

Royal Jelly Ameliorates Oxidative Stress and Tissue Injury in Submandibular Salivary Gland of Methotrexate Treated Rabbits: Immunohistochemical Study

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Abstract: The efficacy of methotrexate (MTX), a widely used cytotoxic chemotherapeutic agent, is often limited by severe tissue injury. Royal jelly has a number of physiological effects, such as anti-inflammatory, antitumor, anti-allergic, and antioxidant activities on various cell types. **Aim:** The purpose of this study was to evaluate the protective effects of royal jelly on methotrexate induced cytotoxicity on rabbit submandibular salivary glands. **Methods:** Thirty male white New Zealand rabbits weighing between 1800 and 2200 g. were divided equally into three groups. Group I (control group) was intraperitoneally injected by 1ml/kg dose of sterile 0.9% saline for 2 weeks. Group II (Methotrexate group) received methotrexate injection. Group III (Methotrexate & Royal jelly group) was administered royal jelly prior to methotrexate injection. Rabbits were sacrificed after 2 weeks of Methotrexate/Royal jelly administration. The submandibular salivary glands were dissected out and prepared for histological and immunohistochemical examinations. **Results:** Light microscopic examination of Methotrexate group revealed deformity of the secretory portions with numerous intracellular vacuoles. Secretory cells revealed deeply stained atrophied nuclei. The excretory ducts appeared dilated with degenerated epithelial lining. Widening of the connective tissue septa was also detected. Some secretory cells were completely degenerated leaving large vacuoles. There were numerous dilated blood vessels engorged with red blood cells. While Royal jelly treated group revealed well defined serous acini having distinct outline and lined by pyramidal cells with rounded basophilic nuclei. Well formed striated ducts were also detected. Immunohistochemical examination of Methotrexate treated group showed significant increase in Fas positive immunoreactivity indicating apoptotic changes. While Royal jelly treated group revealed expression of Fas immunoreactivity that statistically having no significant difference with the control group. **Conclusion:** Administration of Royal jelly produced a protective effect against cytotoxic and apoptotic changes induced by Methotrexate treatment in rabbit submandibular salivary glands.

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1. Introduction

At the beginning of last century, the only treatment available for patients with solid tumors was surgery, associated with high morbidity and mortality. Over the past 40 years, chemotherapy resulted in a progressive improvement in survival rates of patients with malignant neoplasm ⁽¹⁾. Chemotherapy is one of the most widely used interventions for treatment of cancer. Methotrexate (MTX), a structural analogue of folic acid, is widely preferred as a cytotoxic chemotherapeutic agent in the treatment of malignancies and some autoimmune diseases. While the cytotoxic effect of MTX is not selective for cancer cells, it also affects the normal tissues which have a high rate of proliferation, including the hematopoietic cells in the bone marrow and actively dividing cells of the intestinal mucosa ⁽²⁾. Thus, the efficacy of MTX is limited due to its toxic side effects. Studies revealed

that the systemic oxidative stress is an important factors background of the MTX induced toxicity ⁽³⁻⁶⁾. MTX causes differential toxic effects on lipid peroxidation by significant reduction in glutathione (GSH) levels leads to a reduction of effectiveness of the antioxidant enzyme defense system, sensitizing the cells to reactive oxygen species (ROS). MTX may also depress nucleic acid metabolism ⁽⁷⁾. In biological structures, malondialdehyde (MDA) is considered to be the most significant indicator of membrane lipid peroxidation arising from the interaction of ROS with cellular membranes ⁽⁷⁾. The important enzymatic antioxidant defense mechanisms in the tissues are dismutation of superoxide (O_2^-) to form hydrogen peroxide (H_2O_2) and O_2 by superoxide dismutase (SOD), as well as the conversion of H_2O_2 to molecular O_2 and H_2O by catalase (CAT) or conversion of H_2O_2 v to H_2O by glutathione peroxidase (GSH-Px). CAT and

GSH-Px are unique key enzymes scavenging hydroperoxides^(7, 8). These antioxidants are electron donors and react with the free radicals to form harmless products such as water. Therefore, antioxidants protect against oxidative stress and prevent damage to cells⁽⁹⁾. Some natural products, such as lycopene⁽¹⁰⁾, grape seed extract⁽¹¹⁾, green tea⁽¹²⁾, and caffeic acid phenethyl ester⁽¹³⁾, are demonstrated to have a protective role against oxidative stress. Recently, royal jelly (RJ) has received particular attention because of studies that have reported that it is a highly efficient antioxidant and has free radical scavenging capacity^(14, 15). Royal jelly is a secretion produced by the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*)⁽¹⁶⁾. Royal jelly (RJ) is a food mixture that contains protein, glucose, lipid, vitamins, free amino acids and minerals⁽¹⁷⁾. RJ is widely used as a commercial medical product. Previous studies have shown that RJ has a number of physiological effects, such as anti-inflammatory⁽¹⁸⁾, antitumor, anti-allergic⁽¹⁹⁾, and antioxidant activities⁽²⁰⁾. Oral RJ application of 300 mg kg⁻¹ day⁻¹ increases the number of erythrocytes and their diameters; therefore, it could be used as a supportive antioxidant molecule in anemic patients⁽²¹⁾. RJ peptides have a protective effect against lipid peroxidation caused by free radicals, both under *in vivo* and *in vitro* conditions⁽²⁰⁾. In addition, RJ has a protective effect against paracetamol induced liver damage in mice⁽²²⁾. Apoptosis (programmed cell death) is a gene-regulated event related to special morphological changes such as shrinkage of cell, chromatin condensation, and DNA damages⁽²³⁾. Fas is a member of the tumor necrosis factor. A family of transmembrane receptors involved in cell death signaling. It is a cell surface glycoprotein (about 36 KD molecular weight) that involved in mediation of apoptosis (programmed cell death). Fas has three cysteine rich extracellular domains and an intracellular death domain essential for signaling. Ligation of Fas by either agonistic antibody or by its natural ligand transmits a death signal to the target cells potentially triggering apoptosis⁽²⁴⁾. Chemotherapeutic drugs had many side effects so protection against their cytotoxicity is necessary. Therefore, the aim of the present study was to investigate the protective effect of royal jelly in methotrexate-induced oxidative damages and apoptotic changes of rabbit submandibular salivary gland by histological and immunohistochemical methods.

2. Material and Methods:

The study included 30 healthy white New Zealand rabbits weighing between 1800 and 2200 g. During the study, the rabbits were supervised in a 15m² closed balcony system illuminated by a night lamp.

Mean temperature was maintained at 22 ± 3 °C. Appropriate nutrition was provided; the main food sources were green vegetables, carrots, and tap water. Each animal that underwent surgery was anesthetized with 25 mg / kg IM ketamine hydrochloride (Ketalar®) and 5 mg / kg IM xylazine HCl (Alfazyne®, Alfasan International B.V. Woerden, Holland). Submandibular gland excision was achieved surgically. The 30 rabbits were divided into 3 groups, each containing 10 rabbits.

Group I (Control group):

The rabbits received a single intraperitoneal injection of isotonic saline.

Group II (Methotrexate treated group):

The rabbits were intraperitoneally injected with 15 mg/ Kg body weight day after day for two weeks.

Group III (Royal jelly and Methotrexate group):

The rabbits were given Royal jelly at 300 mg /kg/day via intraperitoneal injection for 15 days, before the intraperitoneal injection of 15 mg/Kg Methotrexate.

The doses of RJ used in this study were selected in accordance with previous studies⁽²⁵⁾.

At the end of the experimental period, bilateral submandibular gland excision was performed on rabbits under anesthesia. The submandibular salivary glands were fixed immediately in 10% calcium formol for 12 hours, washed by tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Then:

I- Sections of 6-7 µm were obtained and mounted on clean glass slides and stained with Haematoxylin and Eosin stain for routine light microscopic examination.

II- Sections of 4-5 µm thick were cut and mounted on poly-L-lysine coated glass slides and prepared for Fas immunohistochemical staining for detection of apoptotic changes in the submandibular salivary glands.

Fas Immunohistochemical staining:

Serial sections were cut and mounted on poly-L-lysine coated glass slides. The slides were dried over night at room temperature. Then sections were deparaffinized and hydrated in descending grades of alcohols. The sections were treated with blocking reagent for 5 minutes and washed in phosphate buffer working solution (PBS) for 10 minutes. After pre-incubation with 1% bovine serum albumin for 15 minutes, two to three drops of Fas protein mouse primary antibody were applied to the sections for 1 hour. Two to three drops of monoclonal mouse linking reagent were added to the slides then incubated for 30 minutes. Slides were incubated over night at 28°C in a humidity chamber. Two to three drops of streptavidin enzyme were placed then several drops of the working color reagent (DAB) were placed. The slides were counterstained with Mayer hematoxylin, passed through baths of 95% ethyl alcohol, absolute ethyl

alcohol and xylene respectively. Two drops of Canada balsam were placed on each slide and covers were mounted.

The immunostained sections were examined using:

a) Ordinary light microscope to assess the prevalence of Fas positive immunoreactivity in the submandibular salivary glands.

b) Image analyzer computer system was used to assess the optical density of Fas positive cells and the intensity of the immunostaining. The image analysis was performed using a computer (software Leica Quin500) consisting of color video camera, color monitor, CBU of IBM personal computer connected to the microscope. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The intensity of the reactions within the cells was measured by the optical density in 10 small measuring fields in each specimen using a magnification of 400.

Statistical analysis:

ANOVA-Test was used to compare the mean % values of Fas immunoreactivity between (control group), (methotrexate treated group) and (royal jelly and methotrexate treated group). A *p*-value (*p*< 0.0001) was considered significant.

3. Results

I-Light microscopic results:

Group I (Control group):

The Light microscopic examination of the rabbit submandibular salivary glands of control group showed regular structured acini and ducts in between. The acini were uniform in shape, having narrow lumen and lined by pyramidal secretory cells having rounded basophilic nuclei. The striated ducts were lined by one-layered cubic epithelium having central rounded nuclei. Connective tissue septae that divided the gland into lobes and lobules were also detected (**Fig.1**).

Group II (Methotrexate group):

Histological examination of submandibular salivary glands of Methotrexate treated group showed sign of acini degeneration represented by disfigured lobular structure and loss of normal architecture (amalgamation) of the secretory portions (**Fig. 2**). Some of the secretory elements as well as ductal elements revealed complete degeneration and were completely missed leaving large vacuoles. The lining cells of the acini were indistinct and showed numerous intracellular vacuoles (**Fig.3**). The serous cells showed deeply stained atrophied nuclei. The nuclei of the acinar cells revealed different sizes and shape (polymorphism). Stagnation of secretory material in the striated and excretory ducts was revealed (**Fig 4**). There were large dilated blood vessels engorged with red blood cells (**Fig 5**). The excretory ducts appeared

dilated with proliferation of the epithelial lining, surrounded by degenerated connective tissue (**Fig 6**). Widening of the connective tissue septa with increased fibrosis was also detected in some areas (**Fig 7**).

Group III (Methotrexate & Royal jelly group):

Histological examination of the submandibular salivary glands of Royal jelly treated group revealed marked improvement in cells of acini as well as cells of striated ducts, the acini relatively preserved their shape. The numbers of vacuoles decreased and well formed striated ducts were also detected. The connective tissue septa appeared relatively much closer to that of the control (**Figs 8 &9**).

II- Immunohistochemical results:

Group I (Control group):

Immunohistochemical examination of Fas protein in rabbit submandibular salivary glands of control group revealed negative Fas immunoreactivity in the secretory portions as well as in the duct cells and blood vessels (**Fig. 10**).

Group II (Methotrexate group):

Immunohistochemical examination of Fas protein in rabbit submandibular salivary glands of methotrexate treated group showed intense Fas positive immunoreactivity in the secretory portions, excretory ducts and slightly positive immunoreactivity in striated ducts (**Fig. 11**).

Group III (Methotrexate & Royal jelly group):

Immunohistochemical examination of Fas protein in rabbit submandibular salivary glands of Royal jelly treated group revealed slight Fas immunoreactivity in the secretory portions and duct system (**Fig. 12**).

Statistical analysis using ANOVA - Test to compare between different groups revealed, a significant increase in the mean optical density of the immunoreactivity of Fas protein in methotrexate treated group compared with the control group. Royal jelly treated group showed no significant difference in mean optical density of the immunoreactivity of Fas protein compared with the control group, while it produced a significant decrease in the mean optical density of the immunoreactivity of Fas protein compared with methotrexate treated group (**Table I & Histogram I**).

Table I: The difference in mean Fas optical density of different groups using ANOVA Test.

Group	Fas optical density		
	M±SD	F Value	<i>p</i> Value
I-Control group	44.86±2.24	57.59	<0.0001**
II-Methotrexate group	63.94±4.04		
III-Methotrexate & Royal jelly group	47.09±2.62		

** High significant difference at (*p*<0.001). M1 vs M2 *P*<0.0001 M1 vs M3 non-significant; M2 vs M3 *P*<0.0001

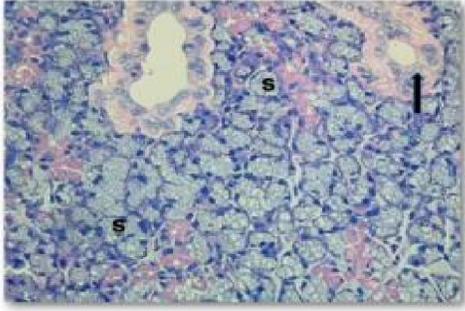


Fig 1: A photomicrograph of rabbit submandibular salivary gland of the Control group showing the normal architecture of salivary acini (S) and striated duct (arrow). (H & E Orig.mag. X 400).

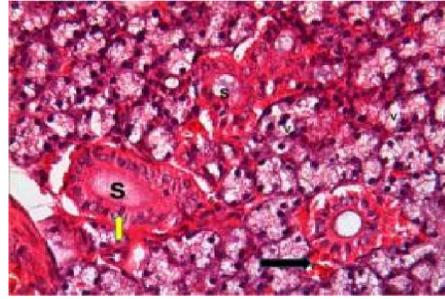


Fig 4: A photomicrograph of rabbit submandibular salivary gland of the Methotrexate treated group showing stagnation of the secretion in the striated and excretory ducts (s), cytoplasmic vacuoles (v), congested blood vessel (black arrow) and different size and shape of the nuclei (yellow arrow). (H & E Orig.mag. X 400).

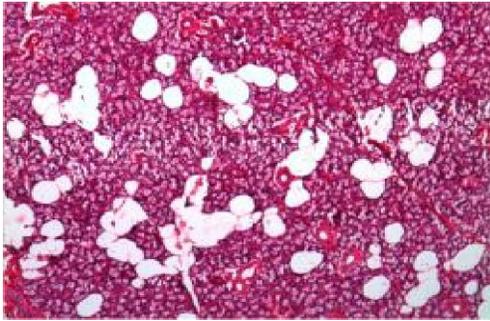


Fig 2: A photomicrograph of rabbit submandibular salivary gland of the Methotrexate treated group showing loss of normal architecture of salivary acini and disturbed lobular structure. (H & E Orig.mag. X 100).

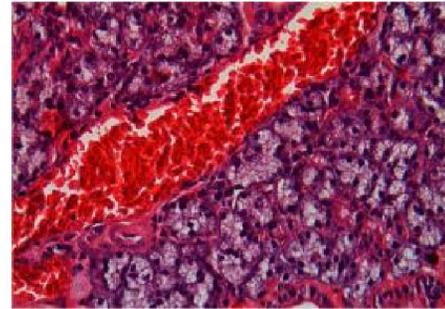


Fig 5: A photomicrograph of rabbit submandibular salivary gland of the Methotrexate treated group showing large dilated blood vessel engorged with red blood cells (H & E Orig.mag. X 400).

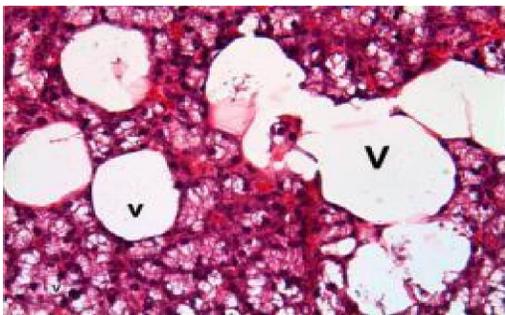


Fig 3: A photomicrograph of rabbit submandibular salivary gland of the Methotrexate treated group showing degeneration of some secretory and ductal elements leaving large vacuoles. (H & E Orig.mag. X 400).

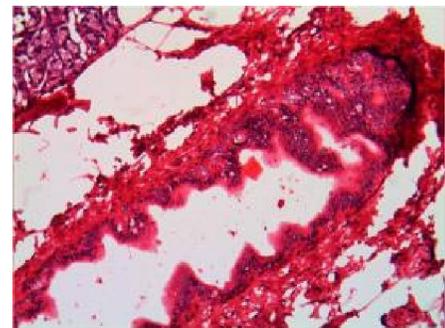


Fig 6: A photomicrograph of rabbit submandibular salivary gland of the Methotrexate treated group showing large dilated excretory duct with proliferation of its epithelial lining. (H & E Orig.mag. X 200).

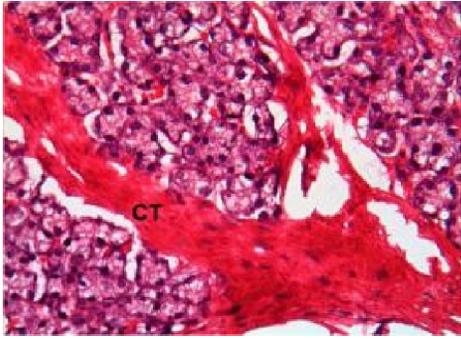


Fig 7: A photomicrograph of rabbit submandibular salivary gland of the Methotrexate treated group showing widening of the connective tissue septa with increased fibrosis (CT). (H & E Orig.mag. X 400).

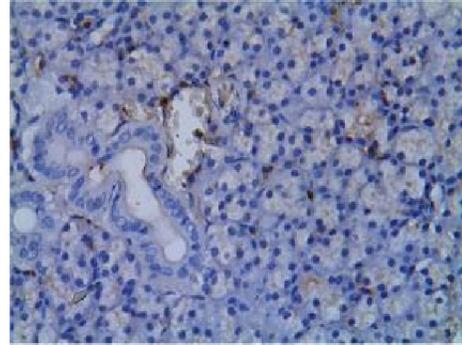


Fig.10: A photomicrograph of rabbit submandibular salivary glands of control group showing negative Fas immunoreactivity in the acinar cells, ductal cells and blood vessels. (Fas Orig. mag. X 400).

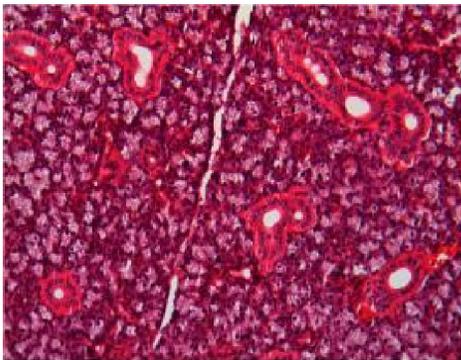


Fig 8: A photomicrograph of rabbit submandibular salivary gland of the Royal jelly treated group showing relatively normal histology and architecture of salivary ducts and acini. (H & E Orig.mag. X 200).

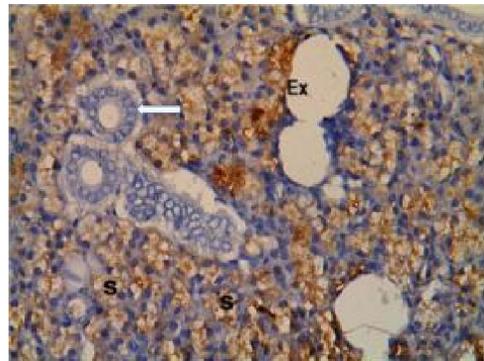


Fig.11: A photomicrograph of rabbit submandibular salivary glands of Methotrexate treated group showing intense Fas positive immunoreactivity in both acinar cells (S), excretory ducts (Ex) and slightly positive in striated ducts (arrow) (Fas Orig.mag. X 400).



Fig 9: A photomicrograph of rabbit submandibular salivary gland of the Royal jelly treated group showing well formed acini and duct lining with decreased number of vacuoles. (H & E Orig.mag. X 400).

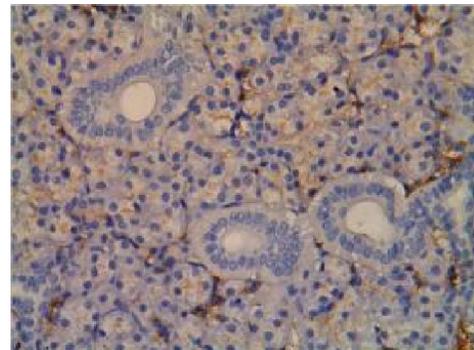
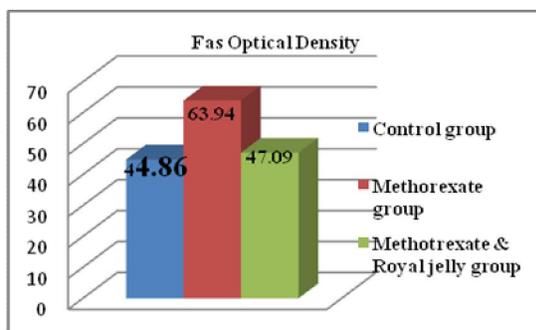


Fig.12: A photomicrograph of rabbit submandibular salivary glands of Royal jelly treated group showing slight Fas immunoreactivity in the secretory portions and duct system (Fas Orig.mag. X 400).



Histogram I: Represents the difference in mean Fas optical density between different groups.

4. Discussion

Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent for several malignancies and various inflammatory diseases. However, the clinical uses of methotrexate have been limited due to its serious adverse effects, which include anaemia, neutropaenia, increase risk of bruising and nausea, that are likely to result in the generation of free radicals and lipid peroxidation^(1,26). In the present study methotrexate injection had adversely affected the histological structure of the rabbit submandibular salivary glands, light microscopic examination revealed disfigurement, enlargement of the serous acini with abnormal architecture and indistinct cell boundaries. The enlargement of the acini might be due to dysfunction of the gland and disturbed salivary secretion leading to accumulation of the salivary secretion in the acini followed by its swelling and enlargement. The striated and excretory ducts showed dilatation with retaining secretion in their lumen. This finding might be attributed to failure of exocytosis as a result of glandular dysfunction. These histological changes in the duct system were in agreement with previous clinical findings. As the use of methotrexate for long term produced salivary gland dysfunction manifested as xerostomia⁽²⁷⁾. This result coincides with previous study reported that methotrexate had the ability to inhibit protein synthesis through depletion of folate cofactor that led to defect in glandular secretion and failure of secretory activity of salivary gland, which may explain the cause of altered secretion and subsequent xerostomia with methotrexate treatment⁽²⁸⁾. The loss of gland architecture and dilation of ducts suggested the pathological effect of chemotherapy on myoepithelial cells embracing them with failure of expelling the secretion into the oral cavity, leading to xerostomia⁽²⁹⁾. In addition, administration of methotrexate decreased the whole mouth and submandibular salivary Na and CL output with no change in K output which resulted from blocking of parasympathetic stimulation of the salivary

glands that leads to defect in Na reabsorption in the duct cells⁽³⁰⁾. Some of acini were completely missed leaving large vacuoles. This might be due to fatty degeneration and aggregation of the lipid degenerative products into large droplets, which were dissolved during in the routine processing of the tissue leaving large empty vacuoles. Similar findings were detected in the small intestine of rats and related to generalized lipidosis induced by methotrexate administration⁽³¹⁾. In the present study, Damaging of the salivary gland (acinar and ductal cell vacuolization and swelling, apoptosis in the acinar cells with pyknosis in the nuclei following methotrexate treatment could be due to several distinct molecular mechanisms; the most important might be related to the free radical damaging effect. These free radicals released during the intracellular metabolism of methotrexate, which interacts with the cell membrane, causing membrane lysis and release of major scavenger enzymes (glutathione based enzymes) such as (glucose-6-phosphate dehydrogenase and glutathione reductase) into the serum. This affects the physiological level of this antioxidant enzyme in serum and gives an indication of an adverse effect of methotrexate on cellular integrity^(32, 33). This finding coincides with other studies on the liver cells, as methotrexate exerted hepatic toxicity, the mechanism by which MTX causes hepatotoxicity results from binding to the enzyme dihydrofolic reductase, thus preventing conversion of folic acid to its active form, folinic acid. This in turn blocks the synthesis of nucleic acids, certain amino acids and indirectly proteins. This might lead to damage of organelles and plasma membranes of hepatic parenchymal cells interfering with their function and allowing leakage of enzymes^(34,35).

Two major pathways are known to be responsible for chemotherapy-induced apoptosis, the death receptor pathway and mitochondrial pathway, respectively involving ligation of death receptor (e.g. Fas) by a ligand (e.g. Fas-L)⁽³⁶⁾ and release of cytochrome c from the mitochondria⁽³⁷⁾. In this study increased density of Fas expression has been immunohistochemically detected mainly in acinar and duct cells. Also the statistical analysis of Fas immunoreactivity showed a significant increase in the mean optical density of methotrexate treated group compared with the control group indicating the apoptotic changes in the secretory cells as well as duct cells. These results emphasized the first pathway, that apoptosis caused by elevated expression of Fas. Royal jelly is a secretion product of the cephalic glands of nurse bees that has been used for centuries for its extraordinary properties and health effects. In current study, royal jelly administration according to the experimental design showed decreased number of vacuoles and significant improvement in cells of acini

and striated ducts, which were significantly altered by Methotrexate administration. Immunohistochemical examination of royal jelly treated group revealed no significant difference in Fas immunoreactivity compared with control group. This could be due to antiapoptotic, antioxidant, and the ability of RJ for scavenging the free radicals that resulted from the methotrexate treatment. Previous study revealed that RJ caused a significant recovery in antioxidant status of glutathione (GSH) and a significant inhibition of malondialdehyde (MDA) production⁽³⁸⁾. These findings were in agreement with another study on mice, where RJ has been found to protect tissue DNA against the oxidative damage. Studying the effect of the RJ diet on mice, it has been showed that after feeding RJ to mice for 16 weeks, the levels of 8-hydroxy-2-deoxyguanosine (an oxidative stress marker) were significantly reduced in kidney DNA and serum and the average life span of mice life expectancy was increased through the mechanism of reduced oxidative damage⁽³⁹⁾. Also, the protective effect of RJ which appeared in this investigation may be attributed to its constituents, such as free amino acids, proteins, sugars, fatty acids, minerals, and vitamins⁽⁴⁰⁾.

5. Conclusions

Methotrexate produced severe degenerative and apoptotic changes in rabbit submandibular salivary glands. Royal jelly produced protection against methotrexate induced cytotoxicity as well as anti apoptotic effect. Therefore, Royal jelly can be used as a protective natural product in cases of chemotherapy treatment, as it decreases its side effects on salivary glands.

Recommendations

RJ is a natural bee product with a great potential for use in medicine. RJ has numerous precious therapeutic properties used from ancient times until today. Some of its biological and therapeutic activities have been confirmed, but scientists have only begun to uncover the many health benefits of this amazing superfood and there are just a few solid evidences for those claims. On the other hand, the chemistry and bioactive compounds RJ are not sufficiently known. Further experimentation (in vitro, in animal research and on clinical trials) and validation would be needed to prove any useful benefit and action mechanism of native RJ and of isolated components as well.

Conflict of Interests

The author has no any commercial interest, financial interest, and/or other relationship with manufacturers of pharmaceuticals, laboratory supplies, and/or medical devices or with commercial providers of medically related services.

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