(Figure 30 A&B)**

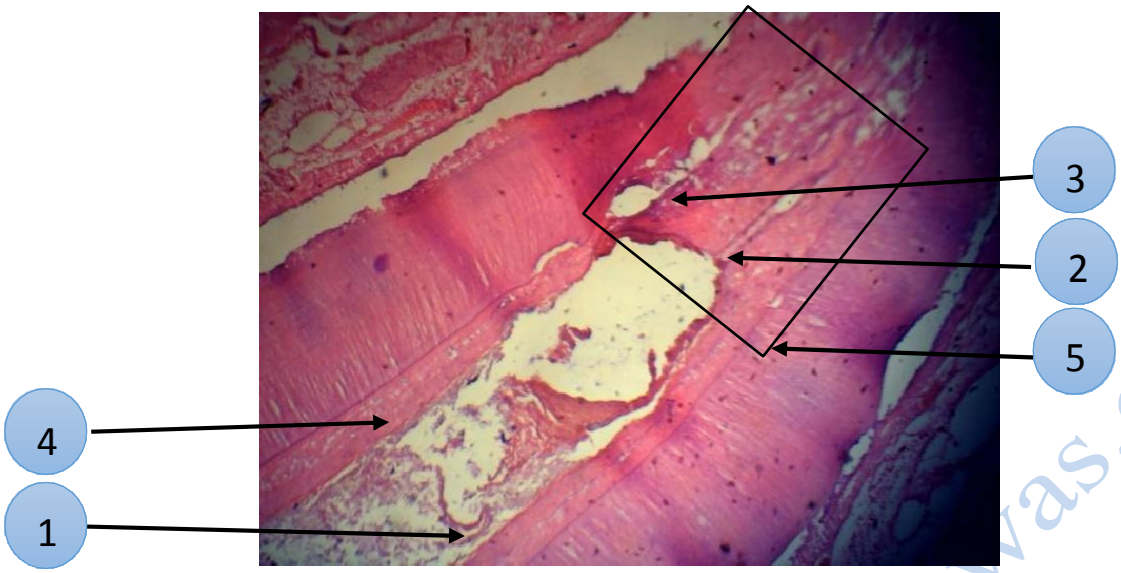


Figure 30 A: Photomicrograph of group 4 animals After 1 week period treated with (ASCs) showing, 1) inflammatory cell infiltrate, 2) coagulated blood vessels, 3) intracellular edema, 4) odontoblast like cells 5) magnified area (H&E100 x).

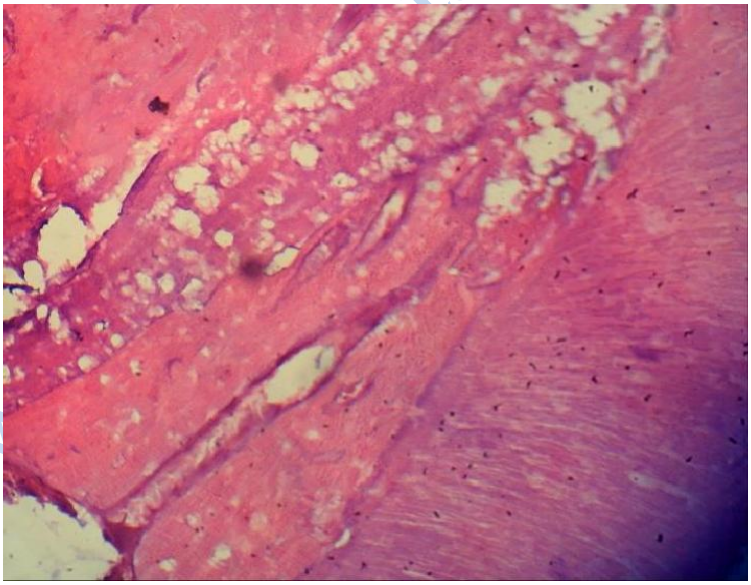


Figure 30 B: Photomicrograph showing higher magnification of the previous figure showing, inflammatory cells, coagulated BVs and intracellular edema (H&E400 x).

After one month evaluation period:

The histologic section stained with H&E revealed that the radicular pulp canal showing internal hard tissue formation that led to narrowing of the canal as well as cementum like tissue deposited in the external part of the root that reduce the dimensions of the open apex but still open at a level considered larger than the previous combination groups treated with addition of SIM. Intra cellular edema and coagulated blood vessels are also present. **(Figure 31A&B)**

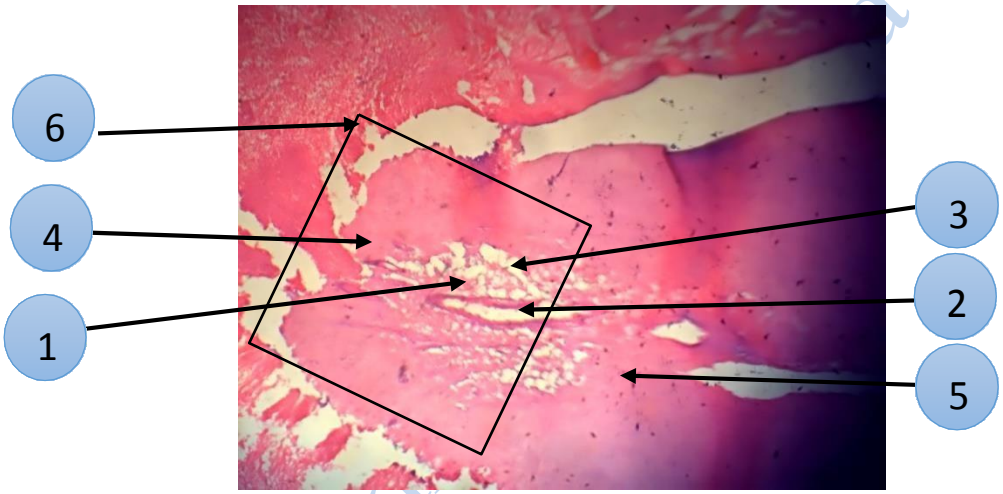


Figure 31 A: Photomicrograph of group 4 animals After 1 month period treated with (ASCs) showing, 1) inflammatory cell infiltrate,2) coagulated blood vessels, 3) intracellular edema, 4) cementum-like tissue and 5) dentin-like tissue 6) magnified area (H&E100 x).

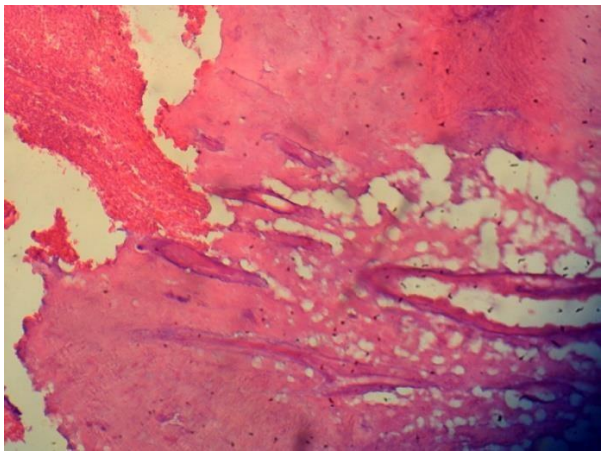


Figure 31 B: Photomicrograph of group 4 animals treated with (ASCs) showing, inflammatory cell infiltrate, coagulated blood vessels, intracellular oedema, odontoblastic differentiation and dilated BVs (H&E400 x)

After three months' period:

The histologic section stained with H&E revealed that the pulp canal showed coagulated BVs, cementum like tissue on the external configuration of the root that reduce the dimensions of the open apex nearly to normal except very small part still open. Internal hard tissue formation by cementum like tissue (ectopic cementum was found reflecting internal ankyloses and narrowing of the canal obviously. **(Figure 32: A&B)**

After 3 months:

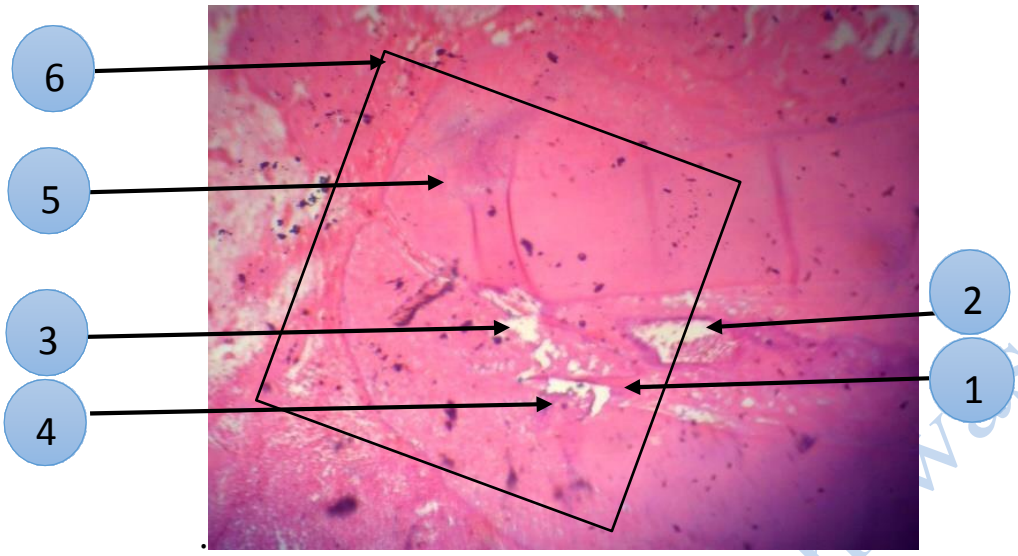


Figure 32 A: Photomicrograph of group 4 animals After 3 months treated with (ASCs) showing, 1) inflammatory cell infiltrate, 2) coagulative dilated blood vessels, 3) intracellular oedema, 4) cementoid tissue, 5) execmentosis like tissue, dilated BVs and mild open apex 6) magnified area (H&E100x)

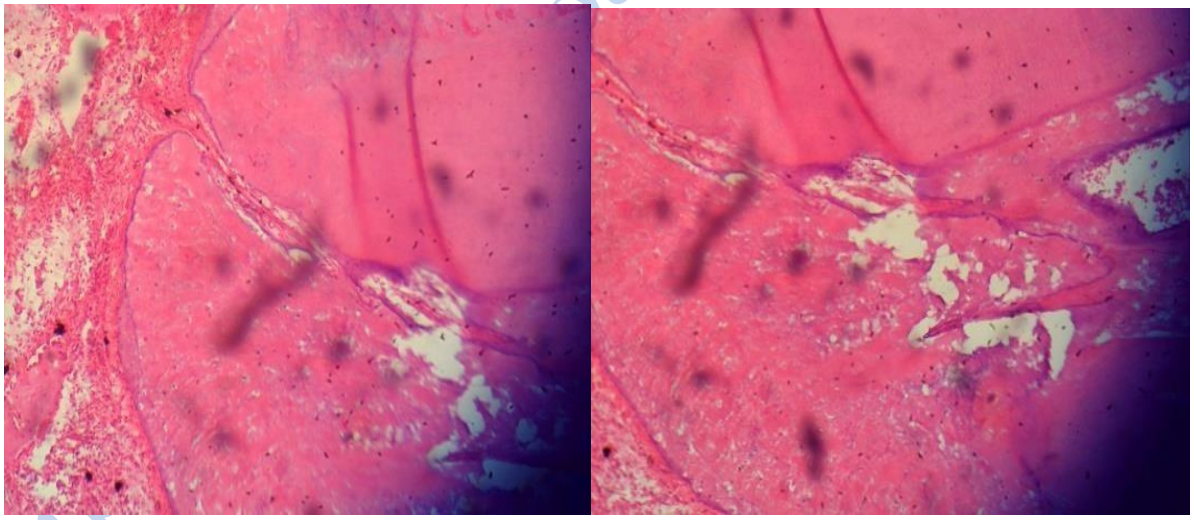
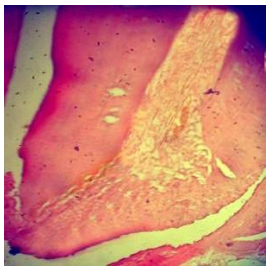
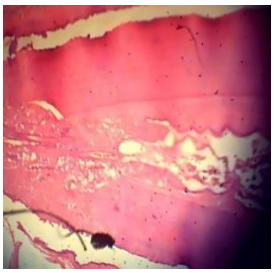
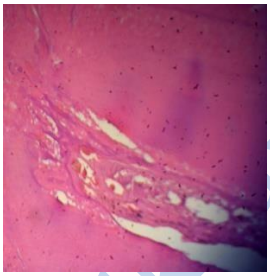
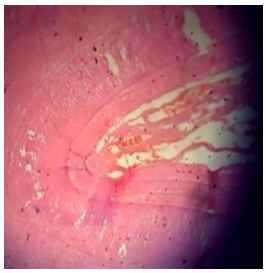
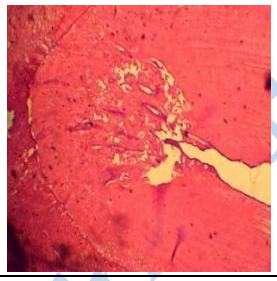
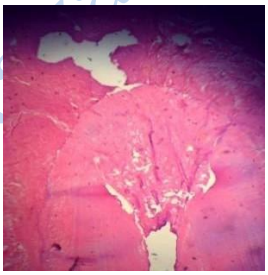
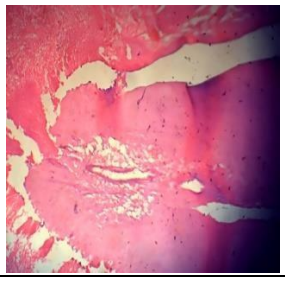
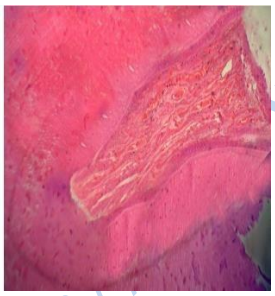
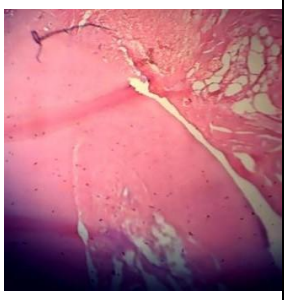
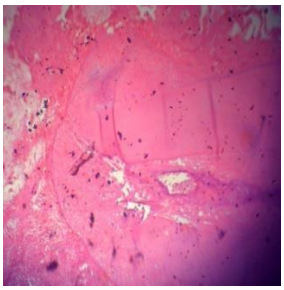


Figure 32 B: Photomicrograph, higher magnification of the previous figure showing, mild inflammatory cells, coagulated BVs and intracellular edema mild open apex. (H&E400 x).

Table (5): Showing histological sections of the four groups over different evaluation periods:

Groups	DPSCs+SIM	ASCs+SIM	DPSCs	ASCs
1 w				
1 m				
3 m				

5.1.2. Correlation between histological changes with time: (Table 6)

- **Inflammatory changes**

There was a **negative direct relation** between DPSCs+SIM, ASCs+SIM, DPSCs and ASCs with time as r = - 0.968, - 0.968, - 0963 and - 0.714 respectively. As time increases, inflammatory changes decreases.

- **Intracanal tissue changes**

There was a **negative direct relation** between DPSCs+SIM, ASCs+SIM with time as r = - 0.968, - 0.963, As time increases, intra canal tissue changes decreases. while no relation between DPCs and ASCs with time.

- **Hard tissue formation**

There was a **positive direct relation** between DPSCs+SIM, ASCs+SIM, DPCs and ASCS with time as r = 0.968, 0.963, 0.714 and 0.963 respectively. As time increases, hard tissue formation increases.

- **Apical closure**

There was a **positive direct relation** between DPSCs+SIM, ASCs+SIM, DPCs and ASCS with time as r= 0.968, 0.963, 0.963 and 0.963 respectively. As time increases, hard tissue formation increases.

Table (6): Correlation between histological changes with time:

	Time of regeneration			
	DPSCs+SIM	**ASCs+SIM**	**DPSCs**	**ASCs**
Inflammatory changes	**-0.968**	**-0.968**	**-0.963**	**-0.714**
Intracanal tissue changes	**-0.968**	**-0.963**	**NR**	**NR**
Hard tissue formation	**0.968**	**0.963**	**0.714**	**0.963**
Apical closure	**0.968**	**0.963**	**0.963**	**0.963**

Pearson correlation (r) value	*significance*
(+) ***1***	***Linear relation***
(+) ***1 - 0.9***	***Very strong relation***
(+) ***0.7 - 0.9***	***Strong relation***
(+) ***0.5 - 0.7***	***Medium relation***
(+) ***0.3 - 0.5***	***Week relation***
(+) ***0 - 0.3***	***Very week or no relation***

In case of (-) signal, this refers to the same significance but in adverse direction

NR= No Relation

5.2. Evaluation of the radiographic findings:

5.2.1. Radiographic comparison among the tested groups after different evaluation periods

Three criteria were compared at different periods, all data and comparisons were represented at **table (7,8,9), figures (33,34&35).**

- **Radiolucency**

For DPSCs+Sim, there was a statistically significant difference between one week, one month and three months as p = 0.00. Better radiolucency decrease was recorded at 3 months followed by one month.

For ASCs+Sim, there was a statistically significant difference between one week, month and three months as p=0.00. better radiolucency decrease was recorded at 3 months followed by one month.

For DPSCs, there was no statistically significant difference between one week, one month and three months as p = 0.317.

For ASCs, there was no statistically significant difference between one week, one month and three months as p=0.616.

For one week, there was no statistically significant difference between four groups as p = 0.413.

For one month, there was no statistically significant difference between four groups as p = 0.012. DPSCs+SIm showed the better radiolucency decrease followed by ASCs+SIM then DPSCs and ASCs.

For 3 months, there was a statistically significant difference between four groups as p = 0.0.00. DPSCs+SIm showed the better radiolucency decrease followed by ASCs+SIM then DPSCs and ASCs.

- **Apical closure**

For DPSCs+Sim, there was a statistically significant difference between one week, one month and three months as p=0.001. better apical closure was recorded at 3 months and one month followed by one week.

For ASCs+Sim, there was no statistically significant difference between one week, one month and three months as p = 0.116.

For DPSCs, there was no statistically significant difference between one week, one month and three months as p=0.791.

For ASCs, there was no statistically significant difference between one week, one month and three months as p = 0.616.

For one week, there was no statistically significant difference between 4 cells as p=0.582.

For one month, there was no statistically significant difference between four groups as p = 0.033. DPSCs+SIm showed the better apical closure followed by ASCs+SIM then DPSC and ASCs.

For 3 months, there was a statistically significant difference between four groups as p = 0.000. DPSCs+SIm showed the better radiolucency decrease followed by ASCs+SIM then DPSC and ASCs.

- **Canal calcification**

For DPSCs+Sim, there was a statistically significant difference between one week, one month and three months as p = 0.001. canal calcification was significant at 3 months and one month when compared with one week.

For ASCs+Sim, there was no statistically significant difference between one week, one month and three months as p = 0.116.

For DPSCs, there was no statistically significant difference between one week, one month and three months as p = 0.761.

For ASCs, there was no statistically significant difference between one week, one month and three months as p = 0.616.

For one week, there was no statistically significant difference between four groups as p = 0.413.

For one month, there was no statistically significant difference between four groups as p = 0.125

For 3 months, there was no statistically significant difference between four groups as p = 0.052.

Table (7) The mean values in radiolucency and standard deviation (SD) when comparing each group after different evaluation periods:

			week	month	3 months	p
Radiolucency	DPSCs+SIM	Mean	0.167 ^{A}	1.000 ^{Ba}	1.667 ^{Ca}	0.000 *
		SD	0.408	0.000	0.516	
	ASCs+SIM	Mean	0.262	0.833 ^{B} ^{b}	1.364 ^{Cb}	0.000 *
		SD	0.395	0.408	0.614	
	DPSCs	Mean	0.205	0.707 ^{c}	1.027 ^{c}	0.315
		SD	0.182	0.415	0.437	
	ASCs	Mean	0.270	0.561 ^{d}	0.937 ^{d}	0.616
		SD	0.107	0.360	0.501	
		p	0.413	0.012*	0.000*	

*** significance,**

small letters for significance between the same column of the same color only,

large letters for significance between the same row of the same color only,

$p \leq 0,05$

Table (8) The mean values in apical closure and standard deviation (SD) when comparing each group after different evaluation periods:

			week	month	3 months	p
Apical closure	DPSCs+SIM	Mean	0.167^{A}	1.167^{B} ^{a}	1.667^{Ca}	0.001 *
		SD	0.408	0.753	0.516	
	ASCs+SIM	Mean	0.262	1.320^{b}	1.364^{b}	0.116
		SD	0.395	0.543	0.614	
	DPSCs	Mean	0.205	1.130^{c}	1.193^{c}	0.791
		SD	0.182	0.509	0.589	
	ASCs	Mean	0.270	0.904^{d}	0.991^{d}	0.616
		SD	0.107	0.347	0.483	
		p	0.582	0.033*	0.000*	

*** significance,**

small letters for significance between the same column of the same color only,

large letters for significance between the same row of the same color only,

$p \leq 0,05$

Table (9) The mean values in canal calcification and standard deviation (SD) when comparing each group after different evaluation periods:

			week	month	3 months	p
Canal calcification	DPSCs+SIM	Mean	0.000 A	0.833^{B}	0.833^{C}	0.001 *
		SD	0.000	0.408	0.408	
	ASCs+SIM	Mean	0.000	0.707	0.707	0.116
		SD	0.000	0.415	0.415	
	DPSCs	Mean	0.000	0.561	0.561	0.761
		SD	0.000	0.360	0.360	
	ASCs	Mean	0.000	0.547	0.547	0.616
		SD	0.000	0.189	0.189	
		p	0.413	0.125	0.052	

*** significance,**

small letters for significance between the same column of the same color only,

large letters for significance between the same row of the same color only,

$p \leq 0,05$

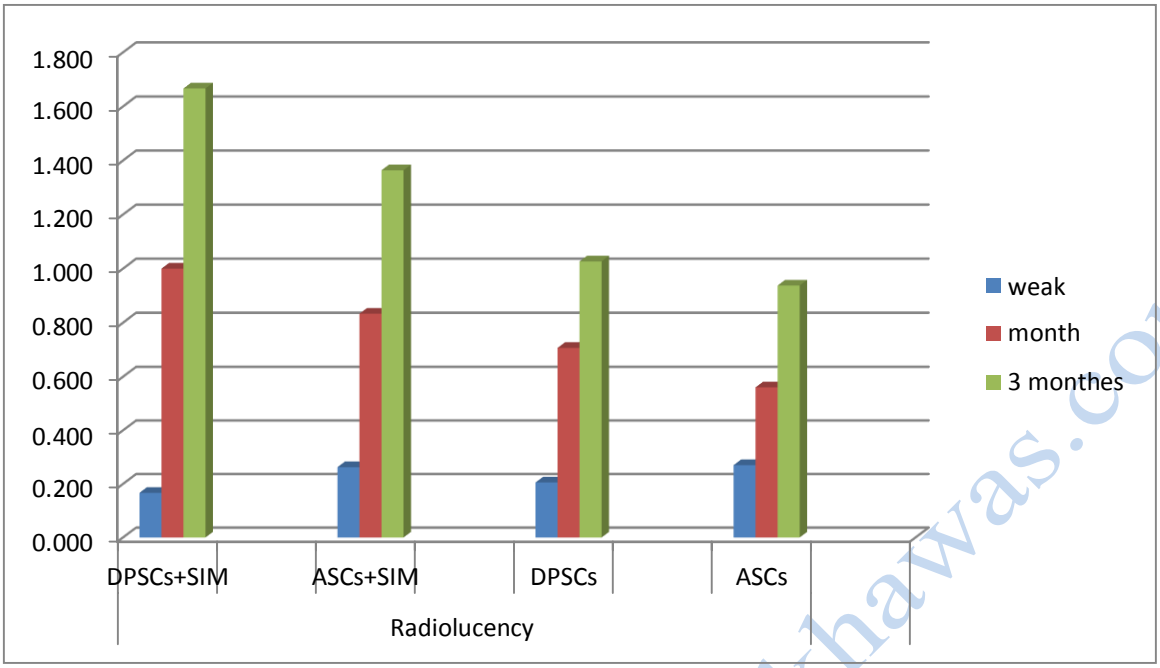


Figure 33: bar chart representing the mean radiolucency changes of the four groups

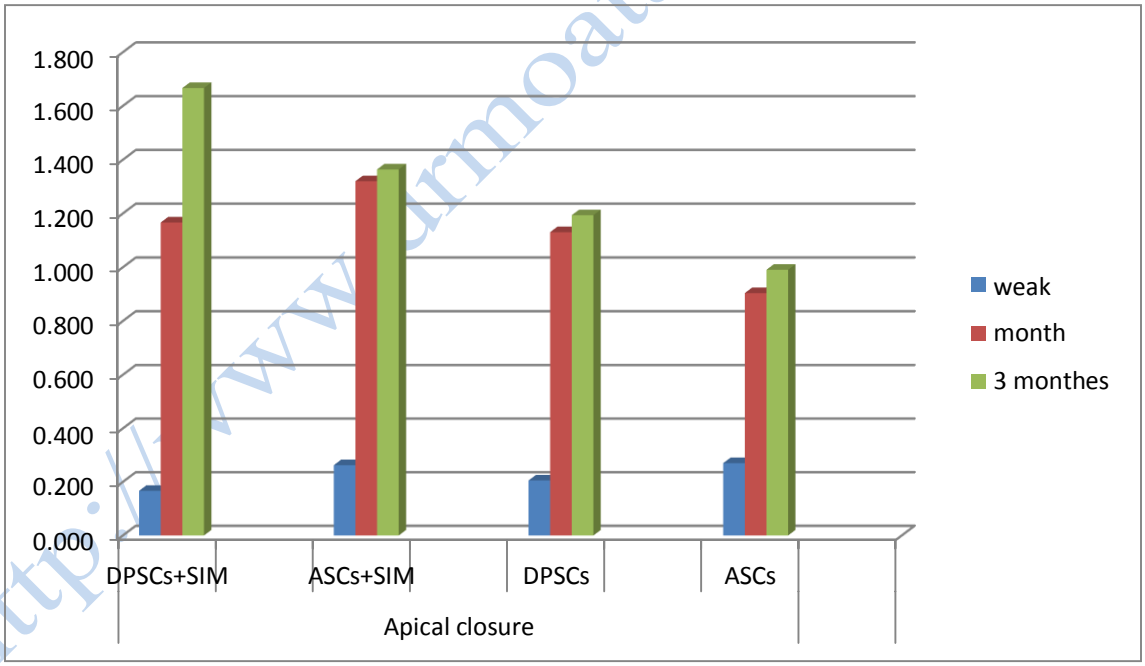


Figure 34: bar chart representing the mean apical closure changes of the four groups

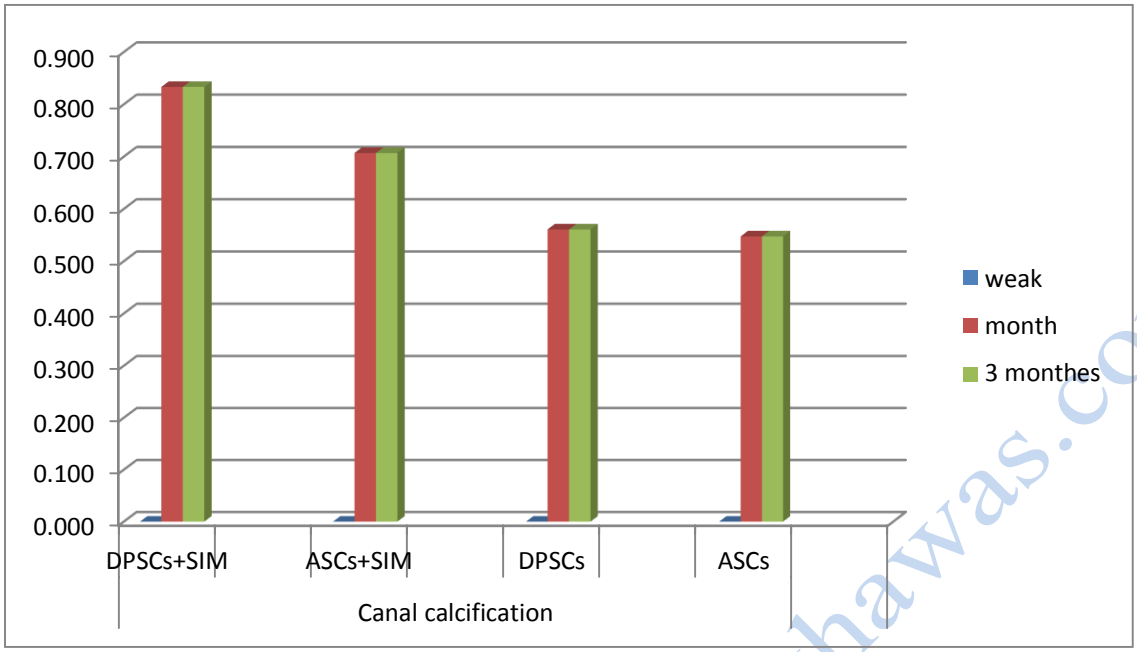


Figure 35: bar chart representing the mean canal calcification changes of the four groups

Group 1: Teeth treated with dental pulp stem cells added to simvastatin

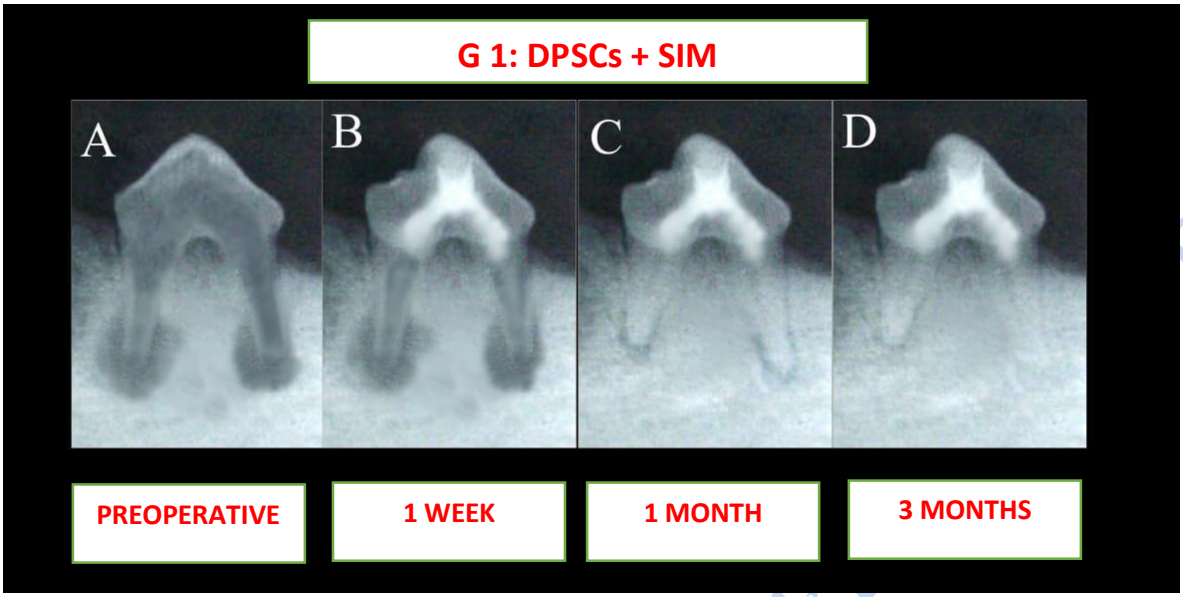


Figure 33: radiographic changes of group 1 over different evaluation periods

Group 1: Teeth treated with dental pulp stem cells added to simvastatin

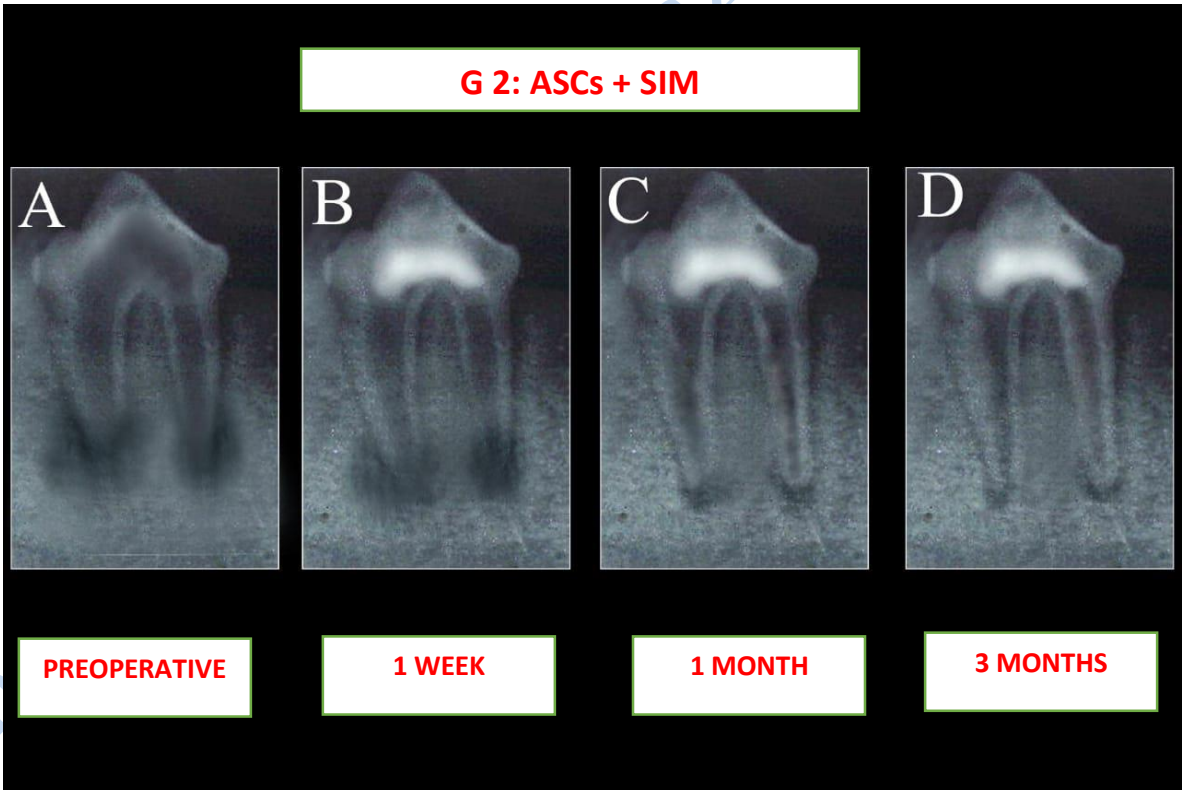


Figure 34: radiographic changes of group 2 over different evaluation periods

Group 1: Teeth treated with dental pulp stem cells added to simvastatin

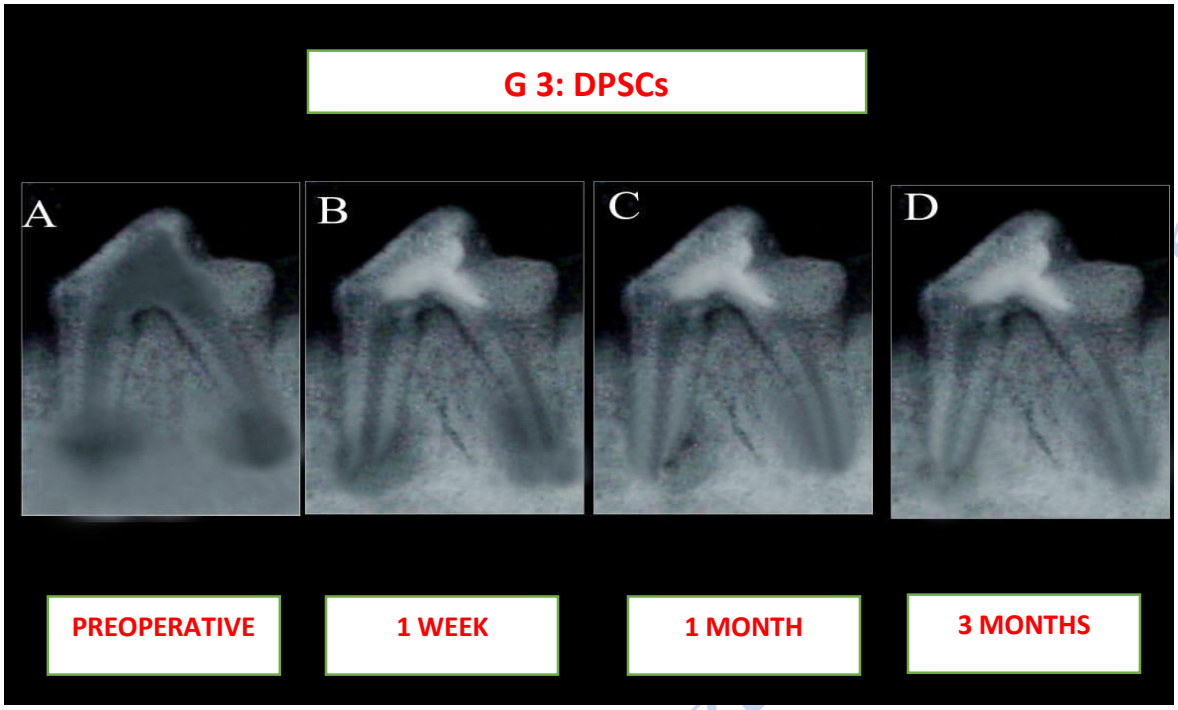


Figure 35: radiographic changes of group 3 over different evaluation periods

Group 1: Teeth treated with dental pulp stem cells added to simvastatin

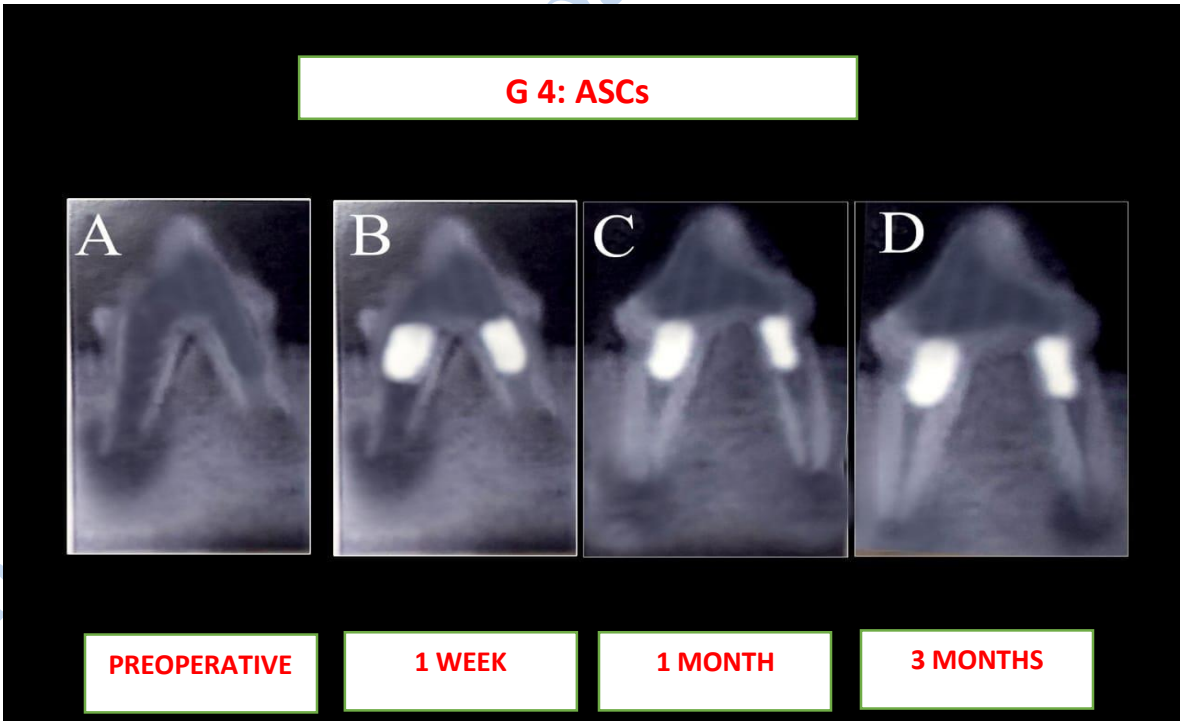


Figure 36: radiographic changes of group 4 over different evaluation periods

5.2.2. Correlation between radiographic changes with time: (Table 10)

- **Radiolucency**

There was a **positive direct relation** between DPSCs+SIM, ASCs+SIM,and DPCs with time as r= 0.968, 0.968 and 0.247 respectively. As time increases, absence of radiolucency increases. No relation between ASCs and time

- **Apical closure**

There was a **positive direct relation** between DPSCs+SIM, ASCs+SIM with time as r= 0.968 and 0.714, As time increases, apical closure increases. while no relation between DPCs and ASCS with time.

- **Canal calcification**

There was a **positive direct relation** between DPSCs+SIM with time as r = 0.714. As time increases, canal calcification increases. No relation between ASCs+SIM, DPCs and ASCs with time.

5.2.2. Correlation between radiographic changes with time: (Table 10)

Time of regeneration				
	DPSCs+SIM	**ASCs+SIM**	**DPSCs**	**ASCs**
Radiolucency	**0.968**	**0.968**	**0.249**	**NR**
Apical closure	**0.968**	**0.714**	**NR**	**NR**
Canal calcification	**0.714**	**NR**	**NR**	**NR**

Pearson correlation (r) value	***significance***
(+) *1*	***Linear relation***
(+) *1 - 0.9*	***Very strong relation***
(+) *0.7 - 0.9*	***Strong relation***
(+) *0.5 - 0.7*	***Medium relation***
(+) *0.3 - 0.5*	***Week relation***
(+) *0 - 0.3*	***Very week or no relation***

In case of (-) signal, this refers to the same significance but in adverse direction

NR = No Relation

6. Discussion

Treatment of immature teeth with necrotic pulps has been considered a big challenge because interruption in root development leads to weak and thin fragile dentinal walls liable to fracture, beside the difficulty to achieve an adequate apical seal using conventional root canal filling techniques [2].

Traditional management of such teeth ranges from induction of apical closure using calcium hydroxide (Apexification), apical plug using bioactive materials such as MTA, to apical surgery with retrograde sealing [165,166].

Recently, regenerative endodontics has gained attention as a biologically based alternative as it can allow for further root maturation in length and thickness by the regenerated vital tissue [167]. To complete this procedure successfully, three components including stem cells, scaffolds and growth factors should be involved in such protocol [7,168].

The present study is an experimental study done to evaluate the treatment outcomes of immature teeth with necrotic pulps of dogs using injectable scaffold of simvastatin in combination with dental pulp stem cells or adipose-derived stem cells. Both radiographic and histologic evaluations were used to overcome the limitations of each method.

An experimental animal model should have comparable anatomical, physiologic, histologic, and pathologic characteristics to the ultimate treatment cohort [169]. The choice of dogs as an animal model for biological experiments in endodontics is based on the fact that they have similar apical repair compared with humans but over a short duration (average one-sixth of human) due to the high growth rate [170]. Also, dogs have a close similarity in radicular structure to immature human teeth in their open apex characteristics [106,171].

Two rooted teeth were selected in order to their accessibility for endodontic procedures and they have average-sized canals for endodontic manipulation. The age of the dogs ranged between 4 and 6 months which was suitable for the study of immature teeth, as premolar teeth are immature at this age range and the animals can withstand general anesthesia and surgical procedures.

Preoperative sedation was used as a chemical restraint for diagnostic and therapeutic procedures to reduce the anxiety of dogs, ensure a smooth induction of anesthesia and to provide a quiet and gradual recovery.

Induction of periapical radiolucency was an indication of the presence of apical periodontitis related to the tested teeth. Periodontitis is generally interpreted to indicate pulpal necrosis and infection. However, radiolucency at the periradicular region cannot be used as a determining factor of total pulp necrosis because vital tissues can be present in pulp chambers, or even in mature permanent teeth associated with periapical radiolucencies [172]. In immature teeth, an open apex provides good communication between the pulp space and the periapical tissues; therefore, it is possible that extended periapical disease can occur while the pulp is only partially necrotic and infected [173].

With regards to root canal disinfection, a concentration of 1.5 % of NaOCL was used as it has the least cytotoxic effect on the stem cells compared to other concentrations [174.175,176].

Furthermore, concerning the formulation of TAP, the American Association of Endodontics (AAE) recommendations suggests the use of minocycline (spectrum of gram+ and gram−), metronidazole (spectrum of anaerobic bacteria and protozoa) and ciprofloxacin (spectrum of gram+ and gram−). Minocycline was replaced with doxycycline due to lack of minocycline availability and this was similar to other

research done in this field [93,177]. The disinfection period was 3 weeks which was recommended by most of the published articles [178,179,180].

Final irrigation was done by 17% EDTA in order to expose growth factors entrapped in the dentin matrix including, bone morphogenic protein 2 (BMP2), Transferring Growth Factor-B (TGF-B) and angiogenic factors as platelets-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF 2) [127,181]. It has been shown that EDTA-soluble factors stimulate matrix secretion, odontoblast differentiation, and tertiary dentin formation [121,182,183]. Rinsing with saline after irrigation was recommended in order to minimize the risk of possible precipitates and to remove residual debris and remain of irrigant [184].

Many authors advocated non instrumentation technique as instrumentation could not only increase fragility of dentin walls but also injure stem cells present in the apical area of these dentin walls. Growth factor that are essential for the regeneration process could also be eliminated by instrumentation [185,186].

With regards to the coronal seal, MTA was used in the coronal part of the root canal because it is a bioactive material that has an excellent sealing property. Coronal MTA is considered the golden standard in this type of research [187].

Stem cells have been isolated from a wide variety of tissues and they lack tissue-specific characteristics. Under the influence of appropriate signals, they can differentiate into specialized cells with a phenotype distinct from that of the precursor.

The use of dental stem cells appears to be more committed to odontogenic rather than osteogenic development [77]. Therefore, DPSCs were used in this study due to their advantages in clinical usage including lower mortality rate, less legal or ethical issues, easy access from extracted teeth, and cryopreservation without losing

their multi-differentiation potential [188,189]. Another important feature of dental pulp stem cells are their odontoblastic differentiation potential as they can be induced *in vitro* to differentiate into cells of odontoblastic phenotype [190,191].

Another type of non-dental adult stem cells used was Adipose-Derived Stem Cells (ASCs) which have several advantages including ease of isolation by local excision or suction-assisted liposuction, relative abundance, rapidity of expansion, high cell yield, rapid in vitro expansion and broad multipotency with differentiation into number of cells lineages including adipo-, osteo-, chondro-cytic lineages that is independent upon serum source and quality [83,192]. Additionally, ASCs have neither ethical nor immune-reactive consideration and might be an alternative cell source for pulp tissue regeneration [193].

There are several techniques have been used for stem cell isolation as outgrowth technique and enzyme digestion methods. In this study, enzymatic digestion method was used as it is widely used in laboratories and preferable to detect, isolate, proliferate and differentiate stem cells. This method gives rise to a more heterogeneous cell culture and allows the isolation of fibroblast-like cells and release of endothelial cells and pericytes [194,195].

Simvastatin (SIM) was used because it is widely prescribed and has a higher bone growth stimulation capability compared with the other hydrophilic Statins [143]. Its long-term systemic administration in humans has been shown to result in increased bone mineral density [142] as well as the anti-inflammatory effect when delivered or applied locally [154,196]. Recently, simvastatin-treated dental pulp stem cells exhibited enhanced odontogenic differentiation and accelerated mineralized tissue formation [13, 197]. Methylcellulose is generally regarded as a nontoxic, non-allergic, non-irritating material and is used as a sustained release vehicle for therapeutic drugs [198,199].

Generally, scaffolds can be divided into (1) **casted** (i.e., fairly rigid and custom-made for specific purposes) and (2) **injectable** (i.e., low viscosity gels that can be delivered and ‘‘molded’’ at the site that requires tissue regeneration). Injectable scaffold is one of the treatment alternatives for regenerative endodontics [(200)].

The use of a hydrogel aimed to enhance revascularization processes via the drug delivery system. The hydrogel acts as a resorbable scaffold and is a preferable candidate for its biosafety and inertness [(201)]. Thus, delivery of the drug was controlled via degradation of the hydrogel carrier not by simple diffusion. It is becoming increasingly evident that the ideal scaffold for dental pulp tissue engineering will be injectable, not casted. This is because of the narrow spaces within the root canal and the complexity of its anatomy, particularly in the apical region so hydrogels can penetrate throughout the root canal system [(202)]. In theory, the hydrogel may promote pulp regeneration by providing a substrate for cell proliferation and differentiation into an organized tissue structure [(203)].They can biodegrade within weeks or months, enabling natural tissue to fill the space occupied previously by the diseased tissue [(204)].

Radiographic examination was done periodically over the different evaluation periods to detect tissue changes (radiolucency, apical closure and canal calcifications). While Histologic examination was done to evaluate (inflammatory changes, intracanal tissue changes, hard tissue formation and apical closure).

Inflammatory changes:

Regarding inflammatory changes of the tested groups (DPSCS+SIM, ASCS+SIM, DPSCS and ASCS respectively) after 1 week ,1 month and 3 months’ evaluation periods, the results showed initial increasing followed by marked reduction along the different evaluation periods. This indicates the progression of

healing of periapical lesion and reduction of inflammatory reaction with time. This mild to moderate inflammation might be attributed to the immediate inflammatory reaction of the periradicular tissues to the treatment protocols. This is in agreement with the findings of inflammatory reaction by **wang et al** [205] and **Pereira MS et al** [206] who advocated the presence of mild inflammatory reaction regardless of the new tissue ingrowth following regenerative procedures. Additionally, Inflammation may provide factors to guide the differentiation of stem/progenitor cells in the healing soft tissue into cementoblasts. Furthermore, hard tissue deposition appears to respond to infection and inflammation and most likely serves as part of the defense mechanism.

On the other hand, the reduction of inflammation was an indication of progressed healing of the periapical lesion over time that is in agreement with **Gomes-Filho et al. 2011** [207]. However, inflammatory changes that were demonstrated through the combination groups (DPSCS+SIM, ASCS+SIM,) described as mild inflammations but that in (DPSCs and ASCs) groups showed moderate inflammation. This discrepancy may be attributed to addition of SIM to these groups. This finding was in agreement with **Lu D et al (2007)** [208] and **Miron.VE et al (2007)** [209] who revealed that, statins have an anti-inflammatory effect in various tissues and this could help restore the inflamed pulp tissue beside their ability to accelerate reparative dentin formation.

Intracanal tissue changes:

Regarding intracanal tissue changes of the tested groups (DPSCS+SIM, ASCS+SIM, DPSCS and ASCS respectively) after 1 week ,1 month and 3 months' evaluation periods, the results showed that DPSCs+SIM group has the highest change followed by ASCs+SIM group then DPSCs group while ASCs group showed the least changes. This may be attributed to the potent regenerative power of SIM

that allow for synergistic effect when added to either DPSCS or ASCS (259).Besides the power of DPSCs as a native progenitor cells that have odontogenic rather than osteogenic development in compared with ASCs [(210)].This is in agreement with other study that demonstrated the proliferative ability and developmental potentials of DPSCs beside their ability to develop into distinct tissues representative of the microenvironments from which they were derived *in vivo*. Also, the potent neurogenicity of dental stem cells may be attributed to their neural crest origin [(211)].

Additionally, SIM-treated DPSCs when introduced into the experimental teeth, pulp tissue formation as well as dentin formation was seen at the roof of the pulp chamber over the primary dentin. This result may be attributed to SIM, which stimulates cell mineralization. Furthermore, the structures and arrangement of root pulp cells were like natural pulp [(164)]. SIM has a regenerative highly potential effect on DPSCs rather than ASCs that support the finding of the present study.

DPSCs showed higher potentiality to give rise dental tissues. These results were in accordance to ***Huang et al*** in **2006** [(212)] who stated that when DPSCs seeded onto dentin, some DPSCs convert into odontoblast-like cells with a polarized cell body and a cell process extending into the existing dentinal tubules. Adversely, ASCs group showed the least intracanal tissue changes that might be due to that ASCs had neither angiogenic nor neurogenic potential in vitro and had a little effect on pulp regeneration unlike pulp cells that are the progenitor of DPSCs [(213)]. This is supported by another study showed that simvastatin promoted odontoblastic differentiation when using DPSCs, as evidenced by the induction of ALP activity, the formation of mineralization nodules, and the expression of dentin-specific proteins such as dentin sialoprotein (DSP) and dentin matrix protein 1 (DMP-1) [(13)]. DSP, which is deposited primarily by odontoblasts, is a specific biochemical marker for functional odontoblasts. Expression of DMP-1 is also considered an important characteristic of odontoblasts.

Regarding ASCs, the results of the present study were in disagreement of another investigation demonstrated the transplantations of bone marrow-derived stem cells and adipose-derived stem cells can induce pulp regeneration in the root canal after pulpectomy in dogs. It is noteworthy that there is enhanced matrix formation and root canal obliteration after adipose cell transplantation. MSCs from different sources are comparable in their regeneration potential in vivo and that their capabilities are independent of their origin [(214)].

One of the most important and critical finding in this study is the appearance of dentin bridge under the MTA that may be attributed to the rgenerative power of MTA due to its bioactivity. This was in concomitant with some investigators who concluded that in the MTA group, a dentin bridge was formed at the root canal orifice, which kept the regenerated pulp from entering the pulp chamber [(82)].

Hard tissue formation:

Regarding the changes in hard tissue formation of the tested groups (DPSCS+SIM, ASCS+SIM, DPSCS and ASCS) after 1 week ,1 month and 3 months' evaluation periods, the results showed that there was a statistically significant changes between different periods.

The information about the hard tissue produced on dentinal walls and the cells responsible for hard-tissue production is still lacking. An animal study revealed that a cementum-like tissue was deposited on the root canal dentinal walls after regenerative endodontic treatment. This tissue was irregular and was assumed to be responsible for root development [(215)]. The results of this study was in agreement with a study done by **Batouli et al.** [(70)] that showed that when DPSCs are seeded onto human dentin surfaces and implanted into immunocompromised mice, reparative dentin-like structure is deposited on the dentin surface. Also some reports done by

Laino *et al.*, 2005 [216] and **d'Aquino *et al.*, 2007** [217] showed that DPSCs have osteogenic potential and may form bone-like structure *in vitro* and *in vivo.*

After one-month evaluation period, DPSCs+SIM group showed that the degenerative alterations gradually resolved. A replacement by regenerative tissues began to appear through a regular arrangement of the pulp tissue architecture. Later on, an improvement of the pulp tissue architecture mimicking the normal pattern was seen after 3 months' evaluation period. These dramatic changes reveal the healing capacity of DPSCs+SIM that might indicate their efficacy and biocompatibility as regenerative materials. These results confirm investigations which reported that DPSCs+SIM causes proliferation of human dental pulp cells and increase the protein expression of osteoprotegerin and alkaline phosphatase activity. Therefore, odontoblast like cells will be produced and pulp-dentin complex will form. Also, one of the unique characteristic of DPSCs is their capacity to form dentin pulp–like tissue when transplanted into immunocompromised mice by using HA/TCP as a carrier [218].

In converse to the present study **Hicok et al.** [88] found that ASCs produced more osteoid when being seeded on hydroxyl appetite tricalcium phosphate (HA/TCP) than cells that were cultured on collagen/HA–TCP composite, indicating that the osteogenic capacity of ASCs could be heavily influenced by the scaffold on which cells were seeded.

Apical closure:

Regarding the changes in apical closure of the tested groups (DPSCS+SIM, ASCS+SIM, DPSCS and ASCS) after 1 week ,1 month and 3 months' evaluation periods, the results showed that there was a statistically significant changes between the tested groups except for ASCs at different evaluation periods with better apical closure recorded for DPSCs+ SIM at 3 months. This may be attributed to SIM ability

to induce odontogenesis and angiogenesis especially when added to DPSCs. This is in agreement with other study that advocated that SIM is known to induce angiogenesis and to regulate the survival and increase neurogenesis of neuronal cells [(11)], indicating the possible effectiveness of SIM in pulp regeneration along with dentin regeneration.

Radiographic evaluation:

Regarding radiographic evaluation, it was clear that the conventional 2-dimensional radiographs did not reflect accurately the actual amount of new grown hard tissues in the canal or at the root apex because of the angulation and image resolution.

Radiolucency:

For DPSCs+SIM and ASCs+SIM, there was a statistically significant difference between 1 week, 1 month and 3 months. The highest decrease in periapical radiolucency decrease was recorded at 3 months followed by 1 month. This may be attributed to the effect of simvastatin that promotes osteoblastic differentiation, enhances ALP production and bone mineralization [(150)], and up-regulates the expression of bone anabolic factors such as VEGF [(219)].

Recent review articles regarding this topic concluded that local delivery of simvastatin (SIM) from biomaterials seems to be more reliable than systemic administration for bone regeneration; however, SIM can either accelerate or retard mineralized neotissue genesis according to the concentration used [(220,221)]. At low concentrations, these drugs feature pleiotropic effects with mesenchymal stem cells, increasing the expression of several osteo/odontoblastic markers, such as dentin sialophosphoprotein (DSPP), dentin matrix acidic phosphoprotein 1 (DMP-1), alkaline phosphatase (ALP), collagen type 1 alpha 1 (Col1A1), osteocalcin, osteopontin, runt-related transcription factor 2, and bone morphogenetic protein 2

(BMP-2), leading to intense mineralized matrix deposition in vitro [222]. Conversely, the higher concentration of SIM may lead to cell death.

The time frame of this study may not have been long enough for complete radiographic healing of periapical radiolucencies. It may take up to 4 years for complete healing of apical periodontitis lesions, before which failure should not be assessed [(223)]. However, **Lenzi and Trope** [(224)] demonstrated that healing of the periapical lesion and production of hard-tissue barrier in the apex after regenerative endodontic treatment can be seen even in the absence of vital tissue inside the root canal space.

Apical closure:

For DPSCs+SIM, there was a statistically significant difference between one week, 1 month and 3 months and better apical closure was recorded at 3 months. These radiographic findings were in agreement with **Thibodeau et al. (2007)** who stated that root elongation and thickening was evident radiographically after regenerative protocols.

However, **Wang et al. (2010)** [(94)] concluded that radiographic findings were not accurate regarding actual increase in root length or thickness. This is with agreement of the results of this study supporting the use of radiographic examination for the confirmation of histologic findings not for accurate measurements. This also indicates that the radiographic measure of thickened canal walls (the measurable indication of successful revascularization available to the clinician) is representative of the actual histological outcome.

Canal calcification:

For DPSCs+Sim, there was a statistically significant difference between 1 week, 1 month and 3 months. Canal calcification was significant at 3 months and 1 month when compared with 1 week. The explanation of this finding may also due to

the combination of DPSCs+SIM where statin promotes osteoblastic differentiation, enhances ALP production and bone mineralization [225], and up-regulates the expression of bone anabolic factors such as VEGF [150]. While Dental stem cells display multidifferentiation potential, with the capacity to give rise to at least 3 distinct cell lineages: osteo/odontogenic, adipogenic, and neurogenic.

Root canal calcification/obliteration is another problem after regenerative endodontic treatment of necrotic immature teeth that is reported also in cases disinfected by calcium hydroxide [226]. Although complete root canal calcification/obliteration is not mentioned as a failure in cases that have undergone regenerative endodontic treatment, it can cause serious challenges in case the involved tooth needs root canal therapy.

Finally, it is the time to reject the null hypothesis of this study that stated there is no significant difference among the experimental groups as the result of this study revealed that the differentiation potential of mesenchymal stem cells from different sources are influenced by the niche of their origin.

7. Conclusions

Within the parameters of this study:

1. Simvastatin aids in regeneration of infected immature dog's teeth with the ability of producing functional dentin-pulp complex.

2. Inclusion of a stem cells seemed to improve the outcomes of attempted regeneration.

3. Dental pulp stem cells have the potential to induce regeneration of infected immature dog's teeth.

4. The outcome of regeneration of immature dog's teeth is evident by apical closure with time.

4. Recommendations

1. Further extended follow up periods are needed to monitor the regenerative process.
2. Further research should be done regarding the use of stem cells, growth factors and scaffolds in regenerative endodontics to improve the revascularization outcomes to a higher level.
3. Further research should be done regarding the use of cone beam computed tomography to evaluate the outcome of regenerative process rather than two-dimensional periapical radiographs.
4. Banking teeth as an autologous stem cell source requires more research to determine the ultimate benefits to the patients. In that way the endodontist could be able to translate research from the laboratory to the clinic for patient benefit.
5. Clinical studies are highly recommended to examine the regenerative protocol used in this study.
6. Progress in the field of stem cell therapy will require the collaboration between clinicians and researchers from diverse fields (e.g., biomaterials, stem cell biology, endodontics) working together toward the goal of developing biological approaches to regenerate dental tissues.

9. summary

Treatment of immature teeth with necrotic pulps has been considered a big challenge because interruption in root development leads to week and thin fragile dentinal walls liable to fracture, beside the difficulty to achieve an adequate apical seal using conventional root canal filling techniques.

Traditional management of such teeth ranges from induction of apical closure using calcium hydroxide (Apexification), apical plug using bioactive materials such as MTA, to apical surgery with retrograde sealing.

Recently, regenerative endodontics has gained attention as a biologically based alternative as it can allow for further root maturation in length and thickness by the regenerated vital tissue [(227)]. To complete this procedure successfully, three components including stem cells, scaffolds and growth factors should be involved in such protocol [(228,229)].

The present study is an experimental study done to evaluate the treatment outcomes of immature teeth with necrotic pulps of dogs using injectable scaffold of simvastatin in combination with dental pulp stem cells or adipose-derived stem cells. Both radiographic and histologic evaluations were used to overcome the limitations of each method.

This study was approved by the Ethical Committee at Faculty of Dentistry, Al Azhar University based on the guidelines and regulations. The procedures were carried out in the Department of Veterinary Surgery, General veterinary hospital in Alabbasyya, Cairo, Egypt. the present study was carried out on 6 adult healthy male mongrel dogs aged from 4 to 6 months and their weight ranged from 12 to 17 kg. Three premolars in each quadrant from 6 healthy mongrel dogs were included in the study summing up the total number of roots to 144 (12 premolars x 2 roots x 6 dogs).

Under dog's sedation, clinical and radiographic examination was done to confirm shedding of the primary teeth with eruption of their permanent successors with incomplete root formation.

According to sample size calculation, number of teeth included within the study was set to be 72 teeth ((two rooted (144 roots))) included within 6 dogs. Teeth were divided into four equal groups according to different treatment protocols used for each experimental group as follows:

Group 1: treated with dental Pulp Stem Cells (DPSCs) added to simvastatin (SIM) hydrogel **(18 teeth)**

Group 2: treated with Adipose-derived Stem Cells (ASCs) added to simvastatin (SIM) hydrogel **(18 teeth)**

Group 3: treated with Dental Pulp Stem Cells (DPSCs) **(18 teeth)**

Group 4: treated with Adipose-derived Stem Cells (ASCs) **(18 teeth)**

Then each main group was subdivided into 3 subgroups according to post treatment evaluation periods as follows:

after 1 week of treatment **(24 teeth)**

after 1 month of treatment **(24 teeth)**

after 3 months of treatment **(24 teeth)**

Extraction of maxillary lateral incisor with vital pulp was done from each dog to isolate dental pulp stem cells. Autologous adipose tissue was harvested from the pad of fat in the abdominal region of each dog. Enzymatic digestion of tissue was done.

Simvastatin (Sigma-Aldrich, St Louis, MO) was prepared 1 day before the surgical procedures. methylcellulose was dissolved in hot triple-distilled water to produce methylcellulose gel, which was used as the simvastatin carrier after it cooled down.

Radiographic examination was done periodically over the different evaluation periods to detect tissue changes (radiolucency, apical closure and canal calcifications). While Histologic examination was done to evaluate (inflammatory changes, intracanal tissue changes, vital tissue formation and apical closure). Summing up and correlating radiographic and histopathologic assessment was done for obtaining comprehensive conclusions regarding the outcome of different treatment modalities.

Statistical analysis was performed with IBM® SPSS® Statistics Version. Data were represented by total number and percentage for each method used in the study. Descriptive statistics was done using one way Annova and Pearson correlation (r) tests for comparing the relation between different groups. Significance was set when P value ≤ 0.05 with confidence level 95 %.

Within the parameters of this study:

1. Simvastatin aids in regeneration of infected immature dog's teeth with the ability of producing functional dentin-pulp complex.

2. Inclusion of a stem cells seemed to improve the outcomes of attempted regeneration.

3. Dental pulp stem cells have the potential to induce regeneration of infected immature dog's teeth.

4. The outcome of regeneration of immature dog's teeth is evident by apical closure with time.

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الملخص العربي

يعتبر علاج الأسنان ضمن القناة الجذرية أنه بجرا ألن االقاط ع في ألرمج الذر يؤدي إيى ج ران رئج وهش ألكون عرل يكسر، بالا صعوب ألنوجق احك م ضرق ايقم ايذروية ايك في ب ستخ ام ألزرجت ملء زرة الذر ايقرج ي. ألراوح اإلدارة ايقرج ي يمثل هذه ألسنان مض ألنريق اإلضال ب ستخ ام هج رو سج ايكيسوم، وايمكوا ت ب ستخ ام ايمواد ايرشنط بجويوجج مثل م دة ام ألي ايه، إيى ايارا ح األعرى مع ايخثم ايرجعي.

في آلوا األخيرة، انتشيك ايتركسج ايتا ي ي االهتمم ب يل بجويوجي ألاه يمكض أن ألسنند يم ي مض اضتوج اياذر في ايطوم وايسنم مض قبل األاسننا اينجوي ايمتا دة.إل م م هذا اإلجراء برا ح، يا إشراك ثالث مكوا ت بم في ذيك ايخالي ايا ذعج وايسقالت وعوامل ايرمو في مثل هذا ايرو ألو وم.

هذه اي راسن هي دراسن ألاريبج أجريك يئجمم ات الج ايعالج مض ألسنن ضمر ايرلنا مع ايرا ايمت ل مض ايكالب ب ستخ ام سنق ي حقض سجمي سنت ألجض في ألر جب مع ايخالي ايا ذعج مض ي األسنن أو ايخالي ايا ذعج ايمشتق اي هرج .واستخ مك ايئجمم ت اإلشنع عج وايئجمم ت ايرسحاج يرار عرى ايوجود ايمرو ل عرى ل طريق.

وق وانؤك ايرا ر األخالق ني رج طا األسنن با مع األهر عرى هذه اي راسن بر ء عرى ايمب د ايئوججهج وايرواالد.ألم ألريجذ اإلجراءات في قسم ايارا ح ايبجطري ب يمسنشيى ايبجطري ايع م ب يعب سج ب يق هرة بمصنر.أجريك اي راسن اين يج عرى 6 مض ايذ ور ايب ياجض األصنن ء مض ايكالب ايموال ايذيض ألتراوح أعم رهم بجض 4 إيى 6 أشهر ووي امم ألراوح بجض 12 إيى 17 ام.ألم ألضمجض ثالث لواحك في ل ربع مض 6 الب صنينج في اي راسن ألرخ ايع د اإلجمي يرا ذور إيى 144. ألنك أله ال ايكرا، ألم إجراء ايين ايسنريري واإلشنع عي يت ج خرع ألسنن األويج مع ب وغ خري المم اي المجض مع ألكويض جذر ضجر مكتمل.ونق ينس ب حام ايجر ، ألم ألعجض ع د األسرن ايم رج في اي راس يتكون ألسرن ايم رج 72 داخل 6 الب.ألم ألقسنجم ألسنن إيى أربع ماموع ت مسن وي ونق يبروألو والت ايعالج ايمخئري ايمستخ م يكل ماموع ألاريبج عرى ايرنو ايت يي:

ايماموع 1: عوياك مع ايخالي ايا ذعج ب يمشتقه مض ي األسرن بإل ف سجمي ست ألجض إيى ايخالي ايا ذعج

ايماموع 2: عوياك مع ايخالي ايا ذعج ايمشتق مض اي هرج بإل ف سجمي ست ألجض إيى ايخالي ايا ذعج

ايماموع 3: عوياك مع ايخالي ايا ذعج يرا األسرن

ايماموع 4: عوياك مع ايخالي ايا ذعج ايمشتق مض اي هرج

ثم ألم ألقسنجم ل ماموع رالجسنج إيى 3 ماموع ت نرعج ونق يئرات ألقجم م بع ايعالج عرى ايرنو ايت يي: بع أسبوع واح مض ايعالج)24 سر (بع شهر واح مض ايعالج)24 سر (بع 3 أشهر مض ايعالج)24 سر (.

ألم استنخراج ايق طع ايا ابي يريك ايعروي مع ايرا اينجوي يع م ايخالي ايا ذعج مض ي األسنن.ألم حصت دا ألاسا اي هرج ايذألج مض وس دة مض اي مون في مرطق ايبطض ثم ألم عمل ايهضم األا يمي بألاسا . ألم إع اد

سجمي ست ألجض قبل يوم واح مض ايعمرجت اياراحج .ألم حل مجثجل سجروى في ايم ء ايس خض ثالثي ايئقطجر إلائ ج هالم مجثجل سجرويوى، ايذي استخ م ن مل سجمي ستألجض بع أن ألم ألبري ه.

ألم إجراء ايين الإشننع عي بشننكل دوري عرى م ا فترات ايئؤجم ايمختري يركشننا عض ألاجرات األاسننا)ايتقشنر الإشنع عي، الإضال ايذروي وألكرس ايؤر ة(.في حجض ألم إجراء ايين ايهسنتويوجي يئؤجم)ايتاجرات االيته بج ، وألاجرات األاسننا داخل ايؤر ة ، وألكويض األاسننا اينجوي والإضال ايذروي .ألم ألرخ وربط ايئؤجم الإشع عي و ايهستويوجي يرنصوم عرى اسئرئ ج ت ش مر بش ن ات لاج طر ايعالج ايمختري.ألم إجراء ألنرجل إحصننالي مع ألمثجل الإحصننءات مض قبل ابع د الإجم يي وايرسنب ايم وي يكل طريق مسننخ م في اي راسنن.ألم إجراء الإحصننءات ايوصننفج يمقرا ايعالق بجض حاموع ت مختري .ألم ألعجض أهمج ايئؤجم $\leq P$ 0.05 مع مسئوا ايثق 95٪.

لحمض مع يم حذه اي راس :

1 .سجمي سئ ألجض يس ع في ألا ي أسرن ايكرا ضجر اير لا ايمص ب مع ايق رة عرى إائ ج مامع يا األسرن ايع جي.

2 .ب ا أن ألضمجض ايخالي اي ا ذعج ينسض ائ لاج من وي ايتا ي .

3 .ايخالي اياذعج يرا األسرن ي يه ايق رة عرى ألنيج ألا ي أسرن ايكرا ضجر اير لا ايمص ب.

4 .إن ائجا ألا ي أسرن ايكرا ضجر اير ل ا والن عض طريق الإضال ايقمي مع مرور ايوؤك.

Regenerative potential of simvastatin on stem cells of different sources in dog teeth: a histologic and radiographic study

Protocol Submitted in partial fulfillment of the requirements for Doctor Degree in Endodontics

By

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Introduction

Treatment of immature non vital teeth represents a big challenge during root canal treatment procedures in which a greater liability of root fracture is evident due to thin fragile walls as well as incomplete disinfection of the large root canal walls with the possibility of bacterial extrusion due to open apex [229].

Traditional management of such teeth ranges from induction of apical closure using calcium hydroxide (Apexification), mineral trioxide aggregate (MTA) apical plug to apical surgery with retrograde sealing [229,229].

Recently, revascularization protocol of immature non vital teeth has been introduced as an innovative treatment protocol in which the introduction of vascularity or angiogensis of root canal system is done. A sequel of stimulation of undifferentiated mesenchymal cells at the periapex, leading to deposition of a calcific material at the apex as well as on the lateral dentinal walls has been demonstrated resulting in complete root maturation. [229]

To complete this revascularization procedure successfully, three components including stem cells, scaffolds and growth factors should be utilized in such protocol[229,229].

Anyhow, restoration of the dentin-pulp complex is still somewhat vague and incomplete as pulp revascularization does not necessarily indicate either repopulation of odontoblasts that align on dentin surfaces or regeneration of the nerve fibers in addition to interstitial fibroblasts and most importantly, stem/progenitor cells that serve to replenish all pulp cells in the regenerated pulp when they undergo apoptosis and turnover [229,229,229,229,].

Regenerative therapy requires the introduction of an agent, which not only hampers tissue destruction but also enhances the regenerative capabilities of the

tissues [229]. Pharmacologic agents offer a great promise in this direction and have been shown to offer a safe and cost-effective alternative to this issue [229].

Simvastatin (SMV), a universally accepted drug used primarily for the treatment of hypercholesterolemia has revealed increase in bone mineral density with long term systemic administration in humans [229]. Recently, simvastatin-treated dental pulp stem cells exhibit enhanced odontogenic differentiation and accelerated mineralized tissue formation [229,229].

Numerous studies already suggested the ability of dental pulp stem cells (DPSCs) to regenerate a dentin-pulp-like complex that is composed of mineralized matrix with tubules lined with odontoblasts, and fibrous tissue containing blood vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth [229].

From the histological point of view, the nature of the tissue formed in the canal space in human revascularized immature permanent teeth with apical periodontitis is speculative because no histologic studies are available [229]. Although human samples are difficult to collect, animal models may shed some light to address this issue [229].

Therefore, the present study will focus on the histological interpretation of the tissues grown into the root canal space after revitalization procedure using different pharmacologic agents. Also, both endogenous and exogenous neural stem cells (NSCs) have been investigated for their capacity to regenerate a damaged nervous system.

Aim of study

The present study will be directed to evaluate the regenerative potential of simvastatin on stem cells of different sources in dog teeth both histopathologically and radiographically.

Materials and methods

Animal model:

Sixty double-rooted premolar teeth in six purpose-bred dogs aged approximately up to 6 months will be selected to be used in this study. All dogs will be bred and housed under similar conditions (22℃ room temperature, 40% humidity and 12 hours daylight cycle) with a standard laboratory diet and water.

Dental treatment:

All the procedures will be done under general anesthesia supplemented with local anesthesia. The pulps of all experimental teeth will be mechanically exposed followed by disrupting the pulp tissue in the canal spaces, without removing it from the canals. The teeth will be monitored radiographically until there will be a radiographic evidence of apical periodontitis as apical radiolucency.

In the second visit, previously infected teeth will re-entered under aseptic conditions of rubber dam isolation with the dogs under general and local anesthesia. Then, cleaning and shaping of the root canals followed by intracanal medication using triple antibiotic paste will be done and intermediate coronal restoration using resin modified glass ionomer will be done

In the third visit, grouping of the teeth will be done according to stem cells source with different materials used as follow;

Group 1: teeth will be treated using simvastatin with Dental pulp stem cells

Group 2: teeth will be treated using simvastatin with adipose derived stem cells

Group 3: teeth will be treated using Dental pulp stem cells

Group 4: teeth will be treated using adipose derived stem cells

Dogs sacrifice:

All of the teeth will be monitored radiographically on a monthly basis before the dogs were sacrificed, and tissues were harvested for histological examination.

Histologic examination:

After scarifying the dogs, sections will be made followed by staining with hematoxylin and eosin for their evaluation regarding tissue types.

Radiographic and histologic examination:

Radiographic examination was done periodically over the different evaluation periods to detect tissue changes (radiolucency, apical closure and canal calcifications). While Histologic examination was done to evaluate (inflammatory changes, intracanal tissue changes, hard tissue formation and apical closure).

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رج طا األسرن,برجض,ايق هرة ج مع

األنهر

ادة تجدد الخاليا الجذعية ذات المصادر المختلفة في أسنان الثلب : دراسة نسيجية و إشعاعية

خط بزث مق م ١ ء مض مقوم ت اينصوم عرى درج اي تـوراه ني عالج اياذور

مق م مض ابطبج١

عمرو عب اوه ب عب اينمج بجومى

بك يورىوس ط١ اييم و االسرر ن ،2004 م ،برجض ، ايق هرة ، ج مع االلـهر م

جسنجر عالج اياذور، 2015 م ،برجض ، ايق هرة ، ج مع االلـهر

م رس مس ع بؤسم عالج اياذور ، رج ط١ االسرر ن ،برجض ، ايق هرة ، ج مع االىهر

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رج ط١ االسرر ن ،برجض ، ايق هرة ج

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لجنة المناقشة والحكم

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األلسرن,برجض,ايق هرة

ج مع األلـهر

د/معتب هلل أحم ايخواص

أستذمس ع بقسم عالج اياذور

رج طا األلسرن,برجض,ايق هرة ج مع

األلـهر

د/ ه اي جمبل فـهمي

أستذ مس ع بقسم بجويوجج اوبم واالسررن

رج طا األلسرن,برجض,ايق هرة ج مع

األلـهر

قدرة السيمفاستاتين علي إعادة تجدد الخلايا الجذعية ذات المصادر المختلفة في أسنان الكلاب : دراسة نسيجية و إشعاعية

رسالة مقدمة من

الطبيبة

عمرو عبد الوهاب عبد الحميد بيومى

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الأزهر

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