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RESEARCH ARTICLE

Evaluation of Caspase 3 as a Target for Apoptosis induced via Chemotherapy in Rats.

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Abstract

Background:
Apoptosis is a systematic cellular process (programed cell death) that occurs in physiological and pathological conditions. It plays an important role in the treatment of cancer as it is a popular target of many treatment strategies. Caspase 3protein is one of the main executioners of apoptosis. Fluorouracil (5-FU) is a chemotherapeutic agent which has been used against cancer several years ago and now there is uprising interests in its usage as an apoptotic enhancer.

Methods:
Sixty albino rats were allotted randomly into 2 equal groups, control group I and group II which received intraperitoneal injection of 5-FU. Oral samples (buccal mucosa, gingiva, labial mucosa, tongues and submandibular glands) were routinely processed for Caspase3 immunohistochemical stain. Blood samples were taken from all rats for Polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR).

Results:
A significant loss of weight and oral tissues inflammation has been noticed in group II. This study also revealed a significant difference in the immunostaining of Caspase 3between two groups. Group II had a moderate reaction for Caspase 3in 60% of its samples while the remaining 40% showed an intense reaction. In contrast, group I revealed a mild reaction. Furthermore, the significant increase in group II Caspase 3 expressions was associated with genotype AA which also was associated with genotype heterozygote GT (71% of the tested rats).

Conclusion:
We concluded that 5-FU injection increases the Caspase protein expressions which enhance the apoptotic activity. Evaluation of Caspase 3 polymorphisms with their haplotypes and amount expression help to define genetic susceptibility to cancer development and response to chemotherapy.

INTRODUCTION

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. An understanding of the underlying mechanisms of apoptosis is important as it plays a pivotal role in the pathogenesis of many diseases. Cancer is one of the scenarios where apoptosis is blocked, resulting in malignant cells survival (Pop et al 2009). The mechanism of apoptosis is complex and involves many pathways. Defects can

occur at any point along these pathways, leading to malignant transformation of the affected cells, tumor metastasis and resistance to anticancer drugs (Ghobrial et al 2005). Despite being the cause of problem, apoptosis plays an important role in the treatment of cancer as it is a popular target of many treatment strategies (Wong RS 2011).

Caspases, which are the executioners of apoptosis, comprise two distinct classes, the initiators and the effectors. Although general structural features are shared between the initiator and the effector Caspases, their activation and inhibition are differentially regulated (Riedl et al 2004). Caspases remain one of the important players in the initiation and execution of apoptosis. Therefore, it is reasonable to believe that low levels of Caspases or impairment in Caspase function may lead to a decrease in apoptosis and carcinogenesis (Wong RS 2011). The execution phase of apoptosis involves the activation of a series of Caspases. The upstream Caspase for the intrinsic pathway is Caspase 9 while that of the extrinsic pathway is Caspase 8 (Devarajan et al 2002). The intrinsic and extrinsic pathways converge to Caspase 3. It then cleaves the inhibitor of the Caspase-activated deoxyribonuclease, which is responsible for nuclear apoptosis (Ghobrial et al 2005).

The human Caspase-3 gene is located on 4q33-q35.1 and possesses 2635 base pairs leading to 7 exons (Kitada et al 1998). Polymorphisms in the CASP-3 gene may influence CASP-3 production and/or activity, thereby modulating the susceptibility cancer. Jang et al 2008 first screened for polymorphisms in the CASP-3 gene by using Single Nucleotide Polymorphisms analysis (SNPs) of genomic DNA samples from 27 healthy Koreans. Then they evaluated associations of these polymorphisms with lung cancer in a case-control study that consisted of 582 lung cancer patients and 582 healthy controls. Individuals with at least one variant allele (heterozygote) of the -928A > G, 77G > A, and 17532A > C polymorphisms were at a significantly decreased risk for lung cancer in comparison to the carriers with each homozygous wild-type allele (Jang et al 2008).

Fluorouracil (5-FU) is a chemotherapeutic agent which has been used against cancer for about 40 years. 5-FU is a fluorinated pyrimidine analog which is used as an anti- neoplastic drug (Tutkun et al 2001).It is a suicide inhibitor and works through irreversible inhibition of thymidylate synthase. It belongs to the family of drugs called antimetabolites. 5-FU was designed, synthesized after the observation that some tumor cells utilize uracil, a pyrimidine base suitable for normal body cells (Chu E 2007). 5-FU acts in several ways, but principally as a thymidylate synthase inhibitor. Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidine, which is a nucleoside required for DNA replication (Correale et al 2003). Being a pyrimidine analogue, 5- FU is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA, finally inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA [Correale et al 2003, Toyoda et al 2000]

The laboratory rat, *Rattusnorvegicus*, was the first mammalian species tamed for scientific research, with work dating back to before 1850 (Lindsey JR. 1979). From this promising beginning, the rat has become the most broadly studied experimental animal model for biomedical research (Jacob J H 1979)

This study is a trial to open new doors into potential of chemotherapeutic anticancer drugs targeting Caspase 3. Furthermore, the usage of both immunohistochemistry and SNPs will be helpful to assess the amount of expression and Caspase 3allelic variations which in favor of apoptotic activity.

Materials and Methods

Subjects

Experimental animals and establishment of animal model: Sixty albino rats weighing 90 ± 9.8gm were obtained from the Experimental Animal Center of Faculty of Medicine, Mansoura University. The rats were maintained on the commercial soft diets and water regimens under standard temperature (22–25°C), ventilation and hygienic conditions. The present study protocol was reviewed and approved according to EU Directive 2010/63/EU for animal experiments. After one week of acclimatization, the rats were allotted randomly into 2 equal groups:

Group I (control group): This group used as a normal parameter.

Group II: This group received intraperitoneal injection of 5-FU (150 mg/kg) for successive 5 days. On the 10^{th} day of the treatment, the rats were deprived of food for 12 hr. then the rats were anesthetized with 20% urethane (ethyl carbamate, NH2COOC2H5) solution, and their buccal mucosa, gingiva, labial mucosa, tongues and submandibular glands were carefully excised, fixed and embedded by paraffin. Then, the samples were routinely processed for haematoxyline and eosin staining as well as for immunohistochemical study.

Blood samples were taken from all rats on EDTA for Polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR). All rats were weighed at the beginning and at the end of the experiment and the weights were recorded. Also, the greatest dimensions of the excised salivary glands were measured by using a ruler. These dimensions represent the sizes.

Immunohistochemistry

A standard avidin-biotin complex method was carried out. Four-micrometer-thick formalin-fixed paraffin-embedded sections were dewaxed in xylene and rehydrated through graded alcohol to distilled water. The sections were subjected to antigen retrieval by boiling in a microwave for 20 min in 0.01 m sodium citrate buffer (pH 6.0). The primary antibody to Caspase-3 (Wuhan boster Biotechnology Co. LTD) was applied at a dilution of 1:100 and incubated overnight at 4°C. After incubation, the slides were rinsed in TBS (3 times for 10 min each) and then incubated with secondary antibody (EnVision detection Kit Peroxidase/DAB Rabbit/mouse, Dako Cytomation) according to the manufacturer's instructions. The slides were rinsed in TBS (3 times for 10 min each) and then stained with 3, 3'-diaminobenzidinetetrahydrochloride (EnVision detection Kit Peroxidase/DAB Rabbit/mouse, Dako Cytomation). Sections were counterstained with Mayer's haematoxyline solution. A negative control reaction with no primary antibody was always carried out alongside the reaction containing sample. The specificity of the Caspase-3 antibody was confirmed by comparison with control antibodies.

Digital image analysis

Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 40 X objective. The images were analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in automated object counting routine for immunohistochemical analysis.

Evaluation of Caspase 3 immunostain

Both cytoplasmic and nuclear staining of different tissue cells was considered. The immunoreactivity was graded according to the number of positively stained cells: ≤ 1% cells with a positive reaction as negative, > 1 ≤ 10 % as mild (+), >10 % ≤ 50 % as moderate (++) and > 50% as intense (+++).

Polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR)

Genomic DNA of Rats was isolated from the whole blood taken on EDTA with Gene JET Genomic DNA purification kit (Fermentas, EU) according to standard protocol provided by the manufacturer. Polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR) was used to identify bi-allelic Caspase 3 gene polymorphisms in two common single nucleotide polymorphisms (SNPs) located in exon 2 and intron 1(rs4647601: G>T, and rs4647603: G>A). PCR reactions were performed in a total volume of 25 µL containing 12.5 µl of Maxima hot start PCR master mix 2X (Thermo Scientific) (containing Maxima hot Taq DNA polymerase, hot start PCR buffer, 400µM of each dNTP, and 4mM Mg+2), 0.1 µl of each primer (100 picomol), and 1µl of extracted DNA (20-50 ng) and completed to 25 µl with molecular grade water. The PCR profile consisted of an initial melting step of 96°C for 5 min; 35 cycles of 96°C for 45 s, 56°C for 40 s, and 72°C for 30 s; and a final extension step of 72°C for 10 min.

The following primers designs were use according to (Chen et al 2008)
Caspase 3 rs4647601: (G>T)
5-′GCGGTAGCGCCGTCCGTTGC-3′ (forward)
5-′ACCGAGCTCCGAGGGCGGGAG-3′ (reverse)
CASPASE 3rs4647603: G>A)(
5-′TGTGTATCCGTGGCCACAGCT-3′ (forward)
5-′GAGAATGGGGGAAGAGGCAGGT-3′ (reverse)

Successful amplification was confirmed by detection of the PCR products on a 2% agarose gel and visualized under UV light, 103 and 132 bp for rs4647601:G>T and rs4647603:G>A, respectively (Fig. 1). The amplified product DNA was incubated with Hpych4V and PvuII restriction enzymes (New England Biolabs) used to digest rs4647601:G>T and rs4647603:G>A, resulting in 84- and 19-bp fragments or 113- and 19-bp fragments, respectively. The digested products were separated using 2.5% agarose gel and detected by ethidium bromide staining. Fragments obtained were 103 Fragments obtained for rs4647601:G>T were ;84 bp and 19 bp to the wild type genotype G/G, 103 bp to the mutant genotype T/T and 103 bp, 84 bp and 19 bp to the G/T genotype. Fragments obtained for rs4647603:G>A ;113bp and 19 bp to the wild type genotype G/G, 132 bp to the mutant genotype A/A and 132 bp, 113 bp and 19 bp to the G/A genotype**.**

Statistical analysis

Data were tabulated, coded then analyzed using Statistical Package for Social Science (SPSS version 17.0). For the analytical statistics, the significance of difference was tested using t-test for parametric data and Mann-Whitney U for non-parametric. Moreover, partial correlation test was used to assess relations between variables. Statistical tests were based on a type 1 error value of 5% (α=0.05) and a power of 0.85 sample size.

Results

General observations

There was a significant loss of weight in all rats receiving 5-flurouracil (group Π) as compared to the control group. The unpaired t-test revealed a significant difference between the weights of the rats of the two groups (Table1) (Fig.2). Also, Salivary glands dissected from (group Π) were found to be smaller in size than those of the control group. Statistical analysis exhibited a highly significant difference between the sizes of the dissected salivary glands of both groups (Table 1) (Fig.2). The oral mucosa of rats of group II was red, shiny and glazy. Some rats of group II exhibited oral ulcerations.

Ordinary histological findings

The tissues dissected from the rats of control group demonstrated structural integrity of their mucosa. It revealed stratified squamous epithelium with normal underlying lamina propria. No inflammatory cells infiltrate was seen, and the basement membrane showed mild or short rete processes. Epithelial atrophy and collagen degeneration were the most common histological changes observed in group II rats. Also, damaged keratinocytes with extensive cytoplasmic vacuoles and hyperchromatic nuclei as well as prominent subepithelial inflammatory cell infiltrate were observed. In addition, numerous congested blood capillaries were detected in lamina propria. The filiform papillae of the tongue showed atrophic changes. The submandibular salivary glands dissected from the control group showed normal gland histology. It showed secretory end piece (serous acini), duct system (intercalated ducts, striated ducts, granular convoluted tubules and excretory ducts) and connective tissue septa which divide the glands into lobes and lobules. The glands dissected from group II animals showed marked degenerative changes of most of their acini as well as their duct system specially striated ducts.

Immunohistochemical and genotyping findings:

The immunostain and genotyping findings are presented in table 2. All sections of group I revealed a mild immune reaction for Caspase-3 (mean =3.923 ± 0.82) (Fig.3&4). Group II exhibited a moderate reaction in 60% of its samples while the remaining 40% showed an intense reaction with an overall mean =40.067 ± 27.165 (Fig.4 &5). Blood vessels were positively stained in all specimens of both groups. Caspase-3 was highly expressed in fibrous tissue of all biopsies while a mild reaction was detected in mucosal epithelium. (Fig. 3&5) For Caspase 3rs4647601: G>T and rs4647603: G>A SNP, four alleles were identified among rats of group II: GT, TT and GA, AA. Genotypes GT and TT were for rs4647601: G>T and GA, AA were for rs4647603: G>A. Homozygote Caspase3 gene polymorphisms TT and AA were expressed in 40% for each (12/30 for each) while heterozygote GT and GA were expressed in the remaining 60% (Fig. 1). Meanwhile, RFLP-PCR of group I revealed GG haplotypes in all rats. In group II, all specimens with genotype AA were associated with genotype GT and intense immunoreactivity (mean of immunostain=71%). In contrast, all specimens with genotype TT were associated with genotype GA and moderate immunoreactivity (mean of immunostain=11.3%). The remaining specimens exhibited GT and GA genotypes and moderate immune reaction (mean of immunostain=34%). All rats of control group had genotype GG and low Caspase 3 immunostain (Tables 2&3). Statistical analysis revealed a significant difference between the two groups in relation to Caspase 3immunostain (Fig. 6) as well as to single nucleotide polymorphisms (SNPs) located in exon 2 and intron 1 (rs4647601: G>T, and rs4647603: G>A) (Tables 3&4). Also, Partial correlation test revealed a highly significant inverse correlation between Caspase 3immunostain and Caspase3 rs4647601: G>T SNP while a highly significant positive correlation between Caspase 3immunostain and Caspase3 rs4647603: G>A SNP was found. Also, a significant positive correlation was found between Caspase 3 gene SNPs located in exon 2 and intron 1 (Table 5).

Table 1: Shows means ± SD of the rats' weights and the sizes of the dissected salivary glands as well as the unpaired t-test for comparison of them.

Parameter	Mean ± SD	P value

	Group I	Group II	
Weight	214 ± 10.2	166 ± 7.7	< 0.0001
Size of salivary gland	1.96 ± 0.17	0.69 ± 0.13	< 0.0001

Table 2: Immunohistochemical expression of Caspase 3 and bi-allalic Caspase 3 gene polymorphisms in two common single nucleotide polymorphisms (SNPs) located in exon 2 and intron 1(rs4647601: G>T, and rs4647603: G>A) in all rats.

Cases	Group I			Group II		
		RFLP-PCR			RFLP-PCR	
	Caspase- 3 Immunosatin	Caspase3 rs4647601: G>T SNP	Caspase3 rs4647603: G>A SNP	Caspase- 3 Immunosatin	Caspase3 rs4647601: G>T SNP	Caspase3 rs4647603: G>A SNP
1	3.25%	GG	GG	74%	GT	AA
2	2%	GG	GG	11%	TT	GA
3	1.5%	GG	GG	71%	GT	AA
4	1.8%	GG	GG	33%	GT	GA
5	3%	GG	GG	15%	TT	GA
6	4.1%	GG	GG	75%	GT	AA
7	4%	GG	GG	30%	GT	GA
8	1.5%	GG	GG	77%	GT	AA
9	2.5%	GG	GG	13%	TT	GA
10	3%	GG	GG	14%	TT	GA
11	3.4%	GG	GG	13%	TT	GA
12	1.7%	GG	GG	11%	TT	GA
13	4%	GG	GG	69%	GT	AA
14	4.2%	GG	GG	67%	GT	AA
15	2.3%	GG	GG	38%	GT	GA
16	3.7%	GG	GG	72%	GT	AA
17	4.8%	GG	GG	37%	GT	GA
18	1.7%	GG	GG	74%	GT	AA
19	4.1%	GG	GG	12%	TT	GA
20	3.3%	GG	GG	10%	TT	GA
21	2.2%	GG	GG	14%	TT	GA
22	3%	GG	GG	13%	TT	GA
23	3.5%	GG	GG	78%	GT	AA
24	5%	GG	GG	64%	GT	AA
25	4.7%	GG	GG	32%	GT	GA
26	2%	GG	GG	62%	GT	AA
27	3.1%	GG	GG	34%	GT	GA
28	1.3%	GG	GG	69%	GT	AA
29	3.3%	GG	GG	9%	TT	GA
30	2%	GG	GG	11%	TT	GA

Table 3: T-test used to determine the significance of difference between the studied groups in relation to Caspase immunostain.

Groups	Number	Mean ± Standard Deviation(SD)	T	Df	P value
Group I	30	3.923 ± 0.82	-7.28	58	.000**
Group II	30	40.067 ± 27.17	-7.28	29.05	.000**

Table 4: Mann-Whitney U used to test the significance of difference between the studied groups in relation to Caspase3 rs4647601: G>T SNP and Caspase3 rs4647603: G>A SNP (RFLP-PCR)

Groups	Mean rank	Z	P value
Group I	15	-7.258	.000**
Group II	45	-7.258	.000**

Table 5: The relations between the different variables in all groups

Variables	R	P value
Caspase 3 immunostain & Caspase3 rs4647601: G>T SNP	-.853	.000**
Caspase 3 immunostain & Caspase3 rs4647603: G>A SNP	.945	.000**
Caspase 3 immunostain & Caspase3 rs4647601: G>T SNP& Immunostain & Caspase3 rs4647603: G>A SNP	.667	.000**

Figure 1: Caspase3 rs4647601: G>T polymorphism genotypes by agarose gel electrophoresis. L: 50–1000 ladder size marker. Lane 1: Caspase 3 GG genotype (wild type) showing a 84 bp band. Lanes 3 and 5 Caspase 3 TT homotype show a 103 bp band. Lanes 2 and 4: CASPAS3 GT heterotype showing 84 and 103 bp bands.

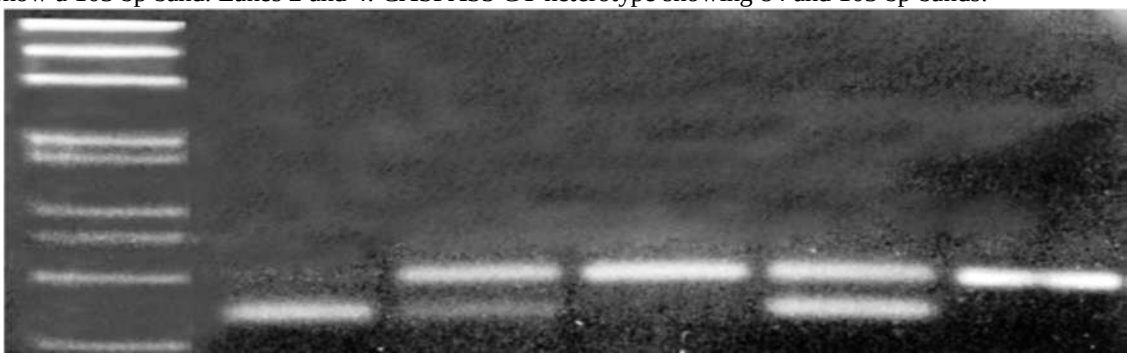


Figure 2: Shows the means ± SD of rat's weights and the sizes of the dissected salivary glands of rats of both groups

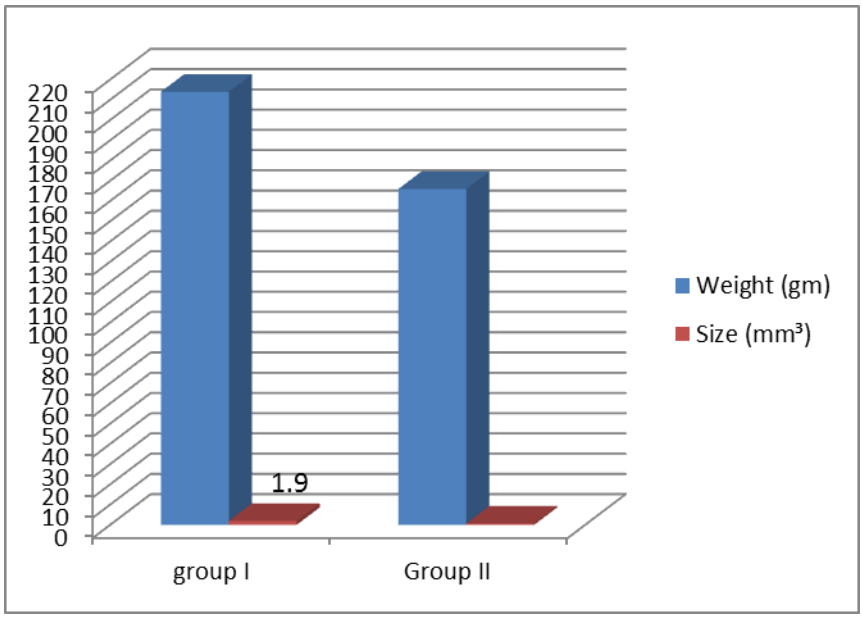


Figure 3: Shows mild expression of Caspase 3 in different oral tissues of rats of control group [fibrous tissue (F), salivary gland (SG) and mucosal epithelium (E)]. Blood vessels are positive in all specimens.

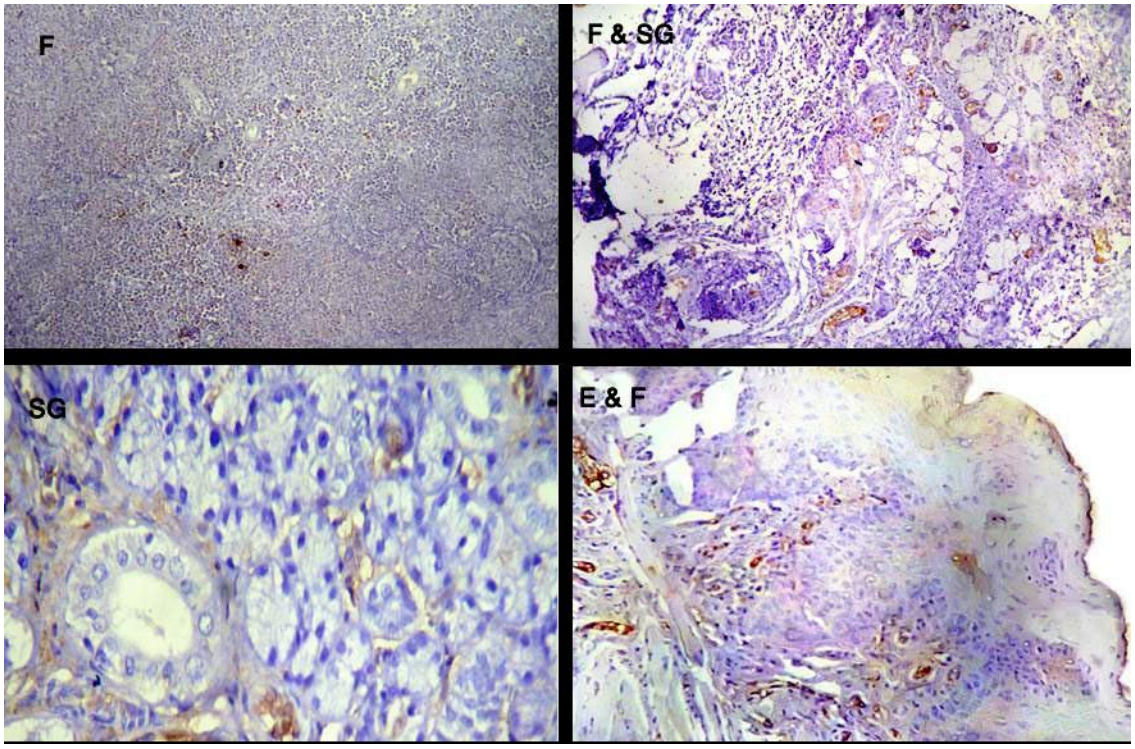


Figure 4: Shows the mean ± SD of Caspase 3 immunostain in the studied groups

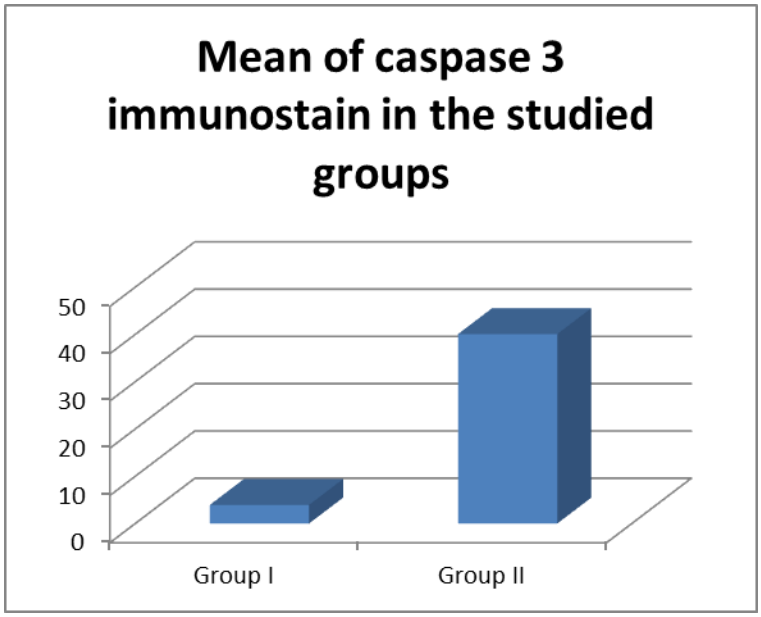


Figure 5: Shows moderate to intense expression of Caspase 3 in different oral tissues of rats receiving 5-FU [fibrous tissue (F), salivary gland (SG) and mucosal epithelium (E)]. Blood vessels are positive in all specimens.

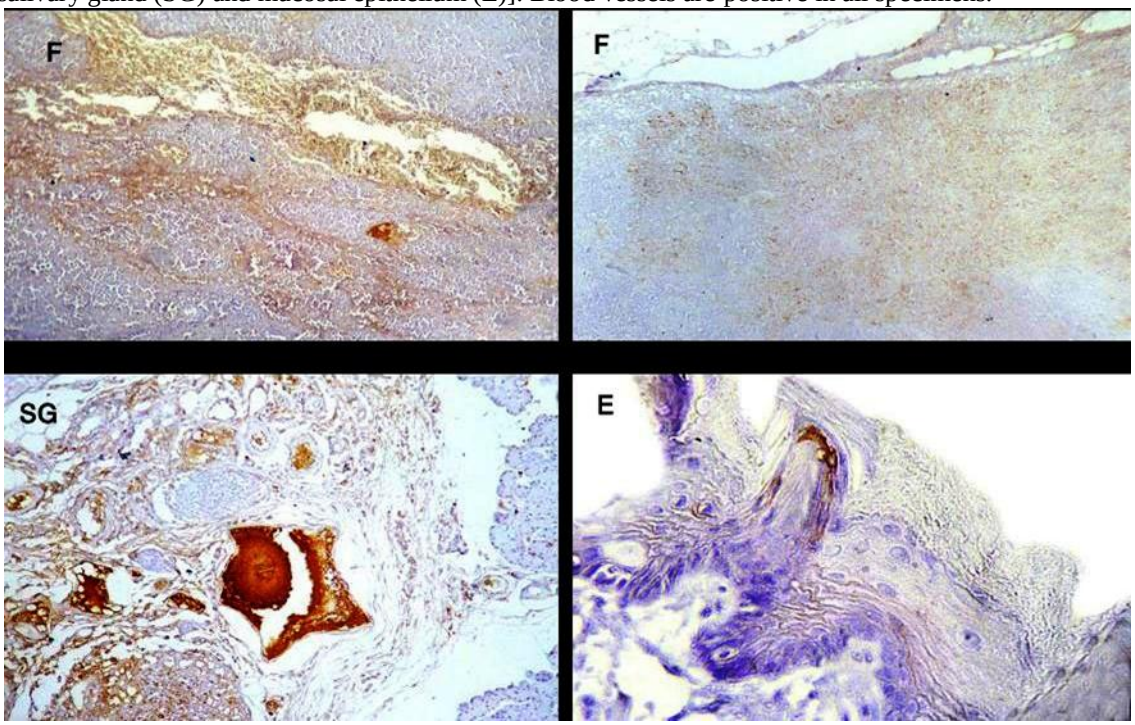
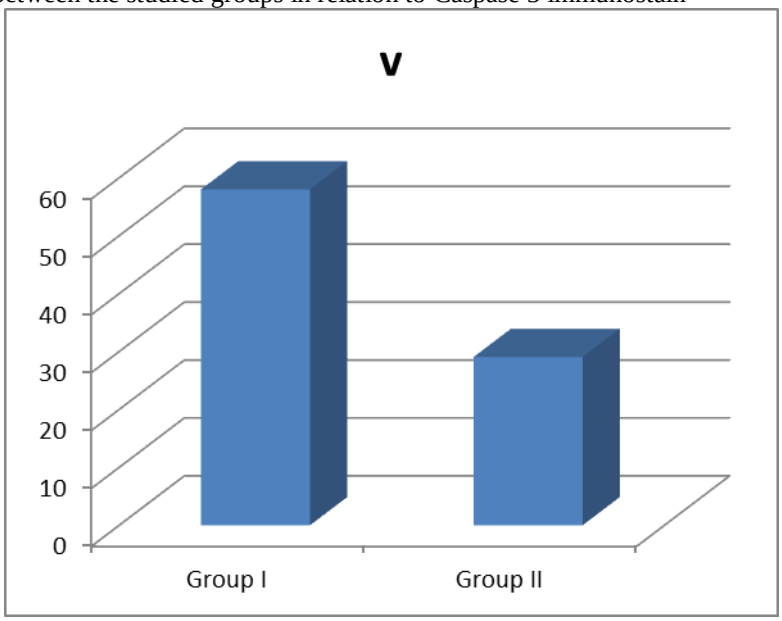


Figure 6: Shows the significant difference between the studied groups in relation to Caspase 3 immunostain



Discussion

In this research, it was found that there was a significant loss of weight in all rats receiving 5-flurouracil (group II) as compared to the control group. These findings are in accordance with other researches which mentioned that this weight loss may be due to chemotherapy induced diarrhea (Xian et al 2004, Bowen et al 2007, Stringer et al 2007, Saegusa et al 2008). Additionally, Bernstein IL, in 1978 indicated that weight loss associated with chemotherapy may be referred to loss of appetite which might be explained by conditioned aversions to food and beverages developed by chemotherapy.

In the present study, the oral mucosa of rats of group II was red, shiny and glazy. Also, some rats of group II exhibited oral ulcerations. This coincides with Guggenheimer et al., 1977, Squier, 1990 whom reported that mucositis is a common complication in patients receiving systemic anticancer chemotherapy, bone marrow transplantation, and local irradiation for tumors in the head and neck area. They indicated that it appears clinically as erythematous or diffuse ulcerative lesions.

Moreover, epithelial atrophy and collagen degeneration were the most common histological changes observed in group II rats. This might be due to the direct inhibitory effects of chemotherapy on DNA replication and mucosal cellular proliferation result in a reduction in the renewal capacity of the basal epithelium (Lotfyet al 2009). These events are believed to result in mucosal atrophy, collagen breakdown, and eventual ulceration (Soniset al 2004, Abayomi et al 2009).

In accordance with Cawley and Benson, 2005 numerous congested blood capillaries were detected in lamina propria of group II studied specimens. This might be attributed to the release of free radicals, modified proteins, and pro-inflammatory cytokines, including interleukin-1B, prostaglandins, and Tumor Necrosis Factor (TNF) during the initial inflammatory/vascular phase of mucositis. These inflammatory mediators cause further damage either directly or indirectly by increasing vascular permeability and angiogenesis, thereby enhancing cytotoxic drug uptake into the oral mucosa (Abayomi et al 2009).

In the current study, salivary glands dissected from (group Π) were found to be smaller in size than those of the control group. The glands dissected from group II animals showed marked degenerative changes of most of their acini as well as their duct system specially striated ducts. Eichhorst et al., 2001and Choyke et al., 1987, were in agreement with these findings reporting that during the first course of chemotherapy, the thymic volume decreased by an average of 43%. The resulting atrophy of submandibular salivary gland in this study might be attributed to the apoptotic and destructive effects of 5-FU upon their acinar and ductal cells.

Like a double-edged sword, every defect or abnormality along the apoptotic pathways may also be an interesting target of cancer treatment. Drugs or treatment strategies that can restore the apoptotic signaling pathways towards normality have the potential to eliminate cancer cells, which depend on these defects to live (Fulda S, 2010).

Assumed that apoptosis is the principle mechanism of chemotherapy- induced regression. Therefore, the rate of apoptosis or proteins that regulate apoptosis may be predictive of responses to chemotherapy (Hickman, J. A., 1992). Our study revealed that group II had a moderate reaction for Caspase 3in 60% of its samples while the remaining 40% showed an intense reaction. In contrast, group I revealed a mild reaction. This is convenient with Adachi et al., 1999who stated that the activities of Caspase 3and Caspase 8 increased when apoptosis was induced by 5-FU. Also, they mentioned that all apoptotic cells showed high Caspase 3activity in these conditions. Moreover, Ohtani et al., 2000 suggested that 5-FU induced apoptosis was mediated by the activation of a Caspase cascade involving Caspase 1, 3 and 8.In consequence, exposure to 5-FU triggered Caspase 8 and 3 cleavages to some extent, but Caspase 9 was hardly cleaved (Bijnsdorp et al 2010). In addition, activation of the Caspases-3 and 7 was increased in 5-FU-treated colon cancer cells compared to negative control (Shang et al 2014). Earlier, Grivicich et al, 2005 indicated that the increase in apoptosis in cells observed with 5-FU treatment could be explained by a disruption in mitochondria membrane potential that induced Caspases activation.

Many studies have analyzed whether alterations to the Caspase 3gene encoding the crucial executioner Caspase-3 might promote human tumorigenesis (Hosgood et al 2008). Our study revealed that group II specimens with genotype AA were associated with genotype GT (heterozygote) and intense immunoreactivity (mean of immunostain=71%). In contrast, all specimens with genotype TT were associated with genotype GA and moderate immunoreactivity (mean of immunostain=11.3%). The remaining specimens immunostain=34%. All rats of control group had genotype GG and low Caspase 3immunostain. Statistical tests revealed a highly significant inverse correlation between Caspase 3immunostain and Caspase3 rs4647601: G>T heterozygote (polymorphism) SNP while a highly significant positive correlation between Caspase 3immunostain and Caspase3 rs4647603: heterozygote G>A SNP was found. These findings might be strengthened by another study analyzing 930 squamous cell carcinomas of the head and neck (SCCHN) and 993 controls, the Caspase 3rs4647601: homozygote TT variant homozygote was associated with an increased risk of SCCHN compared with the GG genotype (Chen et al 2008). This finding was most evident in certain subgroups, including younger (≤56 years) subjects, males, and never smokers. Furthermore, in an analysis of 582 lung cancer patients and 582 controls, individuals bearing at least one allele with a −928A > G, 77G > A, or 17532A > C heterozygote polymorphisms had a significantly decreased risk for lung cancer compared with individuals who were homozygous for the wild-type Caspase 3allele (Jang et, 2008,Hosgood et al, 2008). Besides, multiple myeloma risk was also reduced in individuals with the AG and AA genotypes of CASP9 Ex5 + 32 G > A (Hosgood et al, 2008). An earlier study by the same group found a similar

association between decreased risk of NHL and certain Caspase 3 polymorphisms homozygosis (Lan et al 2007). Interestingly, Caspases-3 mRNA levels in commercially available total RNA samples from breast, ovarian, and cervical tumors were either undetectable (breast and cervical) or substantially decreased (ovarian) and that the sensitivity of Caspase-3-deficient breast cancer cells to undergo apoptosis in response to anticancer drug or other stimuli of apoptosis could be enhanced by restoring Caspase-3 expression, suggesting that the loss of Caspases-3 expression and function could contribute to breast cancer cell survival (Devarajan et al, 2002).

Taken together, the findings of this study suggest that the test group II which taken Fluorouracil showed significant increase in Caspase 3expression and associated with genotype AA were associated with genotype GT (heterozygote) (71% of the tested rats). As a result, evaluation of Caspase 3polymorphisms with their haplotypes and amount expression help to define genetic susceptibility to cancer development and response to chemotherapy. The findings of current work need to be validated in other groups. Additionally, future studies on how Caspase 3polymorphisms affect gene function are needed and a larger case–control study will further clarify the association of SNP with expression of Caspase 3protein and response to chemotherapy.

Abbreviations:
5-FU: Fluorouracil
RFLP-PCR: Restriction Fragment Length Polymorphism-Polymerase Chain Reaction
CASP: Caspase gene
SNPs: Single Nucleotide Polymorphisms analysis
SCCHN: Squamous Cell Carcinomas of the Head and Neck

Authors' contributions:
All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript. All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

Competing interests:
We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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