Stereological and immunhistochemical study on the Submandibular gland of diabetic albino rats

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Abstract: The submandibular salivary gland (SMG), is one of the major salivary glands, that is responsible for secretion of about 80% of the salivary secretion. Diabetes mellitus (DM) is an endocrine disorder that affects glucose homeostasis and results either from insulin insufficiency or sensitivity. It has a deleterious effect on the entire body organs including the SMG. The destructive mechanism of DM is thought to be related to oxidative stresses accumulation. Among the different DM treatment modalities, ozone is claimed to regulate diabetic hyperglycemia and reduce oxidative stresses accumulation. Thus, we hypothesized that ozone could provide a better alternative than insulin in alleviating the diabetic burden on SMG acinar part. Thus, the aim of the present study was to investigate the effect of diabetic induction on rat SMG together with examining the efficacy of ozone and ozone- insulin combination as alternative DM therapies. Forty male albino rats were assigned into 5 groups; control, diabetic, ozone, insulin and ozone-insulin combination. Diabetes was induced by single intraperitoneal injection of 150 mg/kg alloxan. Quantitative analysis of blood glucose was done throughout the entire experimental period. At the end of three months of the experiment, the SMGs were removed and randomly sectioned to estimate the volume- weighted mean volume of the SMG seromucous acini together with their expression of the proliferating cell nuclear antigen (PCNA) in different groups. Our quantitative findings confirmed that ozone and ozoneinsulin combination therapies resulted in better alleviation of diabetic burden on rat SMGs and pancreas than traditional insulin treatment.
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1. Introduction

The submandibular salivary gland (SMG) is one of the three major salivary glands that are generally considered as exocrine glands. The classic physiological role of exocrine SMG is to produce saliva which is an enriched milieu composed mainly of water, electrolytes and biologically active proteins, including growth factors and cytokines (**Amerongen and Veerman, 2002**). Histologically, SMG consists of parenchymal elements, which are well differentiated cells that are arranged into lobes and lobules supported and divided by connective tissue septa (**Paulsen, 2000**). SMG parenchyma has two general types, acinar and ductal which roughly represents the exocrine and the endocrine elements, respectively **(Cook et al., 1994**). Unlike the clear distinction between serous and mucous acini of human SMG, the nature of rat SMG was described as seromucous acini which were neither pure serous nor pure mucous acini **(Curcio et al., 2012, Caldeira et al., 2005, Kitashima et al., 2005).**

Diabetes mellitus (DM) is one of the pathological conditions that disturb the homeostasis of the internal environment. In type 1 DM, pancreatic beta cells are the target of an autoimmune assault, leading to their degeneration and consequently loss (**Klöppel et al., 1985**). Death of pancreatic beta cells cause failure to produce insulin which in turn causes sustained hyperglycemia that affects all organs of the body.

DM adversely affects structure and function of both acinar and duct system of SMG. Initial reports demonstrated that DM led to reduction in SMG weight in rats (**Liu et al., 1969**). Virtually all subsequent studies confirmed these findings (**Chan et al., 1993; Pinkstaff et al., 1996**). Electron microscopy revealed a variety of pathological changes in the acinar part, including a pooling of secretory material, an increase in the number of autophagic vacuoles (**Anderson et al., 1994**) and the accumulation of lipid droplets in the basal regions of serous and mucous acinar cells (**Kamata et al., 2007**).

The exact mechanism of DM destructive effect on SMG is still debatable. However, sustained hyperglycemia is suggested to be one of the main reasons of SMG degenerative changes. This has been supported by studies illustrating that insulin treatment reverse the damaging effects of diabetes and almost restore the normal structure of SMG (**Morris et al., 1992; Take et al., 2007; Mednieks et al., 2009).** Also**, Kasayama et al. (1989)** reported that insulin causes complete recovery of protein levels in the granular ducts, including EGF and NGF. However, important cellular disorganizations continue to exist, especially in the SMG ductal system **(Jose et al.,**

2005). Thus, even prolonged insulin treatment alone appeared to be insufficient to cause complete structural reorganization of salivary glands. It is worth noting that **Reznick et al. (2006**) claimed that the overall diabetic changes in the salivary glands were a consequence of oxidative stress accumulation within the glands. Thus, ozone seems to be an attractive therapeutic alternative and/or additive to treat or reverse the destructive effect of DM on SMG. Ozone (O3) is a triatomic molecule, consisting of three oxygen atoms that seems to have beneficial therapeutic effect on DM. Ozone treatment was found to improve glycemic control and to prevent oxidative stress in streptozotocin induced diabetic rats (**Al-dalain et al., 2001**). In line with these findings, another study reported that there was a decrease in the percentage of damaged pancreatic islets by ozone treatment and that ozone also reduced hyperglycemia (**Martinez et al., 2005).** Surprisingly, pancreatic β-cells were discovered to have some capacity to replenish, even under prolonged autoimmune and metabolic stress caused by type 1DM (**Keenan et al., 2010**). The source of these replenished β-cells appears to be controversial. They may arise from trans-differentiation of pancreatic acinar cells, pancreatic stem cell pool or from bone marrow-derived stem cells (**Manesso et al., 2009)**. Thus, we hypothesized that using ozone treatment could play a role in alleviating the damaging effect of DM on SMG.

2. Materials and Methods

2-1: Animals:

This study was conducted on forty adult male rats with an average body weight of 200- 250 grams. All animals were housed in a temperature-controlled room on a 12-hour alternating light-dark cycle. They were given food and water ad libitum throughout the experimental period, which lasted for 3 months in all groups.

The protocol was first approved and conducted according to *established animal welfare guidelines for the responsible use of animals in research as a part of scientific research ethics recommendation of Research Ethics Committee, Faculty of Dentistry, Tanta University*. The animals will be divided into five equal groups; each group consists of 8 rats.

The groups were divided into one control and four experimental groups. The later include one untreated group and three different treatment modalities namely; ozone, insulin and combination groups.

2-1-1 Control group:

The rats were injected with vehicle (sterile physiological saline) to control the influence of any injection stress on the animals in group 1.

2-1-2 Diabetes induction:

Diabetes was induced by a single intraperitoneal injection of 150 mg/kg monohydrated alloxan dissolved in sterile 0.9% saline (**Diniz et al., 2008**). Animals were fasted overnight then induction of diabetes was done in all four experimental groups (groups 2, 3, 4 and 5).

2-1-3 Insulin administration:

Beginning at the 7th day after alloxan administration subcutaneous injection of, 4-5 units/day, insulin was done in groups 4 and 5 **(Ramamurthy et al., 1974)** throughout the three months experimental period.

2-1-4 Ozone administration:

Intraperitoneal injection of 1 cc of ozone-oxygen gas mixture with concentration of ozone 40 µg/cm3 three times per week was done in groups 3 and 5 throughout the three months experimental period **(Kuryszko et al., 1995**). Ozone-oxygen gas mixture was supplied by Humazone PM generator, GmbH, Kamlst. Germany.

2-2 : Measurement of blood glucose levels:

Blood glucose levels were determined in mg/dl using a digital glucometer (Accuchekr Advantage. Roche Diagnostic. Germany). Blood samples were obtained from the tail vein of the animals. At the beginning of the experiment blood glucose levels were recorded, then 7 days after alloxan administration, to insure induction of diabetes. Then, they were measured every 14 days throughout the entire 3 months of the experimental period to evaluate various treatment modalities. For blood glucose measurements descriptive statistics were calculated as means and standard deviations. Comparison among the study groups was done using analysis of variance (ANOVA). Then Friedman test was used to determine the differences between the readings in each separate group.

2-3 : Tissue Preparations:

At the end of the experimental period rats were euthanized by cervical dislocation. The Submandibular salivary gland of each animal was cut into (3x3x3mm). The samples were fixed in 4% paraformaldhyde for 24 hours. The tissues were put randomly in the embedding media, In order to achieve random orientation of the specimens (**Mayhew et al., 1999**). The sections were cut at 5µm intervals and stained for histological and immunohistochemical examinations using H & E stain and anti-PCNA antibody (Labvision, USA), respectively.

2-4 : Stereological study:

On each cut section five fields were selected in a random fashion. This was done by movement of the microscope's stage in both vertical and horizontal directions (Leica, Germany). Each field was analyzed at a magnification of 400X and a total number of 90-100 acini per animal were examined. A grid system

with lines and associated points (image J) was used to estimate volume-weighted mean volume of the SMG acini using point-sampled intercepts method (**Mandarim-de-Lacerda, 2003; Gundersen et al., 1988; Nielsen et al., 1986**). For each test point which hits an acinus, the intercept (**l**0) was measured at this point.

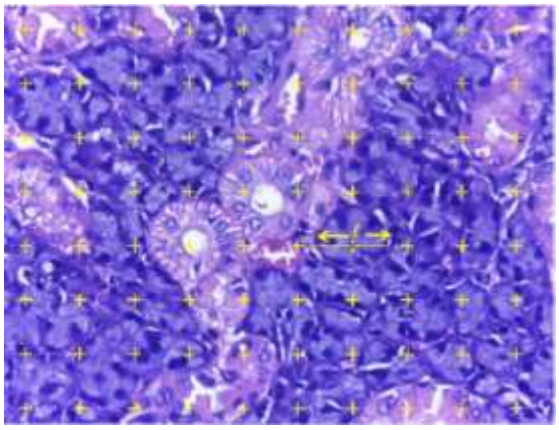
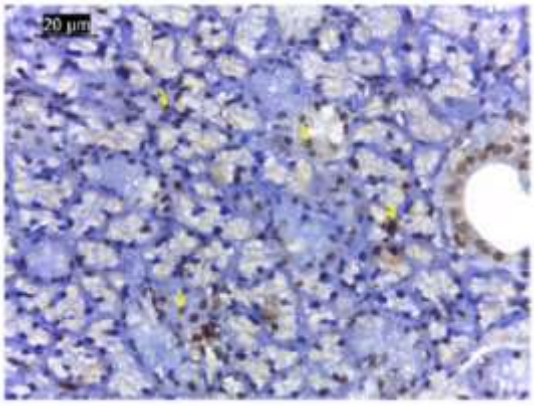
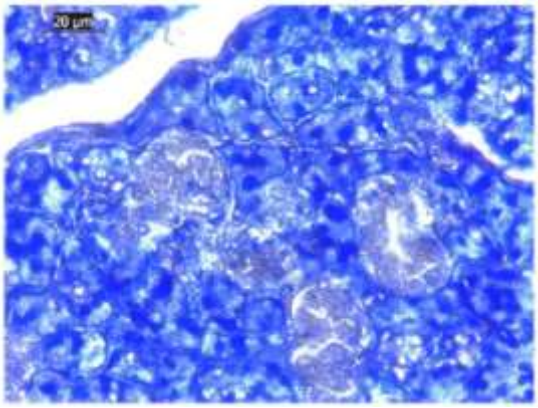


Figure 2-1 light micrograph illustrates the seromucous acini. Using the point-sampled intercepts method, the intercept (**l**0) was measured at this point [H & E stain, original magnification x 400].



A



B

Fig 2-2: light micrograph illustrates (A) positively stained acinar cells (yellow arrows) of control group [Immunoperoxidase staining of PCNA and hematoxylin counterstain]. (B) PCNA expression is absent due to omission of primary antibody as negative control [original magnification x400].

Intercept can be defined as the length of the line passing through the test point from one side of the acinus to the opposite side in an isotropic direction (Fig. 2-1). Then the volume weighted mean volume (Vv) of the acini was calculated using the following equation $V^v = \frac{\pi}{3} \times \ell_0^3 \times M$. M is a correction factor calculated as $(\frac{1}{M})^3$, where is the microscope magnification under which the sections were studied (**Skau et al., 2001**).

2-5 : Immunohistochemistry:

In this study immunohistochemistry of the proliferating cell nuclear antigen (PCNA) was done. Sections were incubated with anti-PCNA antibody (mouse monoclonal primary antibody, clone PC10, Thermo Scientific Labvision) followed by incubation with secondary antibody (biotinylated goat antipolyvalent; Thermo Scientific Labvision). Then, incubation with enzyme labelled streptavidin peroxidase followed by application of DAB substrate with DAB chromogen and incubation until desired reaction is achieved (Lab vision Ultra Vision detection system). Finally, the slides were counterstained with hematoxylin. Negative control was performed following the same previous steps except that the primary antibody was replaced with PBS (Fig. 2-2). The results were examined using light microscope (**Yasser et al., 2012**).

3-Results

3-1 : Blood glucose levels:

The comparison among the five study groups as regards to blood glucose level during the study period. As shown in (table 3-1), the 1st reading represents the mean blood glucose level in mg/dl for the five groups before diabetes induction and before ozone treatment sessions and insulin injection. While the second reading represent the mean blood glucose level after diabetes induction for diabetic, ozone, insulin and combination groups and before starting the ozone treatment sessions for ozone and combination groups and insulin injection for insulin and combination groups. The 3rd to the 7th reading represents the mean blood glucose level after starting the ozone treatment sessions for ozone and combination and insulin injection for insulin and combination groups. The differences among the five study groups as regards to mean blood glucose level in mg/dl was not statistically

significant in the 1st reading (p=0.84). Starting from the 2nd to the 7th reading the difference was statistically significant (p<0.001). In control and diabetic group, from the beginning till the end of the experiment, the changes in the mean blood glucose levels were not statistically significant (p=0.311 and 0.059, respectively). However, in the insulin group the changes in the mean blood glucose levels was statistically significant (P<0.01). In the ozone and the combination therapy groups the changes in the mean blood glucose levels was statistically highly significant (P<0.005) (Figure 3-1).

Table 3-1: Blood glucose levels:

Descriptive							ANOVA	
		Controls	Diabetic	Ozone	Insulin	Combination	F	P- value
Beginning of experiment	Mean	85.333	90.667	87.333	88.333	87.667	0.335	0.848
	SE	2.906	2.963	3.712	4.410	2.186		
After DM induction	Mean	85.000	255.000	251.000	242.667	252.667	182.684	<0.001
	SE	2.887	2.887	0.577	11.392	1.856		
After 4 weeks	Mean	88.000	246.667	186.000	173.333	176.667	24.976	<0.001
	SE	4.933	3.333	8.718	13.642	18.559		
After 6 weeks	Mean	85.667	244.500	110.000	122.667	107.000	182.187	<0.001
	SE	2.963	3.329	1.155	9.333	1.155		
After 8 weeks	Mean	84.667	241.330	69.000	86.000	66.333	594.563	<0.001
	SE	2.906	5.241	0.577	3.055	0.882		
After 10 weeks	Mean	85.333	242.333	69.667	69.000	67.000	750.704	<0.001
	SE	2.906	3.180	2.906	3.055	1.528		
After 12 weeks	Mean	79.333	240.500	69.333	69.000	67.000	621.716	<0.001
	SE	2.963	5.635	1.764	0.577	1.528		
Friedman Test	X2	7.106	12.145	16.922	17.641	17.774		
	P-value	0.311	0.059	0.002 <0.005	0.009 <0.01	0.001 <0.005		

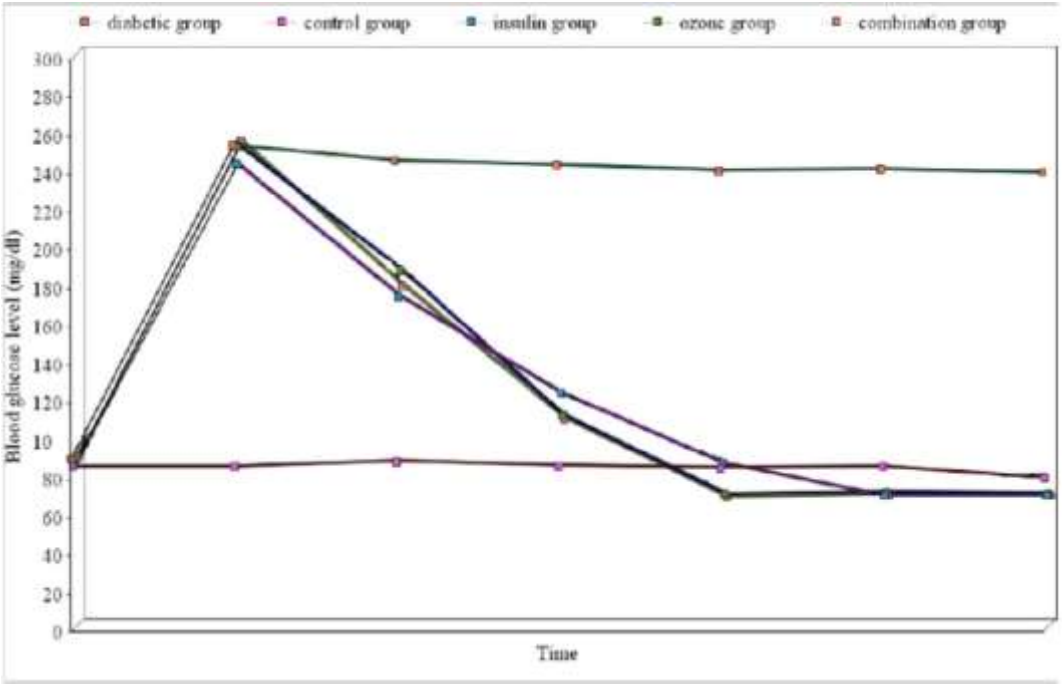


Fig 3-1: Measurment of blood glucose levels

3-2 : Stereological Study:

The results (table 3-2) showed that the volume weighted mean volume of the SMG acini represented significant increase 3 months after initiation of diabetes in diabetic group. The insulin group showed significant decrease from all groups. Although that ozone showed a significant increase from the insulin group, it still depicted a significant decrease from all other groups. On the other hand the combination group depicted similar results to the control (Figure 3-2).

Table 3-2: Changes in volume weighted mean volume (µm3) measurements (Values are mean ± SD)

		Combination group	Insulin group	Control group	Ozone group	Diabetic group
Volume weighted mean volume (μm^3)		710000 ± 3000 [c]	552000 ± 22000[a]	712000 ± 28000[c]	668000 ± 27000[b]	932000 ± 37000[d]
ANOVA	F	111.2				
	P- value	<0.001*				

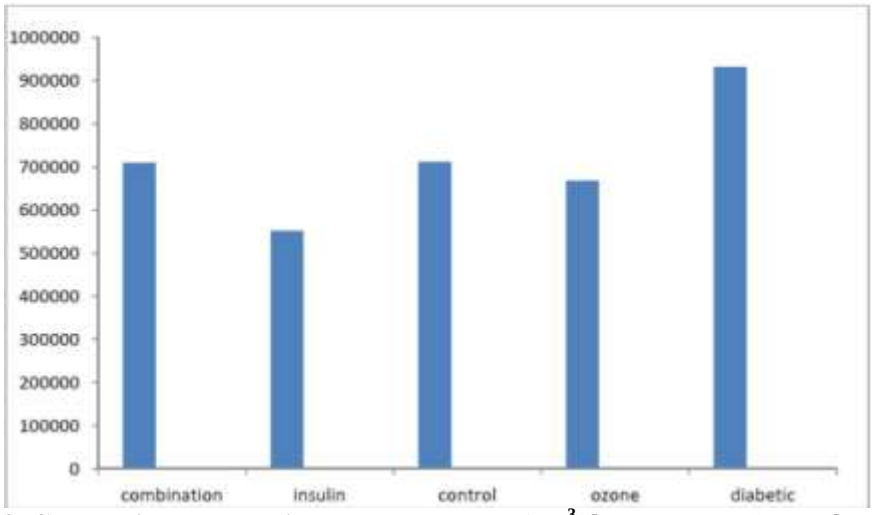


Fig 3-2: Changes in volume weighted mean volume (μm^3) between experimental groups.

3-3 : Immunohistochemical study:

The results (table 3-3) showed that the PCNA expression pattern of diabetic SMG acini represented a highly significant increase than the control group. On the other hand isulin failed significantly to restore the normal PCNA expression. Although the combination group scored the better results followed by the ozone group, there was no significant difference among them and the control. This indicates that the combination of ozone with insulin and ozone therapy had the best results in normalization of PCNA expression levels in comparison with insulin alone (Fig. 3-3).

Table 3-3: Changes in PCNA expression levels in SMG acini (Values are mean ± SD)

		Combination group	Insulin group	Control group	Ozone group	Diabetic group
Acini PCNA%		7.35±3.9 [a]	12.27±4.14[b]	5.78±4.29[a]	10.6±10.59[a,b]	24.55±6.92[c]
ANOVA	F	6.512				
	P- value	=0.002				

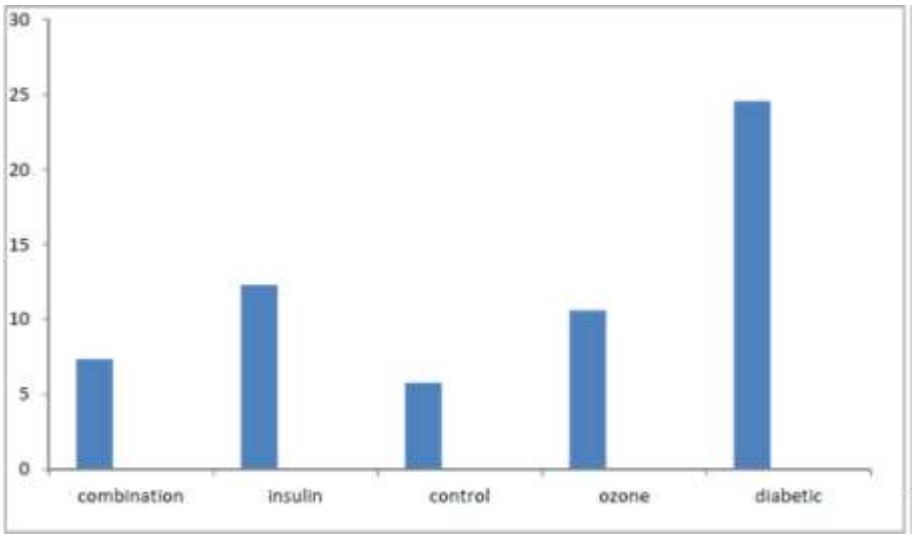


Fig 3-3: Changes in PCNA expression levels in SMG acini between experimental groups.

4. Discussion

Alloxan was used to induce diabetes in rats, since it provokes degeneration of pancreatic beta cells in a process that mimics type 1 DM in humans (**Szkudelski, 2001**). The latter occurs due to progressive autoimmune destruction of pancreatic beta cells, which is partially mediated by free radicals such as ROS (**Ekuni et al., 2010)**. Similarly, lloxan mediates its action via ROS since reduction of alloxan produces dialuric acid that undergoes dismutation to hydrogen peroxide. The action of ROS with a simultaneous massive increase in cytosolic calcium causes rapid destruction of pancreatic beta cells (**Abikshyeet et al., 2012**). Rat normal glucose level is close to 90 mg/dl, whereas levels ranging from 150-200 mg/dl are considered an effective diabetic state (**Muller et al., 2008**). At the beginning of our investigational period, all diabetic rats revealed severe hyperglycemia that persisted, in the diabetic group, throughout the entire experimental period. This was attributed to alloxan cytotoxic action on pancreatic beta cells resulting in diminished insulin production (**Szkudelski, 2001**).

In the present work, we evaluated the efficiency of three different treatment modalities, ozone, insulin and ozone-insulin combination in improving the diabetic burden induced by alloxan administration. Quantitatively, blood samples were analyzed for glucose. Also, to contemplate the role of these treatments in regeneration of rat SMGs, we observed the changes in the volume of seromucous acini. In addition, we confirmed our results by investigating the expression of proliferating cellular nuclear antigen (PCNA). PCNA is a clamp protein known as polymerase-associated protein formed in the early G1 and S phases of the cell cycle and it is involved in DNA repair and replication. Increase in PCNA expression can be associated with stress induced DNA damage in an attempt to bypass it; this has been reported in lungs (**Kyoo-young et al., 2010**) and intestines (**Yuh et al., 2016**).

The diabetic group depicted an elevated blood glucose level throughout the entire experimental period. Before induction of diabetes, at the beginning of the experiment, the mean blood glucose levels depicted statistically insignificant difference among various groups ($p=0.84$). However, starting from the second week, after induction of diabetes, to the end of our experimental period, there was statistically significant ($p<0.001$) differences in the blood glucose levels among different treatment modalities. The present study established that ozone reduced hyperglycemia and exerted glycemic control, this is in agreement with other studies that ozone had an anti-diabetic effect, diminished hyperglycemia and increased antioxidant defense (**Martinez-Sanchez et al., 2005**). The mean blood glucose levels of the ozone and the ozone-insulin combination therapy, were statistically highly significant ($P<0.005$), whereas, the insulin group depicted statistically significant changes in its mean blood glucose levels ($P<0.01$). These results indicated that all of the three different treatment regimes were efficient in lowering blood glucose levels.

Interestingly, our results showed a significant increase, compared to the control, in the volume-weighted mean volume of seromucous of diabetic rats SMG, this is may be explained by the hyperglycaemic effect on cellular water content. The increased glucose level cause electrolytes imbalance either due to impairment of sodium-glucose cotransporters, aquaporins or both (**Lilliu et al., 2015; Delporte, 2014; Wright et al., 2011**) or due to ATP depletion that reduces the activity of the plasma membrane energy dependent sodium pump, resulting in intracellular accumulation of Na^+ and efflux of K^+ causing cell swelling (**Krippeit-Drews et al., 1999).**

Also the diabetic group showed abnormally higher PCNA expression (**Dickinson et al., 2014**). It is well known that the destructive effect of hyperglycemia could be contributed to induction of oxidative stresses (**Jay et al., 2006**). This is done via several mechanisms including glucose autoxidation, formation of advanced glycation end-products (AGEs) and activation of the polyol pathway. Other circulating factors that are elevated in diabetics, such as free fatty acids and leptin, also contribute to increased reactive oxygen species (ROS) generation (**Murphy, 2009**). Oxidative stresses induce cellular injury by enhancing membrane peroxidation and DNA fragmentation (**Pizzimenti et al., 2010**). To eliminate oxidative DNA damage caused by ROS, base excision repair (BER) is activated to repair oxidized bases and single strands breaks (**Meira et al., 2005**). PCNA was found to be mandatory for gap-filling in BER, this makes PCNA essential to repair DNA lesions induced by oxidative stresses (**Yi-Chih et al., 2017),** which may in part explain the elevated level of PCNA expression in the acini of diabetic group in an attempt to bypass the oxidative induced damage.

After the three different therapies, we found that insulin failed to efficiently restore both the normal volume and normal PCNA expression level of the diabetic group acini. This partial recovery might be related to the fact that complications of DM are not completely due to hyperglycemia other reasons are implicated since damage produced by accumulated oxidative stresses remained with obvious great cellular disorganization upon prolonged insulin therapy (**Mizokami et al., 2013**). Also, it is noteworthy that

diabetes results in a decrease in the weights of several endocrine glands, including the thyroid, the adrenal and the pituitary, suggesting that the effects of diabetes on salivary glands might be mediated indirectly through reduced level of circulating hormones other than insulin (**Nishimura et al., 1991**). Thus, prolonged insulin treatment alone seemed to be insufficient to cause complete structural reorganization of SMG.

Interestingly, ozone is one of the most potent oxidizer; it has been reported to be able to induce an antioxidant response that was capable of reversing the chronic oxidative stress (**Frascino et al., 2013**). After intraperitoneal injection of ozone, it dissolves immediately in the water overlaying the epithelium, generating hydrogen peroxide and LOPs in small gradual pulses (**Bocci, 2000**). This repetition of graduated small oxidative stresses induces a multiform adaptive response which exerts a moderate oxidative stress that activates the nuclear transcriptional factor Nrf2. Nrf2 then induces the transcription of antioxidant response elements (ARE), which has triple effects. First, ARE produces numerous antioxidant enzymes, such as SOD, GPx, catalase (CAT) and HO-1. Second, ARE recognize, repair and remove damaged proteins. Third, ARE protects from apoptosis and repair DNA.

This does not only protect cells from oxidation and inflammation but it also may reverse the chronic oxidative stress of DM (**Bocci, 2004, 2013; He et al., 2004**). These results are in accordance with an earlier study by **Sagai et al. (2011)** who found that ozone suppressed another nuclear transcriptional factor kappa B (NFκB), which was activated in case of severe and chronic oxidative stress. NFκB was activated by H2O2, a reactive oxygen species produced during glucose autoxidation and one of the main factors that were responsible for DM cellular destruction. The activation of NF-κB caused an inflammatory response and tissue injury via the production of different inflammatory cytokines, cell adhesion molecules cyclooxygenase1, prostaglandin and nitric oxide synthase (**Martinez-Sanchez et al., 2005**).

This is may explain why the PCNA expression level in the ozone group showed much better results than insulin, as the up regulation of Nrf2 may cause significant down regulation of PCNA expression in an attempt to normalize its level (**Yuh et al., 2016**). Although the seromucous acini of the ozone group showed a major improvement than the insulin group, however, it still depicted a significant reduction in volume than normal size. The beneficial effect of ozone over insulin could be attributed to the ozone triple effects. It could alleviate chronic oxidative stresses accumulated by DM besides its hypoglycemic effect and ability to replenish stem cells from bone marrow to migrate to the needed organs (**He et al., 2004**).

Interestingly, the combination group restored the normal size of the seromucous acini and nearly normalized the PCNA expression level. Thus, we suggested that ozone-insulin combination could be the best therapy in restoring and preserving SMGs from degenerative changes caused by DM. This ozone-insulin combination could act independently and synergistically to reduce hyperglycemia and improved ROS production and stimulate the antioxidant enzymatic activities (**Al-Dalain et al., 2001, Dawe et al., 2007, Caldeira et al., 2005**). Thus, combining the efficacy of insulin as traditional drug with the safe and valid use of ozone verifies the concept of integrated medicine since it correctly associates suitable drugs with the critical stimulus to reactivate the natural defenses **(Caldeira et al., 2005**).

From one hand, ozone treatment could partially reduce the imbalance between the generation of ROS and scavenging enzyme activity. Also, it could enhance antioxidant enzyme activity and reduce overproduction of ROS which together act to protect cell membranes (**Messenger et al., 2003**). On the other hand, insulin could control the deleterious effects of hyperglycemia by stimulating glucose transporters gene expression at the cellular level, by increasing antioxidant enzyme glutathione peroxide levels and decreasing the level of nuclear factor kappa B (**Kim and Egan, 2008; Elefteriou et al., 2005**).

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