

## Histomorphometric Analysis of the Postnatal Development and Growth of Rat Submandibular Glands in Offsprings of Diabetic Mothers

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**Abstract: Background:** Disturbances of the in utero environment may program for disease in later life. Exposure to a diabetic environment in utero is associated with a high risk of obesity and glucose intolerance. **Aim:** The purpose of this study was to determine whether diabetes during pregnancy can adversely affect the development and growth of submandibular salivary gland in the offspring. **Methods** Sixteen adult female rats were divided into two equal groups. Diabetes was induced in one group by alloxan and the other group was control. Both groups become pregnant by mating with four adult male rats. Submandibular salivary glands of 2 & 6 weeks old male offsprings from the two groups were examined by light microscope and morphometric analysis. **Results:** The submandibular glands of the offsprings of diabetic mothers revealed remarkable changes in serous acini and duct system throughout the experimental periods compared to the offspring of the control group. There was a significant increase in the total surface area of acini and ducts of the rat submandibular gland during the two and six weeks of postnatal life in comparison to the corresponding values of the control group. **Conclusion:** Maternal hyperglycemia revealed histomorphological changes in submandibular glands during postnatal period and hence maternal diabetes can be considered a very important risk factor to the development and growth of submandibular glands.

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**Keywords:** maternal diabetes; development; histomorphometric changes; submandibular glands.

### 1. Introduction

Development of the rat salivary glands begins during fetal life and continues well after birth. As a result, both intrauterine events and post-natal changes affect salivary gland maturation. Salivary glands are created by complex and remarkably dynamic processes that yield the final, precisely organized architecture of normal adult glands. Efficient functioning of these salivary glands is essential to produce the approximately half-liter of saliva daily that maintains oral health (Napenas et al., 2009 & Vissink et al., 2010).

At birth, salivary glands are small and completely immature, but marked growth is observed during the initial months of postnatal life when the glands gradually acquire the morphology of the mature organ (Denny et al., 1997). The parenchyma of the rat submandibular glands consists of two structural secretory compartments, the acini and the granular convoluted tubules. The acini are the predominant structures and consist of seromucous cells, whereas the convoluted granular tubules, which are interposed between the intercalated and striated ducts, consist of serous cells (Tandler et al., 2001).

Miclaus et al., (2009) reported that, the acini of submandibular glands of rats had the feature of serous acini, similar but not identical with those from parotid gland. In addition of excretory channels present in

mammalian salivary glands, in submandibular gland of the rats there's one more type of channels, named granular channels. Cells from walls of granular channels contain numerous polymorphous intracytoplasmic granulations which suggest that they have an intense secretory activity. Authors added that granulations from cytoplasm of cells from granular channels are different from those of the acini structure and their secretion is muco- proteic. By mixing serous secretions of acini with that muco-proteic of the granular channels cells, resulted in a mixed final secretion which make us to consider submandibular gland of the rat a mixed particular gland.

Van et al., (2001) reported that pregnancy is a state that allows a life form to develop with the support and protection of its mother's body. The growth and development of the fetus in gestation is partially determined by the genome of the fetus, which produces its own growth factors as well as the majority of its hormones. However, this genetic influence is highly dependent upon interaction with environmental factors. Authors added that one environmental factor vital in the growth and development of the fetus is nutrition. Changes in the plasma nutrient levels in the maternal system have an effect on the fetal plasma composition, and thus affect organogenesis in the fetus. Holemans et al., (1999) suggested that fetal life and all of the developmental

processes that occur during this stage are fundamentally important in the life outcomes of the future individual. Diabetes have an effect in the intra-uterine environment, it also may influence growth rates in neonates.

Experimental and clinical studies have shown that the incidence of diabetes is higher in the offsprings of diabetic mothers than in offsprings of nondiabetic mothers or diabetic fathers **Meigs et al., (2000)**. Exposure to a diabetic environment in utero is associated with a substantially higher risk of obesity, glucose intolerance, and non-insulin-dependent type 2 diabetes mellitus in the offspring **Viana et al., (2000)**.

Therefore, the aim of the present study was to evaluate the relation between the period of growth and the possible histological and morphometric alterations in the area of acini and ducts from submandibular glands under the influence of diabetic environment.

## 2. Material and Methods:

### Animals:

Sixteen adult female albino rats weighting 200-230 gm each and 4-5 months old were used in the present study. Other 4 adult male albino rats were used for breeding. The rats were obtained and housed in the animal house at the Institute of Ophthalmology, Cairo University. All rats had been fed on standardized laboratory balanced diet and given water ad libitum. Animals were divided equally into two groups; control group & diabetic group. All animals received human care in compliance with the national institutes of health criteria for care of laboratory animals.

### Induction of diabetic mellitus:

Diabetes was induced in 8 rats by a single intraperitoneal injection of alloxan 150gm/kg according to **Jelodar et al., 2010** (Sigma Chemical Co., St Louis, MO, USA). The animals fasted 12hours before and after alloxan injection. Rats with blood glucose level above 200 mg/dL as well as polydipsia, polyuria and polyphagia for at least one week were considered as diabetic and selected for the study.

### Experimental design:

The female and male rats of both groups were caged separately (4 females and 1male). Fertilization was confirmed by vaginal smear examination every morning. The presence of vaginal plug was designated as day zero of gestation. Female rats which demonstrated successful breeding were caged separately. The glucose levels during the experiment were typically 400–450 mg/dl in diabetic rats. Twenty Male offspring from both groups were sacrificed in two dates; 2 weeks and 6 weeks after birth.

### Histological examination:

The salivary glands were immediately removed and fixed in a 10% formaldehyde solution for 12 hours, washed by tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Sections of 6-7M were obtained and mounted on clean glass slides and stained with Haematoxylin and Eosin stain for light microscopic examination.

### Morphometric examination:

The haematoxylin and eosin stained sections were examined under the light microscope and the following factors were measured in the submandibular glands of both control and diabetic groups

- 1- The mean total acinar area.
- 2- The mean total ducts area.

This analysis was performed using an image analysis software (Leica Quin 500 plus). The calibration was based on the scale bar on the micrographs (Fig. 1). Mean values  $\pm$ SD of the total area of salivary acini and ducts or granular tubules of diabetic rats were statistically compared with those of appropriate intact acini and ducts of control rats by using ANOVA test. A *p*-value  $p < 0.01$  was considered significant.

## 3. Results:

### Histological results:

#### Control group (Two weeks old offsprings):

Histological examination of the submandibular glands of the offsprings of control group at 14 days showed progressive differentiation of the acinar cell compartment. A single type of acini was observed; these acini were small and variable in size. They were lined by pyramidal cells having a flattened or large ovoid basally situated nucleus and foamy cytoplasm. No lumen was apparent in most of the newly formed acini. Few proacinar secretory portions were detected. Intercalated ducts were observed whose epithelial cells are cuboidal as well as many striated ducts were present by day 14. However, Granular convoluted tubules were not detected. In addition, there is a large amount of loose connective tissue connecting the newly formed lobes and numerous blood vessels (Fig. 2).

#### Control group (six weeks old offsprings):

Light microscopic examination of the submandibular glands at 6 weeks old offspring presented large number of serous acini. They were composed of pyramidal cell with large basally situated nucleus and basophilic cytoplasm. The number of original acini was increased compared with the previous time point (2 weeks). Also an obvious acinar lumen was observed in some acini. The structures of

the glandular lobules were more obvious and all levels of ducts are differentiated and appeared nearly matured. The striated duct lined by columnar cells and excretory ducts lined by pseudo-stratified columnar epithelium. Well formed granular convoluted tubule were detected and lined by columnar cells with abundant acidophilic cytoplasm full of large eosinophilic granules. Loose connective tissues around the glandular lobules and abundant blood vessels were observed (Fig.3).

#### Experimental group (Two weeks old offsprings):

Histological examination of the submandibular gland at 14 days old offsprings of diabetic mothers presented rounded small acinar terminal portions with cytoplasmic vacuoles and more basophilic stain when compared to control group. The nuclei of the acinar and duct cells appeared large, dark and strong basophilic. Intercalated and striated ducts were detected. The excretory ducts were lined by pseudo-stratified columnar epithelium. The striated ducts were lined by a simple low columnar epithelium; no basal striation was observed. Also the acini and striated ducts are enveloped by bundles of fine collagen fibers of the connective tissue septa in different directions. There were also numerous capillaries located closely to the acinar cell membranes. Large number of the terminal tubule cells and proacinar cells were detected without formation proper lumen in most acini (Figs. 4). On the other hand, the parenchyma cells were revealed high mitotic activity and the nuclei were darkly stained (hyperchromatic) with relatively regular size. Intracellular vacuoles were detected in the cytoplasm of most acini (Fig.5). Some excretory ducts showed stratification of their nuclei. The ducts were surrounded with thick fibrous tissue. The ducts were mostly accompanied with dilated blood vessels engorged with red blood cells (Fig.6).

#### Experimental group (six weeks old offsprings):

Light microscopic examination of the submandibular gland at six weeks old offspring of diabetic mothers revealed overcrowding and cytoplasmic vacuolization the secretory acini. High mitotic figures were seen among the acinar cell groups and significant proliferation of ductal epithelium was obvious. Nuclei of the cells of parenchymal element took a heavier stain with more prominent chromatin and were slightly larger than those in the control group. A few granular convoluted tubules were detected. Striated ducts with patent lumen and others having no lumen were found. Also some striated ducts demonstrated cytoplasmic vacuolation. Many striated and excretory ducts showed lumen filled with stagnant secretion. Moreover secretion was detected in the connective tissue septa between lobes and lobules

(Fig.7). The blood vessels associated with the acini and the ducts were dilated and engorged with red blood cells. Vacuolation of the connective tissue stroma was obvious (Fig.8).

#### Morphometric results:

The difference of the mean total acinar area between the control groups of 2weeks and 6weeks old offsprings were  $120 \pm 25.4 - 834 \pm 237$  (respectively). Also the difference of the mean total ductal area between the control groups of 2weeks and 6 weeks old offsprings were  $550 \pm 115 - 1499.44 \pm 495.02$  (respectively) which increased significantly by age. Moreover the difference of the mean total acinar area between the diabetic acini of 2weeks to 6weeks  $658 \pm 184 - 882 \pm 160$  (respectively) was increase significantly. However, the difference of the mean total ductal areas between the diabetic ducts of 2weeks to six weeks  $2032.45 \pm 696.03 - 1825 \pm 310$  (respectively) was decrease significantly (table I & II).

Statistical analysis revealed that, the mean total acinar areas of the two weeks old experimental group were significantly greater than the corresponding values of the control group. (Control acini  $120 \pm 25.4 -$  diabetic  $658 \pm 184$  ( $p < 0.01$ )). However the mean total acinar areas of the six weeks old diabetic group were not significantly greater than the corresponding values of control group nearly equal (control  $834 \pm 234 -$  diabetic  $882 \pm 160$ ). The mean of total duct areas of two weeks old experimental group were significantly greater than the corresponding values of the control group (control  $550 \pm 115 -$  diabetic  $2032.45 \pm 696.03$ ) ( $p < 0.01$ ). Also the mean total duct areas of six weeks old diabetic group were not significantly greater than the corresponding values of control group (control  $1499.44 \pm 495.02 -$  diabetic  $1825 \pm 310$ ) these values are expressed in table (Graph I & II).

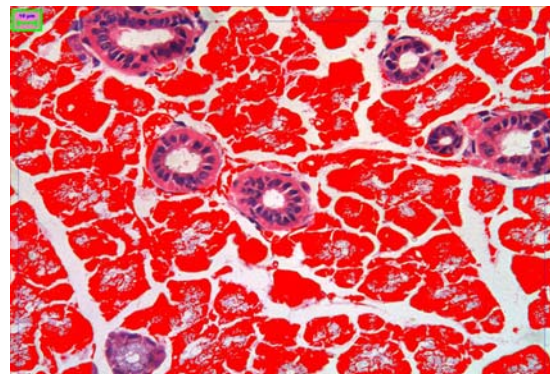


Fig. (1): A copy display as seen on the monitor of the image analyzer showing the measurement of the total surface area of the acini after being masked by red binary color.

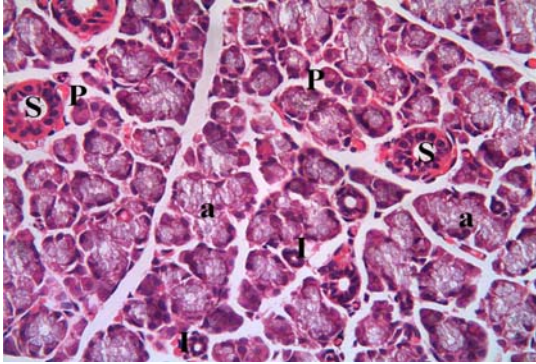


Fig. (2): A photomicrograph of submandibular gland of 2 weeks old offsprings (control group) showing some proacini cells (P), fully formed acini (a), intercalated (I), and striated ducts (S) (H&EX400)

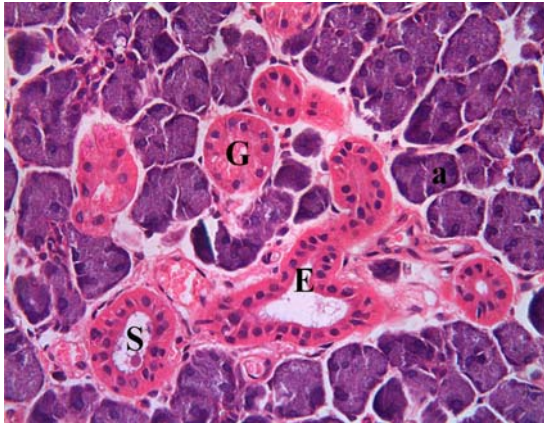


Fig. (3): A photomicrograph of submandibular gland of 6 weeks old offsprings (control group) showing basophilic acinar cells (a), striated (S) & excretory ducts (E) and granular convoluted tubules (G) H&E x 400

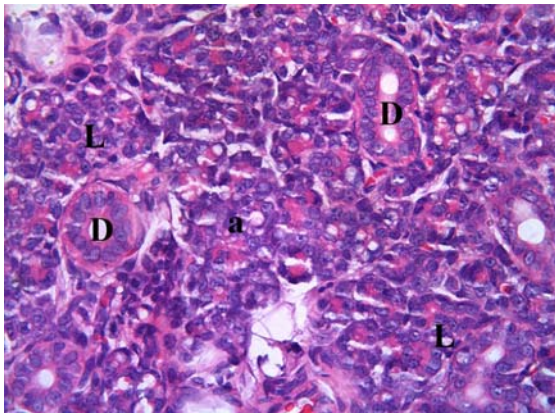


Fig. (4): A photomicrograph of submandibular gland of 2 weeks old offsprings (experimental group) showing ill defined serous acini (a) with variable degrees of cytoplasmic vacuolization, no lumen formed in many acini & ducts (L) and intralobular ducts lined by a non striated, columnar epithelium with large nucleus (D) (H&E x400).

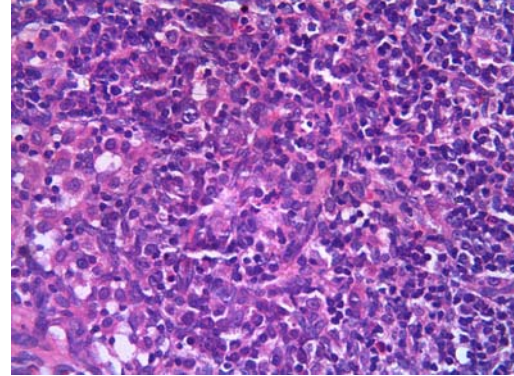


Fig. (5): A photomicrograph of submandibular gland of 2 weeks old offsprings (experimental group): showing: the parenchyma cells with high mitotic activity and loss of acinar form. (H&E x 400).

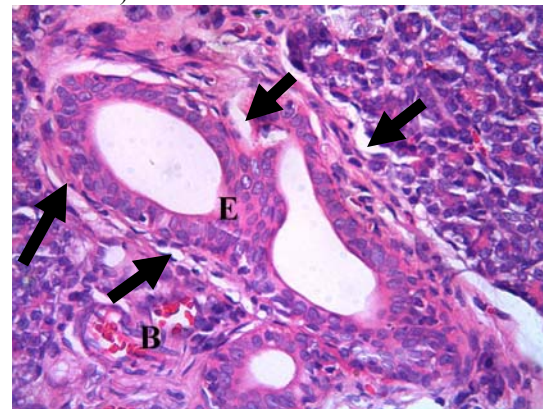


Fig. (6): A photomicrograph of submandibular gland of 2 weeks old offsprings (experimental group) showing: stratification of the nuclei of the excretory ducts (E), vacuolation of the connective tissue stroma (small arrows) and dilated blood vessels (B) (H&E x 400).

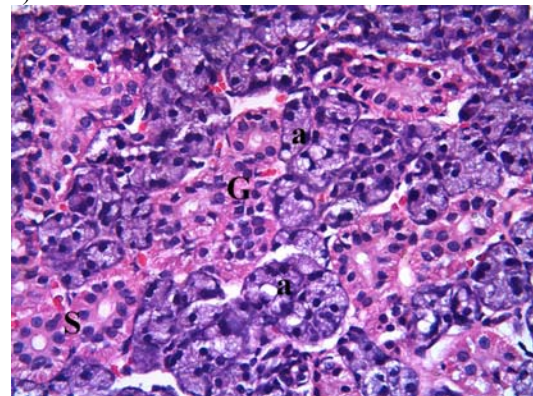


Fig. (7): A photomicrograph of submandibular gland of 6 weeks old offsprings (Experimental group) showing serous acini with variable degrees of cytoplasmic vacuolization (a), striated duct (S) granular convoluted tubules (G) and numerous extravasated red blood cells (H&E x 400)

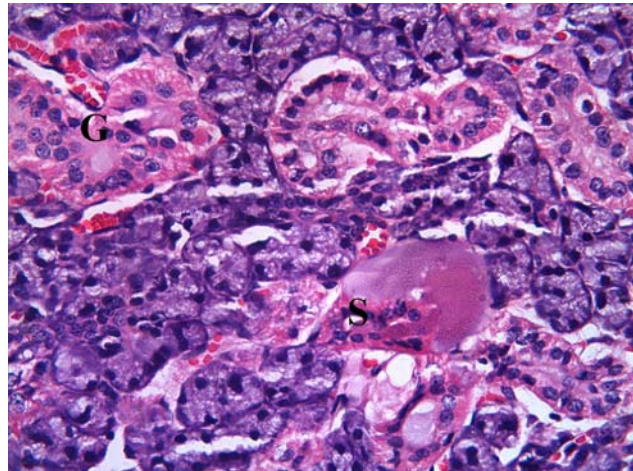
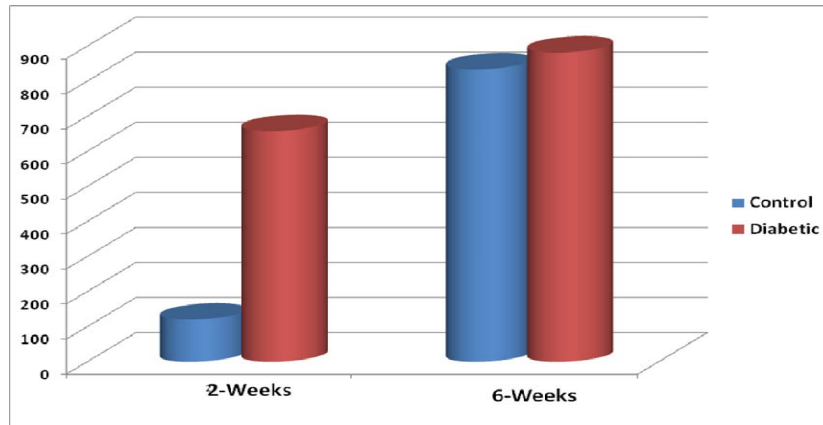


Fig. (8): A photomicrograph of submandibular gland of 6 weeks old offspring (Experimental group) showing striated ducts (S) & convoluted tubules (G) with stagnant secretion inside and outside their lumen (H&E x400)

Table I: Difference in mean total acinar area between different groups using ANOVA statistical test

Group	mean total acinar area		
	M±SD	F-Value	p-Value
Control 2 week	120±25.4	41.87	0.000**
Diabetes 2w	658±184		
Control 6 week	834±237		
Diabetes 6w	882±160		

\*\*High Significant difference, (p<0.01).

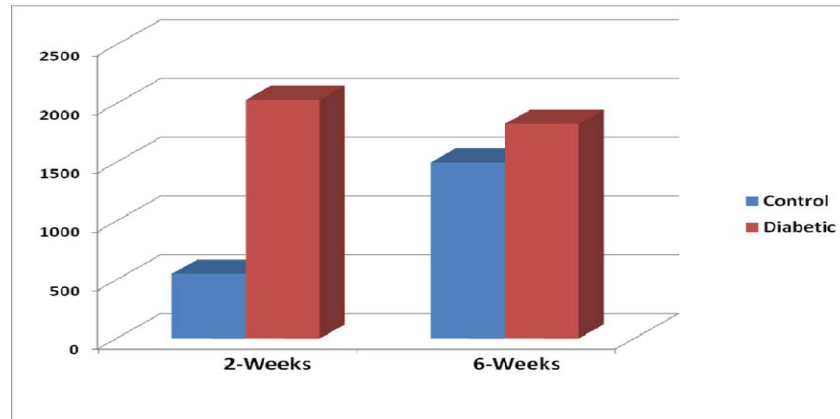


Graph I: Represents difference in mean total acinar area in μm between different groups

Table II: Difference in mean total duct area between different groups using ANOVA statistical test

Group	mean total duct area		
	M±SD	F-Value	p-Value
Control 2 week	550±115	8.418	0.001**
Diabetes 2w	2032.45±696.03		
Control 6 week	1499.44±495.02		
Diabetes 6w	1825±310		

\*\*High Significant difference, (p<0.01).



Graph II: Represents difference in mean total duct area in  $\mu\text{m}$  between different groups

#### 4. Discussion:

Effects of experimental diabetes on rat submandibular glands have been documented, but the effect of diabetes on offsprings' of diabetic mothers was not investigated. To our knowledge this is the first report about histomorphometric study of submandibular glands in offsprings of diabetic mothers.

In the present study, histological examination of the control submandibular gland at 2 weeks old offsprings revealed progressive differentiation of the acinar cells and ductal components. Increase in the number of acini and ducts through mitotic activity. Also 6 weeks old offsprings showed the architecture of nearly mature normal submandibular glands. It consisted of serous acini, and few serous acini with demilune, various types of ducts, granular convoluted tubules and connective tissue stroma. Our findings were in accordance with **Srinivasan and Chang 1975 & Gresik 1980** they reported that the submandibular glands are important secretory organs in vertebrates, consists of terminal portions and a ductal system. Development of rat submandibular glands starts at 14 days in utero and continues after birth. Both proliferation and differentiation remain active until 3–4 weeks after birth. All the parenchymal structures appear during the first 6 weeks. **Pardini & Taga (1992)** reported that, the growth of submandibular gland during postnatal life is the result of two basic mechanisms, an increase in the absolute number of cells through mitotic activity and an increase in individual cell volume. The studies of **Denny et al., (1993)** on growth of submandibular gland cells in rats and mice using 3H-thymidine have shown a significant increase in the number of convoluted granular tubule cells during the ductal phase. This increase was initially due to the differentiation of pre-existing striated duct cells, followed by mitotic activity of recently differentiated cells of the compartment itself, and the proliferation of

intercalated duct cells. The intercalated duct has been suggested as a source of the acinus and the granular duct **Man et al., (2001)**. In addition **Ogawa et al., (2003)** suggested that full maturation of submandibular gland in rodents was completed only after birth. Also the results found in this study represented significant increase in the mean total acinar and ductal areas in both control groups at two weeks and six weeks old offspring.

Proliferation of acinar cells occurred at 2 and 6 weeks offsprings was obvious in diabetic group than control group, and acinar cell hypertrophy continued during the experimental period. Also our morphometric findings demonstrated that the mean total acinar areas of the two weeks old offsprings of experimental group was significantly greater than the corresponding values of the control group this might be due to disturbance in apoptosis rate under the influence of diabetes. These findings were in accordance with those experienced on the pancreas of the offsprings of diabetic rats. **Tomei and Cope (1991)** reported that apoptosis has been implicated as the mechanism responsible for reducing cell numbers during the regression of parenchymal hyperplasia that occurs in many organs during development or cell differentiation. Also **Walker et al., (1992)** stated that apoptosis may also be responsible for reducing the number of terminal tubule cells in the male rat submandibular glands. Moreover, **Kasperska et al., (1996) & Dabelea et al., (2000)** demonstrated that maternal diabetes induces many metabolic and functional aberrations in offspring pancreatic islets that lead to impaired insulin secretion. In addition **Badawoud (2006)** suggest that during pregnancy maternal diabetes stimulates the hypertrophy and proliferation of foetal endocrine pancreas and this effect continues to appear in the early days after birth. They also reported that fetus is confronted with severe intrauterine hyperglycemia which induces fetal islet hypertrophy and  $\beta$ -cell hyperactivity and may result in

early hyperinsulinemia. This overstimulation of fetal  $\beta$  cells limits their adaptation, and they become depleted of insulin granules, and incapable to secrete insulin.

In the present study we chose to treat the animals with alloxan before pregnancy to completely eliminate exposure of the fertilized embryo to the toxin of the alloxan. The offsprings in the diabetic group were less body weight than offsprings of nondiabetic group this finding agrees with **Aerts, et al., (1990)** they reported that  $\beta$ -cell exhaustion results in fetal hypoinsulinemia. Hypoinsulinemia and a reduced number of insulin receptors on target cells lead to a reduction in fetal glucose uptake. The growth of fetal protein mass is suppressed, and fetal protein synthesis is consistently low, leading to fetal microsomia. Postnatal development is retarded, and these offsprings remain small at adulthood and they develop insulin resistance. In addition, **Holemans et al., (1991)** found that the alloxan, when administered at a high single dose, the offspring was remained small throughout the experiment than control group. However **Soulimane et al., (2005)** reported that macrosomia was established through administration to pregnant rat five low doses of streptozotocin starting on day 5 of gestation. This different timing of diabetes induction may explain the divergent of the present results. Moreover **Yessoufou & Moutairou (2011)** suggested that studies in humans maternal diabetes appears to be an important risk factor for fetal obesity or macrosomia. Alterations in macrosomic infants persist postnatally and conduct to several abnormalities including development of insulin resistance, obesity, diabetes, and metabolic syndrome at adulthood.

In the present study lumens of many ducts were stagnated with retained secretory material. These changes might reflect retardation in glandular development and function leading to impaired salivary flow in addition to the reduction in the secretory cells activity. The changes in acinar cells were characterized by formation of many intracytoplasmic vacuoles. This might be due to lipid accumulation within the acinar cells. Accumulation of adipose tissue in the connective tissue stroma was detected. These results were in accordance with **Reuterving et al., (1987)** they found reduction in the structure and function of the rat salivary gland after induced diabetes by long term exposure to alloxan. They have stressed that lipid droplets begin to form in acinar cells of the submandibular glands and the amounts of these droplets are related with the blood glucose levels. **Anderson (1998)** reported that the appearance of lipid in the parenchymal cells of submandibular gland was rapid, beginning within 24 hours after the induction of diabetes, and it persisted indefinitely in the absence of insulin treatment. **Anderson & Garrett (2004)** suggested that lipid accumulation within parenchymal

cells varied with the type of gland and was more pronounced in animals with the highest serum-glucose levels. Secretory cells of parotid and sublingual glands accumulated the greatest amount of lipid. Lesser amounts were present in seromucous acinar cells of submandibular glands. In addition **Padilha et al., 2007** found that alterations in organs resulting from maternal diabetes are related to hyperglycemia and fetal hyperinsulinemia, which affect lipid and protein synthesis. Furthermore, maternal hyperglycemia stimulates fetal growth due to the greater availability of glucose in the blood flow and the regulation of growth factors (**Maayan et al., 2009**).

Our histological results revealed many degenerative changes in the parenchymal element of the submandibular glands in the offsprings of diabetic mothers. This finding was in accordance with **Eriksson & Borg (1993)**. They demonstrated that rat embryos cultivated with medium containing a high concentration of free radical reactive oxygen species ROS show growth retardation and severe malformation. Also **Raza & John (2004)** proposed that the most widely hypothesis for  $\beta$ -cell damage during diabetic pregnancy is the increase of free radical reactive oxygen species (ROS) induce damage to the developing fetus. In neonates of diabetic mothers, ROS levels are higher in several tissues. In addition studies on pancreas by **Junying et al., (2007)** postulate that the damage of  $\beta$  cells in the offsprings of the diabetic group was due to increase in the level of free radical oxygen species during the diabetic pregnancy and the abnormally high glucose levels which prevent the up regulation of certain enzymes during pregnancy, making the  $\beta$  cells more susceptible to damage during the period of maternal diabetes.

The mean total glandular duct areas of six weeks old offsprings of diabetic group was not significantly greater than the corresponding values of control group. Moreover number and development of the granular convoluted tubules was reduced under the effect of diabetes. This finding was in accordance with **Gamal, et al., (2011)**. They found that, diabetes caused significant reduction in the mean count of granular convoluted tubules. In addition, epidermal growth factor (EGF) is produced in rodents mainly by the granular convoluted tubules in submandibular glands. EGF deficiency in diabetes mellitus could be implicated in the complications of the disease.

#### **Conclusion:**

The present study showed that maternal diabetes had a significant effect on offsprings submandibular glands. Maternal diabetes induced different histological and morphometric changes in submandibular gland of young offspring.

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