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HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION FOR THE ROLE OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS TO COUNTERACT THE STREPTOZOTOCIN-INDUCED DIABETIC CHANGES IN THE RAT PAROTID GLAND

Ashraf Mohamad Emran^{1*}, Said Mahmud Hani², Sayed Bakry Ahmed³

ABSTRACT

Objective: the current study was designed to assess the therapeutic effect of bone marrow-derived mesenchymal stem cells (BMSCs) on parotid glands after streptozotocin induced diabetes in rats. **Subjects and Methods:** 36 Sprague-Dawley rats were divided into 3 groups, 12 rats each. Group I: the animals received 0.1 M sodium citrate buffer intraperitoneally, the vehicle of streptozotocin. Group II: diabetes mellitus was induced by single intraperitoneal injection of 60 mg/kg of streptozotocin (STZ) dissolved in 0.1 M sodium citrate buffer. Group III: one day after diabetes induction, single dose of 2×10^6 BMSCs suspended in 0.5 ml of phosphate buffer solution (PBS) was injected via intraglandular route. Animals were sacrificed at 7 or 14 days after BMSCs injection, then histological and immunohistochemical studies were done. **Result:** the experimental stem cell treated group showed better histological features and increased PCNA proliferation more than the untreated group. **Conclusion:** bone marrow derived stem cell is considered as relatively successful method for the treatment of diabetic induced salivary gland dysfunction.

KEY WORDS: Parotid gland, stem cells, diabetes.

INTRODUCTION

Diabetes mellitus (DM) is widespread disease associated with generalized common chronic metabolic disorders characterized by hyperglycemia resulting from defects in the insulin secretion or action ⁽¹⁾ and certain abnormalities in carbohydrate, fat electrolyte and protein metabolism ⁽²⁾.

Alterations in salivary gland composition and hypofunction have been associated with elevated fasting blood glucose concentrations (hyperglycemia) and neuropathy ⁽³⁾. Diabetic state influences glycogen metabolism in submandibular and parotid salivary glands of rats ⁽⁴⁾.

Diabetes and insulin change both the structure and function of rat parotid glands. The parotid glands of diabetic animals are morphologically characterized by extensive intracellular lipid accumulation in the acinar cells with increased lysosomes, duplication and thickening of the basement membranes and crystalloids inclusions⁽⁵⁾.

• Corresponding author: Ashrafemran88@gmail.com

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^{1.} Assistant Lecturer of oral histology, Oral Biology Department, Faculty of Dental Medicine, Al-Azhar University.

Professor of Oral Histology and Pathology, Oral Biology Department, Faculty of Dental Medicine, Al-zhar University.

^{3.} Professor, Department of Zoology, Dean of Faculty of Science, Al-Azhar University.

The STZ is a fungal antibiotic, and its ability to induce both insulin- and non-insulin- dependent DM via β -cell destruction, has been reported previously. It has been reported that a single dose of intravenous or intraperitoneal injection of 40-60 mg/kg of STZ is sufficient to induce insulin dependent DM in adult rats⁽⁶⁾.

A previous study has revealed that the BMSCs can increase the regenerative capacity of the resident salivary gland stem cells that have remained in damaged salivary glands upon degenerative state⁽⁷⁾. So that it seemed that BMSCs could exert direct proliferative effect on salivary gland cells as well as indirect effect via stimulating the survived stem cells resident in the gland. Stem cell therapy for repair and functional restoration of salivary glands could provide long-term and effective solution to damaged tissues induced by degenerative processes due to either the age-related changes or disease⁽⁸⁾. BMSCs showed the ability to differentiate into epithelial cells in vitro and they also showed regenerative power to restore both the lost morphology as well as the function by secreting bioactive factors. These factors are believed to create a repair environment through their antiapoptotic effects, immunoregulatory function and stimulation of endothelial progenitor cell proliferation that helps in vascularization of the damaged tissue⁽⁹⁾.

SUBJECTS AND METHODS

Animals and Experimental Design

The protocol of this study was approved by the bioethics committee of animals from the faculty of dentistry (Al-Azhar university) ⁽¹⁷¹⁻⁶¹⁾. The animal handling and the procedures application were carried out at the house animals of Cairo University. The animals were caged individually with free access to water and food.

The present study was carried out on adult male Sprague Dawley rats with average age around 2 months old and their weight measured 200 to 250gm. There are a strong affections of laboratory animal gender on the ability of STZ to induce DM, where the male laboratory animals, including rats, were more prone to the effect of STZ to induce DM than males⁽¹⁰⁾. The present study has utilized male animals in order avoid the aforementioned obstacle as well as the effects of female sex hormones changes.

Animal Grouping

After 36 hours fasting overnight, they received STZ injection, 36 rats were divided into 3 groups, 12 rats each in the following scheme:

Group I (GI): control group; was considered as the negative control; in which the animals received 0.1 M sodium citrate buffer (SCB) intraperitoneally that is the vehicle of streptozotocin.

Group II (GII): diabetic group, was considered as the positive control in which the diabetesmellitus was induced by a single intraperitoneal injection of 60mg/kg of streptozotocin (STZ) (Sigma Chemical Co., St. Louis, MO) dissolved in 0.1M (SCB)⁽¹¹⁾. The blood samples were examined daily until diabetes was established. The diabetic condition was determined, when after 12 hours fasting, the rats have reached a serum postprandial glucose level of >250 mg/dl ⁽¹²⁾, So that the rats were considered as diabetic and were included into both the diabetic and the subsequent therapeutic groups.

Group III (GIII): stem cells group, one day after diabetic induction as described in group IIBMDSCs with a dose of 2×10^6 cells ⁽¹³⁾ suspended in 0.5ml of phosphate buffer solution (PBS) was injected via intraglandular route in a single injection into the right parotid gland region.

Each group was divided into2 sub-groups A and B according to the time of scarification, A at 7-day, and B at 14 -day after diabetes induction always in the morning (9-11AM) to minimize the effect of circadian rhythms

After 1 and 2 weeks from BMSCs injection, the rats were anesthetized, sacrificed and parotid glands were dissected out, fixed in 10% neutral buffered formalin solution for 72 hours and then microtechnically processed. Sagittal tissue sections were treated with the following techniques: antigen (PCNA).

Histological investigations

The tissue sections were stained with hematoxylin and eosin (H & E) (Mayer's hematoxylin) ⁽¹⁴⁾. for surveiry the histological structure of normal and experimental structure of parotid gland in different experimental groups of animals.

Immunohistochemical investigations for the proliferating cell nuclear antigen (PCNA):

Four to five microns thick sections were cut on tissue adhesive coated slides and tissue sections were stained by commercially available PCNA primary antibody. According to Akyol⁽¹⁵⁾, the staining method proceeded as follows: tissue sections were deparaffinized in two changes of xylenefor 10 minutes each, rehydrated through graded ethanol, then washed in distilled water for 2 minutes, followed by washing in phosphate buffer saline (PBS) for 5 minutes. To block the endogenous peroxidase activity, slides were incubated in a solution of 3% hydrogen peroxide in methanol for 20 minutes, and then washed in PBS for 5 minutes. For antigen retrieval, the slides were heated in a microwave oven at 100°C for three successive trades, 5 minutes each, and then placed in PBS for 5 minutes. Slides were incubated with the primary antibody over night at 4°C. The standard Streptavidin biotinylated peroxidase complex method was performed. The antigen was localized by the addition of diaminobenzdine (DAB) substrate chromogen solution for 5-10 minutes, followed by Mayer's hematoxylin counterstaining.Finally, slides were dehydrated, cleared and finally mounted with purified Canada balsam (DPX-Sigma).

Preparation of bone marrow-derived MSC (Ficoll-Paque technique)⁽¹⁶⁾:

Bone marrow was harvested by flushing the tibiae and femurs of six-week-old male Sprague-Dawley rats with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The nucleated cells were isolated with a density gradient (Ficoll-Paque, Pharmacia)TM and resuspended in complete culture medium supplemented with 1% penicillin- streptomycin. The cells then were incubated at 37°C in 5% humidified CO2 for 12–14 days as the primary culture for formation of the large colonies.

With development of large colonies (80–90% confluence), the cultures were washed twice with phosphate-buffered saline (PBS) and the sticked cells to the base of the bottle were trypsinized with 0.25% trypsin in 1 mM EDTA for 5 min at 37°C. After centrifugation, the cells were resuspended with serum-supplemented medium and incubated in 50-cm² culture flask (Falcon). The resulting cultures were referred to as first-passage cultures ⁽¹⁶⁾ and the MSCs in culture were characterized by their adhesiveness and fusiform shape ⁰.

Image Analysis:

The immunohistochemically reacted tissue sections were digitized using a Zeiss Mirax automated slide scanner with an objective of ×20 magnification. *The proliferating cell nuclear antigen* (PCNA) was evaluated using a score corresponding to the sum of the percent of both the frequency of positively reactive cells and the intensity of reaction. The digital image analysis was performed by importing the Mirax files into the image analyser software Visiopharm Integrater System (VIS).

Statistical analysis:

The data were tabulated, coded then analyzed using the computer program. The SPSS (Statistical package for social science) version 17.0 was used to compare the immune expression of PCNA in salivary gland tissues with the clinical parameters. The measured values were expressed as mean values \pm SD (standard deviation). The statistical importance of the difference in these values between the different groups was estimated using ANOVA followed by post-hoc tukey. The *p* value less than 0.05 was considered as significant.

RESULTS

Histological features of parotid salivary glands in control versus experimental groups of rats.

Control group (GI):

The histological features of parotid gland collected after 1,2 weeks was composed of normal regular parenchyma and connective tissue stroma. The interstitial connective tissue septae, separated the parenchymal tissue into lobes and lobules. The lobes and lobules were mainly composed of tightly packed serous acini, dispersed with some intercalated, striated and secretory ducts. The acinar cells appeared pyramidal in shape with a basally located oval or round basophilic nuclei in deeply eosinophilic cytoplasm. (*Fig. 1*).

Diabetic group (G II):

A. Seven days post diabetes (G II-A):

The general parenchymal architecture of the normal gland was massively destructed at the outmost change, where the vast majority of acini showed severe and massive degeneration with their replacement with collagenous tissue while some areas revealed hyaline degeneration (*Figs.2*). The sparsely remaining acini were widely distributed and appeared atrophied while some of the acini revealed degeneration of the central part and so acquired duct-like appearance. The collagen fibers appeared fused together to form glassy dense hyalinized eosinophilic material and such hyalinization was very common in the fibrous tissue which has been laid down as a reactive replacement for the lost

parenchymal tissue. Theducts appeared degenerated with discontinuity of their cellular lining, whereas some ductal cells showed paranuclear vacuolations. The ductal elements were of significant higher proportion relative to the remaining secretory end pieces (*Figs.2*).

B. Fourteen days post-diabetes (G II-B):

The parotid gland appeared degenerative but less severely than those recorded after seven days post-diabetes. The general acinar architecture was mainly reactively preserved, although some diabetic degenerative changes were also obvious. In general, the proportion of the acini to ducts were closer to normal in contrast to the seven days changes after diabetic induction (*Figs.3*). The most outstanding injurious changes were the paranuclear vacuolations.

Diabetes with stem cells (G III):

A. Seven days post-diabetes (G III-A):

The parotid acini appeared with similar size relative to these in the control group but with occasional mild acinar atrophy and relative absence of the acinar paranuclear vacuolations. The acinar cell nuclei were pleomorphic but some nuclei appeared open faced. The ductal elements revealed intact architecture, while some striated duct cells showed paranuclear vacuolations. Many blood vessels and capillaries appeared in the periductal area whereas some were scattered between the acini (*Fig.4*).

B. Fourteen days post-diabetes (G III-B):

The gland architecture was relatively intact while the parenchymal elements showed more or less changes than those of seven days of the same group as the acinar cell hyperplasia. The acini were mildly atrophic with some acinar cell vacuolations. The duct appeared relatively intact but with some vacuolations in some of the duct cells. (*Fig.5*).



- FIG (1) Parotid gland of control group (G1) showing normal serous acini (arrow), intercalated (arrow head) and striated ducts surrounded by stormed connective tissue (star) (H&E stain orig mag. x400).
- FIG (2) Parotid gland at seven days after diabetes induction (G II-A) showing extensive degeneration of the acinar tissue and its replacement with condensed collagen bundles with occasional focal area of hyaline degeneration (*arrowhead*), while the remaining acini appear severely atrophied (*arrows*) and some other acini acquired duct like appearance (*stars*). (H & E stain, orig Mag. X 400).
- FIG (3) Parotid gland at fourteen days after diabetes induction (G II-B) showing mild acinar atrophy with many paranuclear vacuolations, while the acinar nuclei appear hyperchromatic and pleomorphic. Some areas show circumductal loss of acinar architecture (*arrow*), most other acini appear condensed (*double arrow*). The ducts appear atrophied with some periductal acini appear severely degenerated by vacuolations. (H & E stain, orig Mag. X 400).
- FIG (4) Parotid gland at seven days after diabetes induction and SC injection (G III-A) showing nearly poorly preserved acinar architecture with frequent paranuclear vacuolations(arrow). The acini show cellular hyperplasia, while their nuclei appear pleomorphic with some are hyperchromatic (arrow head). ducts are serounded by rich dilated vessels. (H & E stain, orig Mag. X 400).
- FIG (5) Parotid gland at fourteen days after diabetes induction and SC injection (G III-B) showing of normal acinar architecture (arrow) withoccasional mild acinar atrophy and periductal dilated blood vessels (double arrows) (H & E stain, origMag. X 40

Diabetes with LLLT (G IV).

A. Seven days post-diabetes (G IV-A):

The acini were hypertrophic with scanty or even absence of interacinar spaces. The vast majority of acini acquired ovoid or tubular configuration, while the acinar cell nuclei appeared pyknotic and located closely to the basal cell membrane. The ducts revealed prominent shrinkage that was represented by the development of periductal spaces separating the ducts from their surrounding acini. The ductal lumen appeared collapsed with perinuclear vacuolations in few duct cells (Figs.6).

B. Fourteen days post-diabetes (G IV-B):

The gland revealed few and scattered interacinar spaces denoting mild to moderate acinar atrophy. While some areas were devoid of vacuoles, other areas showed some perinuclear vacuolations in the acini. The acinar cell nuclei appeared pleomorphic with mixture of hyperchromatic and pale staining ones. The ducts appeared with relatively intact architecture, but scanty ones had cellular vacuolations.(*Fig.7*).



FIG (6) Parotid gland at seven days after diabetes induction (G II-A) showing negative immunoreaction (arrow). (PCNA, orig Mag. X 400).

- FIG (7) Parotid gland at fourteen days after diabetes induction (G II -B) showing moderate immunoreaction in many scattered acini throughout the gland and mild immunoreaction in the ducts(arrow). (PCNA, orig Mag. X 400)
- FIG (8) Parotid gland at seven days after diabetes induction and SC injection (G III-A) showing moderate immunoreaction (arrow). (PCNA, orig Mag. X 400).
- FIG (9) Parotid gland at fourteen days after diabetes induction and SC injection (G III-B) showing sever intense immunoreaction in acini and duct (arrow). (PCNA, orig Mag. X 400).

Immunohistochemical features of parotid salivary glands in control versus experimental groups of the rats:

The parotid gland demonstrated mild to moderate immunoreaction to PCNA in laser group (Fig.8,9) compared with stem cell group which demonstrate intense immunoreaction to PCNA inacini and ducts (table 1) and (diagram 1).

Statistical investigations

Statistical analysis for the immunohistochemical reaction of parotid salivary glands in control and experimental groups of rats.

TABLE (1) Comparison of the percent area of PCNA expression in parotid glands between different groups within 7 & 14 days.

	Normal group	Diabetic group	Stem cells group	Р
7 days Post-hoc	0±0	0±0	0.80±0.11	<0.001*
		P1=1.00	P1=<0.001* P2=<0.001*	
14 days Post-hoc	0±0	1.60±0.23	27.50±3.93	<0.001*
		P1=0.39	P1=<0.001* P2=<0.001*	

Data expressed as mean±SD SD: standard deviation P: Probability *: significance ≤0.05 Test used: One way ANOVA followed by post-hoc tukey P1: significance vs Normal group P2: significance vs Diabetic group



Diagram (1): A histogram representing the mean of percent area of PCNA expression in parotid glands in the different groups after 7 and 14 days.

DISCUSSION

The parotid glands demonstrated significant diabetic changes after STZ administration thatwere represented by the degenerative manifestation in both acini and ducts, However, the severity of these diabetic change was variable in the different intervals of the present study. The acinar cell changes of parotid salivary glands showed large numbers of paranuclear vacuoles with shrunken nuclei, loss of acinar structure, severe acinar degeneration with their replacement by collagenous fibrous tissue, while some areas revealed hyaline degeneration; changes that come in accordance with Anderson et al ⁽¹⁷⁾.

The basis of diabetogenic degenerative action of STZ is presumed to be mainly based on DNA destruction, and this ultimate fate is reached through one of the three different mechanisms. The first and the most effective mechanism is DNA alkylation, secondly the release of nitric oxide and finally, the generation of the reactive oxygen species (ROS). The DNA alkylation is regarded as the most effective mechanism in the chemical model of laboratory DM ⁽¹⁸⁾. The DNA alkylation activity of STZ induced toxic effects in β ells of pancreas lead to their death via the transfer of methyl group from STZ to the β cell DNAmolecule. This consequently initiates a chain of events that result in the DNA fragmentation and destruction ⁽¹⁹⁾.

The cytoplasmic vacuoles detected in the present study in either the acinar or duct cells of parotid glands are comparable to those demonstrated in salivary glands following diabetic induction in the rats ⁽²⁰⁾. The cytoplasmic cell vacuolation has been reported to occur in the rat parotid gland whether it is resting or secreting, and it was indicated that the prolonged strong parasympathetic stimulation, especially after acinar degranulation, has increased the tendency foracinar cell vacuolations ⁽²⁰⁾. On the other hand, this cytoplasmic vacuolations have been presumed to reflect physiopathological state in the affairs from the artificial process of stimulating the nerve electrically⁽²¹⁾. Alternatively, the parotid acinar cell cytoplasmic vacuolation has been presumed to occur due to imbalance in the cell calcium, and the condition may be reversible in the early stages⁽²²⁾.

It was reported that the increased cell function is evidenced by the cell componentsproliferation or reorganization in a manner suggesting hyperfunction state in response to certain poisons. For instance, the smooth endoplasmic reticulum proliferates and form complex whorls or gyrations. Both are regarded as adaptive mechanism and interpreted as the cells attemptto increase their ability to detoxify the substance to which they are subjected and evidently cell metabolism becomes increased. This is simulated microscopically by the development of increased macropinocytotic activity resulting in the appearance of numerous cytoplasmic vacuoles ⁽²³⁾.

The early onset period after diabetic induction showed severe diabetic degenerative features of acini that had ultimately led to the disappearance of large area of parenchymal elements of thegland. The absent acini and ducts were replaced by dense fibrous tissue, while the remaining scattered acini were vacuolated and showed signs of degeneration these changes were also reported by Denewar& Amin,2020⁽²⁰⁾. The mechanism underlying collagen abnormalities in diabetic parotid gland is poorly understood. A relationship does exist between STZ-induced diabetes and both ROS and type I collagen organization in parotid gland. Glucose was found to play important role in the accumulation of collagen in the tissues ⁽²⁴⁾. Hyperglycemic state leads to advanced glycation and product formation in matrix components and accelerates the crosslinking between collagen fibers. The increased collagen deposition contributes to increased stiffness of tissues in diabetic subjects because of the changesin both the structure and organization of extracellular matrix (25).

The development of unstained vacuoles attached to the nuclear membrane, simulating negative Golgi image surrounded by deeply eosinophilic cytoplasm were noted by testosterone administration in posterior lingual salivary glands (26). Extensive cytoplasmic vacuolization were noted following oestrogen or progesterone administration in granular convoluted tubules in rat submandibular and parotid glands. The parotid gland acini with diabetic group were higher level of collagen deposition type I collagen occurred around ducts, acini, nerves and in arterial and venous walls and thick densely stained collagen bundles occurred around ducts, whereas thin and delicate fibres were seen around acini and nerves. Diabetes mellitus is metabolic disease affects many organs and in salivary glands an alteration in glycolytic enzyme activity (27) and in antioxidant parameters (28) These changes have been reported in association with diabetes and have also been related to increased collagen deposition described in the heart of diabetic rats⁽²⁴⁾.

Type I collagen content was increased in parotid gland of diabetic rats⁽²⁹⁾, this is in agreement with the findings for heart and kidney in streptozotocin diabetic rat⁽³⁰⁾ The acarbose treatment in diabetic rats reduced the amount of collagen into similar level as the non-diabetic group⁽²⁹⁾.

Positive correlation between the amount of type 1 collagen and the oxidative parameters which was also suggested by Baynes⁽³¹⁾. Acini in parotid gland of diabetic rats were widely spaced and exhibited higher levels of collagen deposition. Type 1 collagen was found in diabetic animals around ducts, acini with staining pattern and are thick and densely stained collagen bundles around ducts but were thin and delicate fibres around acini and nerves⁽²⁹⁾.

In the present study the local administration of bone marrow-derived mesenchymal stem cells (BMDSCs) to parotid gland in diabetic animals has relatively relieved the diabetic manifestations. Concerning one-week interval subgroup, the relief of diabetic features was significant when compared to the same period in the diabetic group G II that was represented by better organization of acinar architecture and less acinar and ductal perinuclear vacuolations. However, some diabetic features including vacuolations and atrophy as well as irregular acinar architecture were still apparent ⁽³²⁾.

The SCs group demonstrated significant expression of PCNA in many acinar and ductal cells, indicating prominent mitotic activity in the vast majority of both acinar and ductal cells. This prominent cellular proliferation was also mentioned in the previous investigation that recorded significant increase in the proliferating cells of BMDSCstreated mice in parotid gland with degenerative manifestations, induced via radiation exposure ⁽³³⁾.

In the present study, the local intraparotid BMSCs injection has led to PCNA proliferation index upregulation as compared with diabetic group. This observation was in accordance with the results recorded in previous study, which noted increased proliferative index as marked by increased PCNA expression and decreased apoptotic index as marked by TUNEL in the irradiated submandibular gland tissues treated with intraglandular BMSCs ⁽³⁴⁾.

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