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Impact of Chamomile on Submandibular Salivary Gland of 5-Fluorouracil Treated Rabbits (Histological and Immunohistochemical Study)

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Abstract

Background and objectives: Chamomile has been used for centuries as an anti-inflammatory, antioxidant, and mild astringent and healing medicine. The purpose of this study was to investigate the potential anticytotoxic effect of chamomile extract on the submandibular glands of 5-FU treated female rabbits.

Materials and methods: Forty female white rabbits weighing between 1.0 kg and 1.5 kg were divided randomly into two groups. Control groups (intraperitoneally injected by physiological saline) consist of distilled water treated group and chamomile extract treated group (100 mg/kg), 10 animals each, and the treatment continue for 16 days. Study groups (intraperitoneally injected by 5-FU) consist also of distilled water treated group and chamomile extract treated group, 10 animals each, and the treatment also continue for 16 days. They were gavaged by distilled water or by chamomile extract in a similar way like control groups, but intraperitoneal injections of the 5-FU at a dose of (4 mg/kg) administered intraperitoneally once daily for 5 successive days. The right and left submandibular salivary glands of all the animals were dissected out and prepared for histological and immunohistochemical examinations.

Results: 5-FU cause signs of acini degeneration and large dilated blood vessels engorged with red blood cells were also seen. A statistically no significant differences were found between saline/distilled water and the other three groups regarding Ki-67 immuno expression ($p>0.05$). Statistical analysis also showed no significant differences present between saline/distilled water and saline/chamomile group in term of rate of apoptosis ($p>0.05$), but this difference were significant ($p<0.05$) between group 5-FU/distilled water and 5-FU/chamomile group, saline/distilled water group and 5-FU/distilled water or 5-FU/chamomile group ($p<0.05$), and saline/chamomile and 5-FU/distilled water or 5-FU/chamomile group.

Conclusion: Chamomile extracts at a concentration of 100 mg/kg/day orally gavaged for 16 days cause some microscopically changes to rabbit's submandibular salivary gland. Cytotoxicity of chamomile extract increased if it's taken with 5-FU by increasing the expression of cleaved caspase-3 immunostaining.

Keywords: Chamomile; Submandibular salivary gland; Chemotherapy; Ki67; Cleaved caspase-3

Introduction

Chemotherapy is one of the most widely used interventions for treatment of cancer. The cytotoxic effect of cancer chemotherapy is not selective for cancer cells, it also affects the normal tissues, the damage and its severity is based on the type, amount and duration of drug used to treat the disease [1]. 5-Fluorouracil (5-FU) is a cytotoxic drug, which is known to have immunosuppressive activities [2]. 5-FU is widely indicated for treatment of malignant tumors, particularly of the breast, colon or rectum, gastric, hepatic, pancreatic, uterine, ovarian and bladder carcinomas [3].

Chamomile is an herb that has been in use since ancient times due to its significant advantages. Chamomile is said to have antioxidant, anti-inflammatory, anti-bacterial and anti-fungal properties [4]. Extracts of chamomile may exert antioxidant effects within human body; their antioxidant activity is due to flavones, flavenols, isoflavones, flavonoids, anthocyanin, cumarin, tannins acid, and isocatechins [5]. Raal et al. founded that in Estonia, chamomile flowers were frequently used as a self medication to treat cold and flu [6]. It is widely used plant for various gastro-intestinal disorders, and commonly used for many human ailments such as hay fever, inflammation, muscle spasms, menstrual disorders, insomnia, ulcers, wounds, rheumatic pain, and hemorrhoids [7]. Chamomile tea is used as a gargle for inflammation of the mucous membranes of the mouth and throat [8]. Inhalation of the vaporized essential oils derived from chamomile flowers is used to relieve anxiety and general depression, and is widely used in cosmetic preparations and in soothing and softening effect on the skin [9].

Antigen KI-67 is a nuclear protein that is associated and necessary for cellular proliferation. Furthermore it has relation with ribosomal RNA transcription [10]. Inactivation of KI-67 antigen leads to inhibition of ribosomal RNA synthesis [11].

Caspase-3 is a member of the cysteine protease family and plays an important role in the regulation of programmed cell death (apoptosis) [12]. Caspase-3 expression has been extensively studied in many cancers. The positive correlation between its expression and a favorable prognosis has been reported in several cancers, suggesting its use as a prognostic marker for cancers [13]. In oral squamous cell carcinomas (OSCCs), increased caspase-3 expression has been shown to inversely correlate with cell differentiation [14]. Cleaved caspase-3, the active

form of Caspase-3, is well known as a marker for cells undergoing apoptosis in both normal tissue and cancer cells [12].

There are three major salivary gland; parotid, submandibular, and sublingual glands as well as minor salivary glands. Salivary glands have essential roles in normal upper gastrointestinal tract function and oral health, including the digestion of starch by the salivary amylase, swallowing, and the maintenance of tooth hard tissues through the production of saliva [15]. Saliva has an important role to keep healthy conditions of the oral cavity. Thus, it was possible that patients with cancer therapy or radiation therapy could be suffering from secretory hypo function as well as difficulty in swallowing, speech and taste [16].

The current study is aimed to investigate the potential anticytotoxic effect of chamomile extract (100 mg/kg/day) on the submandibular glands of female rabbits treated with 5-FU. As variable to evaluate the grade of damage or protection, we used histological and immunohistochemical investigations to clarify its effect on cell proliferation and apoptosis.

Materials and Methods

Forty healthy adult female rabbits (*Oryctolagus cuniculus*) were used in the study. They were around three months old, and weighting (1.0-1.5) kg. All rabbits were housed in the animal house at a College of Medicine, Hawler Medical University, Erbil, Iraq, under similar laboratory conditions in a temperature-controlled environment (21-24°C), with a 12 h light/12 h dark cycle and free access to food and water. The research project was approved by the Research Ethics Committee at College of Dentistry, Hawler Medical University under protocol. The rabbits were randomly divided into two groups (20 rabbit each):

Control groups (intraperitoneally injected by physiological saline)

Consist of distilled water treated group and chamomile extract treated group (10 animals each), the treatment continue for 16 days. In the distilled water treated group, a volume of distilled water equal to chamomile extract was given by gavage needle, while the chamomile extract treated group was gavaged with chamomile extract (*Matricaria chamomilla* organic alcoholic extract-United States-Code HS3751002) at a dose of 100 mg/kg/day administered orally one time daily [17]. A physiological saline (0.9% NaCl) in a similar dose of 5-FU (4 mg/kg) administered intraperitoneally one time daily for 5 successive days.

Study groups (intraperitoneally injected by 5-FU)

Consist also of distilled water treated group and chamomile extract treated group (10 animals each), the treatment continued for 16 days. They were gavaged by distilled water or by chamomile extract in a similar way like control groups. The dose of 5-FU (Kocak farma / Turkey), which was used in the current study was 4 mg/kg body weight administered intraperitoneally one time daily for 5 successive days. This dose was well-tolerated and showing minimal weight loss [18].

Histological and immunohistochemical analysis

At the end of the 16 days, all animals were sacrificed by ketamine over dose (Hameln Pharmaceuticals GmbH, Germany). Specimens were taken from the right and left submandibular gland tissues, and then specimens fixed in 10% neutral buffered formalin for 24 hours then were processed by standard paraffin-embedding methods. Sections were cut at 4 μm, deparaffinized, and then stained with Hematoxylin and eosin (H&E) staining and immunohistochemical staining using monoclonal antibodies to Ki-67 and monoclonal antibodies to cleaved caspase-3.

Cell proliferation was assessed by Ki-67 immunohistochemistry, while the apoptosis was assessed by cleaved caspase-3 immunostaining. This performed using monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, Code No. M 7240 staining system and a monoclonal Mouse Anti-Human cleaved caspase-3 Oncoprotien Clone 124, Code No. 1587, and used with Dako EnVision^{TM}, EnVision^{TM} double staining and LASABTM 2 systems. The staining procedure sections of the instructions included with each detection system were followed.

Positive and negative controls were run simultaneously with biopsy specimen. Positive cells expressing Ki-67 were identified by brown nuclei, while cleaved caspase-3 was demonstrated brown cytoplasmic staining. Five sections were randomly chosen for each animal. Approximately 1000 cells from cell population were counted by two observers at a magnification of 400X and the number of Ki-67 and cleaved caspase-3 positive cells were calculated and the mean values for each group were determined. Mean group values were statistically compared. All microscopic analyses were performed using a light microscope (Olympus, Tokyo, Japan). The level of Ki-67 and cleaved caspase-3 expression was evaluated according to the scoring system of Seleit et al. [19]. The application of this system gives a score ranging from 0 to 3 for degree of positivity: Percentage of positively stained cells (absent: <1%, mild: 1-10%, moderate: 10-50%, strong: >50%).

Statistical analysis

Statistical analysis was performed using Bonferroni Post Hoc test to assess statistical analysis for every individual pair in a group. P value less than or equal to 0.05 was considered statistically significant.

Results

Anatomical and microscopical features

Gross findings revealed that the submandibular gland in rabbits is pyramidal in shape and red-brown in color. Right and left submandibular glands were located medially to the angle of the mandible, and caudal to the sublingual gland. The submandibular gland of rabbit is surrounded by dense regular connective tissue capsule. Fine interlobular connective tissue septae are extended from the capsule dividing the gland tissue into lobules with different shapes and sizes. Each lobule is composed of densely packed acini. The gland is mixed in nature in which their acini are mainly mucous with a restricted extent of serous types. There are few mucous acini capped by serous demilune.

Group-1 (Saline/distilled water treated group)

The acini were uniform in shape and regularly structured. Serous acini are smaller in size than the mucous ones and their lumen are always obscured; the nuclei are large and round in shape, situated in the basal half of the cells. The mucous type showed a small obvious lumen and lined by pyramidal cells possessed oval nuclei rested on the base of the cell. The interlobular connective tissue septae that are separated different lobules revealed the presence of interlobular

excretory ducts. The intralobular ducts such as intercalated and striated ducts are intervening between the acini which are lined by simple cuboidal and columnar epithelium, respectively (Figure 1; A1 and A2).

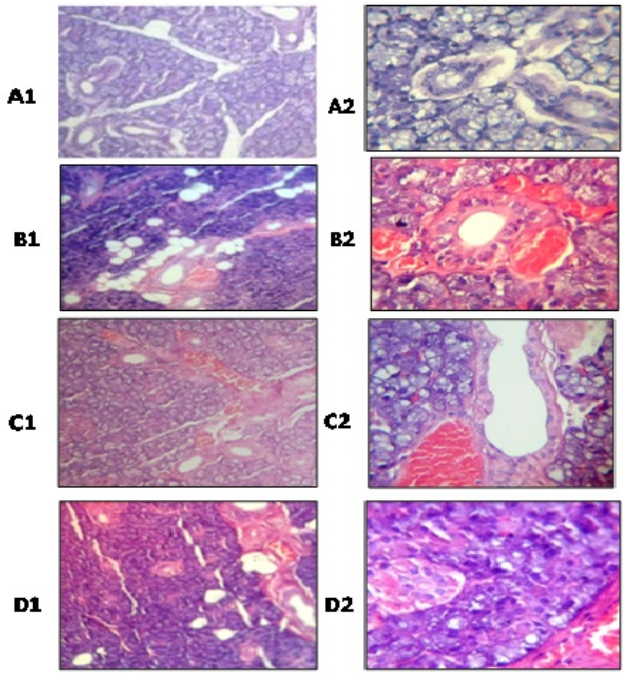

Figure 1: A photomicrograph of submandibular salivary gland of the saline/distilled water group showing the normal architecture of salivary acini and duct (**A1**, H and E × 100; **A2**, H and E × 400). Saline/chamomile group shows normal architecture of salivary acini but with the presence of congested blood vessels. Some of the secretory elements as well as ductal elements revealed complete degeneration leaving large vacuoles (**B1**, H and E × 100; **B2**, H and E × 400). 5-FU/distilled water treated group showing loss of normal architecture of salivary acini; the acinar cells have small pyknotic nuclei and vacuolated cytoplasm (**C1**, H and E × 100; **C2**, H and E × 400). 5-FU/chamomile extract treated group showing more loss of normal architecture of salivary acini and disturbed lobular structure; the acinar cells showing vacuolated cytoplasm and contain nuclei of different sizes and shape. The secretory elements as well as ductal elements revealed complete degeneration leaving large vacuoles (**D1**, H and E × 100; **D2**, H and E × 400).

Group-2 (Saline/chamomile extract treated group)

Histological examination of **the submandibular salivary gland in this** group revealed that the acini were uniform in shape and regularly structured, some ducts showed dilatation, some retaining their secretion in lumen, **and congested blood vessels were also seen. Some of the secretory elements as well as ductal elements revealed complete degeneration and were completely missed leaving large vacuoles;** this suggested the pathological effect of chamomile extract **(Figure-1; B, B2).**

Group-3 (5-FU/distilled water treated group)

Histological examination of submandibular salivary glands in this group showed **stagnation of the secretory material in some ducts.** Signs of acini degeneration **represented by disfigured lobular structure and loss of normal architecture of the secretory portions were seen. Nuclei of the acinar cells revealed different sizes and shape and large dilated blood vessels engorged with red blood cells were also seen (Figure-1; C, C2).**

Group-4 (5-FU/chamomile extract treated group)

Histological examination of the submandibular gland of rabbits in this group showed that the ducts become dilated with discontinuity of their epithelial lining in some areas. Larger number of acini loss their normal architecture and their lining cells were indistinct, scattered small and dark pyknotic nuclei of different size with a **more severe** degrees of cytoplasmic **vacuolization of secretory cells** and a marked vasodilatation of the blood capillaries **were seen. Some of the secretory elements as well as ductal elements revealed complete degeneration and were completely missed leaving large vacuoles** (Figure-1; D, D2).

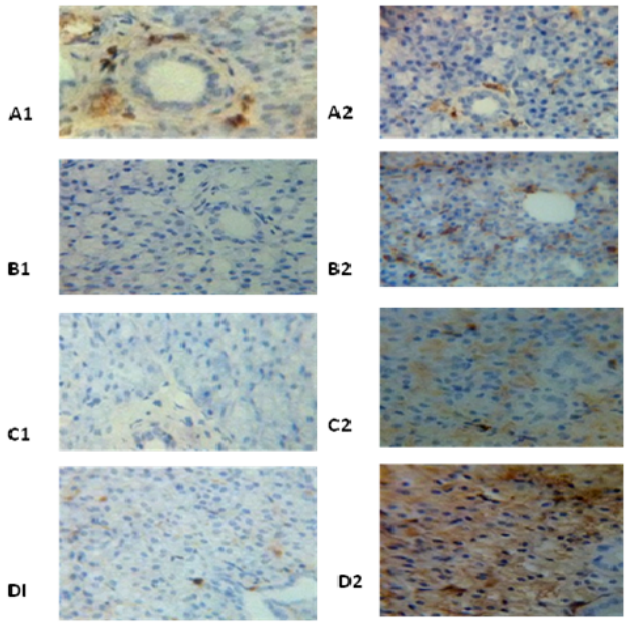

Figure 2: A photomicrograph of submandibular salivary gland of the saline/distilled water group shows **(A1)** negative Ki-67 immuno reactivity in nuclei of cells of ducts and acini and **(A2)** negative cytoplasmic reaction to caspase-3 (immunohistochemistry ×400). Saline/chamomile group shows **(B1)** negative Ki-67 immuno reactivity in nuclei of cells of ducts and acini and **(B2)** negative cytoplasmic reaction to caspase-3 (immunohistochemistry ×400). 5-FU/distilled water treated group shows **(C1)** negative Ki-67 immuno reactivity in nuclei of cells of ducts and acini and **(C2)** mild cytoplasmic reaction to caspase-3 (immunohistochemistry ×400). 5-FU/chamomile extract treated group shows **(D1)** negative Ki-67 immuno reactivity in nuclei of cells of ducts and acini and **(D2)** moderate cytoplasmic reaction to caspase-3 (immunohistochemistry ×400).

Immunohistochemical result

Immunohistochemical analysis using Ki-67 immunostaining to study the cellular proliferation in rabbit submandibular salivary glands of group 1 was negative (0.69 ± 0.02) and most of the positive cells was seen in association with ductal cells. Group 2 and group 3 showed no any positive cells was seen in association with the acinar or ductal cells. While group 4 appear negative (0.01 ± 0.03) for Ki-67 immunostaining.

Immunohistochemical analysis using caspase-3 immunostaining to study the cellular apoptosis in rabbit submandibular salivary glands of group 1 and group 2 revealed negative caspase-3 immunoreactivity in the secretory portions as well as in the duct cells, but group 3 and group 4 revealed mild and moderate caspase-3 immunoreactivity. Statistical analysis showed no significant differences present between group 1 (0.02 ± 0.11) and group 2 (0.86 ± 0.43) in terms of rate of apoptosis ($p>0.05$), but this difference was significant ($p<0.05$) between group 3 (7.65 ± 0.78) and 4 (13.25 ± 1.3), group 1 and group 3 or 4 ($p<0.05$), group 2 and group 3 or 4 ($p<0.05$) respectively (Figure 2).

Discussion

Although the submandibular salivary gland is considered the second largest salivary gland, it produces about 60% of saliva [16], and the time for a drug required to reach the maximum concentration in submandibular saliva was shorter than that in parotid saliva [20], for this reason the present research studied the submandibular salivary gland of the rabbit. Regarded to histology of rabbit's submandibular gland, the result has shown that it is mixed in nature in which their acini are mainly mucous, this result agrees with previous studies [21,22].

In the present study 5-FU injection had adversely affected the histological structure of the rabbit submandibular salivary glands, signs of acini degeneration represented by disfigured lobular structure and loss of normal architecture of the secretory portions were seen. Nuclei of the acinar cells revealed different sizes and shape and large dilated blood vessels engorged with red blood cells were also seen. These results coincides with other studies, which demonstrated that cancer chemotherapy could induce salivary gland acinar degeneration [23,24].

Chamomile is included in the "Generally Regarded as Safe" (GRAS) list by the FDA [25], and according to Medicine Net. com, chamomile seems safe when taken by mouth for short periods of time. The long - term safety of chamomile is unknown. The current study showed that, chamomile exerts cytotoxic effect to submandibular salivary gland at day 16, due to the microscopical changes seen at this day in comparison with the saline/water group. A similar finding was also reported the harmful effect of chamomile when used for long duration in rat intestine by Al-Refai [26], while previous studies have demonstrated the anticancer activity of aqueous and methanolic extracts of chamomile recutita flowers against many human cancer cells [27]. This controversy might have been caused by differences in doses and long duration intake of chamomile.

5-FU and chamomile also effect on cell proliferation and apoptosis of submandibular salivary gland of rabbit, the result showed no significant decrease ($p>0.05$) in the rate of proliferation in group 2, 3, and 4 in comparison with the saline/distilled water group. No significant difference in the rate of apoptosis also present between group 1 and group 2 ($p>0.05$), but this difference was significant ($p<0.05$) between group 3 and 4, group 1 and group 3 or 4 ($p<0.05$), group 2 and group 3 or 4 respectively.

Ozel et al. also observed only slight ductal cellular proliferation and no cellular proliferation in the acinar cells with proliferating cell nuclear antigen (PCNA) antibody staining in any of the groups with or without 5-FU, but the rate of apoptosis was higher in 5-FU treated group according to TdT-mediated dUTP nick end labeling (TUNEL) ($p<0.05$) [18]. It seems that inhibition of DNA synthesis; DNA damage and the production of reactive oxygen species by chemotherapy impair the metabolism in progenitor cells and cause inhibition of mitosis and increase of apoptosis [28]. Cavalieri et al. found that the harmful effect of chamomile may come from its constituents, like bisabolol, volatile oils, anthemic and tannic acid. The α-bisabolol is a small oily sesquiterpene alcohol, it is a pro-apoptotic agent and enhance apoptosis [29]. One study using cadaver skin demonstrated that bisabolol can enhance the penetration of 5-FU [30]. Naveen et al. found that herbal drug associated toxicity can occur because of various factors like drug-herb interactions, coexisting diseases and direct toxicity (dose/duration) [31].

On the other hand, Apoptosis was thought to be the major reason of cell death induced by chemosensitizer. It is now grossly recognized that drug-induced apoptosis may be used to measure the sensitivity of cells to drugs, with an increased rate of apoptosis meaning that the cells have a higher sensitivity to chemotherapy [32,33]. We examined the apoptotic effect induced by chamomile combined with 5-FU or administered individually. In the present study the result showed that chamomile and 5-FU alone significantly induced apoptosis compared with the control, and the combined treatment effects were stronger than the individual effects of chamomile and 5-FU. The 5-FU combine with chamomile could be more efficient to decrease surviving and increase the caspase-3; this indicated that chamomile could enhance the 5-FU cytotoxicity and apoptosis for long duration.

Conclusion

The results of the current study demonstrated that the effect of chamomile at a concentration of 100 mg/kg/day gavaged orally one time daily for 16 days in combination with 4 mg/kg body weight 5-FU administered intraperitoneally one time daily for 5 successive days can enhances rabbit submandibular salivary gland cytotoxicity. This finding could provide a novel insight into long duration of taking chamomile can cause cytotoxicity and damaging effect to rabbit submandibular salivary gland.

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