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THE EFFECT OF BONE MARROW DERIVED STEM CELLS ON AGE CHANGES OF SUBMANDIBULAR SALIVARY GLANDS (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Background: Stem cells are one of the most recent and effective regenerative therapeutic techniques for various tissues damages.

Aim: The purpose of this study was to study the effect of bone marrow derived stem cells injection on age changes of submandibular salivary glands

Methods: Fifteen male albino rats (aging six months and weighing 100-150 grams each) served as group I, were administered saline. Thirty old male albino rats (aging fifteen months and weighing 200-250 grams each) They were fed the standered laboratory diet and divided equally into group II were get infused with saline intravenously and group III were subjected to intravenous infusion by mesenchymal stem cells. Rats were sacrificed after 4 weeks. The submandibular salivary glands were dissected out and prepared for histological and immunohistochemical examinations..

Results: Light microscopic examination of old aged group II, revealed generalized atrophy of the secretory portions with numerous intracellular vacuoles. Secretory cells revealed deeply stained pyknotic nuclei. The granular convoluted tubules showed generalized atrophy with complete loss of their eosinophilic granules. The excretory ducts appeared dilated with degenerated epithelial lining and retained secretion. Widening of the connective tissue septa with chronic inflammatory cells infiltration was also detected. While BM-MSCs treated group III, revealed well defined serous acini having distinct outline. Well formed striated ducts were also detected. Immunohistochemical examination of cytokeratin # 5, 6 & 18 and myosin expression among the studied groups revealed that their immunoreactivity was significantly reduced in the old age rats (Group II) than those of both groups I & III. Also, statistical analysis of bax expression revealed that their immunoreactivity was significantly increased in the old age rats (Group II) than those of both groups I & III.

Conclusion: Intravenously administration of BM-MSCs played a tangible role in ameliorating age related changes in submandibular salivary glands.

KEYWORDS: Aging; Stem cells; Submandibular salivary glands; immunohistochemistry; cytokeratin ; myosin; apoptosis.

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INTRODUCTION

Most biologists define aging as an age-dependent or age-progressive decline in intrinsic physiological function, leading to an increase in age-specific mortality rate **(Fabian and Flatt, 2011).**

Salivary disorders in the aging population usually are caused by systemic disease and their treatment (for example, anticholinergic medications). Numerous medical conditions (such as Sjögren Syndrome, diabetes, Alzheimer's disease and dehydration), medications, head and neck irradiation and chemotherapy can cause or contribute to salivary gland diseases. The increase in xerostomia complaints among elderly people may be due to synergistic effects that occur when aging adults take multiple medications **(Shiboski *et al.,* 2007)**.

Age is accompanied by generalized loss of salivary gland parenchymal tissue. A gradual reduction of up to 30% to 60% of the proportional acinar volume of the major salivary glands has been observed. The lost salivary cells often are replaced by adipose tissue. An increase in fibrous connective tissues also occurs. Changes of the duct system, including an increase in nonstriated intralobular ducts, dilatation of extarlobular ducts, and degenerative and metaplastic changes have been observed **(Nanci *et al.,* 2008)**.

Recent research has extensively shown the potential of bone marrow- (BM-) derived mesenchymal stem cells (MSCs) for regenerative therapies in various organs including the heart **(Li and Ikehara, 2013).** The effects from this approach are dependent on their potency of secretion of beneficial cytokines and growth factors for tissue repair/regeneration and immunomodulation and/or their differentiation for regenerating damaged organs **(Patel *et al.,* 2013).**

Since the first clinical trial of BMC injection in 1995 (Lazarus *et al*., 1995), more than 2,000 patients have been administered with allogeneic or autologous MSCs for the treatment of various diseases, including graft-versus-host disease, hematologic malignancies, cardiovascular diseases, neurologic diseases, autoimmune diseases, organ transplantation, refractory wounds, and bone/cartilage defects **(Lalu *et al.,* 2012).**

MATERIAL AND METHODS

Sixty male albino rats were housed in specially designed wire cages and maintained under good ventilation. They were fed on standardized balanced laboratory diet and were supplied drinking water ad-libitum.

Fifteen albino rats six weeks old were used for isolation and culture of bone marrow stem cells at the Biochemistry and Microbiology Unit, Faculty of Medicine, Cairo University.

The other forty five rats were divided equally into three groups, fifteen rats for each group as follow:

Group I (Adult group):

Consisted of fifteen adults albino rats (aging six months and weighing 100-150 grams each) got infused with saline intravenously, were served as control group.

Group II (Old age group):

Consisted of fifteen albino rats (aging fifteen months and weighing 200-250 grams each) got infused with saline intravenously.

Group III (Stem cells treated group):

Consisted of fifteen albino rats (aging fifteen months and weighing 200-250 grams each) were subjected to intravenous infusion by mesenchymal stem cells. The cell numbers were calculated by the hemocytometer. For I.V. administration, 1.5×10^3–10^6 cells/rat in 0.2 ml phosphate buffer saline were slowly injected into one tail vein of the anesthetized rat over a 2-minute period using a 22-gauge needle **(Guo *et al*., 2011),**

After 4 weeks the rats were sacrificed by CO_2 inhalation. The submandibular salivary glands were dissected free, fixed in calcium formol, washed by

tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax and stained with:

- **Hematoxylin and eosin (Hx. & E) stain:** for histological evaluation.
- **Masson's trichrome stain:** was used to detect any possible changes in the stromal elements of the submandibular salivary glands.
- **Immunohistochemical demonstration of:**

A) Cytokeratin #5, 6 & 18 using monoclonal mouse anticytokeratin antibody.

B) Myosin using monoclonal mouse antimyosin antibody.

C) Apoptosis using monoclonal antibax antibody. Histological and immunohistochemical results were examined using:

a) Ordinary light microscope for routine histological examination and to assess the prevalence of cytokeratins 5, 6 & 18, myosin and bax immunoreactivity in the submandibular salivary glands (colour developed by DAB).

b) Image analyzer computer system to measure the optical density of the immunostain. We performed the images analysis using a computer (software Leica Quin 500) consisting of color video camera, color monitor and CPU Of IBM computer connected to the microscope. The image analyzer was first calibrated automatically to convert the measurement unit from pixcels to actual micrometer units. The intensity of the reaction within the cells was determined by measuring the optical density in 5 small measuring fields in each section using a power of magnification of 400 X. After grey calibration, the image was transformed into a grey delineated image to choose the areas showed positive reactivity with accumulation of all grades of reactivity (i.e. minimum, maximum and median grey). Areas of positive reactivity were masked by a blue binary color. Then the mean values were calculated for each case.

Statistical analysis

The analysis of variance (ANOVA) test was used to compare the mean values ±SD (standard deviation) of the immunostaining intensity of cytokeratin, myosin and bax between the different groups.

RESULTS

I- Light microscopic results:

A) Hx. & E. stains results: (Fig. 1)

Group *I* (Adult control group): Showed the architecture of a normal gland. It consisted of a majority of serous acini. The striated ducts were lined with columnar cells characterized by their basal striations, centrally placed rounded nuclei and deeply stained acidophilic cytoplasm. A lot of granular convoluted tubules were detected. They were large sized lined by columnar cells with abundant acidophilic cytoplasm full of large eosinophilic granules. Connective tissue septa that divided the gland into lobes and lobules were also detected.

Group *II* (Old age group): Showed Generalized atrophy of the serous acini cells with pycnotic nuclei. Generalized degeneration and amalgamation of the granular convoluted tubules cells with vacuolization and loss of their eosinophilic granules. Cytoplasmic vacuolization also has been detected in serous acini**.** The excretory duct has an interrupted epithelial lining with stagnant secretion. Dilatation of blood vessels with extravasated RBCs.

Chronic inflammatory cells infiltration was found in the gland lobules. There are also replacement of some serous acini by the mucous type. Widening of the interlobular and interlobar fibrous connective tissue septa with fibrosis and fatty degeneration.

Group III (Old age group treated with BM-MSCs):

The histological examination of the submandibular salivary gland did not show the prominent extensive features of aging manifested in the previous group. The acini showed normal

structure with homogenous cytoplasm and there were also decrease in the cytoplasmic vacuolization.

The duct system appeared with normal architecture. The lining cells were continuous, regular and no significant change in cells shape and size**.** The connective tissue stroma appeared similar to the control group I.

B) Masson's Trichrome stain results: (Fig. 2)

Group I (Adult control group): Showed the architecture of a normal reticular connective tissue stroma consisting of collagen fibers.

Group II (Old age group): Thick dense bands of collagen fibers were seen radiating and extending throughout the whole parenchymal tissue.

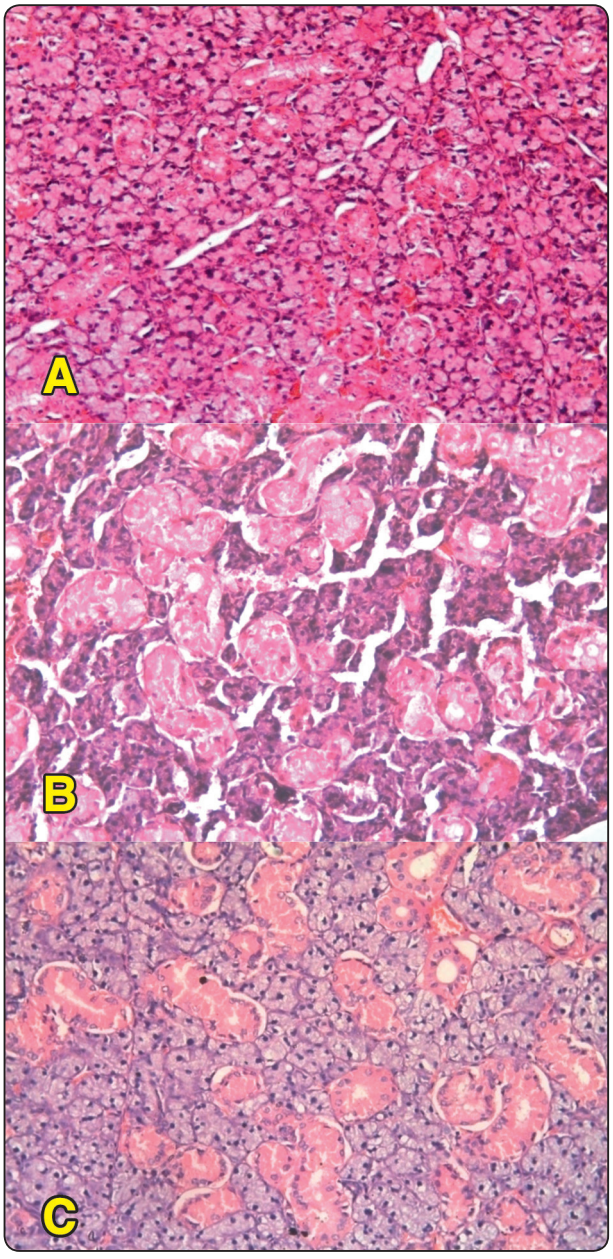


Fig. (1): A Photomicrograph of the submandibular gland of adult rats of group I (A), old rats of group II (B) and old rats treated with MSCs of group III (C). (Hx. & E. X200)

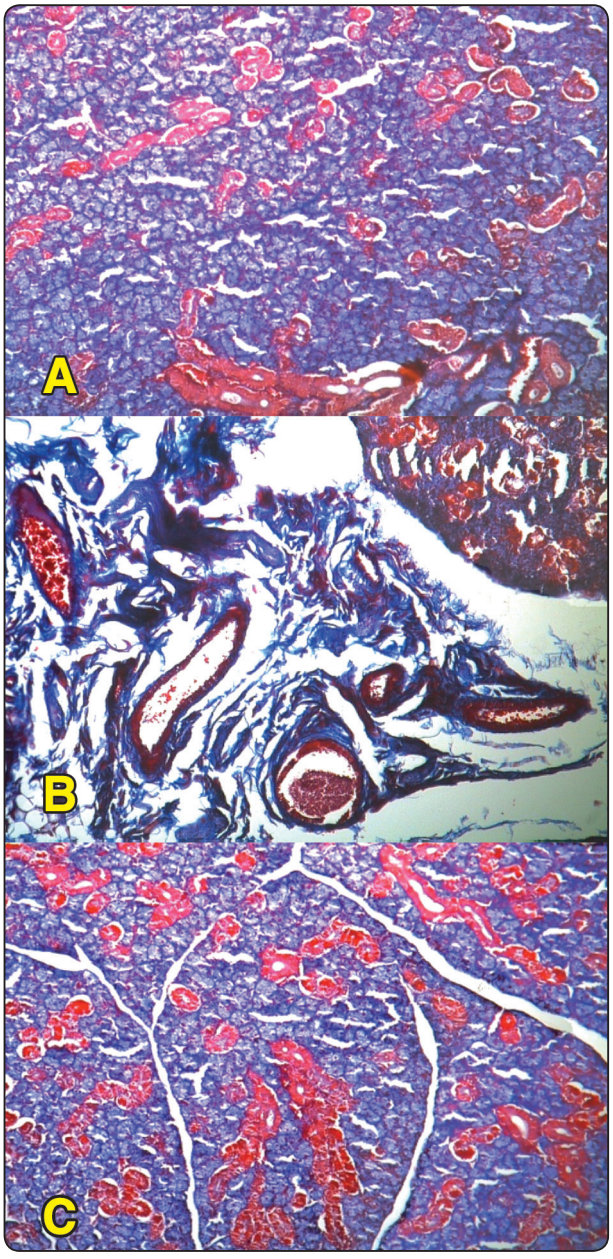


Fig. (2): Photomicrograph of the submandibular gland of adult rats of group I (A), old rats of group II (B) and old rats treated with MSCs of group III (C). (M.T. X100)

Group III (Old age group treated with BM-MSCs): Only thin and regular connective tissue septa which divide the gland into lobes and

II- Immunohistochemical results: (Fig. 3)

Immunohistochemical localization of cytokeratin # 5,6 & 18 :

Revealed moderate cytokeratin immunoreactivity in the acinar cells, striated and granular convoluted tubules as well as the myoepithelial cells in the adult group I, but in the old age group II, they showed negative immunoreactivity. In the old age rats treated with the bone marrow derived stem cells group III, they showed moderate immunoreactivity.

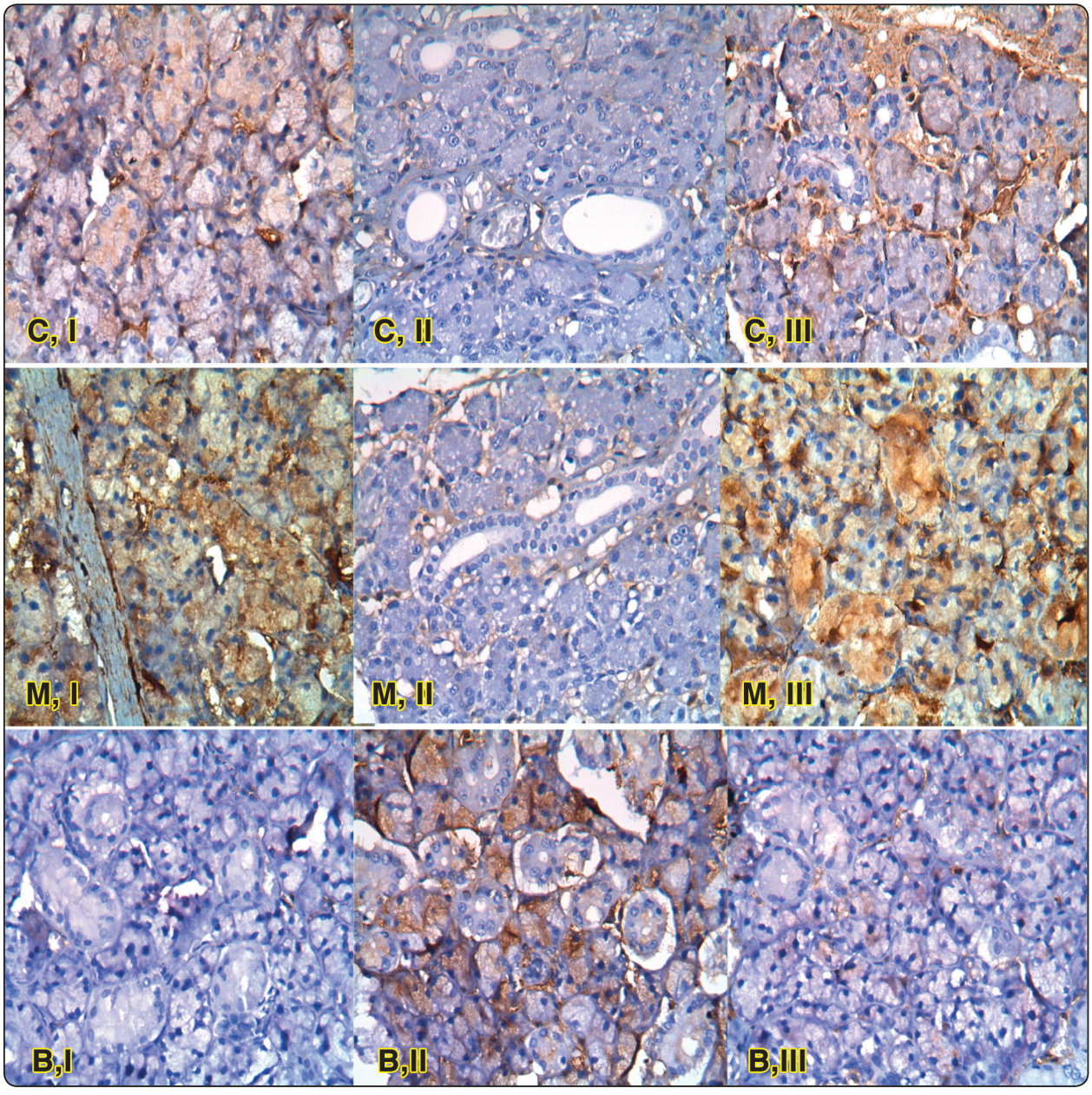


Fig. (3): Photomicrograph of the immunoexpression of the Cytokeratin 5,6 & 18 in the submandibular gland of group I (C,I), group II (C,II) and group III(C,III), Myosin in group I (M,I), group II (M,II) and group III(M,III) and Bax in group I (B,I), group II (B,II) and group III(B,III) (X 400).

Immunohistochemical localization of myosin :

Revealed moderate to strong myosin immunoreactivity in the the acinar cells, striated and granular convoluted tubules, also around the acini and secretory ducts representing the myoepithelial cells in the adult control group I, but in the old age group II they showed mild immunoreactivity. In the old age rats treated with the bone marrow derived stem cells group III they showed moderate to strong myosin immunoreactivity.

Immunohistochemical localization of bax:

Control group revealed negative Bax immunoreactivity in the secretory portions and duct system ,but in rats of old group II showed strong Bax immunoreactivity in the secretory portions as well as in the duct system. In the old age rats treated with bone marrow derived stem cells group III showed mild Bax immunoreactivity.

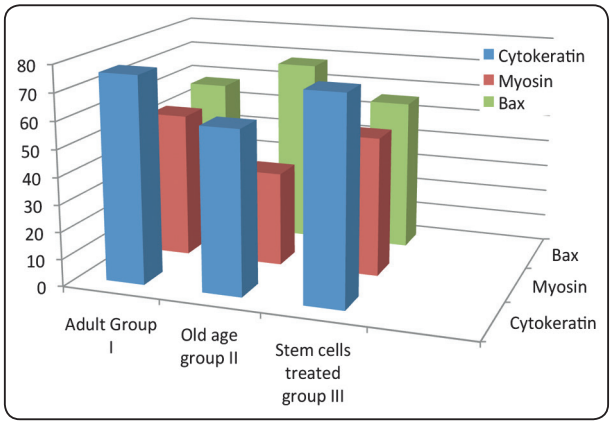
III- Statistical results: (Table I & graph I)

Statistical analysis of cytokeratin # 5, 6 & 18 and myosin expression revealed that their immunoreactivity was significantly reduced in the old age rats (Group II) than those of both groups I & III. But their immunoreactivity was not significantly decreased in groups III compared to group I. Also, statistical analysis of bax expression among the studied groups revealed that their immunoreactivity was significantly increased in the old age rats (Group II) than those of both groups I & III. But their immunoreactivity was not significantly decreased in groups III compared to group I.

TABLE (I) The difference in mean optical density of submandibular salivary glands between Adult, Old and Stem cells treated groups using ANOVA Test

Group	Cytokeratin optical density		
	M±SD	F-Value	p-Value
I-Adult group	76.008±0.32	12.48	<0.001*
II-Old group	60.062± 1.57		
III-Stem cells treated group	75.204± 9.71		
Group	Myosin optical density		
	M±SD	F-Value	p-Value
I-Adult group	53.442±11.47	10.54	<.002*
II-Old group	34.706±2.99		
III-Stem cells treated group	51.23±2.92		
Group	Bax optical density		
	M±SD	F-Value	p-Value
I-Adult group	57.914±0.83	204.99	<0.0001*
II-Old group	68.294± 0.51		
III-Stem cells treated group	56.014± 1.49		

**** Significant difference at (p<0.001)***



Graph I: Represents the difference in mean optical density between different groups

DISCUSSION

Regarding our work and the above mentioned our results of old age group II compared with that of adult control submandibular salivary glands, there were agreed with **Lasisi *et al.,* (2014)** who found that the salivary glands in old rats group showed acinar cell atrophy, and higher periductal fibrosis levels than those in young and adult groups.

Piantanelli *et al.,* (1980) revealed that the beta-adrenoceptor alteration during aging may play a major role in the age-dependent impairment

Regarding the histological results of BM-MSCs treated group, there were noticeable enhancements of the acinar and ductal cells than old aged group II rats. That were agreed with **Lin *et al.,* (2011)** who showed that the transdifferentiation of bone marrow stem cells into acinar-like cells in vivo was also noted. They also demonstrated that cell therapy with bone marrow stem cells that help in functional regeneration of salivary glands.

BMDCs provide a beneficial effect on the saliva production. BMDCs positively affect blood vessels stability and regeneration in irradiated salivary glands. Also, BMDCs provide an immunomodulatory activity in mice with Sjögren's-like disease. While the exact mechanisms by which BMDCs improve organ functions remain controversial (**Tran *et al.,* 2010).**

It is known that MSCs express and secrete Stromal cell-derived factors 1, vascular endothelial growth factor, and other cytokines important for angiogenesis **(Sorrell *et al.,* 2009)**.

In our study the immunohistochemical results of expression of cytokeratins # 5,6 and 18 in the submandibular salivary glands of the control group showed that almost all serous acinar cells, ductal cells lining as well as the myoepithelial cells, gave moderate staining reactivity which was in agreement with the results of **Draeger *et al.,* (1991)**. Also **Ruzankina and Brown, (2007)** demonstrated the absence of proliferative CK6+ regions together with atrophy of ductal and acinar tissue in the older mouse meibomian glands, that may point to an absence or senescence of progenitor cells and decreased renewal of both basal acinar and ductal epithelial cells.

Regarding the expression of myosin in the submandibular salivary glands of the control group, almost all serous acinar cells and ducts cells lining gave moderate staining reactivity which was in agreement with **Stoeckelhuber*, et al*., (2012)** who demonstrated that myosin was expressed in the basal part of the acinar cells with a medium to strong intensity. The staining intensity decreased in the direction of the lumen.

By aging the immunoreactivity of the acinar and ductal cells was decreased than those of adult rats, that indicate decrease intracellular myosin contents that is in accordance with **Nair, (2005)**

At this regard, important improvement have been made by work of **Madeira A. *et al*. (2012)** showed that the proliferative and clonogenic potential of BM-MSCs were seriously affected, as well as their proteome profile, mainly for proteins in the categories "Structural components and cellular cytoskeleton", "Folding and stress response proteins", "Energy metabolism", "Cell cycle regulation and aging" and "Apoptosis", which were in agreement with our immunohistochemical results that demonstrated an enhancement of cytokeratin #5, 6 & 18 and myosin immunoexpression in both acinar and ductal cells as well as myoepithelial cells.

Regarding the immunohistochemical results of bax, there was increase in the expression of bax protein in group II old rats while it was negative in adult rats, which revealed the increase of apoptosis in acinar and ductal cells lining. While in group III rats, salivary glands treated with BM-MSCs sections their immunoreactivity is decreased, which revealed the decrease of apoptosis. These results were in agreement with **Sumita *et al.,* (2011)** as they

found that the histological analysis shows that SGs of treated mice with BM-MSCs, demonstrated an increased level of tissue regenerative activity such as blood vessel formation and cell proliferation, while apoptotic activity was increased in non-transplanted mice.

CONCLUSIONS

Intravenously BM- MSCs infusion improved histological and immunohistochemical picture of the rat's submandibular salivary glands with de-crease the apoptosis rate and enhanced expression of both intracellular cytokeratin # 5, 6 & 18 and myosin of the acinar and ductal system cells in the MSCs treated group than those in the old age group.

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