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Effect Of Vitamin C On Submandibular Salivary Gland Of Methotrexate Treated Rats

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Abstract

Introduction: chemotherapeutic drugs induce changes in parotid and submandibular salivary glands. Methotrexate, the widely used chemotherapeutic drug decreases the gland weight, RNA and amylase content. Antioxidants protect the cells from oxidative stress of many chemicals directly by removing the free radicals.

Aim of work: The aim of this study was to examine the effect of vitamin C as an antioxidant on the salivary glands of rats treated with Methotrexate.

Materials and method: 50 healthy adult male albino rats were used in this study. Freely fed animals were divided into three groups, **Group I**, the control group included 10 rats were intraperitonially injected by I ml / Kg dose of sterile 0.9% NaCl for 4 weeks , **Group II**, the Methotrexate treated group included 20 rats were intrapretonialy injected with 15 mg / Kg Methotrexate drug dilated in 0.95 NaCl day by day for four weeks and **Group III**: the Vitamin C and Methotrexate treated group included 20 rats received Vitamin C intramuscularly at the rates of 200 mg per kg body weight, once a day for four weeks before the intrapretonial Methotrexate injection of 15 mg/kg drug diluted in 0.9% NaCl day by day for two weeks. At the end of the experiment, rats were scarified, the Submandibular gland was dissected. For transmission electron microscopic examination (TEM) glutaraldehyde fixed fresh tissue was subjected to ultrastrctural study by embedding in epoxy resin. Semithin sections were cut 1 um thick using ultra microtome and stained by Toluidine blue. The specimens were then retrimmed to the selected area and ultra thin sections (50 nm) were cut and processed for TEM examination.

Results: Examination of Submandibular gland of Methotrexate group showed loss of architecture of the gland with disarrangement of acini. Mucoserous cells showed marked swelling and altered secretory granules. Striated ducts showed marked decrease in mitochondrial number, basal cytoplasmic vacuoles and apical accumulation of granules in the cells. Vitamin C supplemented group showed marked improvement in cells of acini as well as cells of striated ducts, the number of vacuoles decreased and the number of mitochondria increased and secretory granules became normal.

Conclusion: vitamin C intake improves the architecture of acini, the degenerative changes, and increased cellular activity in salivary glands in rats receiving Methotrexate as a chemotherapeutic drug.

Introduction

Methotrexate is folic acid antagonist, and is considered to be one of the most important antimitotic or cytotoxic drugs. It is commonly used as a cancer chemotherapeutic drug. It has a wide therapeutic use including, leukemia, psoriasis, non neoplastic skin diseases ,and has been employed as immunosuppressive agent in many diseases and in organ transplantation. It binds to dihydrofolate reductase, resulting in a decreased pool of reduced folic coenzymes necessary for nucleic acid synthesis. In the oral cavity, Methotrexate has been reported to increase whole saliva albumin concentration (Izutsu *et al.*, 1981), decrease the buffer capacity of saliva (Schum *et al.*, 1979), and decrease the output of salivary IgA (Courts and Mueller, 1983) in patients undergoing treatment for systemic diseases affecting normal as well as diseased cells. In animal studies, Methotrexate has been demonstrated to cause doserelated histopathological alterations of rat salivary glands, including

vacuolization and swelling of both acinar and duct cells, and a reduction in secretion granules (Romaniuk *et al.*, 1983). Recently much public and scientific interest has been directed toward the molecules that protect from any free radicals or antioxidants. They protect the cells from oxidative stress directly by removing the free radicals in a scavenge manner or by promoting the activity of free radical eliminating enzymes, Vitamin C (ascorbic acid) is one of the most available potent water soluble antioxidant agents (Block, 1991). It is believed to be involved in the vital antioxidant processes in human. It can counteract the damaging effect of oxygen in the tissues. Free radicals can induce local injury by reacting with lipids and nucleic acids leading to membrane damage and generation of lipid peroxide (Bulger, 1998).

Material And Methods

50 healthy mature male albino rats were utilized in this study. Their age was about five months and their body weight ranged from 200 – 250 grams. Animals received food and water ad libitum. Animals were divided into three groups: **Group I**: The control group, 10 rats were intrapretonialy injected by I ml / Kg dose of sterile 0.9% NaCl through the time of the experiment. **Group II**: Methotrexate treated animals: included 20 rats were intrapr-etonialy injected with 15 mg / Kg Methotrexate drug dilated in 0.95 NaCl day by day for four weeks. **Group III:** the Vitamin C and Methotrexate treated group included 20 rats received Vitamin C intramuscularly at the rates of 200 mg per kg body weight, once a day for four weeks according to kraoze *et al*,(2002) before the intrapretonial Methotrexate injection of 15 mg/kg drug diluted in 0.9% NaCl day by day for two weeks This dose was based upon a study of (Branski *et al.*, 1979) and (Kedziora *et al.*, 2003). The used chemotherapeutic drug was in the form of Methotrexate sodium. The used rat dose was equivalent to 0.250 mg /kg body weight calculated according to Paget's formulae (1964) and Kedziora *et al.*, (2003). The duration of the experiment was two weeks, the maximal rat survival time.

Histological examination:

At the end of the experiment, rats were scarified, the Submandibular gland of both sides was dissected using the Stereomicroscope and immediately fixed for transmission electron microscopy, in 2.5% glutaraldehyde at 40 c° for 2 hours, rinsed with cacodylate buffer solution and post fixed in 2% osmium tetroxide with cocodylate buffer solution at 40C for 2 hours. The specimens were, then dehydrated in graded concentrations of ethanol and embedded in epoxy resin. The blocks were trimmed under the binoculare microscope with a razor blade to a truncated cone, Semi thin sections were cut one um thick using ultra microtome by the aid of glass knives. The semithin sections (one um thick) were stained by Toluidine blue and examined for general orientation with light microscope. The specimens were then retrimmed to the selected area and ultra thin sections (90 nm) were cut and picked up on copper grides. Ultrathin sections stained with uranylacetat and lead citrat and tannic acid were examined with (T.E.M.) Drury and Wallington (1980).

Results

Microscopic examination of semithin sections of submandibular gland of control group revealed that the stroma was composed of well developed connective tissues capsule, which sends septa dividing the parynchyma into lobes and lobules of secretory mucouserous acini (fig.1). With TEM Striated ducts predominate the duct system and were interspersed among the secretory acini. Also, Myoepithelial cells were seen inbetween the acini(fig.7). mucoserous acini were spherical in shape and composed of pyramidal shaped cells surrounding a narrow lumen and contained round and flat dark nuclei that located in the basal third of the acinar cells(Fig.1&6). However the striated ducts were lined by single layer of law columnar cells with centrally located oval nuclei and possessing clear basal striation of elongated mitochondria located inbetween the basal cell membranr infoldings (Fig.12). Examination of Submandibular gland of Methotrexate group showed loss of architecture of the gland with

disarrangement of acini (Fig.2, 3, 8 &9). Both cells of mucoserous acini showed marked swelling and ultered secretory granules. (Fig.16 &17). The presence of abnormal large cells in submandibular glands with faint vacuolated cytoplasm and large atypical nuclei were observed. (Fig.9.).The number of ducts as well as myoepithelial cells in the methotrexate treated group was apparently numerous (Fig.2, 7 & 9). Striated ducts showed marked decrease in mitochondrial number, basal cytoplasmic vacuoles and apical accumulation of granules in the cells (Fig.3,13&15). Vitamin C supplemented group showed marked improvement in cells of acini as well as cells of striated ducts(Fig.4,5,10&11), the number of vacuoles decreased and the number of mitochondria increased and secretory granules became normal (Fig 14).

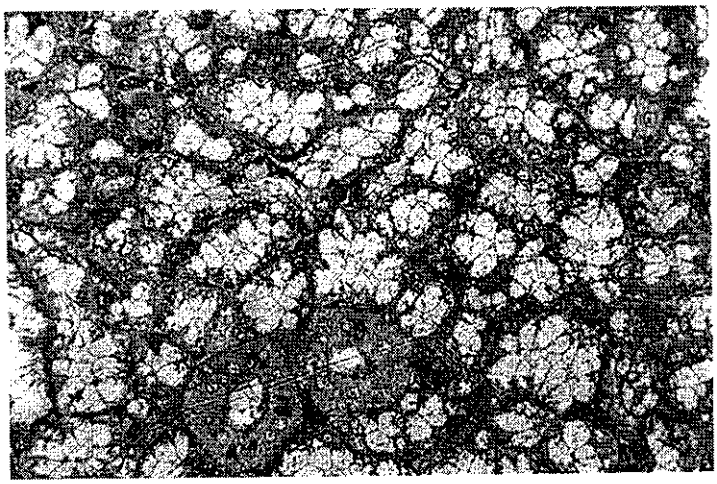

Fig. (1) Semithin section of submandibular gland of contol group showing normal mucoserous acini and striated ducts. (Toluidin blue x 400)

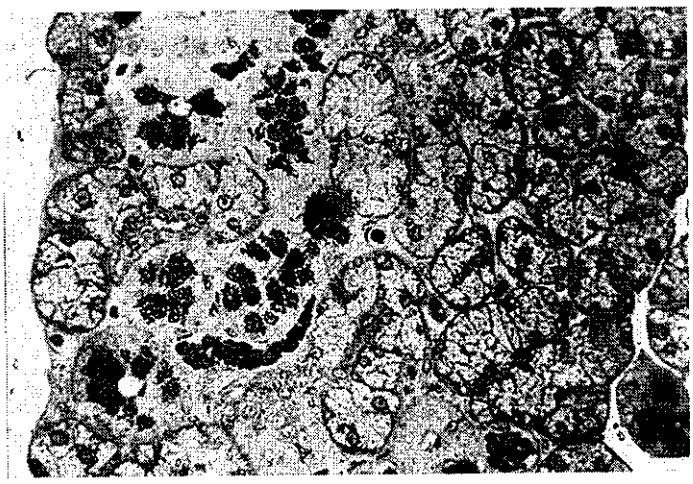

Fig. (2) Semithin section Submandibular gland of Methotrexate group showing loss of gland architecture, swelling of mucoserous acini and accumulation of granules in the cells of striated ducts. (Toluidin blue x 400)

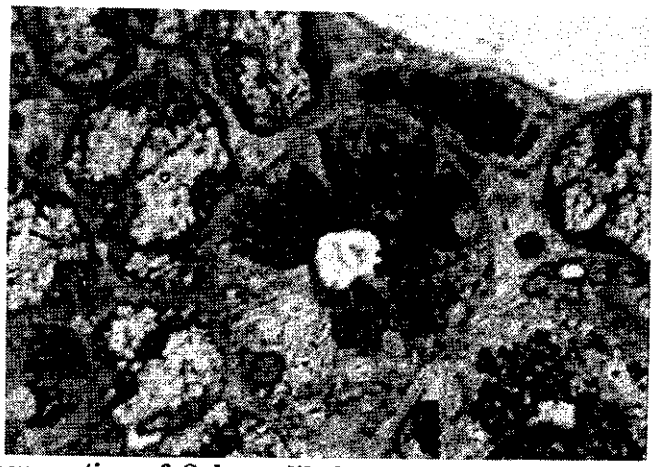

Fig. (3) Semithen section of Submandibular gland of Methotrexate group showing swelling of mucoserous acini and accumulation of granules in the cells of striated ducts. (Toluidin blue x 1000)

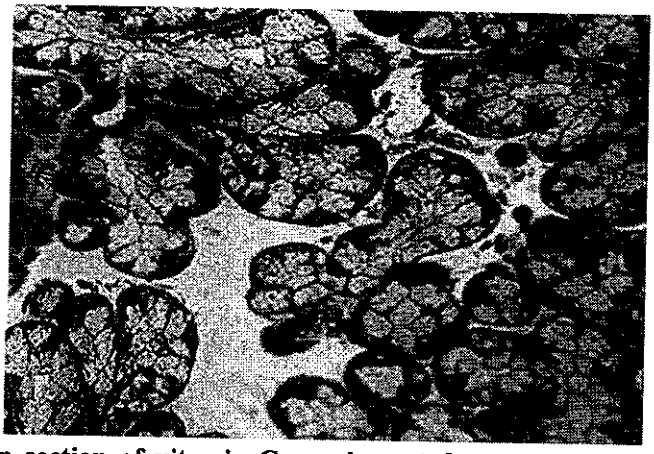

Fig. (4) Semithin section of vitamin C supplemented group showing improvement of architecture of acini and accumulation of granules in the cells of striated ducts. (Toluidin blue x 400)

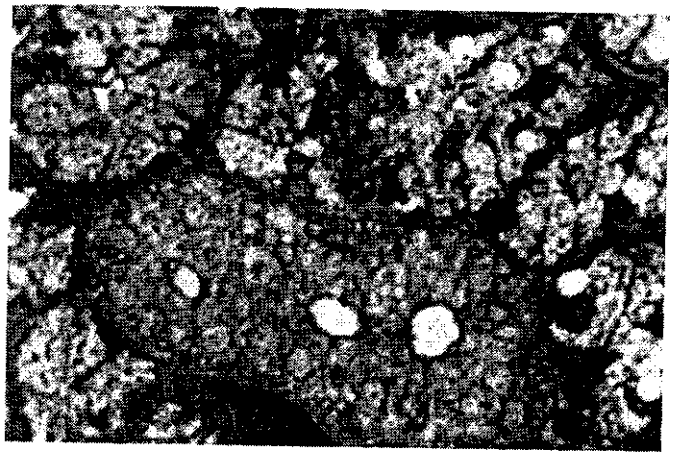

Fig. (5) Semithin section of vitamin C supplemented recovery group showing improvement of acini and of striated ducts. (Toluidin blue x 1000)

Fig.(6)E/M photomicrograph of submandibular gland of control group showing normal cells of mucuserous acini . serous cells are basal with rounded nuclei and dark secretory granules ,mucous cells are large with basal flat nuclei and crowded cytoplasmic mucus granules. (X 4000)

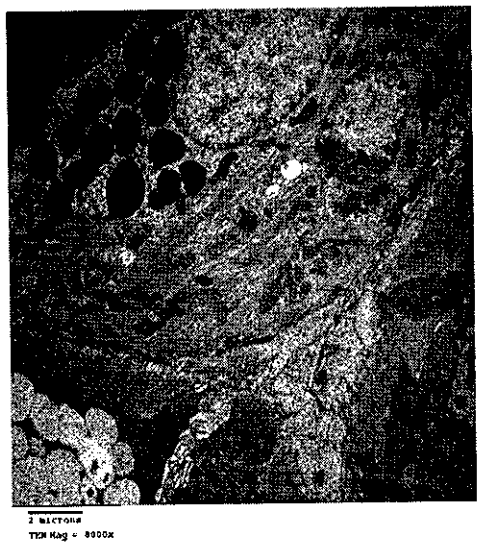

Fig.(7) E/M photomicrograph of submandibular gland of Methotrexate group showing Myoepithelial cell inbetween mucus and serous cells . (X 4000)

Fig. (8) E/M photomicrograph of submandibular gland of Methotrexate group showing disarranged mucoserous cells with vaccuolation . (X 2000)

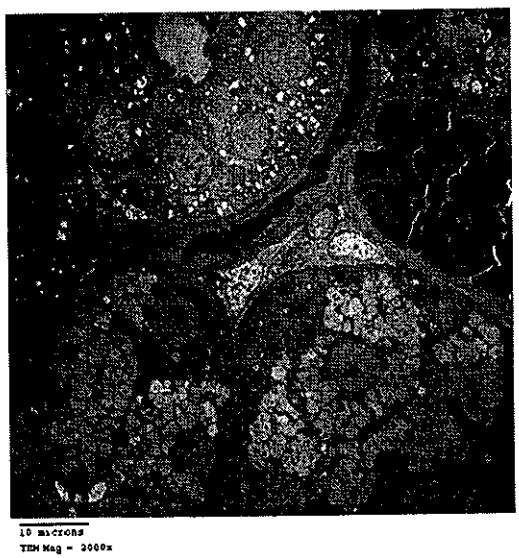

Fig.(9) E/M photomicrograph of submandibular gland of Methotrexate group showing striated duct with vaccuolation (arrow) middle B.V.and myoepithelial cells lower mucoserous cells with atypical nuclei and ultered secretion . (X 2000)

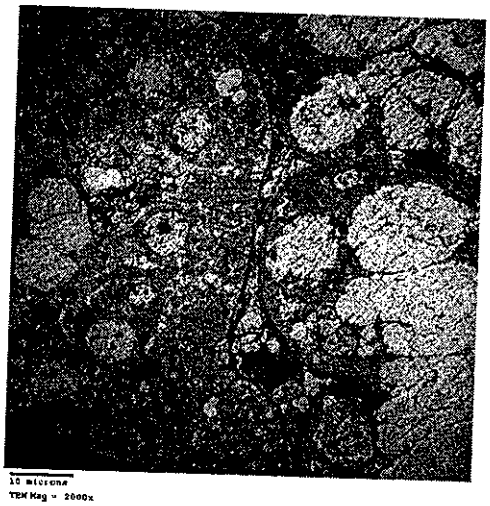

Fig. (10) E/M photomicrograph of submandibular gland of vitamin C supplemented group showing mucoserous cells with increased secretion and cells of striated duct with less vacculation. (X 2000)

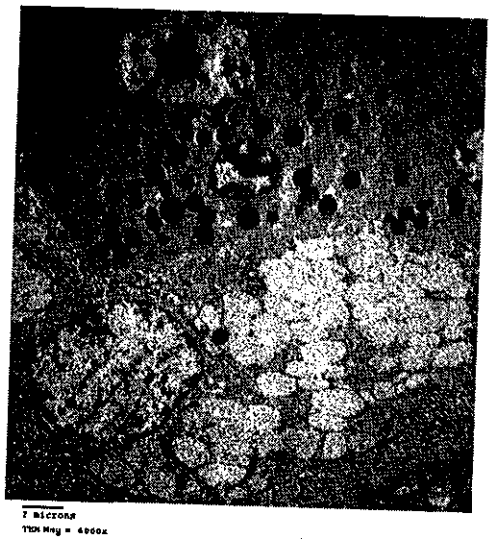

Fig. (11) E/M photomicrograph of submandibular gland of vitamin C supplemented group showing mucoserous cells with increased secretion . (X 2000))

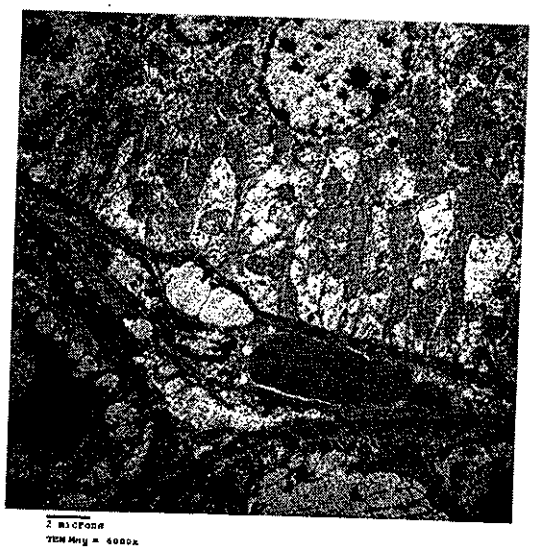

Fig. (12) E/M photomicrograph of submandibular gland of control group showing normal striated duct cells with normal elongated mitochondria arranged basal inbetween membrane infoldings . (X 6000)

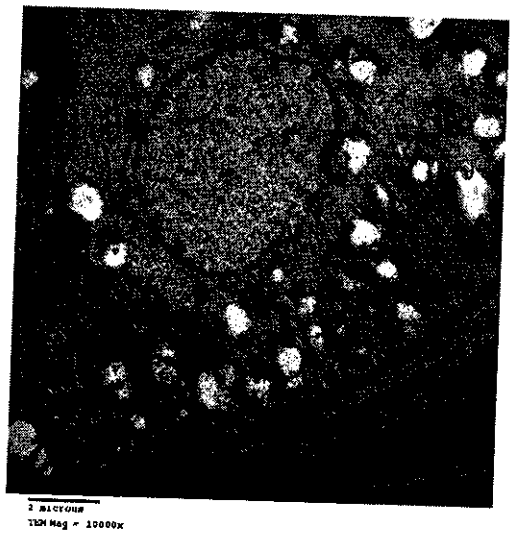

Fig. (13) E/M photomicrograph of submandibular gland of Methotrexate group showing striated duct cells with cytoplasmic vacuoles and few mitochondria . (X 2000)

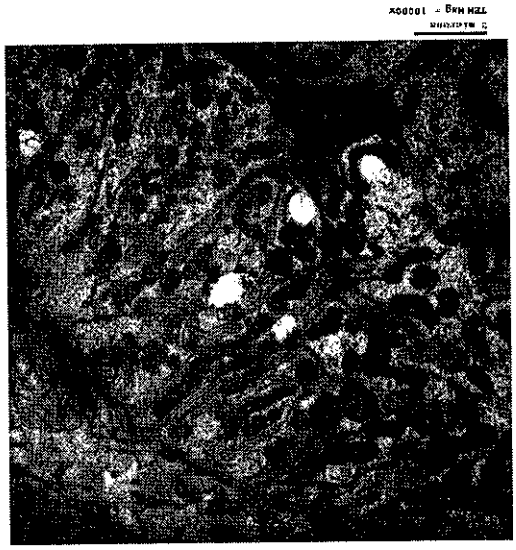

Fig. (14) E/M photomicrograph of submandibular gland of vitamin C supplemented group showing striated duct cells with normal mitochondria and minimal cytoplasmic vaccuoles .
(X 8000)

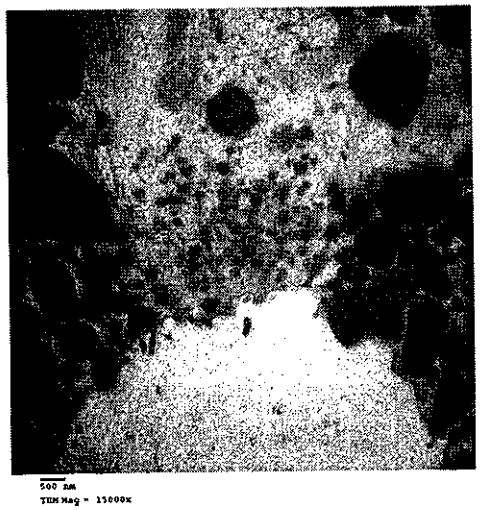

Fig. (15) E/M photomicrograph of submandibular gland of Methotrexate group showing the surface of striated duct cells with apical cytoplasmic granules in the center small immature granules and adjacent large mature granules. (X 2000)

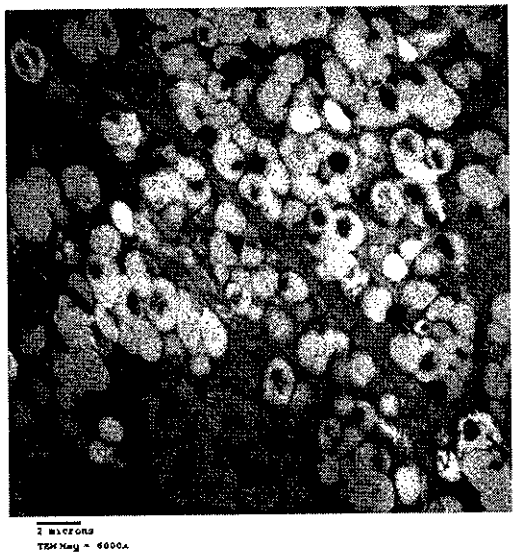

Fig.(16) E/M photomicrograph of submandibular gland of Methotrexate group showing mucous cells with vaccuolation and altered secretion.
(X 6000)

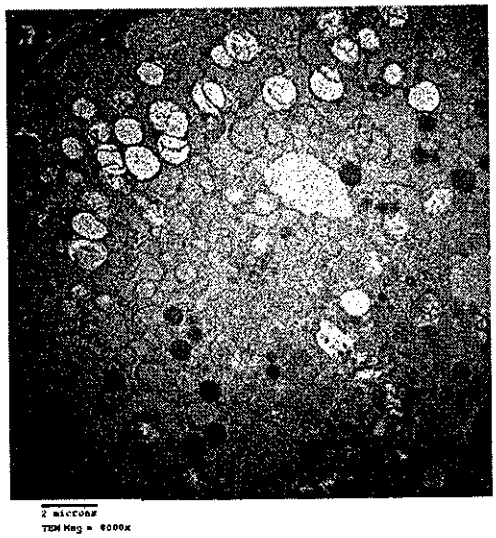

Fig.(17) E/M photomicrograph of submandibular gland of Methotrexate group showing serous cell with vaccuolation and altered secretion.
(X 6000)

Discussion

In general, damage to the salivary glands is a well known sequela of chemotherapy in both human and experimental animals. Experimental data on the pathomechanisms of chemotherapy induced damage are still doubtful. In the present study the histological examination was focused on the submandibular gland, the common affected gland with chemotherapy in most reported researches. In the present study the semithin sections of rat submandibular gland treated with Methotrexate showed atrophy of the acini. Several investigators have reported similar toxic effects of Methotrexate on the gastro-intestinal tract (Phillip *et al.*, 1950 and El Dareer *et al.*, 1981). Muller *et al.*, (2006) reported that major problems of chemotherapy are necrosis of salivary glands and taste disorders. Methotrexate treatment decreases food intake. Some investigations have demonstrated a relationship between diet and salivary gland function and (Schneyer and Hall, 1976; Johnson, 1982; Johnson and Sreebny, 1982), This was anticipated, since several investigators have reported local toxic effects of Methotrexate drug on the gastro-intestinal tract (Phillips *et al.*, 1950 and El Dareer *et al.*, 1981). As atypical nuclei are reversible and could not be explained as a malignant change .Methotrexate affected the DNA content of gland cells. Mcbrid *et al.*, (1987). Mcbride and Siegel (1988), found that Methotrexate decreased the rat submandibular salivary gland RNA and amylase content . Damaging of the salivary glands following Methotrexate treatment could be due to several distinct molecular mechanisms; the most important might be the free radical damaging effect, All cytotoxic drugs generate some free radicals which induce cell damage through the release of cytochrome C from mitochondria ,Solary *et al.*, (2000) and Hsu (2006). Heaney *et al.*, (2008) reported that the mechanism for action of chemotherapy includes inhibition of protein kinase. Methotrexate ability to inhibit protein synthesis through depletion of folate co-factors can account for the defect in glandular secretion and failure of performing the secretory activity of salivary glands may explain the cause of altered secretion and subsequent xerostomia with Methotrexate treatment Carolyn *et al.*, (2000).

The crowded number of ducts in the present investigation was more numerous and this might be due to the decrease in number of acini cused by early tissue atrophy which indicated the early effects of chemotherapy on the acinar cells .This finding was supported by Fajardo and Berthrong, (1981), which noted that duct cells was the last sensitive part of salivary gland to external stimuli. The loss of gland architecture, and dilatation of striated ducts suggested the pathological effects of chemotherapy on myoepithelial cells embracing them with failure of expelling the secretion into the oral cavity leading to xerostomia. Cutler *et al.*, 1974, reported that compression of secretory cells by myoepithelial cells possibly play a role in modification of salivary secretion as do in both acini and striated ducts. Striated ducts appeared studded with altered secretion ,this stored secretion may be caused by changing in secretion chemistry rather than myoepithelial cell damage. Methotrexate treatment reduces the output of Na and Cl in submandibular saliva with no changes in K output. This was suggested by Thaysen *et al.* (1954), as a decreased rate of sodium transfer into the precursor solution and a defect in sodium reabsorption in the duct cells. The presence of large cells in submandibular glands was characterized by faint vacuolated cytoplasm and large atypical nuclei. The marked increase in the acinar cell size suggested being due to the beginning of healing processes and recovery of the glands. These results proposed the pathological effects cell organelles found basal and lateral to the nucleus specifically Golgi complexes causing a decrease in enzymatic activity at the basal cell membrane of acinar & ductal cells, surrounding basement membrane and myoepithelial cells embracing them. This suggestion has been supported by many investigators.

Vitamin C supplemented group

showed marked improvement in cells of acini as well as cells of striated ducts, the number of vacuoles decreased and the number of mitochondria increased and granules became normal. The antioxidant effect of vitamin C may prevent the release of harmful free radicals as well as of cytochrome C from mitochondria. Block, (1991). And Heaney *et al*, (2008). Also, Ashour, (1998) reported that antioxidants improve the architecture of acini, the degenerative changes, with increased cellular activity. In conclusion the use of antioxidants like vitamin C could be used safely in large doses in cases of treatment with chemotherapy, as it decreases the side effects on salivary glands.

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التاثير الواقى لفيتامين ج على الغدة اللعابية التحت فكية فى الفئران المعالجة بعقار الميثوتريكسات

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الغدد اللعابية سريعة التاثر بالادوية المستخدمة فى علاج الاورام السرطانية وتهدف كثير من الابحاث الى محاولة تقليل الاثار الجانبية على نسيج الغدد اللعابية باستخدام الاغذية الطبيعية وبعض المواد المستخلصة منها ذات الفائدة وهى ماتعرف بمضادات الاكسدة . وقد هدفت هذه الدراسة الى اختبار التاثير الواقى لفيتامين ج على الغدد اللعابية تحت الفك بعد استعمال عقار الميثوتريكسات المضاد للسرطان وقد استخدم لذلك خمسون من ذكور الفئران البيضاء البالغة متوسطة الوزن 200 – 250 جم للفار قسمت الى مجموعات حقنت المجموعة الاولي تحت الجلد بمحلول ملح كلوريد الصوديوم 0.9 ٪ (المذيب) يوميا طوال فترة التجربة واستخدمت كمجموعة ضابطة للمقارنة . بينما قسمت المجموعة الثانية والتى حقنت بمادة الميثوتريكسات بتركيز 15 مجم لكل كجم وزن داخل تجويف البريتون لمدة 15 يوما الي مجموعتين حقنت احداهما بفيتامين ج يوميا بالعضل بجرعة 200 مجم لكل كجم وزن لاختبار التاثير الواقى للفيتامين كمضاد للاكسدة على نسيج الغدة اللعابية بينما تركت المجموعة الاخرى بدون حقن فيتامين ج . وبعد انتهاء مدة التجربة تم تخدير الفئران واستخراج الغدد اللعابية تحت الفك وتم اخذ عينات لتمريرها بالطريقة المعتادة لفحصها بواسطة الميكروسكوب الالكترونى النافذ. وقد أظهرت النتائج وجود تغيرات هستولوجية مختلفة مع استعمال الميثوتريكسات حيث اظهرت المجموعة المختبرة بالميثوتريكسات ضمورا بالغدة وقد صاحب ضمور الوحدات المفرزة بالغدة تغييرا فى طبيعة الافراز وشكله بالاضافة الى التغيير فى احجام الانوية و فقدان الشكل الهيكلي للغدة وظهرت بعض أماكن احتقان بالشعيرات الدموية في النسيج , وقد اظهرت انابيب الغدة انتفاخا ملحوظا و نقصا في السيتوبلازم وانكماشا فى الانوية كما ظهرت فجوات داخل السيتوبلازم وكذلك نقصا فى عدد الميتوكوندريا بالخلايا . اما خلايا الوحدات المصلية فقد اظهرت تباينا فى الحجم بسبب انتفاخات فى السيتوبلازم وكذلك تباينا فى احجام الانوية . وقد لوحظ تحسنا متواضعا فى خلايا نسيج الغدة اللعابية حيث استعادت خلايا الغدة نشاطها في صورة زيادة فى معدل افراز المخاط بالخلايا , وقد لوحظ التحسن ايضا فى خلايا الانابيب حيث اختفت الفجوات وزاد عدد الميتوكوندريا.