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EFFECT OF BONE MARROW- DERIVED STEM CELLS ON APOPTOSIS OF DENTAL PULP CELLS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT

Aim: the aim of the present investigation was to study the effect of mesenchymal bone marrow stem cells (BMSCs) on the histological structure of the dental pulp of rats with Streptozotocin-induced diabetes mellitus using hematoxylin & eosin and Masson's trichrome stains. Furthermore, the apoptotic activity of dental pulp cells was detected through Caspase-9 localization.

Material and Methods: Thirty, three- month old, adult male albino rats, 180-200 gram weight were used in this investigation. They were divided into three groups. **Group I**: 10 animals served as controls. **Group II**: 10 animals subjected to a single intraperitoneal injection of Streptozotocin in a dose of 60 mg/kg body weight for induction of diabetes mellitus that was verified by detecting hyperglycaemia (200mg/dl or more) in a blood sample. **Group III**: 10 animals treated the same as those of group II and one week later these animals were intravenously injected with BMSCs. At the end of the experiment which lasted for two months, the animals of the different groups were euthanized. Their jaws were dissected, decalcified and processed for hematoxylin and eosin, Masson's trichrome stains and immunohistochemical stain for caspase-9 to study the dental pulp of the experimental rats.

Results: The present work revealed that the treatment of diabetic rats with single intravenous injection of bone marrow stem cells resulted in amelioration of the degenerative and apoptotic .effect of diabetes on the pulpal tissues

Conclusion: Induction of diabetes in albino rats has degenerative effect on pulpal tissues accompanied by increased expression of caspase-9 (apoptotic marker). Intravenous injection of BMSCs resulted in regeneration of pulp tissues and decreased apoptosis as detected by low caspase-9 expression.

KEYWORDS: stem cells, apoptosis, dental pulp, diabetes, caspase-9.

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INTRODUCTION

Diabetes mellitus is the most common endocrine disorder affecting a wide range of population[1]. It represents a group of diseases of heterogeneous etiology, characterized by chronic hyperglycaemia and other abnormalities in carbohydrate, lipid and protein metabolism which are caused by deficiency of insulin effect[2]. Diabetes is classified etiopathogenetically into two main categories; type I and type II diabetes. Patients of type I diabetes suffer from complete insulin deficiency. Individuals at high risk for developing this type could be detected by serological tests for autoimmune diseases involving pancreatic island and some genetic markers. On the other hand, patients suffering from type II diabetes have resistance to insulin action accompanied by impaired insulin compensatory mechanism. Type II diabetes is more prevalent and it could develop various diabetes-related complications even before the time of being diagnosed[3]. Carbohydrate intolerance could result in another form of diabetes called gestational diabetes that manifests variable severity of hyperglycaemia. This type is usually detected during pregnancy[4]. There are other specific forms of diabetes caused by; genetic defects of the beta cells or insulin action, diseases involving the exocrine pancreas or the endocrinal system in addition to drug or chemically induced diabetes[5].

Despite the decrease in diabetes-related complications in the past two decades, the continued increase in diabetes prevalence resulted in huge load on societies[6]. Higher red blood cell distribution width (RDW) values are accompanied by increased incidence of developing cardiovascular and renal diseases in American adult diabetic patients [7]. The deteriorating effect of hyperglycaemia on the vascular system results in the most serious complications associating diabetes. In general, the complications resulting from damaging large vessels include coronary artery disease, peripheral arterial disease, and stroke. While those due to affection of microvasculature include diabetic nephropathy, neuropathy, and retinopathy[8].

Dental pulp is connective tissue with specific microcirculatory system. Pulp vasculature has significant reparatory abilities intending to preserve pulp vitality. Due to microcirculatory system disorders, treatment of affected dental pulp in patients with diabetes mellitus (DM) is additional challenge. Ilic [9] in his study on diabetes mellitus and reparative response of dental pulp found that changes in the function and levels of growth factors in various diabetic tissues including dental pulp could be the main causative effect of DM on pulp reparative response.

Apoptosis is one of the most studied mechanisms of cell death. It is a genetically regulated process of cell elimination, and plays important roles in both morphogenesis and pathogenesis of multi-cellular organisms[10]. It is stimulated by various factors including physical and mechanical stresses [11]. Stress stimuli propagate apoptosis cascade through the release of cytochrome C from mitochondria followed by the activation of apoptosome which in turn activate caspase-9. Caspase-9 is one of the initiator caspase that has the ability to activate other procaspases such as pro-caspase-3. Once activated, caspase-3 inactivates several cellular targets and induces DNA cleavage[12].

All multicellular organisms harbour a specialized group of cells called stem cells. Stem cells are biologically undifferentiated and possess various degrees of potency and plasticity. They have unique abilities of proliferation, self-renewal, and differentiation to other cell types in addition to tissue regeneration. [13].

Stem cell classifications vary according to their source or potency. Based on their source, they are either embryonic stem cells (pluripotent) derived from blastocyst or adult stem cells with variable origins (endodermal, mesodermal, ectodermal or cancer).

While according to their potency, there are five types. First, the totipotent stem cell (zygote, spore, morula) can give rise to an entire functional organism. Second, the pluripotent stem cell (embryonic stem cell) can give rise to all tissue types but not an entire organism. Third, the induced pluripotent stem cell is artificially derived from a non-pluripotent cell (adult somatic cell) by inducing a "forced" expression of specific genes. Fourth, the multipotent stem cell (progenitor cell) can give rise to a limited range of cells within a tissue type (hematopoietic stem cell and mesenchymal stem cell). And finally the unipotent stem cell is a precursor cell[14].

Stem cells divide and differentiate into several types of specialized cells including odontoblasts, neural progenitor cells, osteoblasts, chondroblasts and adipocytes[15]. They could be used in drug testing and discovery of more drugs. In addition, stem cells drugs are developed to treat multiple diseases as the use of umbilical cord stem cells in the treatment of some neural disorders[16].

Bone marrow–derived stem cells are called hematopoietic stem cells (HSCs) or hemocytoblasts. They are the gold standard in stem cell applications. Through haematopoiesis, they can differentiate into all other blood cells either myeloid or lymphoid. They are mesodermal in origin and located in the red bone marrow. The definition of hematopoietic stem cells has evolved since HSCs were first discovered in 1961 [17, 18]. Promising results have been documented for using HSC transplants in the treatment of cancers and other immune system disorders[19].

It has been a growing interest recently in use of non-medication methods in management of medical diseases as diabetes. Therefore, the current study aimed to evaluate the possible curative effect of stem cell therapy in improving the histological changes in the dental pulp of diabetic rats experimentally induced by Streptozotocin.

MATERIAL AND METHODS

Thirty, 3 month old, adult male albino rats with body weight ranges from 180-200 gram were used in this investigation. They were housed in rat cages (five rats per cage), and labeled with numerical numbers and kept in well ventilated animal house of the faculty of dentistry Suez Canal University, at regular temperature 27-30°C, 12 hours natural light and 12 hours darkness. The animals were fed with dry rat pellet and allowed drinking water adlibitum. Animals were divided randomly into three groups as follows: **Group I**: 10 animals served as controls. **Group II**: 10 animals were subjected to a single intraperitoneal injection of streptozotocin (STZ) in a dose of 60 mg/kg body weight for induction of diabetes mellitus[20]. Verification of induction of diabetes mellitus was carried out by detecting hyperglycaemia (200mg/dl or more) in a sample of blood collected in heparinized capillary tubes from the retro-orbital plexus of veins of 12 hours fasting animals. **Group III**: 10 animals were treated the same as those of group II. One week after the verification of diabetes induction, the animals were subjected to single intravenous infusion of mesenchymal bone marrow stem cells. The cell number was calculated by haemocytometer for intravenous administration $1.5x10^3$-10^6 cells per rat in 0.2 ml phosphate buffer saline and slowly injected into the tail vein of anaesthetized rat over two minutes period using a 22 gauge needle [21] to study the effect of the injected mesenchymal bone marrow stem cells on the dental pulp cells of diabetic rats.

Preparation of mesenchymal bone marrow derived stem cells

Additional 10 male albino rats, 6 weeks old, were used for isolation and culture of bone marrow stem cells at biochemistry department faculty of Medicine, Cairo University.

Dulbecco's modified Eagle's medium (DMEM, Gibco/ BRL) supplemented with 10% fetal bovine

serum (Gibco/BRL) was used to flush the tibiae and femurs of the euthanized rats for harvesting the bone marrow cells [22]. Through density gradient [Fico 11/paque (Pharmacial)] the nucleated cells were isolated and resuspended in complete culture medium supplemented with 1% penicillin-Streptomycin (Gibco/BRL). Cells were then incubated in normal culture condition; at 37°C in a humidified atmosphere of 5 % CO2 and 95 % air. Once cultures reached 80-90% confluence (usually within 12-14 days), the cells were harvested by trypsinization using 0.25% trypsin in 1mm EDTA for 5 minutes at 37°C after washing with phosphate buffer saline twice [23]. After centrifugation, cells were resuspended with phosphate buffer saline. The mesenchymal stem cells (MSCs) in culture were characterized by their adhesiveness and fusiform shape and by detection of CD29, one of the surface markers of MSCs, by qRT-PCR [24, 25].

At the end of the experiment which lasted for two months, the animals of the different groups were euthanized. Their jaws were dissected out, separated into right one and left one, fixed in 10% neutral buffered formalin, processed to be embedded in paraffin. Six microns thick sections were cut and stained with H&E for histological examination, Masson's trichrome stain for collagen evaluation and immunohistochemical detection of Caspase-9 in the dental pulp of the experimental rats.

Caspase-9 immunohistochemical staining:

Tissue sections were deparaffinized in xylene, rehydrated in graded alcohols. Antigen retrieval was done in microwave for 3 min at 80°C followed by 10 min at 20°C. Caspase-9 staining was carried out using Rabbit monoclonal antibody to caspase-9 (GTX112888, GeneTex, Irvine, CA) diluted 1:100. Staining detection was carried out using Power-Stain™ 1.0 Poly HRP DAB Kit for Mouse + Rabbit (REF 54-0017 Genemed Biotechnologies, CA, USA) according to the manufacturer's instructions. Stained tissue sections were photographed using the Olympus BX50 microscope (Olympus America, Centre Valley, PA) with an attached Olympus E330-ADU1X Camera (Japan).

RESULTS

I-Histological Results:

Hematoxylin & Eosin and Masson's trichrome stains:

1-Group I: Control group:-

The pulp tissue of control specimens was formed of loose vascular connective tissue bound with a layer of odontoblasts arranged at the periphery of the pulp core and had processes extending to dentin. Collagen fibers were embedded in an amorphous ground substance. The pulp contains vascular and neural elements (Figure 1, A and B). The collagen fibers of the pulp revealed normal distribution with strongly positive staining reactivity to Masson's trichrome stain (Figure 2, group I).

2-Group II: Streptozotocin induced diabetic rats:

The pulp showed granulation tissue formation, vasodilatation and marked inflammatory cell infiltration. Separation of the odontoblastic layer from the dentin was frequently seen with massive odontoblastic degeneration, loss of organization of the remaining degenerated cells with marked narrowing of pulp space (Figure1, C, D, E, and F). The collagen fibers of the pulp of diabetic rats revealed degeneration, with weak positive reactivity to the Masson's trichrome stain (Fig.2, group II).

3-Group III: Streptozotocin induced diabetic rats treated with bone marrow –derived stem cells:

The pulp showed almost complete regeneration of its cellular, vascular and neural elements. There was apparent reorientation of odontoblasts and newly formed pulp core structure(Figure 1, G,H). The collagen fibers revealed moderately to strongly positive reaction to Masson's trichrome stain and became more organized and integrated(Figure2, group III).

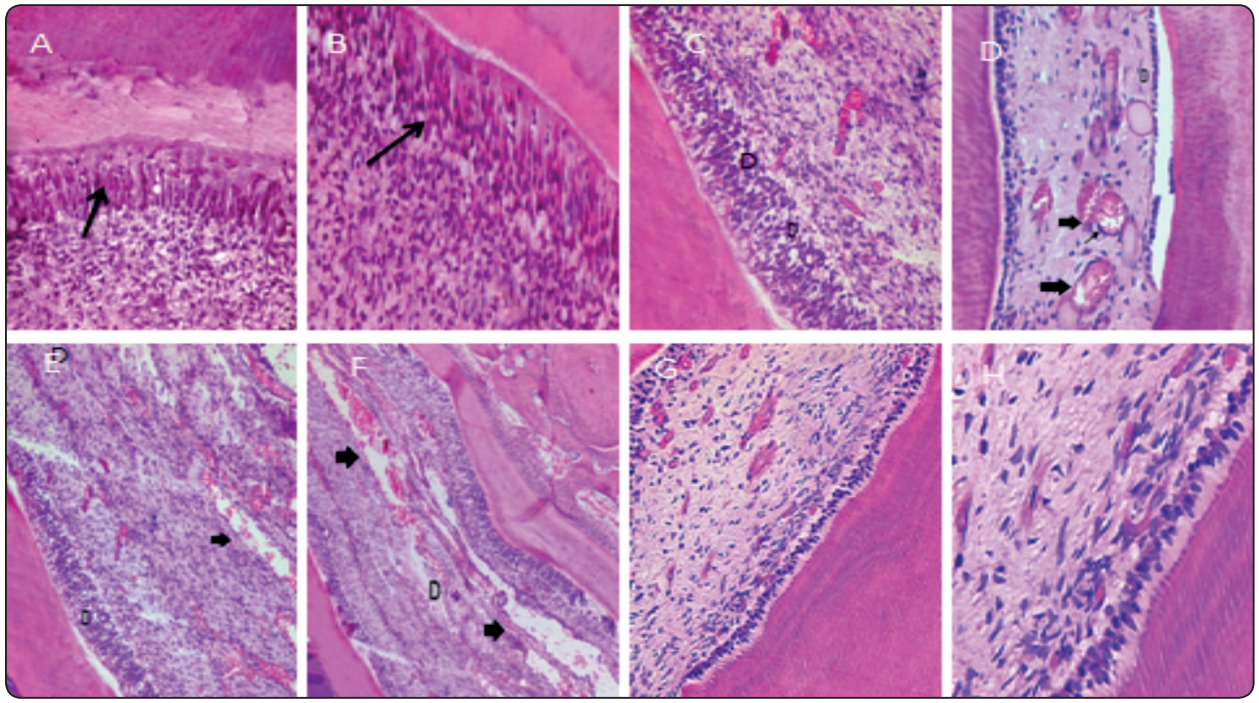

Fig. (1): A photomicrograph showing the pulp tissue of different groups. A, B showing the pulp of control group with normal histological structure (H&E. orig. mag. 200,400). C, D, E, F showing the pulp of group II animals with massive odontoblastic degeneration (D) and separation of the odontoblastic layer from dentine. Pulp core showing degeneration, widening of the blood vessels with stagnation of blood cells (arrow) and massive inflammatory cell infiltration. (H&E. orig. mag. 100, 200). G, H showing the pulp of diabetic rats treated with bone marrow–derived stem cells showing almost complete regeneration of its cellular and vascular elements as well as apparent reorientation of odontoblasts and newly formed pulp core structure. (H&E. orig. mag.100, 200).

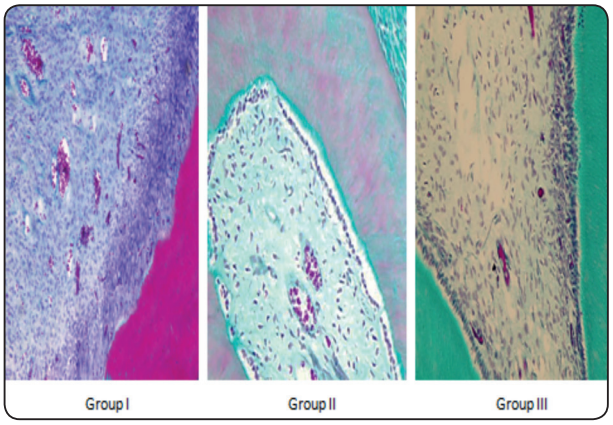

Fig. (2): A photomicrograph of the pulp tissue of different groups stained with the Masson's trichrome stain. Group I showing strongly positive reactivity of the collagen fibers with Masson's trichrome stain. Group II animals of diabetic group showing weak to moderate positive reactivity to the Masson's trichrome stain. Group III animals of diabetic rats treated with stem cell showing moderate to strong positive reactivity to the Masson's trichrome stain (Masson's trichrome, Origin Mag. 100)

II-Immunohistochemical results:

Caspase-9 localization:

1-Group I: Control group:-

The dental pulp of control group rats incubated with caspase-9 polyclonal antibody showed negative to weakly positive staining reaction of the dental pulp cells, fibers & blood vessels to caspase9 (Figure 3, group I).

2-Group II: Streptozotocin induced diabetic rats:

The dental pulp of group II rats incubated with caspase-9 polyclonal antibody showed moderately to strongly positive staining reaction of the dental pulp cells, fibers & blood vessels to caspase-9 (Figure 3, group II).

3-Group III: Streptozotocin induced diabetic rats treated with bone marrow –derived stem cells:

The dental pulp of group III rats incubated with caspase-9 polyclonal antibody showed negative to weakly positive staining reaction of the dental pulp cells, fibers & blood vessels to caspase-9 (Figure 3, group III).

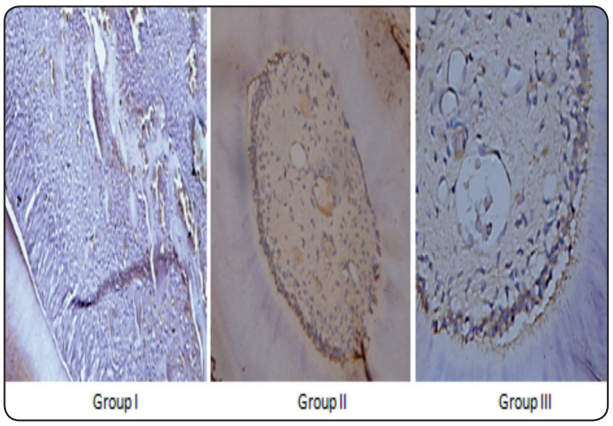

Fig. (3): A photomicrograph of the pulp tissue of different groups incubated with caspase-9 polyclonal antibody. Group I, pulp of control group rats showing negative to weakly positive staining reaction to caspase-9. Group II, the pulp tissue of diabetic rats showing moderately to strongly positive staining reaction of pulp cells, fibers & blood vessels to caspase-9. Group III showing negative to weakly positive staining reaction of pulp cells, fibers & blood vessels to caspase-9 (Caspase-9, Origin. Mag.100).

DISCUSSION

Animal models have been used for studying many diseases in order to discover the structural changes in different tissues. In this study streptozotocin was used to induce diabetes in albino rats. Streptozotocin (STZ) is a permanent diabetogenic compound, produced by the gram positive soil bacterium *Streptomyces achromogenes* that exhibits broad spectrum of antibacterial properties. STZ functions through destruction of pancreatic β-cells of the laboratory animals. The toxic effector mechanism of STZ starts with its decomposed products and the free radicals generated, which destroy the pancreatic βcells by alkylating DNA, impairing mitochondrial system and inhibiting OGlcNAcase[26]. The STZ-induced diabetes in albino rats was successfully used by many researchers [27-29].

The changes detected in the pulp histologically in response to diabetes induction included degeneration of the odontoblastic layer that was detected as well by Cintra *et al* in 2017[30].In addition to the deteriorating effect of diabetes on the odontoblasts, it resulted in vasodilatation and increased inflammatory cell infiltrate. Our results are in agreement with Bender and Bender who in their histological studies showed increased thickness of blood vessel basement membrane, reduction in collagen level of intercellular substance and signs of chronic inflammation and angiopathy [31].

These findings were also reported in the periodontal ligament of STZ-treated rats[32]. Moreover, collagen degeneration was detected in the pulp in the form of weak positivity to Masson's trichrome. This finding is in agreement with Catanzaro's group who reported decreased collagen concentration in the pulp extracted from STZ-treated rats[27].

Regarding caspase-9 which is one of the initiator caspases in cell apoptosis [33], its expression was weak in the normal control pulp while it was strongly expressed in the diabetic pulp. This finding is in agreement with previous researchers who detected increased expression of caspase-9 in response to diabetes in various tissues [34-36]. The activation of caspase-9 in apoptotic pulp tissues was reported as well by other researchers in response to different stimuli [37, 38].

Using stem cells for treatment of several illnesses is an interesting search point for regenerative medicine. Many clinical trials have been carried out in order to investigate their efficacy in treatment of diseases affecting different organs. Great results have been achieved but the underlying

mechanisms are not clearly understood [39, 40].With respect to diabetes mellitus, stem cell therapy is an interesting research field. Many studies have been conducting and reported amelioration of hyperglycemia[41] and its complications in various organs and tissues[42]. Injection of stem cells in diabetic rats resulted in improvement of pancreatic and kidney functions [43], cardiac dysfunction [44] and the neurologic manifestations [29, 45]. Different stem cell injection routes were compared in the literature. Single intravenous dose of stem cell was effective in treatment of diabetic rates while intraperitoneal injection of the same dose was not effective [46]. In addition, injecting diabetic rates with single intravenous dose resulted in greater improvement in the neuropathic symptoms compared to intramuscular single injection [45].

In this study, treatment of diabetic rats with single intravenous injection of bone marrow stem cells resulted in amelioration of the degenerative effect of diabetes on the pulpal tissues. Reorientation of the odontoblastic layer and the regeneration of pulp core structure were clear by hematoxylin & eosin stain. Collagen fibers were also more organized as detected by Masson's trichrome stain. Similar improvement was reported in the periodontal tissues of the diabetic rats when treated with stem cells [32]. The structural improvement in the pulp in response to stem cell treatment was confirmed by the modulation of apoptosis as detected by decreased expression of caspase-9.

In summary, it could be postulated that the induced hyperglycemia in STZ-rats resulted in degeneration in different tissues through apoptosis caused by increased protein glycation [34-36, 47].A process affected the bone marrow stem cells as well[48]. Injecting the diabetic rats with stem cells resulted in improvement of the symptoms that could be achieved through stem cell differentiation[49], paracrine action[50-52] or compensation of the apoptotic stem cells.

CONCLUSION

Induction of diabetes in albino rats has degenerative effect on pulpal tissues accompanied by increased expression of caspase-9 (apoptotic marker). Intravenous injection of BMSCs resulted in regeneration of pulp tissues and decreased apoptosis as detected by low caspase-9 expression.

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