Title: Stereological Estimation and Zonal Distribution of the Hepatotoxic Effects of Doxorubicin on the Female Albino Rat (Rattus Norvegicus)

Article type: Original Article

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Acknowledgment: Jimmy Gakure, Felix Mburu, Noel Odero

Financing: Self-funded

Conflict of interest statement by authors: No conflict of interest

Compliance with ethical standards: Work covered in this manuscript has been conducted with the ethical approval from The Biosafety, Animal Use and Ethics Committee, Faculty of Veterinary Medicine (Reference Number: FVM BAUEC/2021/286).

Authors Contribution Statement:

Conceptualization, K.N, and T.C.; Methodology, K.N, and T.C.; Investigation, K.N.; Writing – Original Draft, K.N.; Writing – Review & Editing, V.K.; Funding, K.N.; Resources, K.N.; Supervision, A.P., B.O., and J.M.

Manuscript word count: 2951

Abstract word count: 247

Number of Figures and Tables: 8

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Discussion Points:

- Hepatic Effects of Doxorubicin- An Overlooked Adverse Effect?
- Doxorubicin Induced Damage to the Hepatic Histoarchitecture
- What does doxorubicin do to the organization of the liver?
- Effects of doxorubicin, the first line agent for breast cancer, on the Hepatic Histoarchitecture
- Double edged effects of doxorubicin in cancer management

Dates
Submission: 10/22/2022
Revisions: 04/04/2023, 12/09/2022
Responses: 07/20/2023, 01/04/2023
Acceptance: 08/06/2023
Publication: 08/09/2023

Editors
Associate Editor/Editor: Francisco J. Bonilla-Escobar
Student Editors: Amaan Javed, Manas Pustake & Patricio García-Espinosa
Copyeditor: Sebastian Diebel
Proofreader:
Layout Editor:

ABSTRACT.

Background

Doxorubicin is an antineoplastic agent widely indicated for a variety of cancers. One of its adverse effects is hepatotoxicity which presents with hepatocyte necrosis, sinusoidal dilation and fibrosis. However, there remains a dearth in the quantification and zonal distribution of this damage.

Methods

Twenty-three adult female Wister albino rats were placed into baseline, control and experimental group receiving 2.5mg/kg bodyweight doxorubicin intra-peritoneally thrice weekly for 3 weeks. Rats were sacrificed on days 0, 7, 14 and 21 and livers harvested for processing. Masson's Trichrome was used in staining 7 µm thick sections. Images were taken and analyzed via STEPanizer, and data entered into SPSS for analysis.

Results

Rats treated with Doxorubicin had increased liver to body weight ratios from 5.00% at baseline to 6.15%, 6.69% and 7.56% on days 7, 14 and 21 ($p=0.090$). There was a decrease in hepatocyte densities from 51.88/mm^2 to 48.61/mm^2, 46.65/mm^2 and 42.24/mm^2 on day 7, 14 and 21 ($p=0.779$). Collagen fiber deposition increased from 0.12±0.06 cm^3 to 0.47±0.55 cm^3, 1.64±0.11 cm^3 and 1.88±0.24 cm^3 on days 7, 14 and 21 ($p=0.009$). Deposition was greatest periportally and least pericentrally. Volume of sinusoidal spaces increased from 5.46±0.50 cm^3 to 5.49±0.15 cm^3, 5.53±0.24 cm^3 and 5.50±0.17 cm^3 on days 7, 14 and 21 respectively ($p=0.827$). Sinusoids were larger pericentrally than periportally.

Conclusion

Doxorubicin administration is associated with an increase in volume density of fibrotic tissue and sinusoidal spaces but decrease in hepatocytes. The quantitative changes presented may facilitate histopathological grading of doxorubicin-induced hepatotoxicity.

Key Words: doxorubicin, hepatotoxicity, liver, stereology

INTRODUCTION.

Doxorubicin is a widely indicated antineoplastic agent for a variety of cancers including breast cancer, bladder cancer, thyroid cancer, Kaposi's sarcoma, lymphoma, soft tissue sarcoma, multiple myeloma and acute lymphocytic leukemia. It is the first line agent for metastatic breast cancer as well as for metastatic and locally advanced un-resectable soft-tissue sarcoma.[1] It is administered intravenously in dosage regimens specific to the cancer type and progression.[2] With regards to its mechanism of action, doxorubicin generates free radicals during its metabolism in the liver. These free radicals disrupt normal cellular physiology and subsequently may cause toxicity in multiple organs mainly in the heart, kidneys and liver.[3] Previous studies on Doxorubicin induced hepatotoxicity have been descriptive and remain short of stereological and zonal data.[4,5]

Stereology is a growingly applied quantitative method used for the estimation of 3D parameters.[6] Because 2D profiles do not adequately depict object sizes and quantities, they are prone to inaccuracies when used in morphometric research.[7] As a result, significant mistakes might be made when interpreting quantitative data from 2D profiles. Stereology provides a solution to this by providing strong mathematical methods to eliminate bias and thus accurately make 3D estimations provided sampling, randomization and isotropy are streamlined.[8] Additionally, quantitative data that can show tiny variations in the volume or number of chosen parameters can be obtained using stereological procedures.[7] This is of high significance in analysis of liver biopsies which continue to be the gold standard for classifying liver damage.[9] Consequently, efforts have been made over the past few years to precisely quantify necrotic, fibrotic,[10] steatotic[11] and cancerous tissue,[12] both in clinical and experimental studies.[13] This study, therefore, sought to estimate and zonally determine the distribution of the toxic effects of doxorubicin on the hepatic stroma and parenchyma of the female Albino rat over a 3-week period of Doxorubicin administration.

METHODS

The study was of quasi-experimental design where female albino rats were used. These were obtained from the Department of Zoology and were kept and studied at the Department of Zoology Animal House. The harvested specimens were processed at the Department of Human Anatomy. These rodents were the preferred animal models for research due to their genetic, physiological, and anatomical similarity to humans, ease of maintenance, short life cycle and their small size.[14]

Sample size calculation was done using the formula by Charan & Biswas, 2013[15] using a statistical significance of 0.05, a power of 80%, a smallest meaningful difference of 1.4% and the standard deviation of 0.87% for mean liver fibrosis derived from a study by Yi et al, 2012[16]. A total of 23 rats were used of which two were baseline, six were controls and fifteen experimental.

Ethical approval for the study was sought from the Faculty of Veterinary Medicine (*Reference Number: FVM BAUEC/2021/286*). The study was conducted and the animals were handled according to the guidelines provided by the committee.

Adult female albino rats of three months of age were used in the study upon selection by convenience sampling technique. Doxorubicin's commonest indication is for breast cancer, and breast cancer is more common in females than males, thus female rats were used to account for the protective effects of estrogen on the liver[17]**.** Rats with any visible pathology or abdominal injuries were excluded. The sampling technique involved randomly assigning all the rats a number between 1 and 23 using non-repeating numbers generated by Intel® Digital Random Number Generator Software. The rats were labelled using picric acid on their fur and random selection applied to split them into 3 groups, where group A was the baseline group, Group B was the control group that was administered normal saline intra-peritoneally while group C was the experimental group that was administered Doxorubicin Intra-peritoneally (IP). The rats were housed in standard cages floored with wooden shavings which were replaced every two days. The cages were placed in a room with a normal 12-hour light/dark diurnal cycle. The rats were kept in their cages for 3 days for acclimatization after which intervention was begun. They were provided with standard rat pellets and water ad libitum during the study period. No additional intervention was given to reduce chemotherapy induced distress.

Each rat in the experimental group received 2.5 mg/kg body weight of Doxorubicin intra-peritoneally thrice weekly for three weeks (at an interval of 48 hours) to correspond with the intravenous route and timeline of administration of Doxorubicin in humans without posing toxicity to the rats. Animals in the control group received zero point five milliliters of normal saline intra-peritoneally thrice weekly as a sham. IP injections were done using a 31-gauge needle to prevent iatrogenic injury.

Tissue harvesting was done after weighing the rats using an electric measuring scale and euthanizing them by placing them in sealed containers with 1% halothane (1-3%) soaked in cotton wool. Death was confirmed by the absence ocular reflexes. Then a longitudinal incision in the midline of the body was made and normal saline used to flush out all the blood. Thereafter, 10% formal saline was infused by the trans-cardiac method to start

tissue fixation. The liver was harvested from each rat, absolute volumes calculated using Scherle's method and stored in formal saline. Systematic uniform random sampling method was used to get the liver segments. The liver was sliced across the lobes into 16 equal parts. The parts were rearranged into a diamond shape with smaller pieces arranged on either side of the largest piece of liver[9]. Following this, the 2nd piece was selected and thereafter every 3rd piece. A total of 5 pieces per liver were picked for histological processing.

The liver pieces obtained were placed in specimen bottles containing 10% formalin for at least 24 hours in order to preserve the tissues. Following fixation, they were dehydrated in ascending concentrations of alcohol, then cleared in toluene then infiltrated with paraffin wax. The embedded tissues were blocked for sectioning. They were cut into 7 µm thick sections. Every fourth section of the ribbon was selected and floated in a warm water bath to enhance spreading. The sections were fished from the water bath onto a gelatinized glass slide. They were dried at 38°C for 24 hours, then de-waxed, re-hydrated and stained using Masson's Trichrome.

Out of the 10 stained sections, 5 even sections were chosen for histomorphometric analysis. Photomicrographs were taken at x400 magnification using a Richter Optica™ digital photomicroscope (Model UX1) connected to Motic Images Plus 3.0 for stereological analysis. Three images were taken per slide- one of the periportal region, one of the midzonal region and one of the pericentral region. The photomicrographs were analyzed using STEPanizer Stereology Tool Version 1.0. **The e**stimation of volume densities and hepatocyte densities was done using Cavalieri's principle of point counting [8]. The histological regions were analyzed using a superimposed 100-point grid over the photomicrographs (Figure 1). The volume densities of the histological components were calculated and averaged in order to reduce bias. Absolute volumes were then calculated by multiplying the volume densities with the liver volumes.

Data obtained were keyed into the Statistical Package for Social Sciences software (version 28.0) for statistical analysis. Hepatocytes were expressed in numbers/mm^2 and fibrotic tissue and sinusoidal spaces were expressed as absolute volumes. The data were grouped into three groups: A, B and C. The Shapiro-Wilk test and a visual examination of the histograms and box plots produced from the data were used to determine whether the data were normal. Although the Shapiro Wilk test indicated a normal distribution, the histograms displayed skewed distributions. Thus, non-parametric tests were employed. Kruskal Wallis test was employed to check for statistically significant differences over time in both the control and experimental groups over the study period. A Dunn Bonferroni post-hoc test was carried out. Mann Whitney U tests were carried out to assess for significant differences between the control and experimental group on each of the perfusion days. A p value of 0.05 or lower was regarded as significant. Photomicrographs were used to demonstrate the histological findings.

RESULTS.

General health of Study Animals

Following Doxorubicin administration, the study animals developed diarrhea, roughness in their fur coat, mucosal inflammation and tremors. They exhibited hypoactivity and reduced food intake. No discernible gross changes in livers of the experimental rats were noted when compared to the control and the baseline groups.

Liver to Body Weight Ratio (LBWR)

The liver to body weight ratios of the control animals increased slightly over time ($p=0.180$) with those of the experimental animals increasing more rapidly ($p=0.090$). The difference in LBWR between controls and experimental groups was statistically significant ($p=0.029$).

Collagen fiber density

Doxorubicin administration resulted in deposition of collagen fibers in the periportal areas as well as within perisinusoidal spaces that increased with time ($p=0.009$). This is illustrated in Figures 2 and 3 below. Bridging fibrosis also developed and was most defined on day 21. In contrast, the stroma of the control group hardly had any differences in collagen fiber volumes from baseline tissues. The experiment and control groups thus, had statistically significant differences in collagen fiber volumes ($p<0.001$). Table 1 displays the means, standard deviations, medians, interquartile ranges and p values of the control and experimental groups over time.

Sinusoidal space density

Doxorubicin administration resulted in an increase in volumes of sinusoidal spaces over time ($p=0.827$). This is illustrated in Figure 4 below. Sinusoids in the pericentral area were larger than those in periportal areas. In contrast, sinusoids in control tissue did not have distinct differences in sinusoidal densities from baseline tissue ($p=1.000$). The experimental and controls groups did not have statistically significant differences in sinusoidal volumes ($p=0.667$). Table 2 displays the means, standard deviations, medians, interquartile ranges and p values of the control and experimental groups over time

Effects of Doxorubicin on the Hepatic Parenchyma

The control rats displayed normal liver histoarchitecture. However, the experimental rats had distortions in their parenchyma. There was marked degeneration with disruption of the cord-like arrangement of hepatocytes. There was also infiltration of deeply basophilic leukocytes in the periportal area and regions of focal necrosis in the pericentral area. The hepatocytes nearer the central veins were more vacuolated than those in the periportal areas.

Hepatocyte densities declined progressively with minimum numbers recorded in the 3rd week ($p=0.779$). This is illustrated in Figure 5 below. The periportal areas had a higher concentration of hepatocytes relative to the pericentral areas. On the other hand, hepatocyte densities for the control rats remained similar in number to those in baseline tissue ($p=0.867$). The differences between control and experimental values were however, not statistically significant ($p=0.178$). Table 3 displays the means, standard deviations, medians, interquartile ranges and p values of the control and experimental groups over time (Table 3).

DISCUSSION.

The findings in this study are suggestive of a temporal increase in the deposition of collagen fibers and in sinusoidal dilatation but decrease in hepatocyte densities as discussed below. These structural changes may be helpful in grading of toxicity via liver biopsies, provide clarity on the zonal distribution of structural changes, and pave way for studies to determine strategies to reduce the severity of Doxorubicin induced hepatotoxicity.

General health of Study Animals

The experimental rats in this study developed diarrhea, roughness in their fur coat, mucosal inflammation and tremors. These effects may be due to an inhibition of multiplication of otherwise rapidly proliferating cells of the gastrointestinal tract, skin and bone marrow by doxorubicin [18].

Liver to Body Weight Ratio

Doxorubicin treatment resulted in an increase in the LBWR compared to that of the control rats. The increase in liver weight observed in this study following Doxorubicin is similar to the findings by Salouege et al., (2014) who found an increase in organ to body weight ratios of the heart, liver, spleen and kidneys following Doxorubicin administration in rats. The increase in liver weights corresponds to augmentative hepatomegaly which is a compensatory regenerative process following hepatocyte necrosis [20]. The initial phase of hepatomegaly is hypertrophy of the existing hepatocytes then later hyperplasia if regeneration is still incomplete. In this study, hypertrophy of hepatocytes could explain the increase in LBWR since a reduction in hepatocyte densities precluding regenerative hyperplasia of hepatocytes was observed. Hepatomegaly is a common adverse effect of chemotherapy and may culminate in severe liver injury and even liver failure if not controlled [21].

Collagen Fiber Density

Doxorubicin administration resulted in an increase in collagen fiber deposition in the perisinusoidal and periportal areas over time. Perisinusoidal fibrosis is postulated as being a result of hepatocyte stellate cell (HSC) activation by the reactive oxygen species (ROS) released during Doxorubicin metabolism. The activated HSC transform into highly proliferative myofibroblasts-like cells with a greatly enhanced capacity to synthesize ECM components including type I and III fibrillary collagen, laminins and fibronectin[22]. The periportal and bridging fibrosis may be due to the activation of portal fibroblasts to form portal myofibroblasts which are pronounced for their matrix deposition and contractility [23]. Placement of groups of contractile cells in collagen type I matrices leads to compaction and alignment of the collagen between them, creating the appearance of bridging fibrosis. This may explain the realignment of connective tissue fibers seen in bridging fibrosis. Advancing of liver fibrosis may result in nodular regeneration, cirrhosis, and portal hypertension and often requires liver transplantation [22].

Sinusoidal Space Dilation

Doxorubicin administration resulted in an increase in the sinusoidal space density. Injury to sinusoidal endothelial cells by ROS leads to embolization of endothelial cells and blood cells in sinusoidal spaces. This blocks venous outflow, resulting in hepatic congestion and subsequent sinusoidal dilatation. This is followed by sinusoidal obstruction syndrome characterized by fiber deposition in the sinusoids by activated HSC and obliteration of central venules[24]. The result is the loss of fenestrations and development of basement membranes by sinusoidal endothelial cells in a process of capillarization, forming channels with larger calibers[25].

Sinusoidal obstruction syndrome can progress into regenerative nodular hyperplasia or may normalize with time after cessation of chemotherapy[21]. Sinusoidal obstruction causes congestion, hepatomegaly, fluid retention, jaundice and ascites, and becomes fatal in 20-50% of patients on high dose chemotherapy [26].

Effects of Doxorubicin on the Hepatic Parenchyma

Periportal leukocyte infiltration following doxorubicin administration as observed in this study may due to an increase in recruitment of immune cells via chemotaxis following hepatocyte injury and death [27]. Hepatocyte vacuolation following Doxorubicin administration as observed in this study, was associated with larger nuclei, and is postulated as being a marker of senescence. It is present in a variety of acute and chronic liver diseases. However, the exact pathophysiology behind the vacuolation is unclear and is suggested as being the result of hydropic change [28].Focal necrosis, in association with lymphocytes, as observed in this study, describes a continuum of lobular injury [29].

Decrease in hepatocytes following doxorubicin administration as observed in this study, may be a result of hepatocyte necrosis and apoptosis. The FR released during doxorubicin metabolism reacts with hepatocyte lipids, proteins and nuclei acids causing mitochondrial dysfunction and lipid peroxidation which induces apoptosis. Following cell death, regeneration of hepatocytes is also impaired as doxorubicin inhibits topoisomerase II activity and thus inhibiting cell division [30]. The result is a decline in hepatocyte numbers and distortion in the radial organization of cords. Severe hepatocyte apoptosis and necrosis may culminate into liver failure.

Limitations and Delimitations

This study may have had some possible confounders such as inter-animal differences in the absorption and metabolism of doxorubicin. This was, however, minimized by the use of in-bred rats which are genetically similar. Also, stress due to intraperitoneal injections may have affected the hepatic histoarchitecture. This was standardized by the administration of normal saline intraperitoneal injections in the control group. In addition, tissue shrinkage during tissue processing may have altered the normal parameters. However, errors due to tissue processing were carried through all measurements.

Strengths of the Study

1. The histoarchitecture of the Albino rat liver very closely resembles that of humans.
2. Intermittent dosage forms administered make it analogous to Doxorubicin therapy in humans.
3. Sacrifice at the end of each week enabled establishment of temporal effects

Conclusion

Doxorubicin administration is associated with an increase in the volume densities of fibrotic tissue and sinusoidal spaces, and decrease in hepatocyte densities. The quantitative structural changes further corroborate Doxorubicin-induced hepatotoxicity and may facilitate histopathological diagnosis of hepatotoxicity.

SUMMARY - ACCELERATING TRANSLATION

Title

Stereological Estimation and Zonal Distribution of the Hepatotoxic Effects of Doxorubicin on the Female Albino Rat (Rattus Norvegicus)

Main Problem

Doxorubicin is a chemotherapeutic agent widely indicated for a variety of cancers. One of its side effects is liver toxicity which presents with cellular death, vascular dilation and fibrosis. However, there has remained a dearth in the quantification and zonal distribution of this liver damage.

Aim

To quantify and zonally determine the distribution of the hepatotoxic effects of doxorubicin on the female Albino rat

Methodology

Twenty-three adult female Wister albino rats were placed into 3 groups: baseline, control and experimental. The experimental group received 2.5mg/kg bodyweight of doxorubicin intra-peritoneally thrice weekly for 3 weeks. The control group received 0.5 ml normal saline intra-peritoneally thrice weekly as a sham. Rats were then sacrificed on days 0, 7, 14 and 21 and their livers harvested for processing and analysis.

Results

Rats treated with Doxorubicin had increased liver to body weight ratios from 5.00% at baseline to 6.15%, 6.69% and 7.56% on days 7, 14 and 21 ($p=0.090$). There was a decrease in hepatocyte densities from 51.88/mm^2 to 48.61/mm^2, 46.65/mm^2 and 42.24/mm^2 on day 7, 14 and 21 ($p=0.779$). Collagen fiber deposition increased from 0.12±0.06 cm^3 to 0.47±0.55 cm^3, 1.64±0.11 cm^3 and 1.88±0.24 cm^3 on days 7, 14 and 21 ($p=0.009$). Deposition was greatest periportally and least pericentrally. Volume of sinusoidal spaces increased from 5.46±0.50 cm^3 to 5.49±0.15 cm^3, 5.53±0.24 cm^3 and 5.50±0.17 cm^3 on days 7, 14 and 21 respectively ($p=0.827$). Sinusoids were larger pericentrally than periportally.

Conclusion

Doxorubicin administration is associated with an increase in volume density of fibrotic tissue and sinusoidal spaces but decrease in hepatocyte densities. The quantitative changes presented may facilitate histopathological grading of doxorubicin-induced hepatotoxicity.

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FIGURES AND TABLES.

Figure 1. STEPanizer Grid for Estimation of Volume Densities

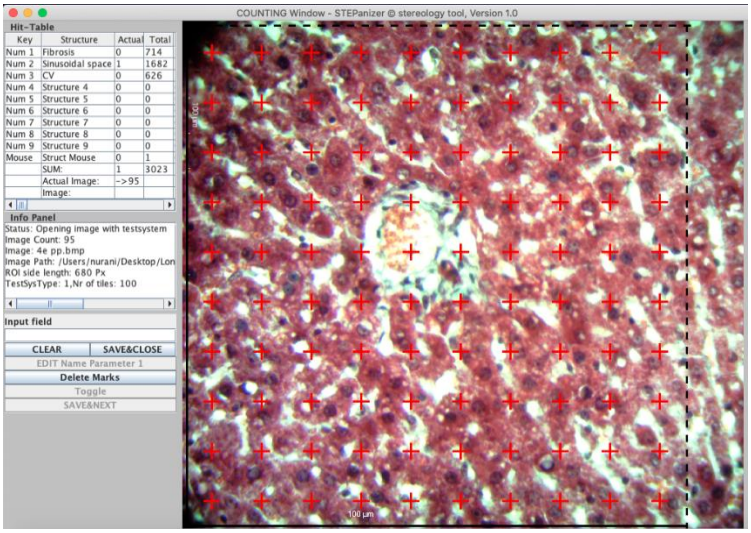
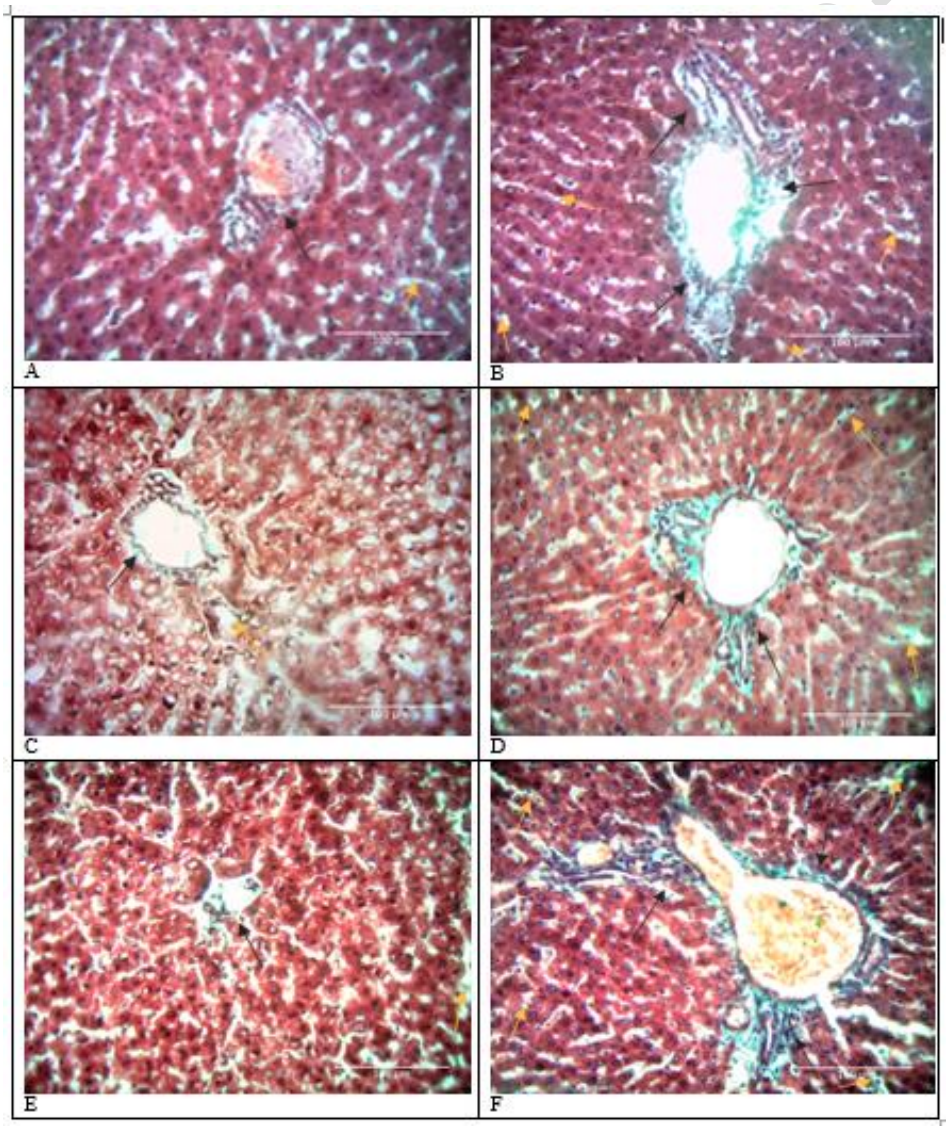


Figure 2. Collagen Fiber Profile in Rat Livers



LEGEND:

Figure 2A-F: Collagen Fiber Profile in the Rat Livers. Stain: Masson's Trichrome, Magnification: X 400.

Figure 2A: Photomicrograph of the liver of a control rat on day 7 of the study. There are a few collagen fibers (yellow arrows) interspersed between the hepatocytes in the perisinusoidal spaces. The black arrows point at collagen fibers around the portal triad.

Figure 2B: Photomicrograph of the liver of a rat treated with Doxorubicin on day 7 of the study. There are a few collagen fibers (yellow arrows) interspersed between the hepatocytes in the perisinusoidal spaces. The black arrows point at collagen fibers around the portal triad.

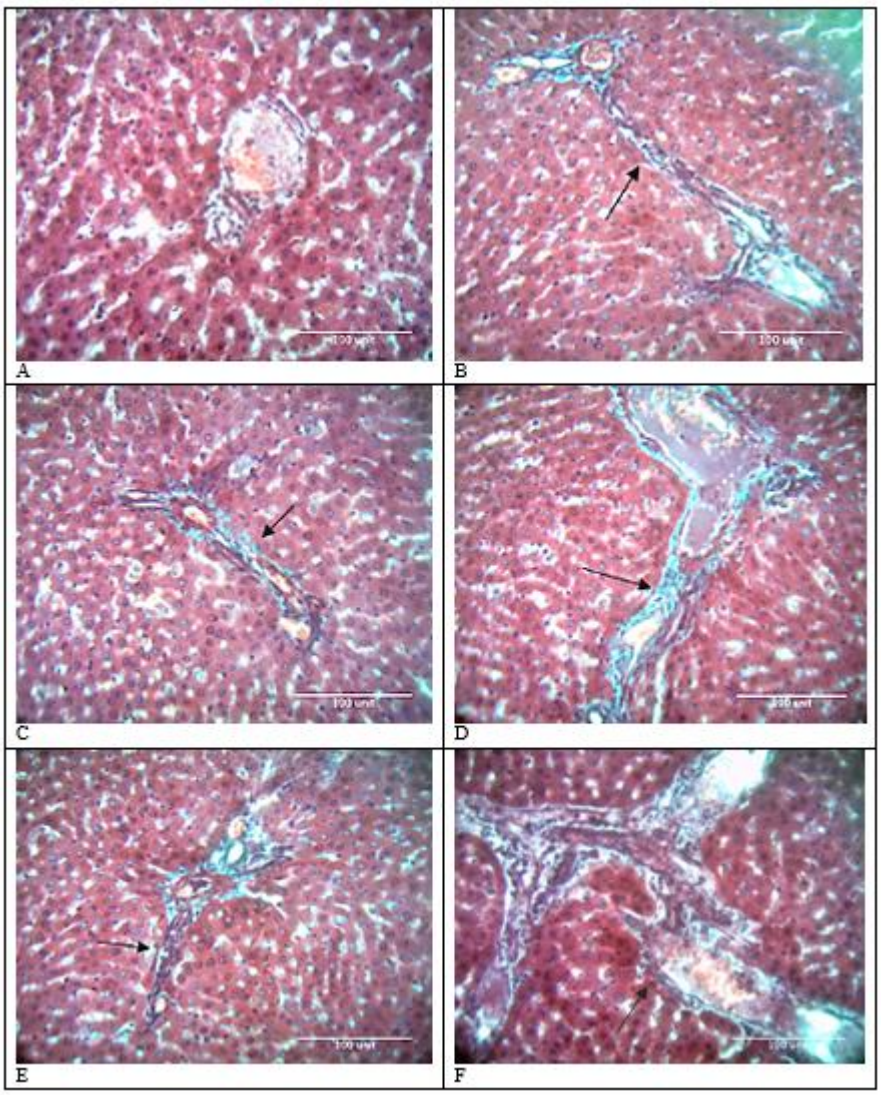
Figure 2C: Photomicrograph of the liver of a control rat on day 14 of the study. There are a few collagen fibers (yellow arrows) interspersed between the hepatocytes in the perisinusoidal spaces. The black arrows point at collagen fibers around the portal triad.

Figure 2D: Photomicrograph of the liver of a rat treated with Doxorubicin on day 14 of the study. There are collagen fibers (yellow arrows) interspersed between the hepatocytes in the perisinusoidal spaces. The black arrows point at collagen fibers around the portal triad.

Figure 2E: Photomicrograph of the liver of a control rat on day 21 of the study. There are a few collagen fibers (yellow arrows) interspersed between the hepatocytes in the perisinusoidal spaces. The black arrows point at collagen fibers around the portal triad.

Figure 2F: Photomicrograph of the liver of a rat treated with Doxorubicin on day 21 of the study. There are more collagen fibers (yellow arrows) interspersed between the hepatocytes in the perisinusoidal spaces. The black arrows point at collagen fibers around the portal triad.

Figure 3: Comparison of Bridging Fibrosis in Control and Experimental Rat Livers



LEGEND: Figure 3A-F: Bridging Fibrosis in the Rat Livers. Stain: Masson's Trichrome, Magnification: X 400.

Figure 3A: Photomicrograph of the liver of a control rat treated on day 7 of the study. There was no bridging fibrosis. Only some collagen fibers were present around the portal triad

Figure 3B: Photomicrograph of the liver of a rat treated with Doxorubicin on day 7 of the study demonstrating setting in of bridging fibrosis (black arrows).

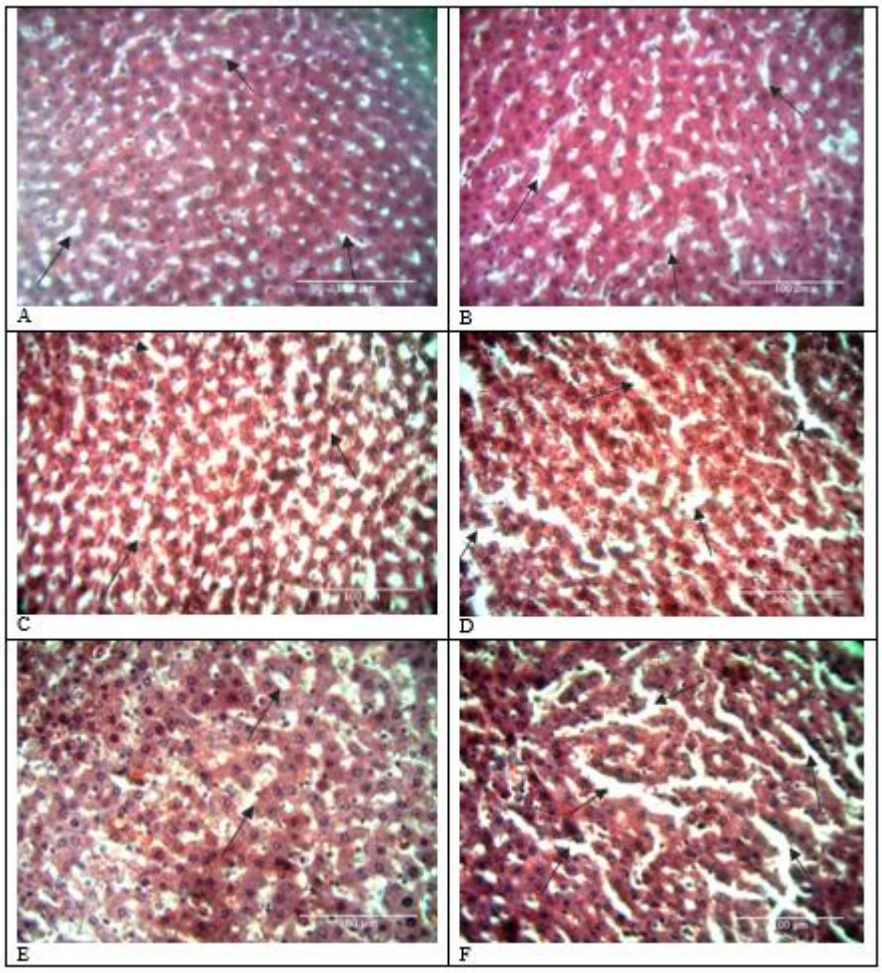
Figure 3C: Photomicrograph of the liver of a control rat on day 14 of the study demonstrating some bridging fibrosis (black arrows).

Figure 3D: Photomicrograph of the liver of a rat treated with Doxorubicin on day 14 of the study demonstrating some bridging fibrosis (black arrows).

Figure 3E: Photomicrograph of the liver of a control rat on day 21 of the study demonstrating some bridging fibrosis (black arrows).

Figure 3F: Photomicrograph of the liver of a rat treated with Doxorubicin on day 21 of the study demonstrating the most extensive bridging fibrosis (black arrows).

Figure 4: Comparison of Sinusoidal Spaces in Control and Experimental Rat Livers



LEGEND: Figure 4A-F: Sinusoidal Spaces in the Rat Livers. Stain: Masson's Trichrome, Magnification: X 400.

Figure 4A: Photomicrograph of the liver of a control rat on day 7 of the study. The relatively small sinusoidal spaces are illustrated with black arrows.

Figure 4B: Photomicrograph of the liver of a rat treated with Doxorubicin on day 7 of the study. There are relatively larger sinusoidal spaces (Black arrows).

Figure 4C: Photomicrograph of the liver of a control rat on day 14 of the study. Sinusoidal spaces, pointed at by black arrows, are smaller than those in Figure 9D.

Figure 4D: Photomicrograph of the liver of a rat treated with Doxorubicin on day 14 of the study. There are larger and more distorted sinusoidal spaces (Black arrows).

Figure 4E: Photomicrograph of the liver of a control rat on day 21 of the study. Sinusoidal spaces, pointed at by black arrows, are smaller than those in Figure 9F.

Figure 4F: Photomicrograph of the liver of a rat treated with Doxorubicin on day 21 of the study. Sinusoidal spaces, pointed at by black arrows, are the largest and most distorted.

Table 1. Volume of Fibrotic Tissue at different time periods

Day	Group	Volume of Fibrosis (cm^3)	
		Mean ± SD	Median (IQR)
0	Baseline	0.12 ± 0.08	0.12
7	Control	0.14 ± 0.03	0.14
	Experimental	0.47 ± 0.12	0.45 (0.36-0.59)
14	Control	0.13 ± 0.06	0.13
	Experimental	1.64 ± 0.24	1.54 (1.50-1.83)
21	Control	0.17 ± 0.11	0.17
	Experimental	1.88 ± 0.55	1.97 (1.37-2.35)

Table 2. Volume of Sinusoidal Spaces at different time periods

Day	Group	Sinusoidal Spaces (cm^3)		P value (vs control) Exact Sig. [2*(1-tailed Sig.)]
		Mean± SD	Median (IQR)	
0	Baseline	5.46 ± 0.70	5.46	-
7	Control	5.14 ± 0.56	5.44	1.000
	Experimental	5.49 ± 0.34	5.52 (5.15-5.80)	
14	Control	5.15 ± 0.21	5.45	0.857
	Experimental	5.50 ± 0.54	5.67 (5.04-5.95)	
21	Control	5.14 ± 0.45	5.44	0.857
	Experimental	5.50 ± 0.39	5.40 (5.18-5.88)	

Table 3. Hepatocyte area densities at different time periods

Day	Group	Hepatocyte area densities (no./counting area)		P value (vs control) Exact Sig. [2*(1-tailed Sig.)]
		Mean ± SD	Median (IQR)	
0	Baseline	11.41 ± 2.24	11.41	-
7	Control	11.32 ± 1.09	11.32	0.571
	Experimental	10.69 ± 1.03	10.56 (9.74-11.71)	
14	Control	11.28 ± 0.16	11.27	0.381
	Experimental	10.26 ± 0.65	10.13 (9.71-10.88)	
21	Control	11.05 ± 0.72	11.05	0.857
	Experimental	9.29 ± 4.43	6.52 (5.85-14.12)	

Figure 5. Change in hepatocyte densities over time