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Histological and Immunohistochemical Study of Macrophages in Salivary Glands of Control and Isoproterenol Treated Adult Male Albino Rats

Dr. Hussein Hammami*
Dr. Ezzat Abdul Aziz El-Dreiny**
Dr. Najlaa Ibrahim Sarhan***

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□ ABSTRACT □

Salivary glands are important effecter site in the mucosal immune network that possesses monocytic population which regulates and mediates the immune response contributing to the protection of oral surface. Isoprenaline is a sympathomimetic beta adrenergic. It is structurally similar to epinephrine but acts selectively on beta receptors. This work was carried out in order to study the macrophages in the major salivary glands in control and isoproterenol treated adult male albino rat. Salivary glands specimens were processed for histological study by LM & EM and stained immunohistochemically for detection of macrophage using CD68**.** LM examination of the major salivary gland sections of isoproterenol treated group revealed wide monocytic infiltration in their connective tissue septa. Immunohistochemical study displayed significant increase in the CD68 positive cells in the submandibular and specifically and more frequently in the sublingual salivary glands. Several forms of macrophages in different functional states were identified in the form of resident, adherent migrating, activated and giant macrophages. EM examination revealed many activated macrophages. It could be concluded that population composition of salivary macrophage is different in salivary glands, and its role in mucosal oral immunity.

Keywords: Macrophage; Salivary Gland, Isoproterenol, CD68. Albino Rat.

*Professor, Pathology & Histology Department, Faculty of Dentistry, Tishreen University, Lattakia, Syria.
** Professor, Head of Histology Department, Faculty of Medicine, Tanta University, Tanta, Egypt.
***Assistant professor, Histology Department, Faculty of Medicine, Tanta University, Tanta, Egypt.

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دراسة نسيجية وكيميائية نسيجية مناعية للبلعميات الكبيرة في الغدد اللعابية الشاهدة والمعالجة بالأيزوبروترينول في ذكر الفأر الأبيض البالغ

الدكتور حسين علي حمامي*
الدكتور عزت عبد العزيز الدريني**
الدكتور نجلاء إبراهيم سرحان***

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□ ملخّص □

تلعب الغدد اللعابية دوراً كبيراً فى المناعة الموضعية للفم وذلك لاحتوائها على نسبة كبيرة من الخلايا أحادية النوى. يعد الأيزوبرينالين (أيزوبروترينول) أحد الأدوية التى تماثل الأدرينالين فى تركيبه وعمله. لقد أجرى هذا البحث لدراسة بلعميات الغدد اللعابية الضابطة والمعالجة بالأيزوبروترينول فى ذكر الفأر الأبيض البالغ. تم أخذ عينات الغدد اللعابية لفحصها بالمجهر الضوئى والإلكتروني والكيمياء النسيجية المناعية باستخدام CD68 للكشف عن هذه البلعميات. أظهر المجهر الضوئى للمجموعة المحقونة بالأيزوبروترينول وجود ارتشاح خلايا وحيدات النوى كثيرة فى النسيج الضام، كما لوحظ زيادة ذات دلالة إحصائية فالتفاعل المناعى للبلعميات CD68 فى الغدد التحت فكية وخصوصاً في الغدة تحت لسانية. كما لوحظ وجود خلايا وحيدة النواة ملاصقة للغشاء البطانى للأوعية الدموية وخلايا مهاجرة ونشطة وعملاقة. كما أظهر المجهر الإلكتروني وجود العديد من البلعميات النشطة.

نستنتج من هذه الدراسة اختلاف في توزع مجاميع البلعميات فى الغدد اللعابية. وهذا يعطى مؤشراً هاماً على دور هذه الغدد فى المناعة الموضعية لمخاطية الفم.

الكلمات المفتاحية: البلعميات الكبيرة،الغدد اللعابية، إيزوبروترينول،CD68 ، فأر أبيض.

* أستاذ – قسم النسج والتشريح المرضى– كلية طب الأسنان– جامعة تشرين– اللاذقية– سورية.
** أستاذ – قسم النسج – كلية الطب– جامعة طنطا– طنطا– مصر.
*** أستاذ مساعد – قسم النسج – كلية الطب– جامعة طنطا– مصر.

Introduction:

The mouth is the largest natural opening in the human body, and is a major component in the mucosal barrier system. It has its own immune barriers, which is called the oral immune system (Antonio,2008). Three pairs of major salivary glands; parotid, submandibular and sublingual glands were distinguished and described to be the main component of the oral immune system (O'Sullivan et al., 2001). They harbor antigen-presenting cells which may function in the generation of local mucosal immunity of the mouth cavity. Although the functions of these cells have yet to be completely described, they appear to regulate some aspects of normal tissue physiology and influence significantly the expression of immune and inflammatory responses. Macrophages interact with extracellular products, internalize proteins and polysaccharides and present antigen to lymphocytes. (Stasulis & Hand, 2003and Estecondo S, Codón M S & Casanave E M 2005). The distribution of mononuclear phagocyte in normal salivary gland tissue and their influence on salivary function have not been completely described.

Isoprenaline (Isoproterenol) is a sympathomimetic beta adrenergic agonist medication. It is structurally similar to epinephrine (adrenaline) but acts selectively on beta receptors. Its primary use is for bradycardia and heart block as it induces positive chronotropic and inotropic effects. It can be used as an inhalation aerosol to treat asthma by relaxing the airways to increase airflow. It is also supplied in ampoules and in sublingual pill form for treatment of chronic bronchitis and emphysema (Reynold J F (1996)). Rats treated with isoproterenol have been widely used to study salivary gland physiology and biochemistry because isoproterenol, increases salivary gland DNA synthesis and cell division and increases saliva flow (Denny and Denny, 1981). It also induces synthesis of novel proteins in salivary gland like cyctatins and enhances expression of B_1 and B_2 adrenoreceptor (Bedi, 1989 and Li et al.,2009).

Isoproterenol has been used in clinical trials to treat salivary hypofunction, xerstomia and acinar cell atrophy resulting from radiation therapy (Li et al.,2009) The effects of isoprenaline on salivary glands have been extensively studied in rodents. It was proven in some experimental studies that isoproterenol induces a mild inflammatory response within the submandibular glands that is not observed in normal glands (Cohen et al., 1995). The potential inflammatory changes and subtle alterations in the macrophage subpopulations could have an important impact on the physiological function and immune regulation of these glands.

Based on these facts, this work was carried in order to study the number, distribution and structure of the macrophages in the major salivary glands in control and isoproterenol treated adult male albino rat.

Materials and Methods:

The present study was carried out in histology department faculty of medicine Tanta University from September third to December tenth 2010 on 20 adult male albino rats, weighing from 180 to 200 gm. They were housed in clean properly ventilated cages under the same environmental conditions with free access to food and water throughout the whole period of the experiment. The animals were divided into two groups:

Group I (Control group): included 10 animals that were further subdivided into two subgroups (five rats each):

Subgroup (i) kept without treatment throughout the whole period of the experiment.

Subgroup(ii) received 1 ml physiological saline intraperitoneally (the vehicle of isoproterenol).

Group II (isoproterenol treated animals): included 10 animals that received 20mg/Kg /day (Sigma Chemical Company St Louis,MO,USA) by intraperitoneal injection once daily for 10 consecutive days between 8-9 a.m. This regimen of treatment was carried according to Cohen et al., (1995).

At the appropriate time, all animals were sacrificed under anaesthesia and perfused with 4% Paraformaldehyde in 0.1 M sodium phosphate buffer (pH7.4) containing 2.5 glutraldehyde solution. After perfusion, specimens of the glands were obtained by careful dissection of the skin from the ventral and lateral surfaces of the neck and from the side of the head as far as the corners of the mouth. The parotid gland is triangular in shape, its apex lying behind the base of the ear. The submandibular gland is long and oval gland lying on the ventral aspect of the neck. The sublingual gland is present craniolateral to the submandibular gland. For light microscopic study, specimens of the glands were fixed in 4% paraformaldehyde for preparation of paraffin blocks. Five μm sections were cut and stained by Hematoxylin and Eosin (Drury & Wallington, 1980).

For CD-68 immunohistochemical study, the sections were deparaffinized in zylene, rehydrated and incubated with 3% hydrogen peroxide to block the endogenous activity of peroxidase. Microwave–assisted antigen retrieval was performed for 20 minutes. Sections were then incubated overnight at 4 Cº with primary rabbit anti-rat CD-68 antibody, a specific marker of macrophages (Neo Markers Fremont CA, Lab Vision). After washing with PBS (phosphate buffer solution), sections were incubated with biotinylated goat anti rabbit secondary antibody for 30 minutes and then with streptavidin peroxidase conjugate. Sections were then washed with PBS, and incubated with diaminobenzidine (DAB) chrmogen to detect immunoreactivity. Counterstaining was performed by Mayer's haematoxylin. CD-68 positive cells will be visualized as brown coloration of the cytoplasm of the macrophage. Negative controls were done using the same steps except that phosphate buffered saline was applied instead of the primary antibody (Bancroft & Cook , 1994).

For electron microscopic examination, the glandular specimens were immediately immersed in 2.5% phosphate buffered gluteraldehyde (pH 7.4) at 4̊ C for one hour and processed to be examined by EM. Specimens were washed three times in phosphate buffer solution and post fixed in 1% phosphate buffered osmium tetraoxide for one hour, then dehydrated in ascending grades of ethanol. After immersion in propylene oxide, the specimens were embedded in epoxy resin mixture. Semithin sections (1μm) were cut, stained with toluidine blue and examined by light microscopy. Ultrathin sections (80-90nm) were obtained and stained with uranyl acetate and lead citrate, and examined and photographed using JEOL transmission electron microscopy in EM Unit, Faculty of Medicine, Tanta University (Bozzola & Russel, 1999).

Quantitative Morphometric Measurement and Statistical Analysis:

The number of CD-68 positive cells was counted in ten non overlapping fields from each slide of different glands of the different animals of each control and isoprotrenol treated rats at X400 magnification using the Leica Qwin 500 C image analyzer computer system (Leica Imaging System LTD., Cambridge, England) in Faculty of medicine Al-Azhar University and were expressed as cell number per μm^2. Values were represented as mean $\pm$ standard deviation. Statistical analysis was performed using student's t-test, where

the results were considered statistically significant if $p<0.05$ and highly significant if $p<0.001$.

Results:

Light microscopic results

Control group

Light microscopic examination of hematoxylin and eosin stained sections of the major salivary glands of the control group revealed that they were consisted of series of branched ducts terminating in acini of pure serous nature in parotid glands (Figs.1) and serous acini with mucous tubules capped with serous demilune in submandibular glands (Figs.2) and mucous tubules with serous demilune in sublingual glands (Figs.3). Connective tissue septa of the examined glands of the control group revealed absence of mononuclear cellular infiltration in parotid (Fig.1). Some mononuclear cellular infiltrations were encountered in submandibular gland (Fig.2) and many in sublingual glands (Fig.3). Toluidine blue stained semithin sections of the same group revealed very few resident macrophages in the connective tissue septa of the parotid (Fig.4). Submandibular and sublingual glands of the same group revealed apparent increase in the number of the resident macrophages with special predominance in sublingual gland in comparison to the parotid (Figs.5&6). Resting or fixed macrophages were seen with irregular outlines bearing numerous processes and oval or kidney shaped nucleus. (Fig.4), while motile macrophages were more rounded (Fig.5).

The immunoperoxiades staining of the normal salivary glands with CD68 confirmed the previous findings and demonstrated positive dendritic cells with relatively oval nuclei, little cytoplasm and numerous slender processes. Apparently, few of them were detected in parotid gland (Fig.7) while, normal submandibular and sublingual salivary glands exhibited many CD68 positive cells in the connective tissue septa and at the periphery of the acini (Figs.8&9).

Isoproterenol treated group

Light microscopic examination of H&E stained sections of the major salivary glands of the isoproterenol treated group revealed presence of many areas of mononuclear cellular infiltrate in the connective tissue septa and in between the acini of the parotid gland mainly and specifically of the submandibular and sublingual salivary glands (Figs10,11&12). Semithin sections of the same group revealed that macrophages were prominent cells in the mononuclear cellular infiltrate in the three major salivary glands. Few active macrophages were observed in the connective tissue septa of the parotid mainly perivascular (Figs. 13). Connective tissue septa of the submandibular and sublingual glands displayed large number of active macrophages characterized by apparent increase in their size and elongation of their processes in the perivascular and interacinar connective tissue (Figs. 14&15).

CD68 reaction of three major salivary glands of the same group confirmed the previous findings, where few CD68 positive cells were encountered in the connective tissue septa of the parotid gland (Fig.16). Many CD68 positive cells were observed in the submandibular and (Fig.17) more frequent in the sublingual glands (Fig.18). Adherent and emigrating macrophages from the nearby blood vessels were also observed in the submandibular salivary glands (Fig.19). Furthermore, multinucleated giant macrophages with abundant cytoplasm were more prominent in the submandibular and sublingual salivary glands (Figs.19&20).

Electron microscopic results

Electron microscopic examination of the macrophages of the control group of the salivary gland revealed the well known electron microscopic character of the macrophage. They showed irregular outlines and many pseudopodia with oval or kidney shaped nucleus. The cytoplasm contained mitochondria and few phagocytic vacuoles. (Fig.21). Moving macrophages were characterized by withdrawal of most of their processes except few of pseudopodia displaying long sleeve like extension (Fig.22). Macrophages of the salivary gland of isoproterenol treated group revealed apparent increase in its size with numerous slender villous projection, ridges, pits, and indentations. The cytoplasm displayed multiple fused mitochondria and many phagocytic vacuoles (Fig.23). Some cells were seen in the attachment phase of phagocytosis where their cellular processes trying to enclose the foreign particle (Fig.24).

Morphometric and statistical studies

Morphometric analysis revealed non significant increase in the CD68 positive cells in the parotid gland of the isoproterenol treated group in comparison to the control group. Submandibular and sublingual salivary gland displayed respectively significant and highly significant increase in the CD68 positive cells in comparison to their correspondents of the control group. The immunoperoxiadase staining of the salivary glands with CD68 was demonstrated in table (1) and diagram (1).

Table (1): The mean number of CD 68 positive cells in the rat salivary glands of the different studied groups.

Glands	Parotid gland		Submandibular gland		Sublingual gland	
Group	Control	Iso-proterenol	Control	Iso-proterenol	Control	Iso-proterenol
Mean	34	66	75	120	90	167
±SD	8.36	9.87	12.74	18.93	14.52	23.80
T. test	2.351		4.365		7.861	
p. value	0.058 NS		0.022*		0.001**	

SD=Standard deviation. NS=Non significant.
***= significant **=Highly significant**

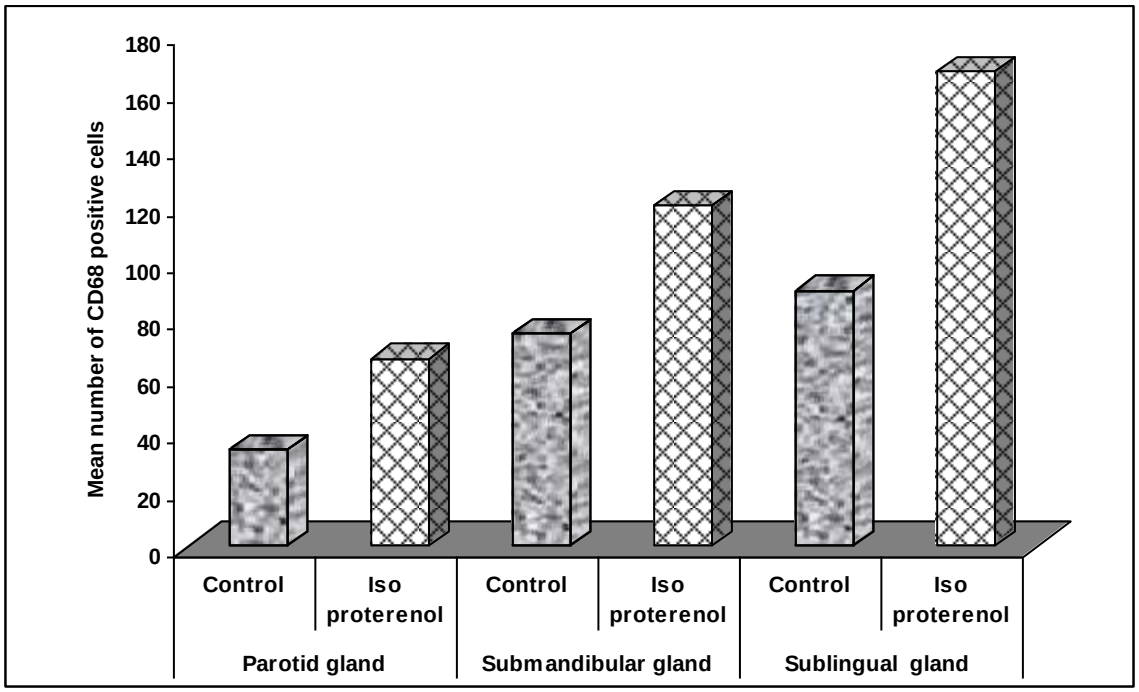

Diagram (1): The mean number of CD 68 positive cells in the rat salivary glands of the different studied groups.

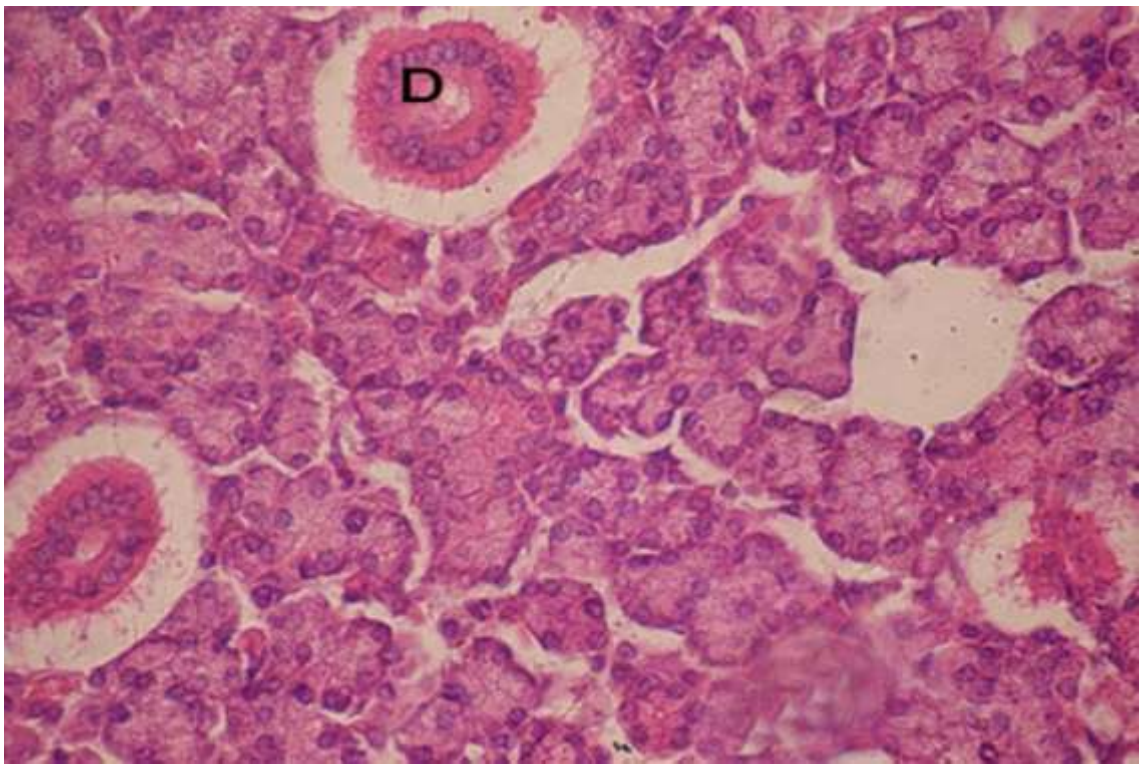

Fig.(1): A photomicrograph of paraffin section of the parotid gland of the control group showing multiple serous acini and striated ducts (D). Notice absence of mononuclear cellular infiltration in the connective tissue septa (H & E x 400).

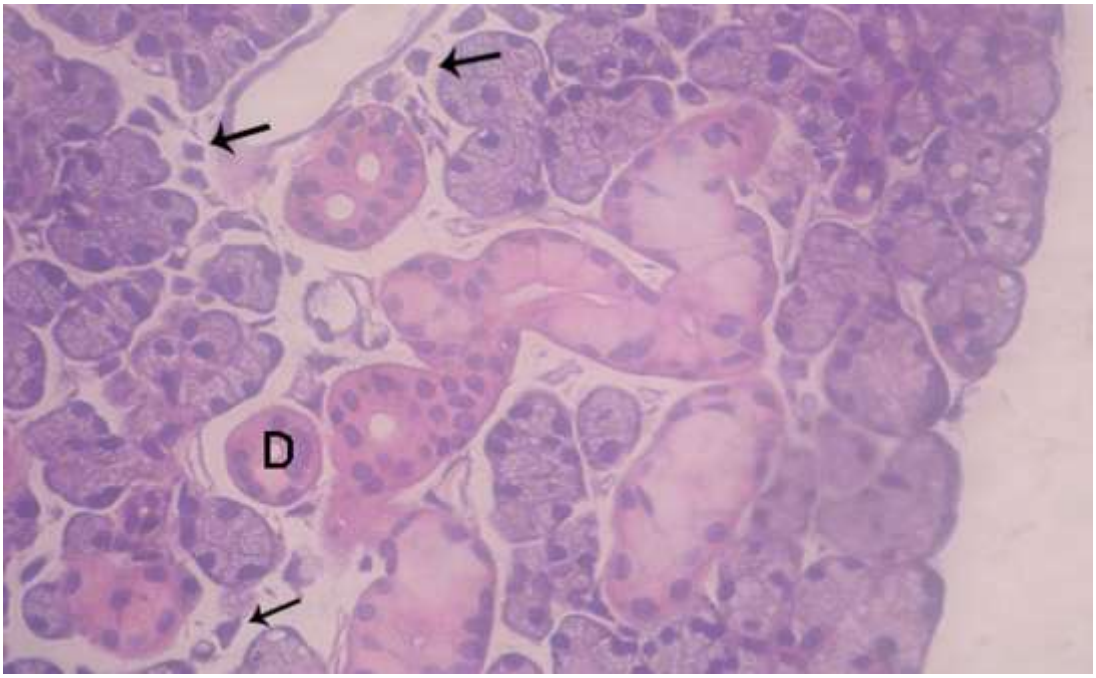

Fig.(2): A photomicrograph of paraffin section of the submandibular salivary gland of the control group showing serous and mucous acini and striated ducts(D). Notice mononuclear cells in the connective tissue septa (→) (H & E x 400).

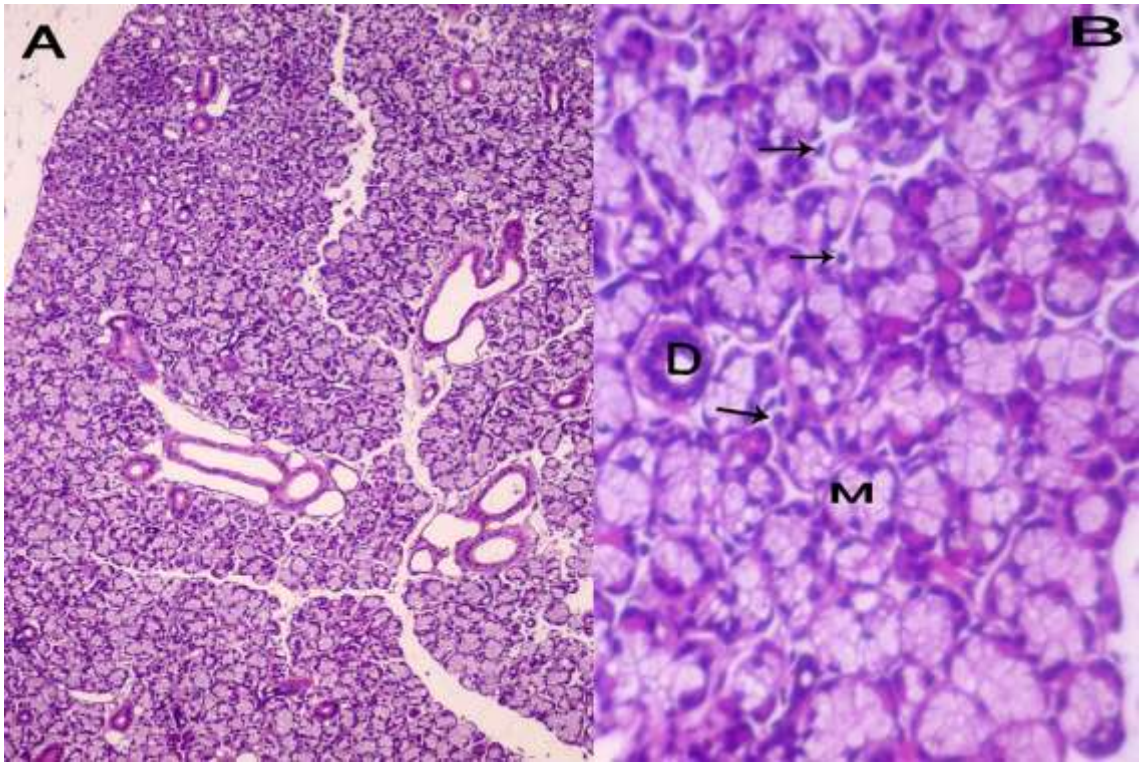

Fig.(3): A photomicrograph of paraffin section of the sublingual salivary gland of the control group showing multiple mucous acini (M) with serous demilune and striated duct (D). Notice mononuclear cells in the connective tissue septa (→)(H & E. A x 200 and B x 400).

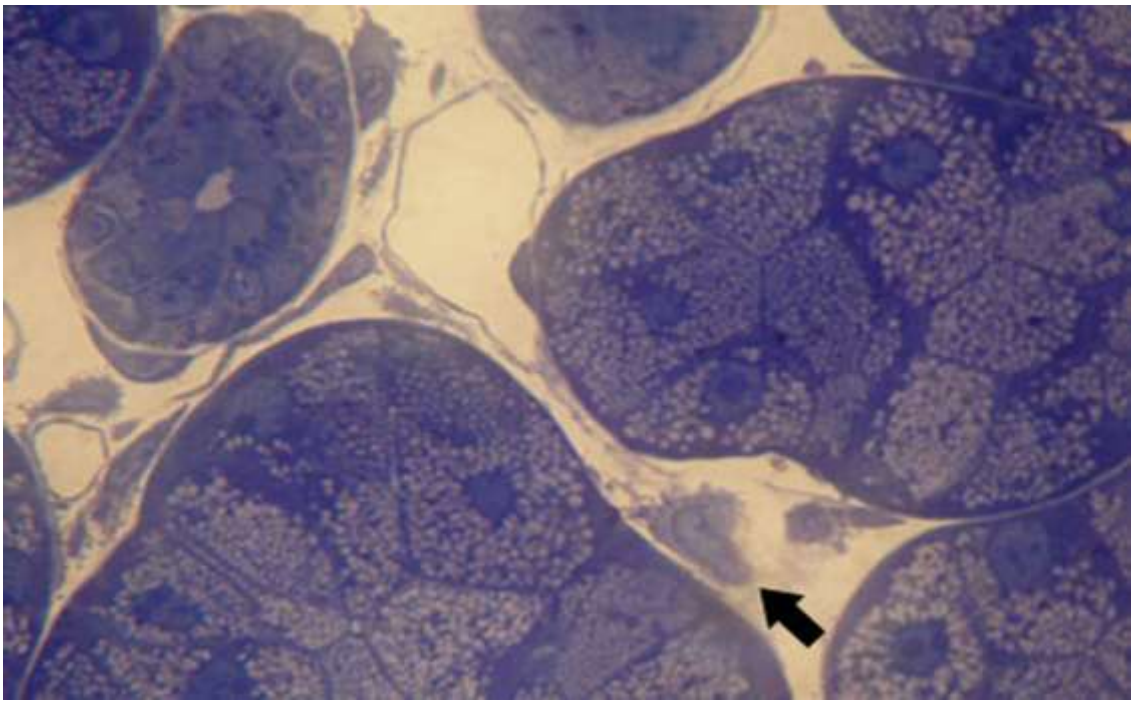

Fig.(4): A photomicrograph of a semithin section of the parotid gland of the control group showing a macrophage with irregular outline and oval or kidney shaped nucleus in the connective tissue septa (→) (Toluidine blue x1000).

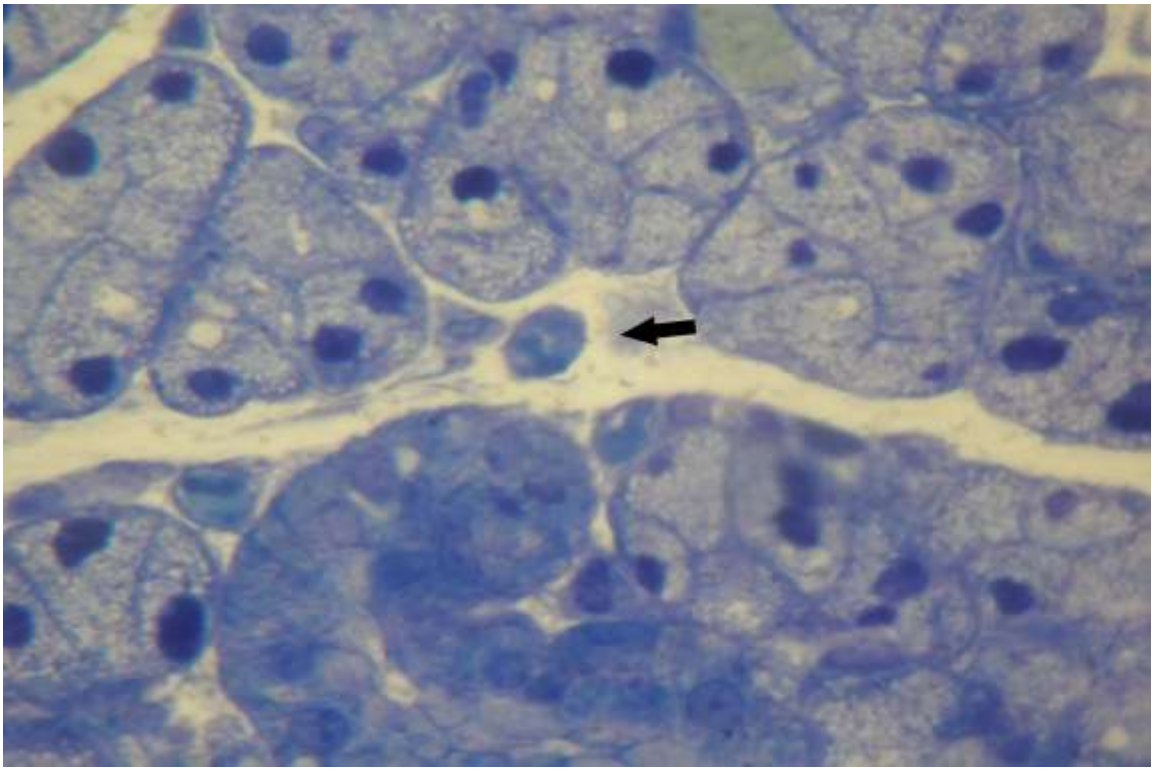

Fig.(5): A photomicrograph of a semithin section of the submandibular salivary gland of the control group showing a macrophage with smooth rounded outline in the connective tissue septa (→) (Toluidine blue x 1000).

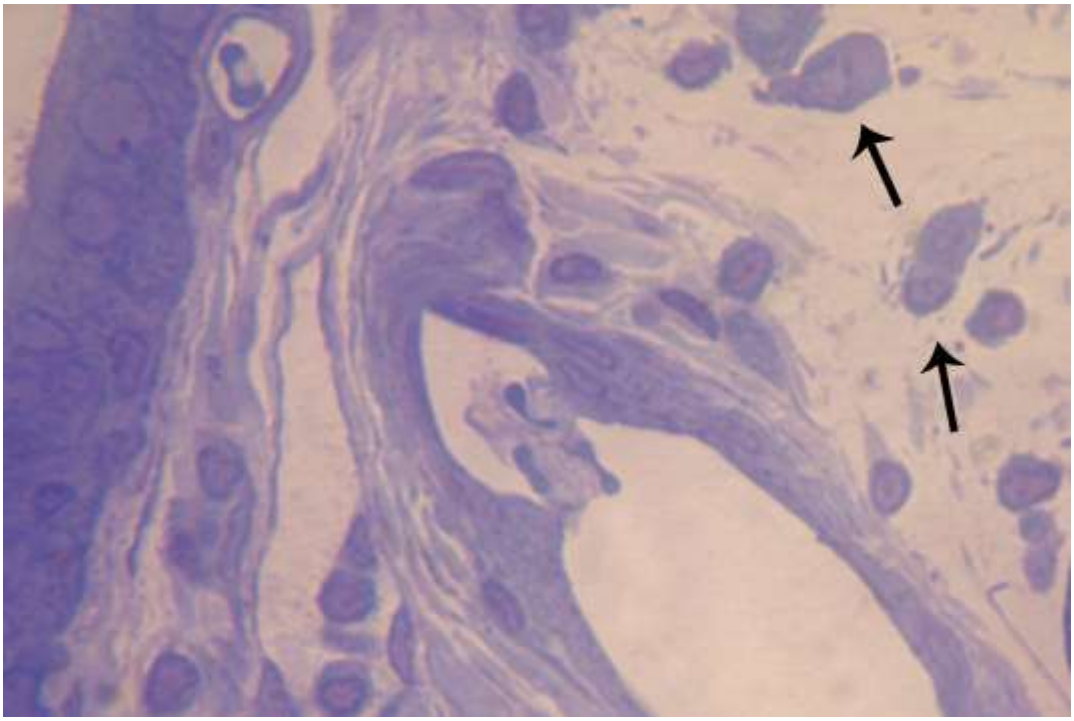

Fig.(6): A photomicrograph of a section of the sublingual salivary gland of the control group showing macrophage in the connective tissue septa (→) (Toluidine blue x 1000).

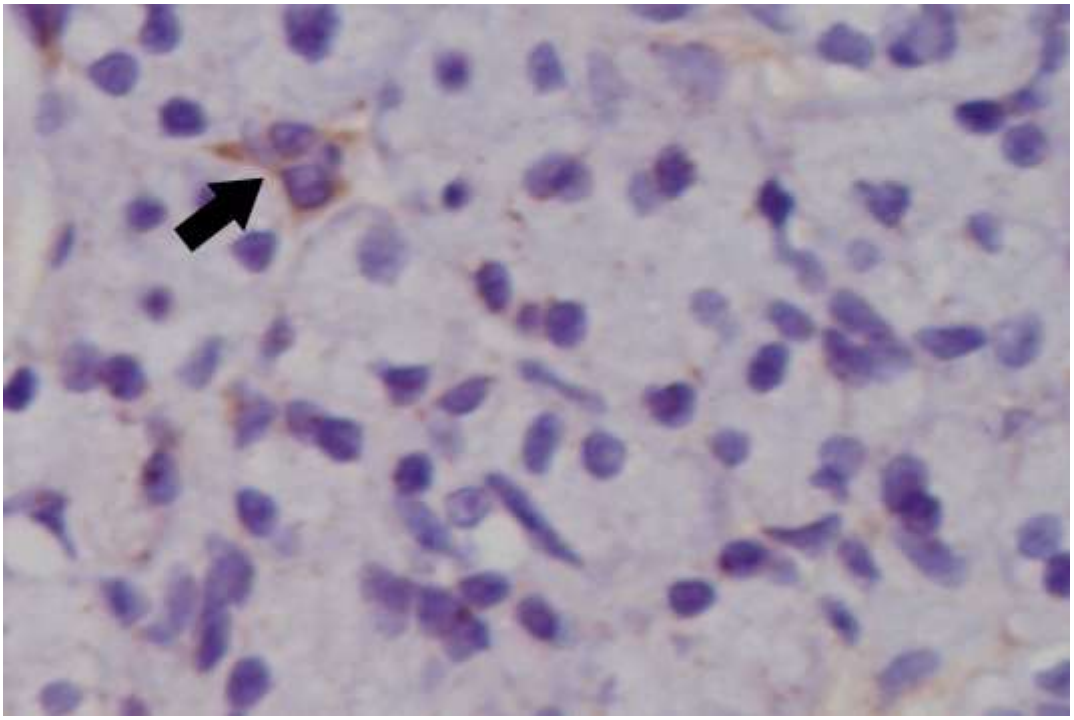

Fig.(7): A photomicrograph of a section of the parotid gland of the control group showing CD68 positive cell (→) (CD68 immunostaining x1000).

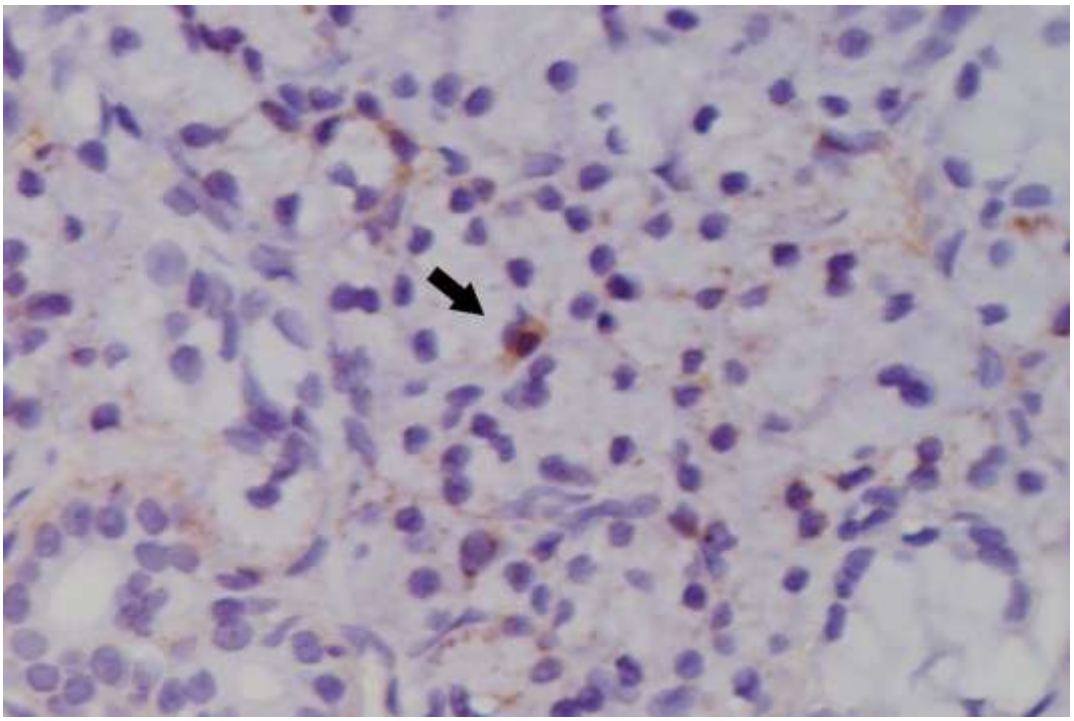

Fig.(8): A photomicrograph of a section of the submandibular salivary gland of the control group showing CD68 positive cell(→) (CD68 immunostaining x1000).

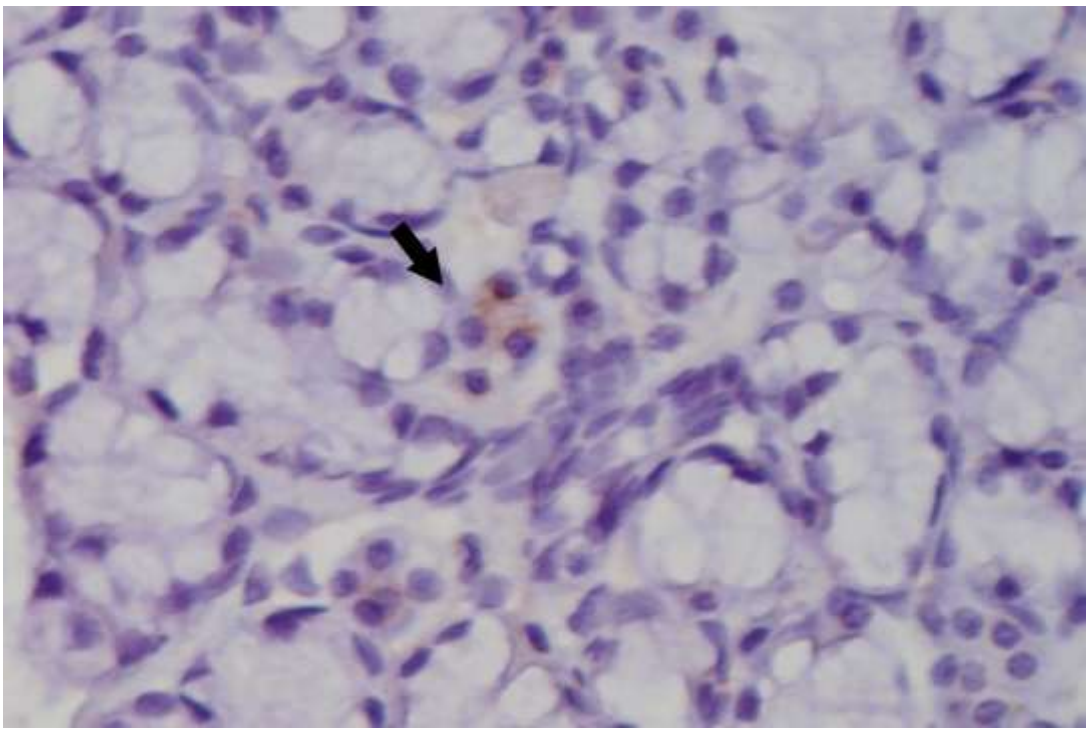

Fig.(9): A photomicrograph of a section of the sublingual salivary gland of the control group showing positive CD68 cell in the connective tissue septa (CD68 immunostaining x1000).

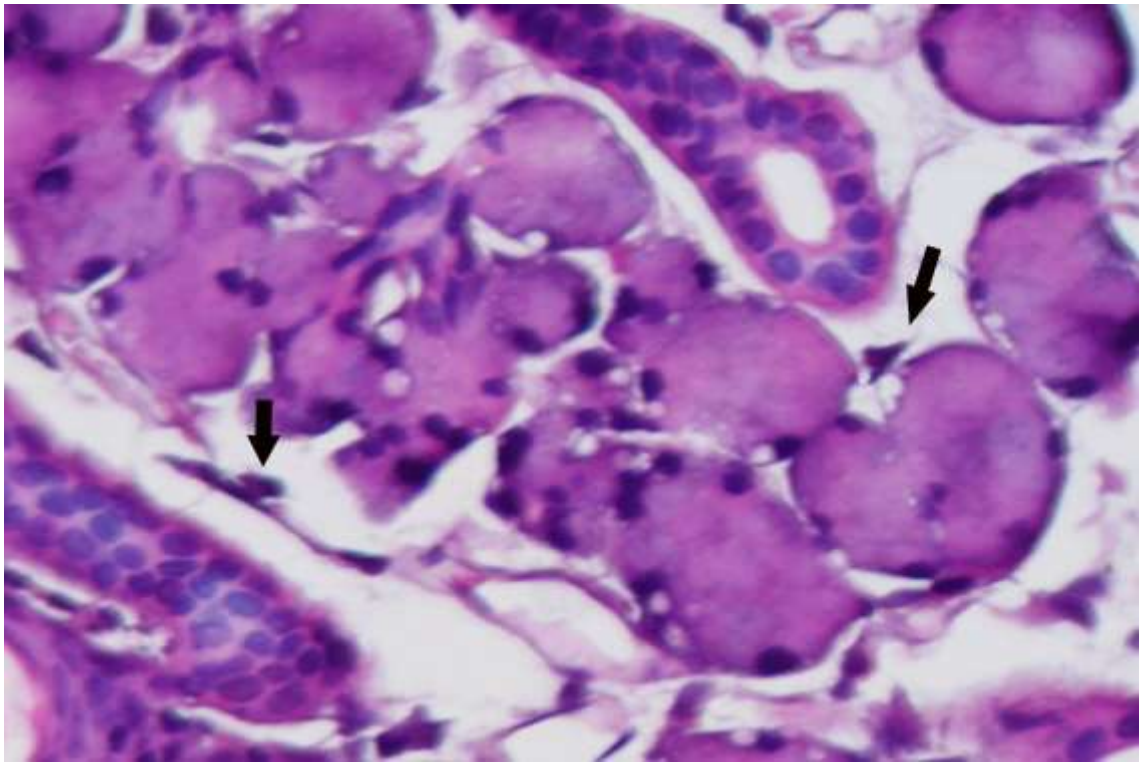

Fig.(10): A photomicrograph of a section of the parotid gland of the isoproterenol treated rats showing very few mono nuclear cells(→) in the connective tissue septa (H & E x 1000)

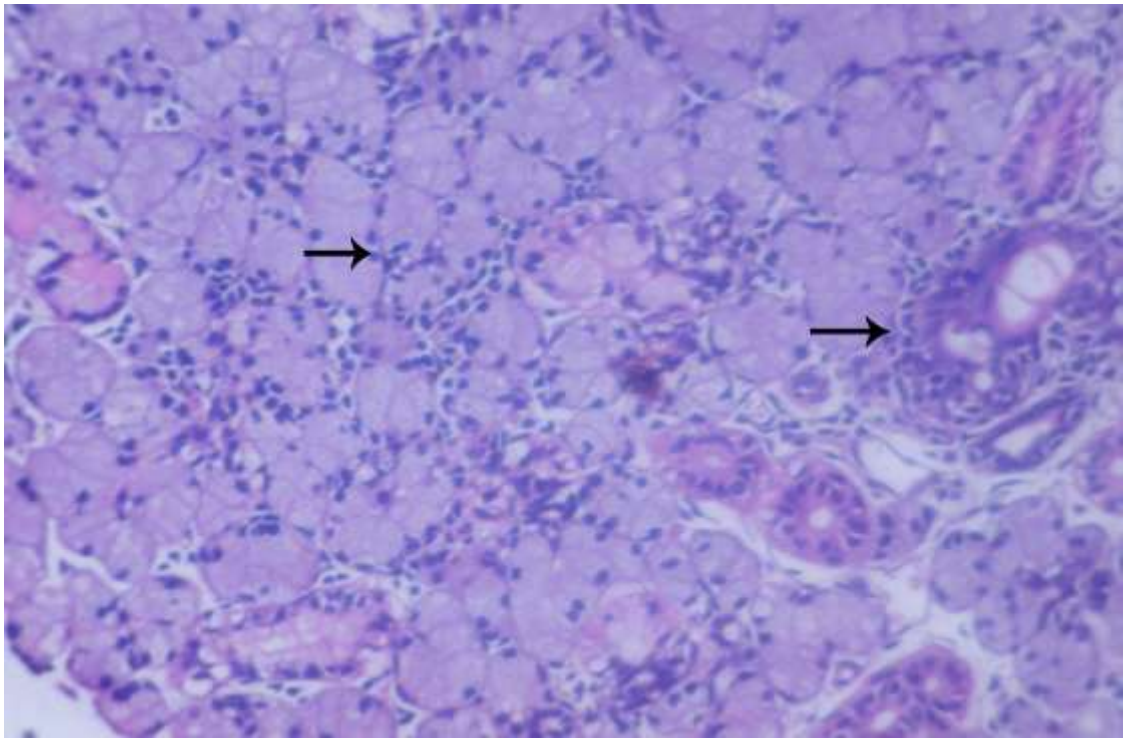

Fig.(11): A photomicrograph of a section of the submandibular salivary gland of isoproterenol treated rats showing extensive areas of mononuclear cellular infiltration in the interlobular and in the periacinar connective tissue (→)(H & E x 400).

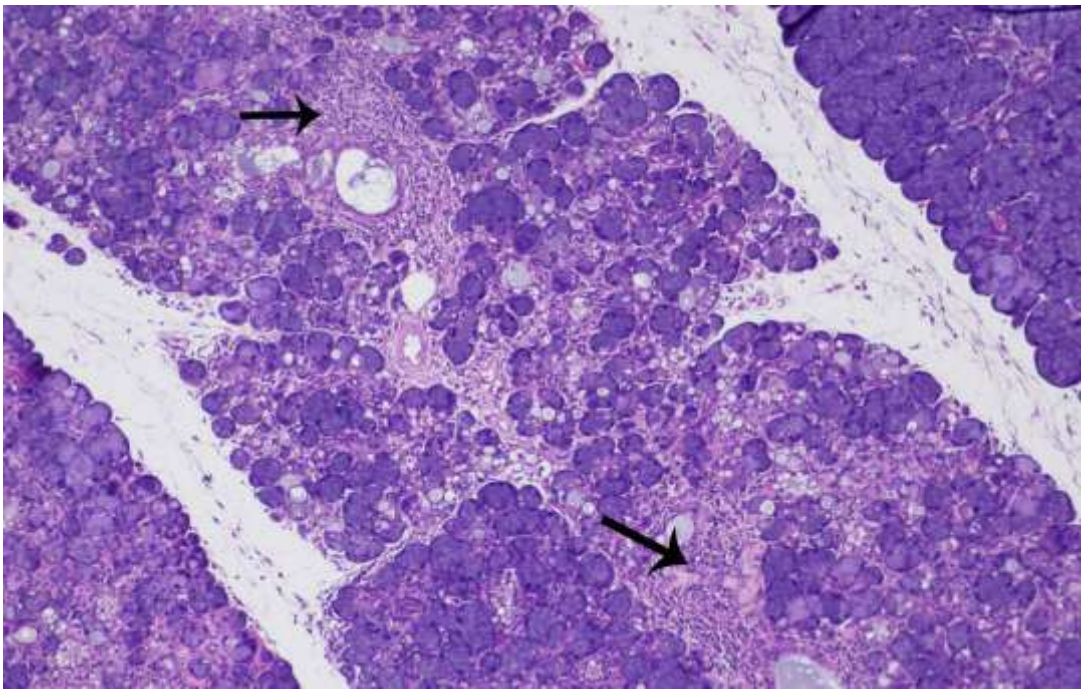

Fig.(12): A photomicrograph of a semithin section of the sublingual salivary gland of isoproterenol treated rats showing extensive mononuclear cellular infiltration (→) in the interlobular and in the periacinar connective tissue (H & E x 200).

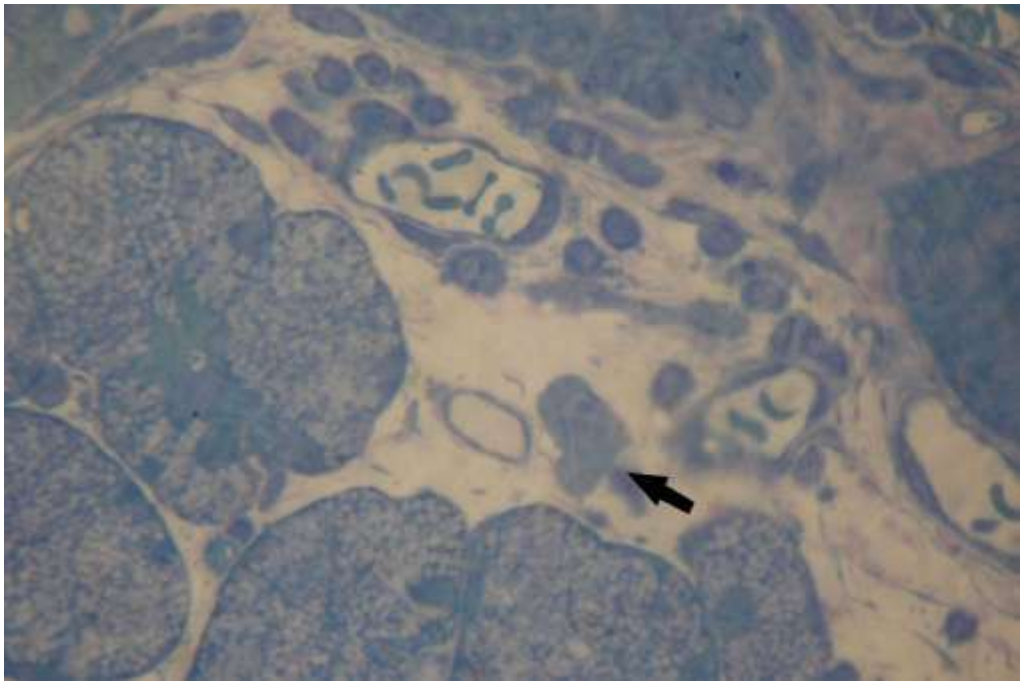

Fig.(13): A photomicrograph of a semithin section of the parotid gland of the isoproterenol treated rats showing activated macrophage (→) in the connective tissue septa (Toluidine blue x1000).

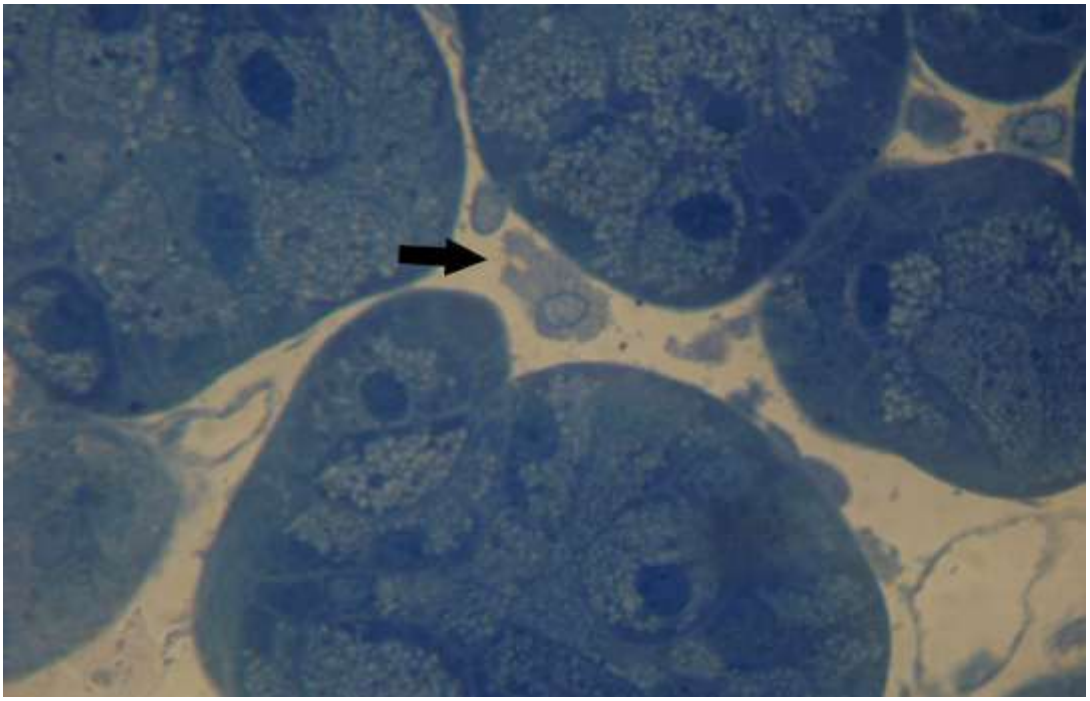

Fig.(14): A photomicrograph of a semithin section of the submandibular salivary gland of the isoproterenol treated rats showing activated macrophage (→). (Toluidine blue x 1000).

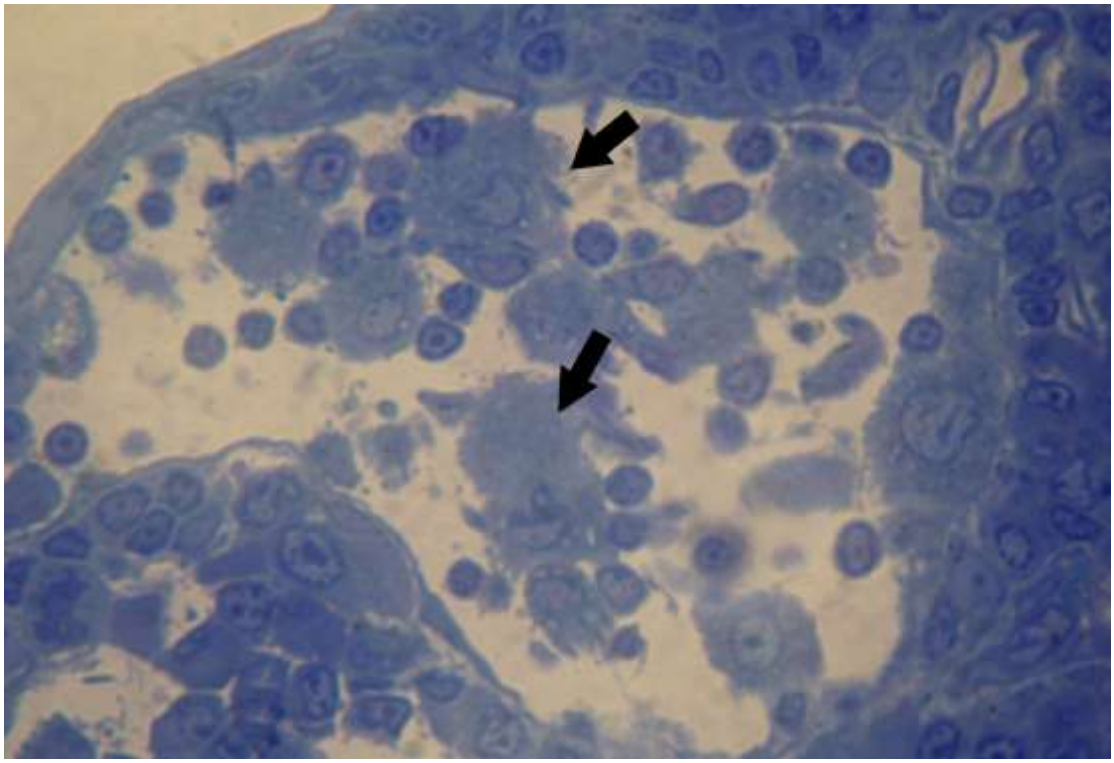

Fig.(15): A photomicrograph of a semithin section of the sublingual salivary gland of the isoproterenol treated group showing many activated macrophages in the connective tissue septa(→). (Toluidine blue x 1000).

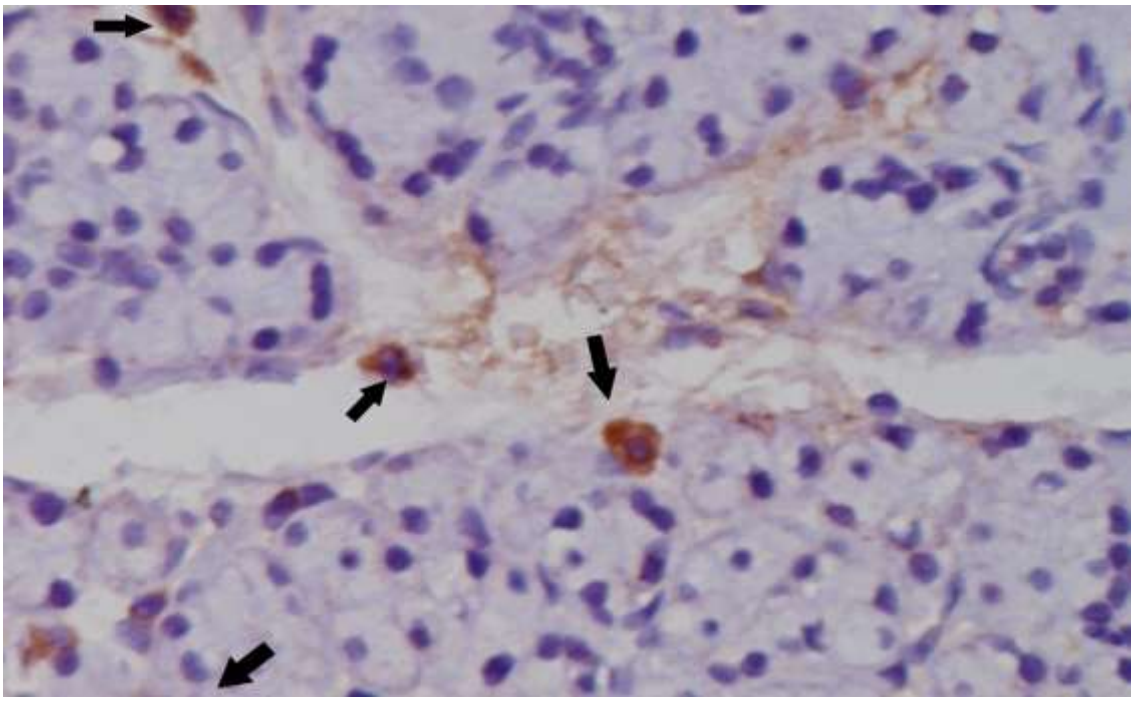

Fig.(16): A photomicrograph of a section of the parotid of the isoproterenol treated rats group showing positive CD68 cells in the connective tissue septa(→)(CD68 immunostaining x1000).

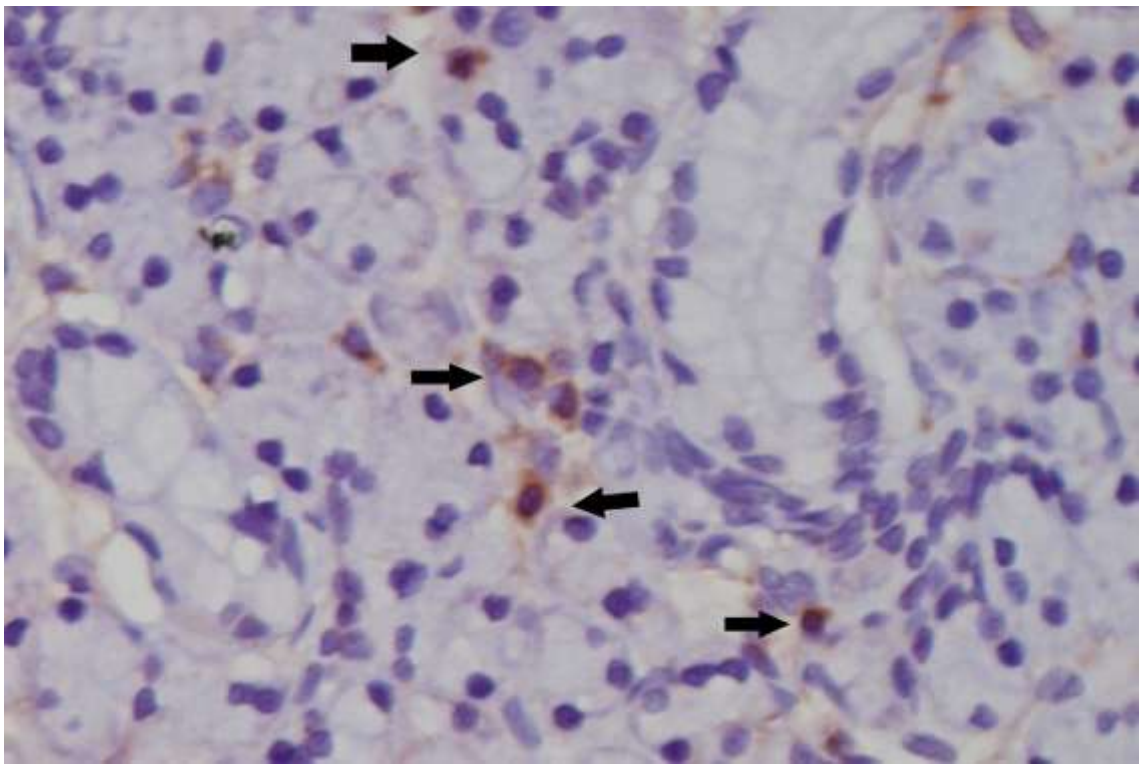

Fig.(17): A photomicrograph of a section of the submandibular salivary gland of the isoproterenol treated rats showing many positive CD68cells in the connective tissue septa(→)(CD68 immunostaining x1000).

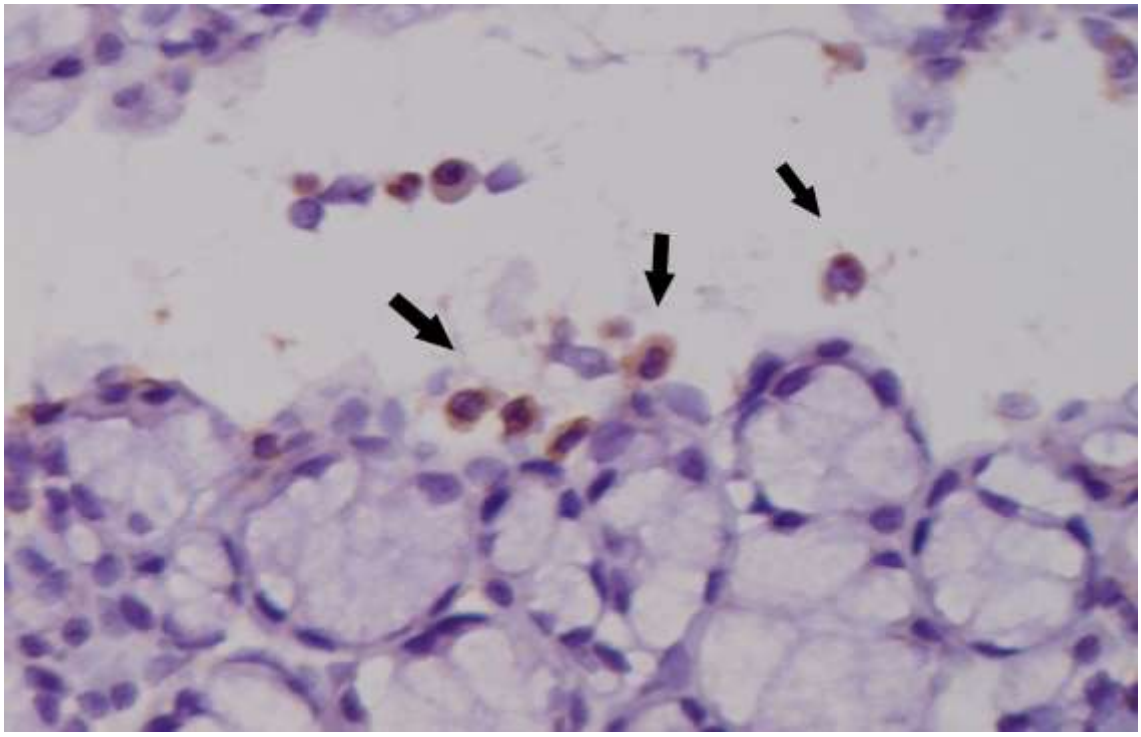

Fig.(18): A photomicrograph of a section of the sublingual salivary gland of the isoproterenol treated rats showing many positive CD68 cells in the connective tissue septa(→).(CD68 immunostaining x1000). .

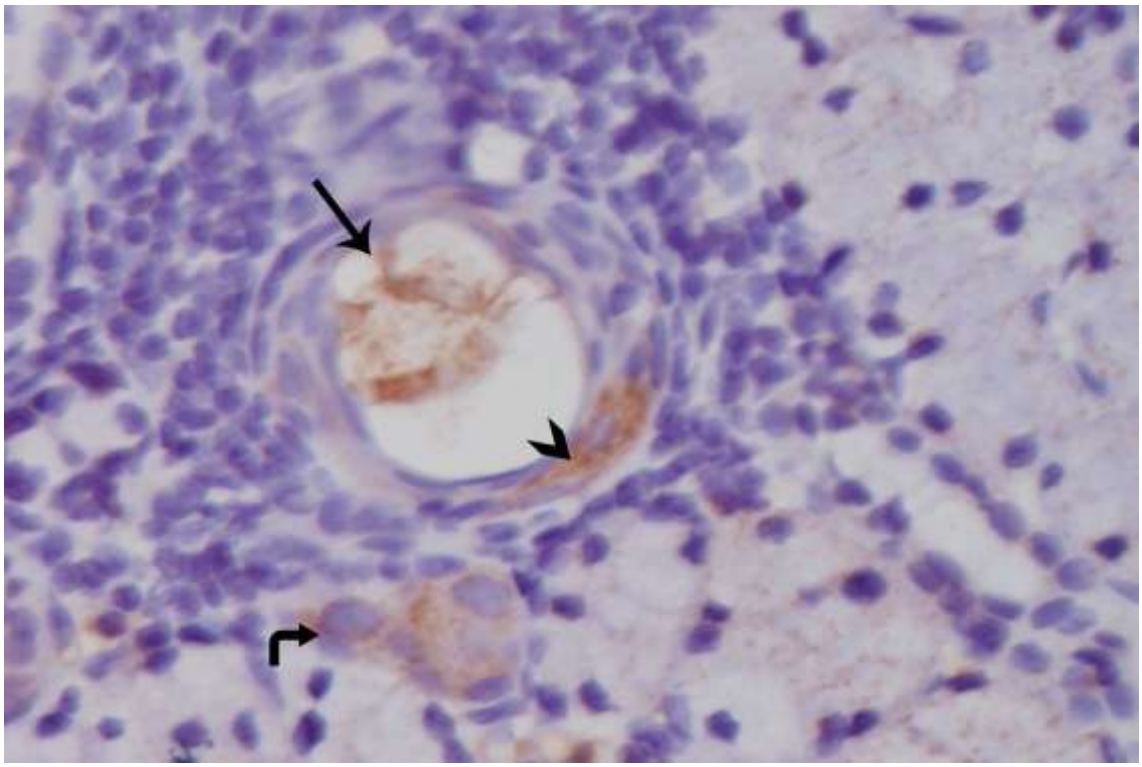

Fig.(19): A photomicrograph of a section of the submandibular salivary gland of the isoproterenol treated rats showing positive CD68 cells both adherent (→) to the lumen of the blood vessels and migrating one from the blood vessels (►) into the connective tissue. Notice giant macrophage (curved arrow) (CD68 immunostaining x1000).

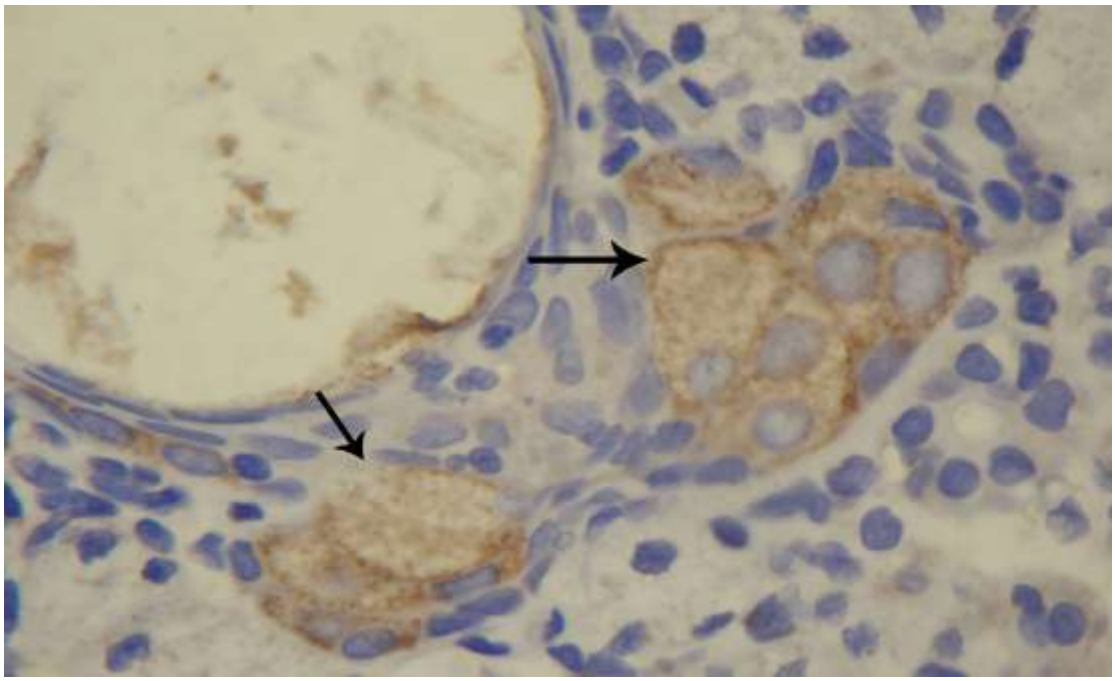

Fig.(20): A photomicrograph of a section of the sublingual salivary gland of the isoprotrenol treated rats showing positive CD68 giant macrophages in the perivascular connective tissue septa (CD68 immunostaining x1000).

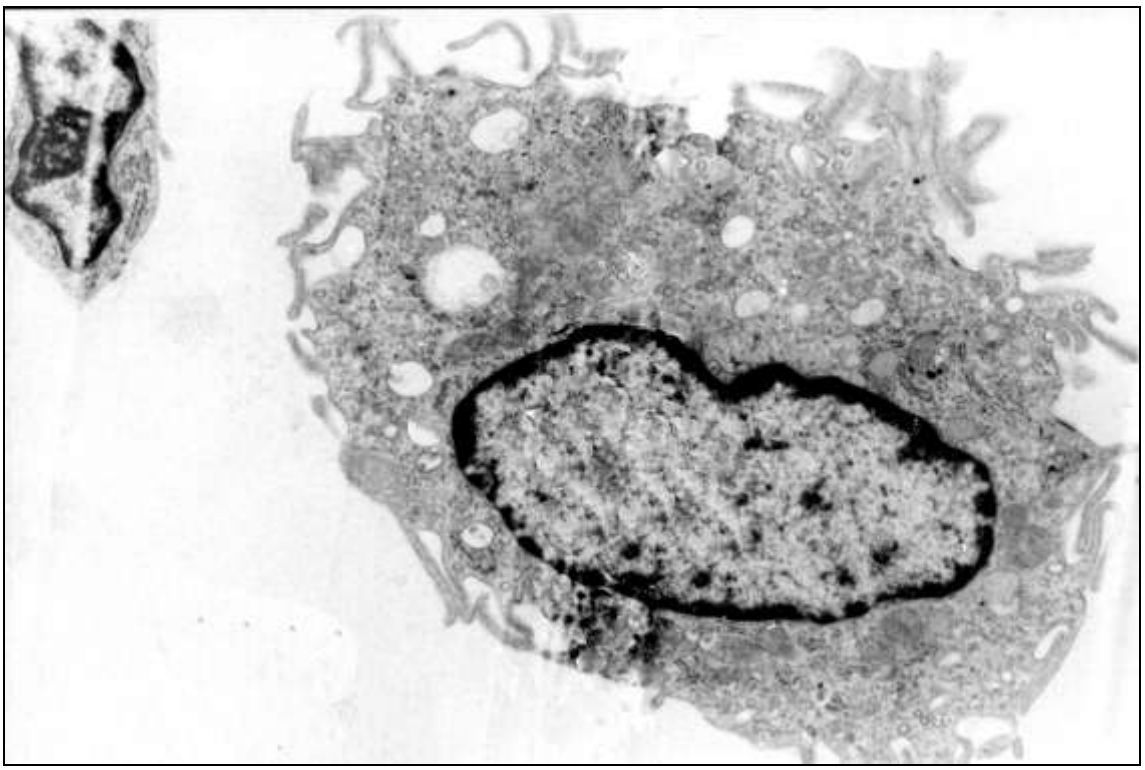

Fig.(21): Electron micrograph of the salivary gland of the control group showing a resident macrophage having irregular outlines and many pseudopodia with oval or kidney shaped nucleus. The cytoplasm contains mitochondria and few phagocytic vacuoles (X6000)

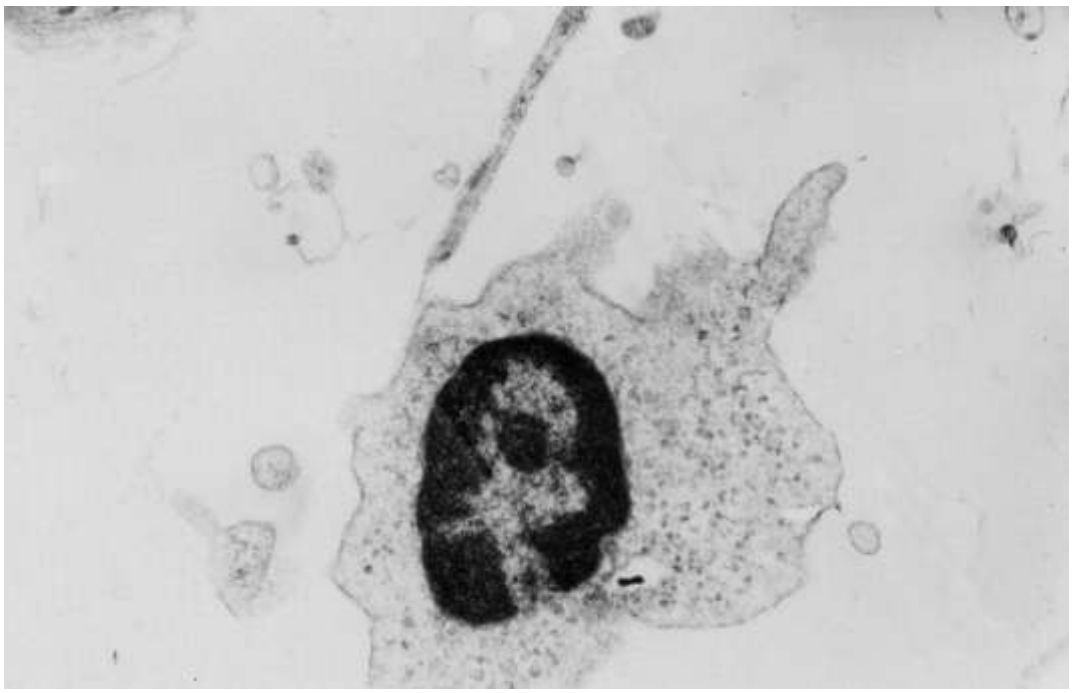

Fig.(22): Electron micrograph of the salivary gland of the control group showing a moving macrophage characterized by withdrawal of most of its processes except few of pseudopodia displaying long sleeve like extension (X1000).

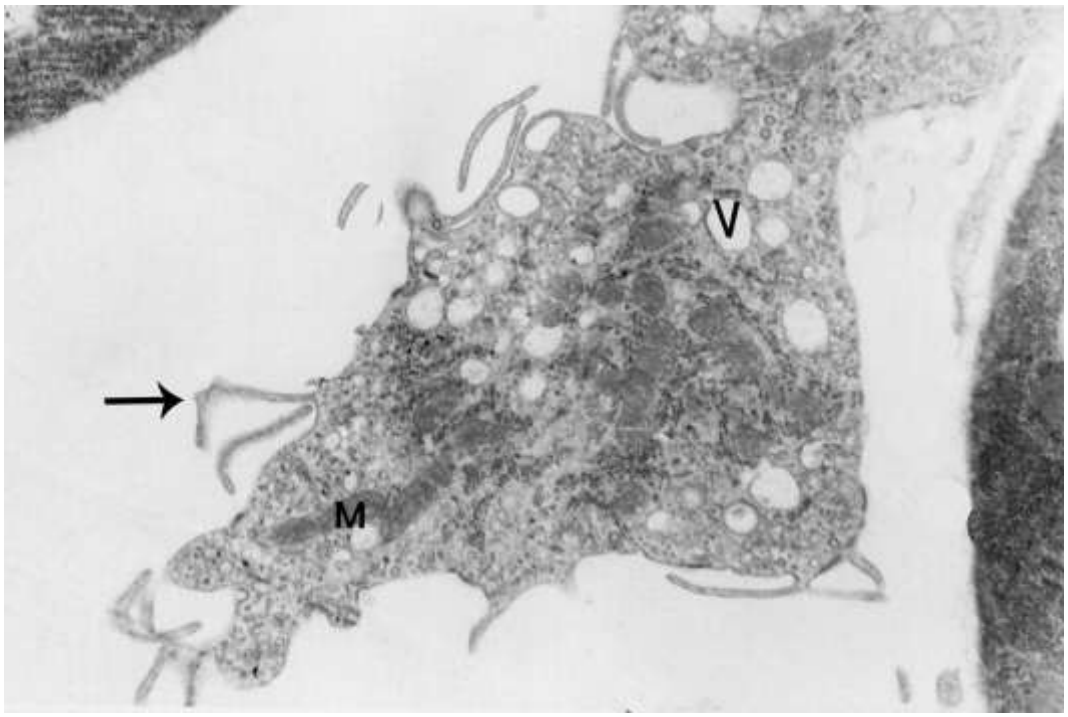

Fig.(23): Electron micrograph of the salivary gland of the isoproterenol treated group showing large sized activated macrophage with numerous slender villous projection(→), multiple fused mitochondria and many phagocytic vacuoles (X6000)

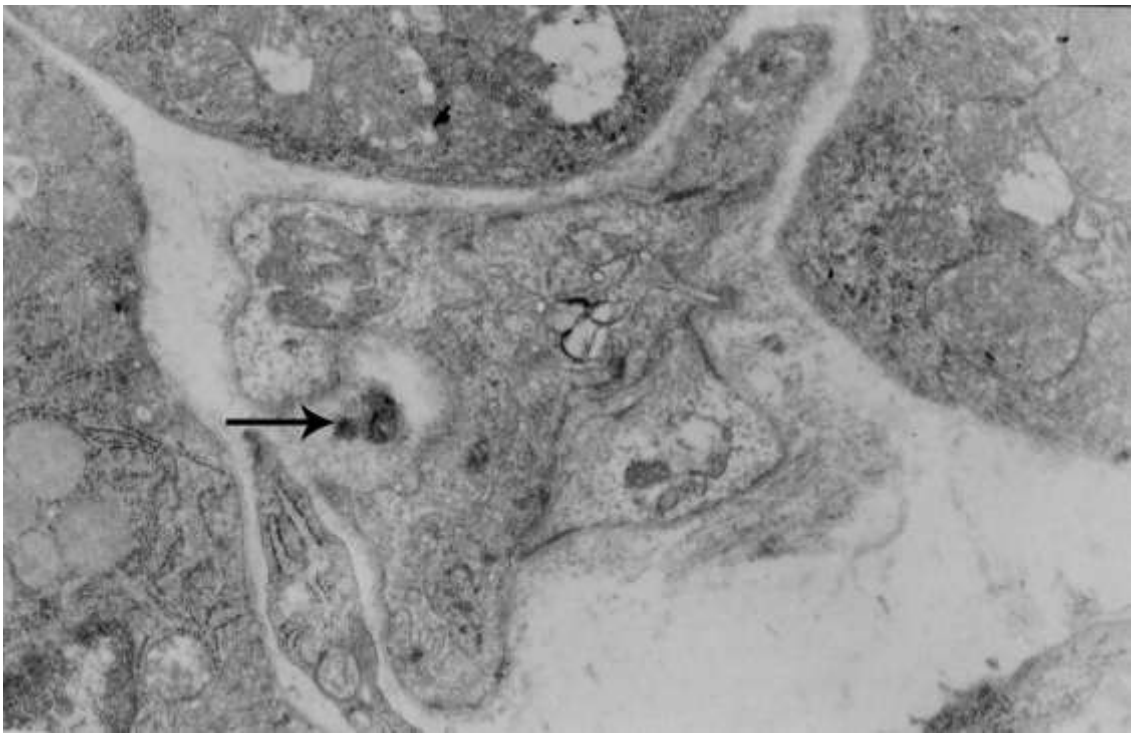

Fig.(24): Electron micrograph of the salivary gland of the isoproterenol treated group showing activated large sized macrophage with numerous large processes, Notice cellular processes trying to enclose the foreign particle (→) (X8000)

Discussion:

The present study revealed that macrophages are present within the interstitial tissue of the normal parotid, submandibular and sublingual glands of adult male albino rats. Also, isoproterenol administration once daily for ten days induced activation and increased infiltration of macrophages into the connective tissue septa mainly of the submandibular and specifically of the sublingual salivary gland in comparison to the parotid gland. Several forms of macrophages in different functional states were identified in the form of adherent, migrating, resident, activated and giant macrophages.

Phagocytic infiltration of major salivary glands induced by isoproterenol was evidenced formerly in the research work of Cohen et al., (1992) who reported 20 folds increases in resident macrophages of the submandibular and sublingual glands. They reported mild inflammatory response in the treated rats and mentioned that resident macrophages comprise the predominant type.

The increased number of CD68 positive cells in the submandibular and frequently in the sublingual glands in the isoproterenol treated group was similar to the previous findings of Cohen et al., (1995) who attributed this increase to the differentiation of recently arrived blood monocytes resulting from expression of adhesion molecules as well as from proliferation of resident macrophages. Also, immobilization of macrophages within the site of infiltration by the effect of cytokines and oxidized lipids was another theory postulated by Mitchell et al.,(2006). Similar influx of CD68 positive cells was not observed in the parotid gland due to differences in the population composition of macrophages in normal rat parotid, submandibular and sublingual glands. Furthermore, treatment with isoproterenol alters the phenotypic composition and expression of salivary macrophage in a characteristic manner for each gland. These observations suggested that

immunoregulatory mechanisms may operate differently in these glands to inflammatory stimuli (Cohen etal.,1995). Also, submandibular and sublingual salivary glands are anatomically in close association with oral mucosa in comparison to the parotid gland, making them continuously in direct contact with foreign antigens. Beckenkamp (1985) also studied the cellular distribution of lymphocytes and immunocytes in the major and minor salivary glands showing marked difference in the cellular distribution in these sites. Massive infiltration with lymphocytes and immunocytes was 27% in the sublingual gland then 8% in the submandibular gland and lastly 6% in the parotid gland.

A trial to understand the underlying mechanism responsible for the presence of different forms of macrophage, Cotran et al., (1994) mentioned that isoproterenol has a direct effect on the wall of the blood vessels producing vasoconstriction followed by vasodilatation leading to congestion and extravasation of blood cells. Congestion leads to slowing of circulation, increasing the permeability of the microvasculature and out pouring of the inflammatory fluids and extravastaion of monocytes. Adhesion of the monocytes to the endothelium occurs by interaction between complementary adhesion molecule (integrin, selectin etc) on macrophage and endothelium. Monocyte transmigration (diapedesis) occurs through the interendothelial junctions traversing the basement membrane and move towards the site of injury along gradient of chemotactic agents. When they become activated they secrete numerous biologically active products by chemical mediators (Kumar et al., 2005)

Activated macrophages seen in the present study may be produced by a variety of stimuli including cytokine (IFN-ɣ) secreted by sensitized T lymphocytes, chemical mediators and by complement system. When they become activated, macrophages enlarge in size, number and complexity of intracellular organelles (increase number of mitochondria, lysosomes and phagocytic vacuoles). Two major classes of activated macrophages were described; the first is the classically activated macrophages and the second is the alternatively activated macrophages. Classically activated macrophages promote inflammation, extracellular matrix (ECM) destruction, and apoptosis, while alternatively activated macrophages promote extracellular matrix construction, cell proliferation, and angiogenesis. Although both phenotypes are important components of both the innate and adaptive immune systems, the classically activated macrophage tends to elicit chronic inflammation and tissue injury whereas the alternatively activated macrophage tends to resolve inflammation and facilitate wound healing (Stein *et al*.,1992 and Gordon,1999).

Activated macrophages respond to injurious agents by secreting TNF and IL-1 and chemokines (chemoattractants-chemokine). TNF and IL-1 act on the endothelial post capillary venule and induce expression of several adhesion molecules and chemical mediators. Ongoing adhesion molecule and chemotactic factor expression are responsible for continued recruitment of monocytes and macrophages accumulation at the site of inflammation.(Madge and Pober, 2001).

Giant macrophages were one of the prominent histological finding in salivary glands of the isoproterenol treated rats. This was explained by Thoenes et al.,(1983) and confirmed by Vigenry (2000) where they represented strong evidence in favour of the hypothesis that giant cells do not form by endomitotic processes but rather through fusion of certain precursor cells. Recently, **Moreino** etal.,(2007) added that IL-4 which is produced by T helper factor 2 lymphocyte(TH2), NK cells, mast cell, eosinophil and basophil plays a critical role in regulating the differentiation and functional activity of

mononuclear macrophage lineage induces expression of E-cadherin which is critical for the formation of giant macrophage and dendritic cell-specific transmembrane protein.

CONCLUSION:

From the present study, it could be concluded that population composition of salivary macrophages is different in normal rat parotid, submandibular and sublingual glands and that treatment with isoproterenol induces significant increase in the number of CD68 positive cells in the submandibular and frequently in the sublingual salivary glands. These findings suggest that the normal sublingual gland is the best adapted of the major salivary glands to play a part in mucosal oral immunity. Further researches are recommended for tracing the cellular distribution and structural characterization of other mononuclear cells in these glands including plasma cell, B& T lymphocytes and mast cells.

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