The histological and histochemical changes of the rat's liver induced by 5-fluorouracil

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Abstract

The main histological and histochemical changes in the rat's liver tissue induced by intraperitoneal injection of 5-FU were investigated in this study. Twelve adult female rats were divided into two groups of 6 rats each, group I was given 2 ml/kg body weight of normal saline for 7 consecutive days and served as the control group, while group II was given 20 mg/kg of 5-fluorouracil intraperitoneally for 7 consecutive days. Specimens of the liver tissue from the two groups were taken and prepared for light microscopic examination. Results showed the appearance of some histological changes in the5-FU recipient group (group II) compared to the control group (group I) including congestion of central vein, dilatation and congestion of the hepatic sinusoids, vacuolar degeneration of the hepatocytes, loss of normal histological architecture of the liver, in addition to sever fatty changes and apoptosis of the hepatocytes. Histochemical examination of the liver of the treated group revealed a marked decrease in the carbohydrates including glycogen manifested as weak positive reaction to PAS and Best's carmine stains compared to the control group in addition to marked increase in the activity of alkaline phosphatase manifested as a strong reaction to Gomori's alkaline phosphatase stain as compared to the control group. We conclude that 5-fluorouracil has toxic effects on the liver tissue causing vacuolar degeneration of the hepatocytes, sever fatty changes and apoptosis.

Keywords: Vacuolar degeneration; Apoptosis of hepatocytes**;** Congestion**;** fatty changes
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التغيرات النسيجية والكيمياء نسيجية التي يحدثها عقار الفايف فلورويوراسيل في كبد الجرذان

منى زهير الحمداني و عبد الجبار ياسين الحبيطي

فرع التشريح، كلية الطب، جامعة الموصل، الموصل، العراق

الخلاصة

تضمنت هذه الدراسة معرفة التغيرات النسيجية والكيمياء نسيجية في نسيج كبد الجرذان التي يحدثها عقار الفايف فلورويوراسيل عند الحقن البريتوني. اثنا عشر من اناث الجرذان البالغة قسمت الى مجموعتين ستة في كل منهما. اعطيت المجموعة الاولى ٢ مليلتر من المحلول الملحي العادي لكل كيلوغرام من وزن الجسم لمدة سبعة ايام متتالية واعتبرت مجموعة السيطرة. بينما اعطيت المجموعة الثانية ٢٠ مليغرام لكل كيلوغرام من عقار الفايف فلورويوراسيل عن طريق الحقن البريتوني لمدة سبعة ايام متتالية. اخذت نماذج من نسيج الكبد من المجموعتين وتم تحضيرها للفحص بالمجهرالضوئي.أظهرت النتائج وجود بعض التغيرات النسيجية في المجموعة المعاملة بعقار الفايف فلورويوراسيل (مجموعة II) بالمقارنة مع مجموعة السيطرة (مجموعة I) تضمنت احتقان في الوريد المركزي، توسع واحتقان في الجيبانيات الكبدية، تنكس خلايا الكبد مع فقدان الشكل النسيجي الطبيعي للكبد، فضلا عن تغيرات دهنية شديدة مع وجود نخر في خلايا الكبد. الفحص الكيمياء نسيجي للكبد في المجموعة المعاملة بعقار الفايف فلورويوراسيل اظهر تفاعل ضعيف مع ملّون شيف - حمضِ البيروديك و صبغة البستسكارمين بسبب انخفاض كمية الكربوهيدرات بضمنها الكلايكوجين اضافة الى زيادة فعالية انزيم الفوسفاتيزَ القاعدي مقارنة مع مجموعة السيطرة. ان عقار الفايف فلورويوراسيل له تأثير سام على خلايا الكبد فهو يسبب تنكس خلايا الكبد مع تغيرات دهنية شديدة ونخر في خلايا الكبد.

Introduction

Chemotherapy involves the use of chemical agents to combat neoplasm. However, it does not distinguish between the neoplastic and normal healthy cells in the body including those lining the digestive and respiratory tracts (1).

5-fluorouracil (5-FU) is a chemotherapeutic drug commonly used as a part of many cancer treatment protocols, it is a potent antimetabolite that acts during the S- phase of the cell cycle (2). It is activated by thymidine phosphrylase into fluorodeoxyuridylate (5 fluoro 2'deoxyuridine 5'monophosphate, 5-FdUMP) which inhibits thymidylate synthase, thus blocking DNA synthesis that leads to imbalanced cell growth and ultimately cell death. 5-FU is also converted to 5-fluorouridine monophosphate (5-FUMP) and can be incorporated into RNA and interfered with RNA processing and function (3). The trade names of 5-FU are Efudex, Carac, Fluroplex, it is used in the treatment of various malignancies including gastrointestinal, breast cancer, head and neck cancers, basal cell carcinoma of skin (as a cream) and in the ophthalmic surgery (4).

5-fluorouracil is metabolized mainly in the liver and has a half-life of about 10 minutes (5). Dihydropyrimidine dehydrogenase (DPD) found in the liver and it is the initial and rate-limiting enzyme in 5-FU catabolism, therefore 5-FU should be used with a great care in patients who are known or suspected to have a DPD deficiency as they are at a greater risk of 5-FU induced toxicity (6). The common clinical adverse effects of 5-FU include myelosuppression, diarrhea, vomiting and mucositis (7). However, in the last decade, cardiotoxicity and neurotoxicity had been reported (8,9) in addition to its hepatotoxicity (10).

Since there are few previous work and insufficient informations concerning the effects of 5-FU on the histochemical activity of the hepatic tissue, thus the present work had been conducted to clarify the histological changes induced by 5-FU which had not been recorded before by other investigators and to record the histochemical changes induced by 5-FU within the hepatocytes.

Materials and methods

Twelve healthy adult female Wistar albino rats of the same age group (2.5-3) months and weight (200-250g) were obtained from the Animal House of the Experimental Research Unit, College of Medicine, University of Mosul, Mosul. The animals were housed in a standard condition at a room temperature of about 24C^{o} and all animals were allowed for free access to laboratory pellet foods and tap water drink.

The experiment was conducted in the accordance of the ethical guidelines and internationally accepted principles for laboratory use and care in animal research.

The body weight of each rat was recorded at the beginning of the experiment before the injection of 5-FU and recorded again at the end of the experiment just before killing of the animals.

The animals were randomly and equally distributed into two groups consisting of 6 animals for each group; Group I: Each animal of this group was given 2 ml/kg body weight daily of normal saline by intraperitoneal injection for 7 consecutive days and served as a control group. Group II: Each animal of this group was given 5-FU 20 mg in 2ml normal saline (as a carrier solution) per kg body weight daily by intraperitoneal injection once daily for 7 consecutive days.

One day after the last injection of the two groups, all the animals were dissected under light ether to extract the liver from each animal. The weights of extracted liver from each animal was recorded then all the liver tissues were fixed in 10% neutral buffered formalin solution for about 24 hours. Small pieces of about 2-3 mm in size were cut from each liver and dehydrated in ascending grades of ethanol (70%, 90%, 100%). Clearing was done by xylene and embedded in paraffin wax. Serial sections of about (4-5) microns in thickness were collected from each paraffin block using Reichert's Rotatory Microtome. The tissue sections were stained with Harris Hematoxylin and Eosin (H&E) stain according to Kim *et al.* (11). Then the stained sections were examined using Compound Photo Microscope and some micrographs were taken from some sections using BEl Photonice microscope.The sections were prepared according to Bancroft *et al.* (12).

For histochemical reaction, the following stains were used: Periodic Acid Schiff's (PAS) stain to detect the carbohydrates, glycogen and mucin (13,14) in the cytoplasm of the hepatocytes and in the extracellular spaces. Best's carmine stain to detect glycogen in the cytoplasm of the hepatocytes..We have modified the method used by Kumar and Chakrabarti (15) to test for Best's carmine activity. Gomori's Alkaline Phosphatase-Cobalt method to detect alkaline phosphatase activity within the cytoplasm of the hepatocytes and in the extracelluar spaces (16).

Statistical analysis

Statistical analysis was performed by SPSS version 20 for windows software. Data were presented as mean±SD and were analyzed using one-way Analysis of Variance (ANOVA) followed by Bonferroni multiple comparisons for post-hoc analysis to compare the animals' mean body weight before and after injection of 5-FU for 1 week (17). The cutoff point for statistical significance was set at 0.05.

P- values$\leq$ 0.05 were considered to be significant whereas P- values $>$ 0.05 were considered to be non significant.

Results

Physical Observations

The animals of the control group stayed alive till the end of the experiment. They were active, responded very quickly to stimuli, and they had good appetite. The sites of injections showed no swelling or inflammation (picture 1) whereas the animals of group B became less active and gathered themselves at one corner of the cage on the 3rd day of the experiment and onward, their appetite was greatly reduced. Some rats had frequent diarrhea with ulcerations around the eyes and mouth and loss of furring of the skin which was recorded on the 7th day of the experiment (picture **2**).

Picture 1: Normal appearance of rat from group I looks healthy and active.

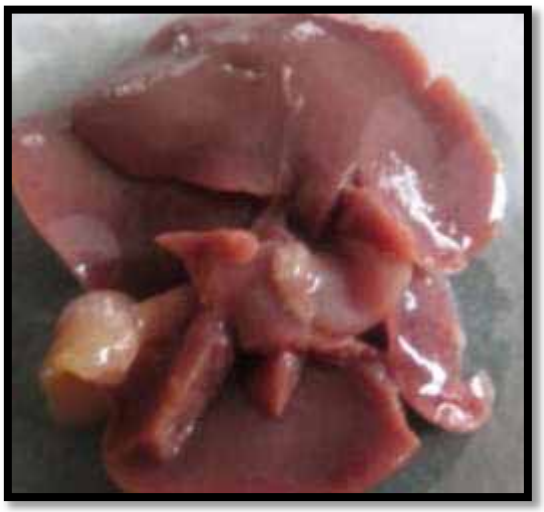
Picture 2: General appearance of rat from group II looks inactive with ulcerations around the eyes.

Histological findings

Macroscopically

The liver of the control group (group I) appeared dark red, lobulated with hepatic artery, portal vein and bile duct traverse the liver at porta hepatis (picture 3). The liver sections of the control group showed: Hepatocytes are arranged in form of cords radiating from the central vein. The cells are relatively large, polyhedral in shape with prominent rounded nuclei (some are binucleated) and eosinophilic cytoplasm (Fig. 1). The plates of hepatocytes are separated by hepatic sinusoids which are lined by endothelial cells and Kupffer cells and drain into the central vein (Fig. 1). Normal portal triads containing branches of hepatic artery, portal vein and a branch of bile duct (Fig. 2).

The liver of the treated group (group II) showed abnormal severely congested lobes (picture 4). The liver sections of the group II showed: Congestion of the central vein with dilatation, congestion and clot formation within the sinusoids (Fig. 3). Loss of normal liver architecture with disruption of the hepatic cords, sever vacuolar degeneration of the hepatocytes around the central vein seen as clear lipid vacuoles deposited in the cytoplasm of the hepatocytes and displace the nuclei to the periphery of the cells. Some hepatocytes with pyknotic nuclei and other binucleated cells were seen (Fig. 4). Sever fatty changes of the hepatocytes in the form of cytoplasmic lipid droplets (microvesicular steatosis) in the midzonal region (Fig. 5). In the portal area, congestion of the portal vein with mononuclear inflammatory cells infiltration, and destruction of the epithelium lining the bile ducts (Fig. 6).



Picture 3: liver of the control groups appears dark red and lobulated

Picture 4: liver of group II showing sever congestion.

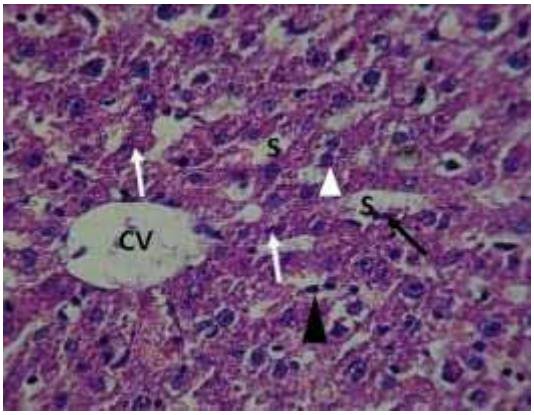


Fig. 1: Photomicrograph from rat's liver of group I (control group) showing the central vein (CV), plates of hepatocytes (white arrows) some are binucleated (arrow head), hepatic sinusoids (S) with endothelial cells (black arrow) and Kupffer cells in their lining epithelium (black arrow head) (H&E X 600).

Histochemical findings

Periodic Acid Schiff's (PAS) stain showed a strong positive reaction (magenta color) in the cytoplasm of the hepatocytes around the central vein in the control group (Fig. 7) as compared to the treated group which showed a weak positive reaction in the cytoplasm of hepatocytes in the midzonal region associated with fatty changes (Fig. 8). Best's carmine stain showed strong positive reaction in the cytoplasm of the hepatocytes of the control group (Fig. 9) as compared to the treated group which showed a weak positive reaction in the cytoplasm of hepatocytes around the central vein associated with vacuolar degeneration of some hepatocytes (Fig. 10). Gomori's alkaline phosphatase stain showed a weak positive reaction of alkaline phosphatase in the cytoplasm of the hepatocytes around the central vein in the control group (Fig. 11) as compared to the treated group which showed a strong positive reaction seen as black staining of the cytoplasm of the hepatocytes in the midzonal region associated with fatty infiltration (Fig. 12).

Body weight and liver weight

Results were expressed as mean±SD, very high significant reduction (P =0.001) of the animals' mean body weight at the end of the experimental period was observed in the treated group (group II) compared with the control group (group I) (Table 1). In addition, A significant reduction (P =0.01) of the animals' mean liver weight was observed in the treated group (group II) compared with the control group (group I) (Table 2).

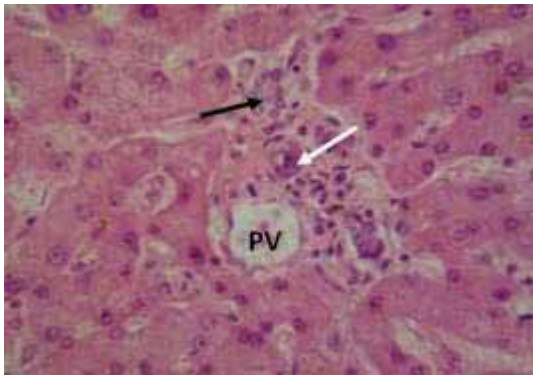


Fig. 2: Photomicrograph from rat's liver of group I (control group) showing the portal area containing hepatic artery (white arrow), portal vein (PV) and bile duct (black arrows) (H&E X 600).

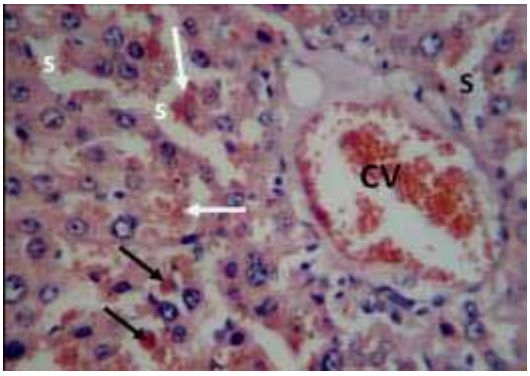


Fig. 3: Photomicrograph from rat's liver of group II showing congestion of central vein (CV) with dilatation of sinusoids (S), congestion (white arrow) and clot formation (black arrows) (H&E X 600).

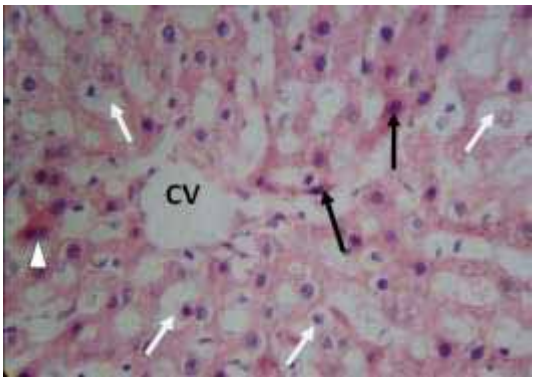


Fig. 4: Photomicrograph from rat's liver of group II showing sever vacuolar degeneration (white arrows) of the hepatocytes around the central vein (CV), some hepatocytes with pyknotic nuclei (black arrows) and other binucleated cells (arrow head) (H&E X 600).

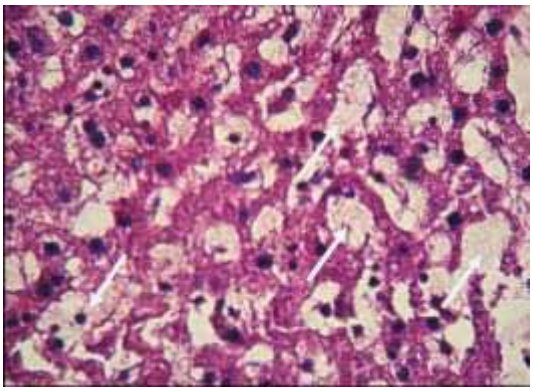


Fig. 5: Photomicrograph from rat's liver of II showing sever fatty changes of the hepatocytes in the form of cytoplasmic lipid droplets (microvesicular steatosis) in the midzonal region (white arrows) (H&E X 600).

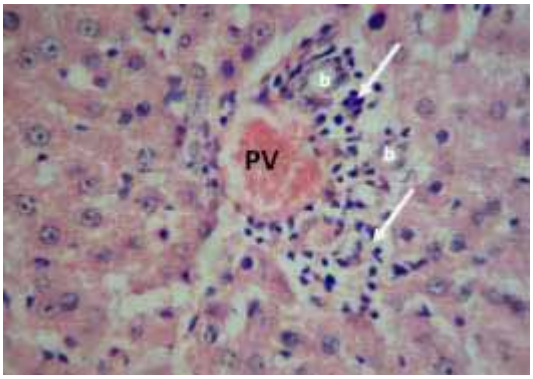


Fig. 6: Photomicrograph from rat's liver of group II showing congestion of the portal vein (PV), mononuclear inflammatory cells infiltration (white arrows), destruction of the epithelium lining the bile ducts (b) (H&E X 600).

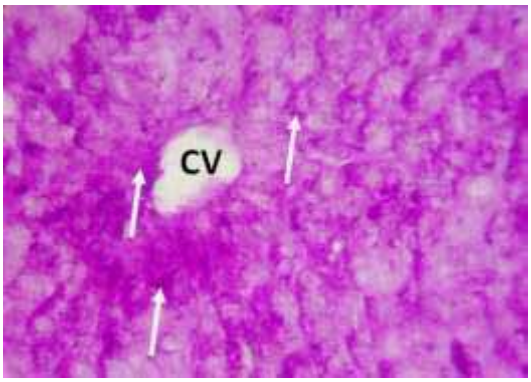


Fig. 7: Photomicrograph from rat's liver of group I (control group) showing strong positive reaction (magenta color) to PAS stain in the cytoplasm of the hepatocytes (white arrows) around the central vein (CV) (PAS X600).

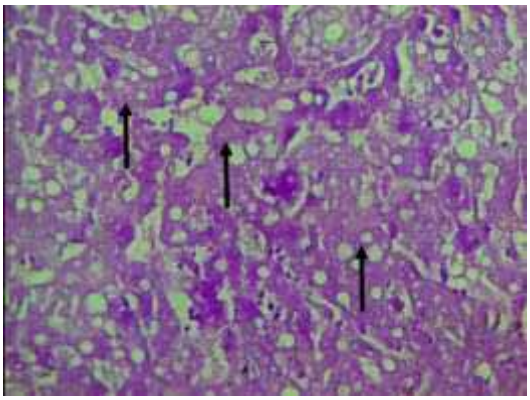


Fig. 8: Photomicrograph from rat's liver of group II showing weak positive reaction to PAS stain in the cytoplasm of hepatocytes in the midzonal region associated with fatty infiltration (black arrows) (PAS X600).

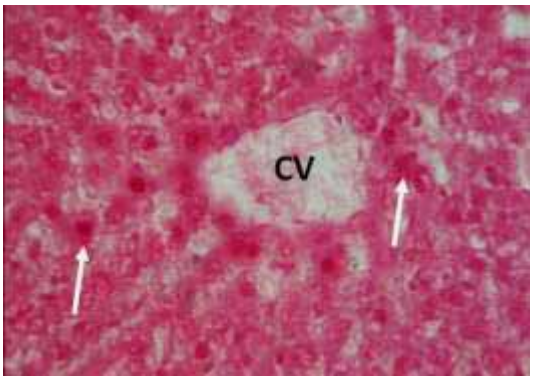


Fig. 9: Photomicrograph from rat's liver of group I (control group) showing strong positive reaction to Best's carmine stain in the cytoplasm of the hepatocytes (white arrows) around the central vein (CV) (Best's carmine X 600).

Table 1: Mean and standard deviation of the body weight (in gram) of the control (group I) and treated group (group II) which received 5-FU for one week

	Groups	N	Body weight (Mean ± SD) Before injection	Body weight (Mean ± SD) in grams	Statistical significance	P-values
A	Control	6	155 ± 16.43	158.5 ± 17.78	A vs. B = (VHS)	0.001
B	5-FU	6	160.5± 69.72	106.6 ± 39.39		

SD= Standard deviation; S=Significant (P≤0.05); NS=Non-significant (P>0.05); VHS= very high significant (P=0.001); vs=versus.

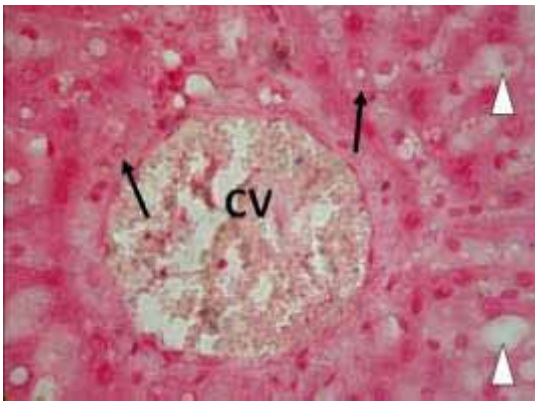


Fig. 10: Photomicrograph from rat's liver of group II showing weak positive reaction to Best's carmine stain in the cytoplasm of hepatocytes (black arrows) around the central vein (CV) associated with vacuolar degeneration of some hepatocytes (arrow heads) (Best's carmine X600).

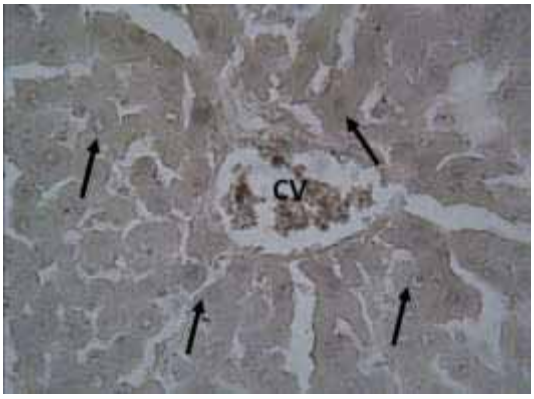


Fig.11: Photomicrograph from rat's liver of group I (control group) showing weak positive reaction of alkaline phosphatase in the cytoplasm of the hepatocytes (black arrows) around the central vein (CV) (Gomori's alkaline phosphatase X600).

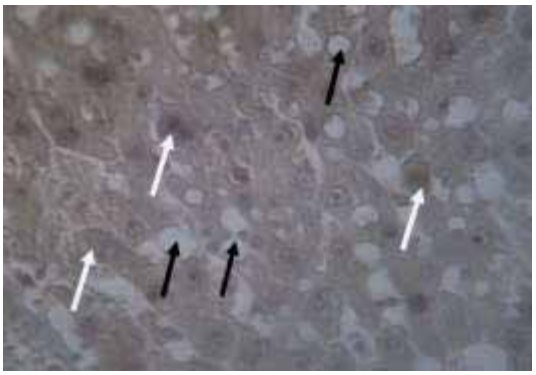


Fig. 12: Photomicrograph of rat's liver of group II showing strong positive reaction of alkaline phosphatase in the cytoplasm of the hepatocytes in the midzonal region (white arrows) associated with fatty infiltration (black arrows) (Gomori's Alkaline phosphatase X600).

Table 2: Mean and standard deviation of the liver weight (in gram) of the control (group I) and treated group (group II) which received 5-FU for one week

	Groups	N	Liver weight (Mean ± SD) in grams	Statistical significance	P-values
A	Control	6	10.40 ± 1.11	A vs. B = (S)	0.01
B	5-FU	6	8.66 ± 0.74		

SD= Standard deviation; S=Significant (P≤0.05); NS=Non-significant (P>0.05); vs=versus.

Discussion

5-Fluorouracil (5-FU) is a widely used chemotherapeutic agent, it has a great efficacy in a variety of human malignancies. However, the clinical use of 5-FU has been limited because of its undesirable side effects (18).

In the present study, the animals were injected by intraperitoneal 5-FU since the intraperitoneal approach allows very high concentrations of 5-FU to deliver into the peritoneal cavity without increasing the risk of systemic toxicity (19). The best carrier solution commonly used with

intraperitoneal 5-FU is HAES-Steri (neotype 6% hydroxyethyl starch) carrier solution since it delays the absorption of 5-FU from the peritoneal cavity and this provides the advantage of local regional killing of tumor cells which are exposed to 5-FU for longer time (20). Despite of this fact we used the physiological normal saline (0.9% sodium chloride) as a carrier for 5-FU in order to get a quicker absorption from the peritoneum and to achieve a higher effects of the drug (19). The drug 5-FU had not been reported to cause liver damage when it was given orally, and few reports indicated its hepatotoxicity when it was given intravenously (21), but it was found to induce severe hepatotoxicity associated with number of abnormalities after intraperitoneal injection (22).

The animals of group II showed very high significant reduction in their body weight. These results are in agreement with those reported by Cheah *et al.* (7) and Stringer *et al.* (23) who mentioned that intraperitoneal injection of 5-FU significantly reduced rat's body weight, food intake, and faecal output and suggested that 5-FU induced weight loss might be due to oral mucositis which is a painful condition associated with inflammation and ulceration affecting the mucosa of the mouth and causing a difficulty in eating and drinking with the resultant weight loss which was observed in approximately 85% of patients.

In this study, marked histological changes were observed in the liver of rats treated with 5-FU included marked disruption of hepatic cords, fatty changes in the hepatocytes in the form of cytoplasmic lipid droplets (microvesicular steatosis), vacuolar degeneration of the hepatocytes and some hepatocytes showed pyknotic nuclei.

The loss of the normal architecture where the hepatocytes are arranged in chaotic manner and lose their cord like arrangement around the central vein observed in our study is nearly similar to the finding of previous worker on giving Cisplatin by intraperitoneal injection (18,24). The dilatation and congestion of the central vein, portal vein and sinusoids seen in our work might be resulted from inflammatory reaction provoked by some chemical mediators like histamine and prostaglandin secreted from mast cells and some other inflammatory cells which cause vasodilatation and local increase of blood flow associated with engorgement of capillary bed which become more permeable to the fluid rich in proteins thereby increasing blood viscosity and red blood cells aggregate forming blood clots, these pathological events are reflected microscopically as numerous dilated blood vessels packed with erythrocytes with congestion and clotting (25).

The present study showed fatty changes of the hepatocytes in the form of cytoplasmic lipid droplets (microvesicular steatosis) in the midzonal region.Such finding might be a form of hepatic injury induced by 5-FU which causes an alteration in the fat metabolism leading to lipid accumulation in the hepatocytes particularly affecting the hepatocytes in the centrilobular and midzonal region which are less resistant to injury since they receive less enriched blood with nutrients and oxygen than those in the periportal area. Similar fatty changes had been noticed in the liver tissues of rats which were treated with Cisplatin (26) and after Adriamycin injection (27).

The present work showed marked vacuolar degeneration of hepatocytes which might be explained due to disruption of fat metabolism in addition to an increased formation of lipoproteins that are converted into triglycerides which in turn infiltrated in the liver causing cytoplasmic vacuolation seen as clear lipid vacuoles which displace the nuclei to the periphery of the cells. A nearly similar effect in the liver induced by Rifampicin and Isoniazide had been noticed before (28) and also by the effects of acetaminophen on the liver tissue (29).

Another important finding in the present study is apoptosis which is usually evidenced by diminished cell size, pyknotic nuclei with condensed nuclear chromatin and reduced cytoplasmic volume (1,30). This observation is similar to what has been noted by Rash and Abdella (31) who noticed that Doxorubicin induced widespread apoptosis in the mice liver.

During hepatic ischemia a few apoptotic cells had been frequently seen in the liver of the control group (i.e. normal liver) which is usually ranges from 0.01% to 0.3% of the hepatocytes (32) but when a larger number of hepatocytes are undergoing apoptosis, the process may switch to secondary necrosis which may become indistinguishable from oncotic necrosis (33). Furthermore, apoptosis is a common feature of hepatotoxicity following the administration of hepatotoxins particularly thiocetamide and acetaminophen which were proved to induce a significant deteriorations in the liver (34,35) also it precedes necrosis as in the hepatotoxicity induced by Cisplatin (29).

All the above structural changes in the liver are attributed to dose related toxicity, oxidative stress and reactive oxidative species (36-38), or could be explained due to hyperbilirubinaemia which interferes with the secretion of bilirubin, consequentaly causing cholestasis, increased fat oxides and modification in the hepatic transaminase enzyme activity (28). In their study Mora *et al.* (39) noted that the decrease in the antioxidant enzymes activity is attributed to the use of chemotherapy like Cisplatin. Moreover, chemotherapy can induce severe side effects even at the effective therapeutic doses (40). In addition tissue changes due to chemotherapy may reduce the conversion of Hb to meta Hb which suppresses the capacity of carrying oxygen and eventually leads to cell death (41).

The second part of this study showed the histochemical changes in the liver following administration of 5-FU.The Periodic Acid Schiff's (PAS) test is usually used to demonstrate the carbohydrates including glycogen and

mucin (13,14). In the current study, the hepatocytes of the treated group showed weak positive reaction (magenta color) as compared to the control group. This means that 5-FU causes a depletion of the stored carbohydrates from the liver as a result of oxidative stress on the liver caused by the drug. There is no previous histochemical study on the liver after treatment by chemotherapy to compare with, while giving Nandrolone decanoate to rabbits resulted in an increase in the mucopolysacchrides contents of the liver i.e. stronger reaction in the treated group compared to the control (42).

The Best's carmine stain is used to detect the glycogen contents of the liver tissue. Since the fixative penetrates the tissue from one direction only, it will push the glycogen ahead from its position until reaching the cell membrane and it can go no further i.e streaming artifact (14).We have modified the method used by Kumar and Chakrabarti in (15) who immersed the intact tissue in 10% formalin solution for18 hours to get proper fixation and better results in histochemical reaction to Best's carmine stain to identify the glycogen contents of the saccus vasculosus of butter catfish. We failed to achive the optimum results on using their method, thus we tried fixation at longer time and succeeded to get an optimum results after 24-30 hours of fixation. We can't explain this discrepancy except due to climate and temperature variations during fixation and staining of the paraffin sections or probably due to the use of different organs.

Our study showed weak positive reaction to the Best's carmine stain in the hepatocytes around the central vein which indicated a reduction of glycogen content in the liver due to defects in its synthesis as a consequence of degeneration of the hepatocytes and damage of mitochondria with reduction in the amount of ATP (43). There is no previous study to compare with except that belong to rabbits received anabolic steroid which is quite different from chemotherapy (41).

Gomori's method was used to demonstrate alkaline phosphatase histochemically in the liver tissue, it blackens the cell cytoplasm and the degree of blackness is correlated with the activity of the enzyme present (15). A marked increase in the activity of alkaline phosphatase appeared in the liver of the treated rats especially in the degenerated areas mainly due to fatty infiltration which was markedly observed in this group.A similar finding had been demonstrated by previous worker (44).

Conclusion

We conclude that the use of 5 FU in the treatment of some tumors has serious effects on the structure of the liver tissue resulting in vacuolar degeneration of the hepatocytes, fatty changes with apoptosis and mononuclear cells infiltration.

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