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Insights from HPLCG and Histopathology: Measurement of DNA Damage and Hepatotoxicity Related to N-nitrosodimethylamine

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ABSTRACT
Due to the wide spread occurrence of NDMA through water sanitation process, their formation in food and pharmaceutical products.This study is necessary to investigate the DNA damaging effect and hepatotoxicity related to NDMA exposure. The study include 45 adult male rats classified into three groups. Group I represent negative control treated with distilled water I.P for 3 months. Group II treated with 3mg/kg NDMA I.P for 3 months and Group III treated with 1.5 mg/kg NDMA I.P for 3 months. After completion of study periods, liver tissue and blood sample were collected for analysis. The result reveals NDMA groups cause DNA damage of hepatocyte in comparison to control, also showed a significant ($P<0.05$) increase in serum total bilirubin and prothrombin time (PT) with a dose dependent effect. However, a significant ($P<0.05$) decrease in serum albumin level in groups treated with NDMA when compared to control. Liver tissue reflect several pathological condition related to NDMA ranging from hepatocyte degeneration, proliferation, necrosis and hepatic fibrosis. Moreover, severe proliferation of bile duct also noticed along with granulomatous inflammatory reaction and thick bands of fibrous tissue noted with Masson stain in liver tissue exposed to NDMA. Nitrosamine can be consider a carcinogenic agent related to DNA damaging effect of hepatocyte and their hyper-proliferative condition.

Keywords: NDMA, Hepatotoxicity, Prothrombin time, Hepatic fibrosis.

INTRODUCTION

N-Nitrosamines represent a group of compounds well known for the strong carcinogenic properties exhibited by many of its members, as well as their pervasive presence in various aspects of the human environment including atmosphere, water sources, food and medications (Georgescu et al.,2024). N-nitrosodimethylamine (NDMA) is considered to be probable human carcinogen according to the International Agency for Research on Cancer (Wang et al.,2022).

Various molecular mechanisms such as genetic mutations and altered RNA expression may evolve precancerous lesions or promote tumor development with NDMA exposure (Khan et al.,2023). Furthermore, the production of unstable intermediate react with DNA causing their alkylation, liver tissue damage and initiate fibrogenesis (Suvorov et al.,2023). However, chromosomal and genetic damage along with failure of DNA repair mechanism result from free radical production and oxidative stress caused with NDMA metabolism, ultimately resulting in cancer (Blum et al.,2023).Liver cancer developed from wide range dose of NDMA exposure (Gomm et al.,2021). Continuous exposure to NDMA exacerbate oxidative stress causing reinforce of hemorrhagic necrosis and destruction of hepatocyte architecture (Snodin et al.,2024).

NDMA revealing a hidden danger that underscores the importance of understanding their metabolic pathways (Asejeje et al.,2023). Because nitrosamine lack the essential properties to cause cancer unless they are metabolized with CYP450 enzyme that increase their potential to induce cancer.Over the past few years, nitrosamine have become a significant concern after numerous widely used drugs were found to comprise unsatisfactory levels of these impurities, leading to recall (Parr and Joseph, 2019). Beyond valsartan an angiotensin II receptor blockers, this issue has extended to other drugs such as ranitidine and metformin causing widespread recalls and shortage of many pharmaceutical products (Jain et al.,2020 ; Yoon et al.,2021).

Reversed-phase HPLC (RP-HPLC) has become an essential tool in the separation and analysis of proteins and peptides and because of its ability to separate proteins of nearly identical structure (Al-Wafi, 2018); global DNA methylation levels were assessed using RP-HPLC technology (Li et al., 2016).Therefore, this study isdesigned to

evaluate the hepatocyte DNA damaging effect related to NDMA use through HPLCG technique along with hepatotoxicity.

MATERIAL AND METHODS

Experimental animals

The study involve 45 adult male rats with (190-200 gm) weight, housed in plastic cage in animal house of College of Veterinary Medicine / University of Baghdad. The animals kept 2 weeks for acclimatization with standard condition.

Study design

The animals were randomly divided into three groups as follows:
G1: control group,
G2: 1\10 LD_{50} NDMA (3mg\kg) I.P for three consecutive days every week for 3 months (Al-Sabaawy and Al-Kaisie,2021).
G3: 1\20 LD_{50} NDMA (1.5 mg\kg) I.P for three consecutive days every week for 3 months.

Parameters of study

With completion of study periods, all animals sacrificed and blood sample collected from them for biochemical analysis. Liver tissue also collected for measurement of DNA damage and for histopathological analysis.

a. Measurement of DNA damage

1- **DNA extraction**
DNA sample were extracted from liver tissue through using (SIMEX^{TM} DNA extraction kit, Iran).

2- **HPLCG analysis**
Analysis of DNA sample with RP-HPLCG with a SYCAM S-600 instrument chromolith RP−C18E, (225 × 4.6 mm) column, particle size 0.5 µm, with a 15µl injection volume and a flow rate 0.5 ml/min. The wavelength was 260nm, temperature was 35^{o}C and mobile phase solvent A, H2O 95% and solvent B; Acetonitrile (MeCN) 5% with0.1% TFA (Nahi, 2016).

b. Biochemical analysis

The biochemical parameters analyzedinvolved total bilirubin (TB) and albumin (ALB) through fully automated veterinary chemistry analyzer Seamaty-120V (Adeleke and Adaramoye, 2017; Kim et al., 2018; Ahmed and Mohammed, 2022). On the other hands, prothrombin time were measured manually by administering BioClot-PT kit (Khalaf and Salih, 2023).

c. Histopathological examination

Liver tissue preserved in 10% formalin and processed for histopathological assessment withH&E stain according to (Alkaisie et al., 2021; Aghetaaet al., 2023) and Masson trichrome stain according to(Hung et al., 2020).

Statistical analysis

The data analyzed with SPSS version 22 and presented as Mean ± SD. The differences between groups is considered significant when ($P<0.05$).

Ethical approval

Animals processing was done according to the ethical committee of Veterinary Medicine College\ Baghdad University, with approval (NO: P.G\2198).

RESULT

1. DNA damage

The effect of NDMA on DNA damage shown in the table (1) and represented as retention time at which peaks of DNA appear.

Table 1: Effect of NDMA on DNA damage.

Group	Retention time (min.) Mean ± SD
Control	6.961 ± 0.04 **a**
2nd group (3mg/kg)	6.760 ± 0.01 **b**
3rd group (1.5mg/kg)	6.750 ± 0.02 **b**
LSD	**0.04**

The retention time (Rt) of 2nd and 3rd group are significantly ($P<0.05$) decreased when compared to the control group, however, the two groups do not show any significant differences in their retention time.

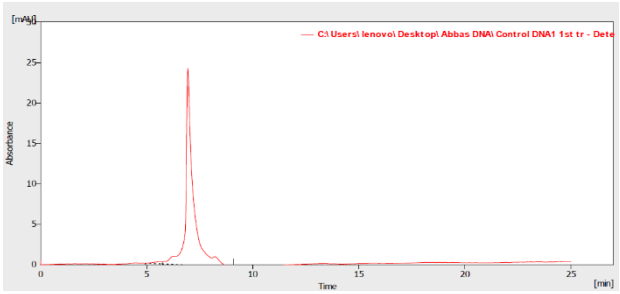

Figure 1: HPLC chromatogram of hepatocyte DNA of control group with peak at retention time (7.01) min.

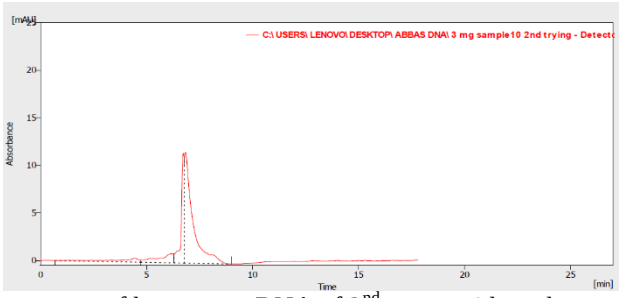

Figure 2: HPLC chromatogram of hepatocyte DNA of 2nd group with peak at retention time (6.73) min.

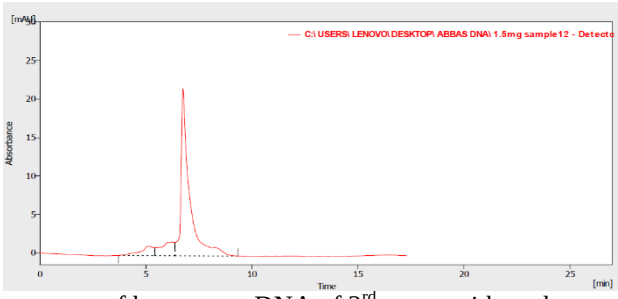

Figure 3: HPLC chromatogram of hepatocyte DNA of 3rd group with peak at retention time (6.74) min.

2. Biochemical study

There is a significant (P<0.05) increase in serum total bilirubin level in the 2nd and 3rd treated group (0.81 and 0.78) respectively as compared to the control. However, there is no significant differences between treated study groups.Albumin concentration represent a significant (P<0.05) decrease in 2nd group (15.75) when compared to control. On the other hands, there is no significant changes between 2nd and control group. Last result reveals a significant (P<0.05) increase in PT among all study groups when compared to each other (27.3 and 24.3) respectivelyin comparison to control group (21) and as presented in table (2).

Table 2: Effect of NDMA on biochemical parameters.

Parammeter / Group	T.S.B Mean ± SD	Albumin Mean ± SD	PT Mean ± SD
Control	0.60 ± 0.16 b	21.03 ± 3.41 a	21 ± 1.89 c
2nd group (3mg)	0.81 ± 0.09 a	15.75 ± 2.38 b	27.3 ± 1.96 a
3rd group (1.5mg)	0.78 ± 0.11 a	19.52 ± 1.01 a	24.3 ± 2.73 b
LSD	0.16	3.04	2.74

3. Histopathological study

Liver tissue of 2nd group showed area of hepatocyte proliferation, atrophy and necrosis along with vascular congestion. Severe proliferation of bile duct is noticed, infiltration of inflammatory cells with formation of cirrhotic hepatic lobules and interlobular hemorrhage. Moreover, presence of granulomatous inflammatory reaction with central hepatic necrosis surrounding with fibrous tissue and mononuclear cells. The 3rd group also reveals vacuolation and necrosis of hepatocyte, characteristic inflammation with bile duct hyperplasia, bile pigment accumulation and edema as showed in figure (4A-F) H&E stain.

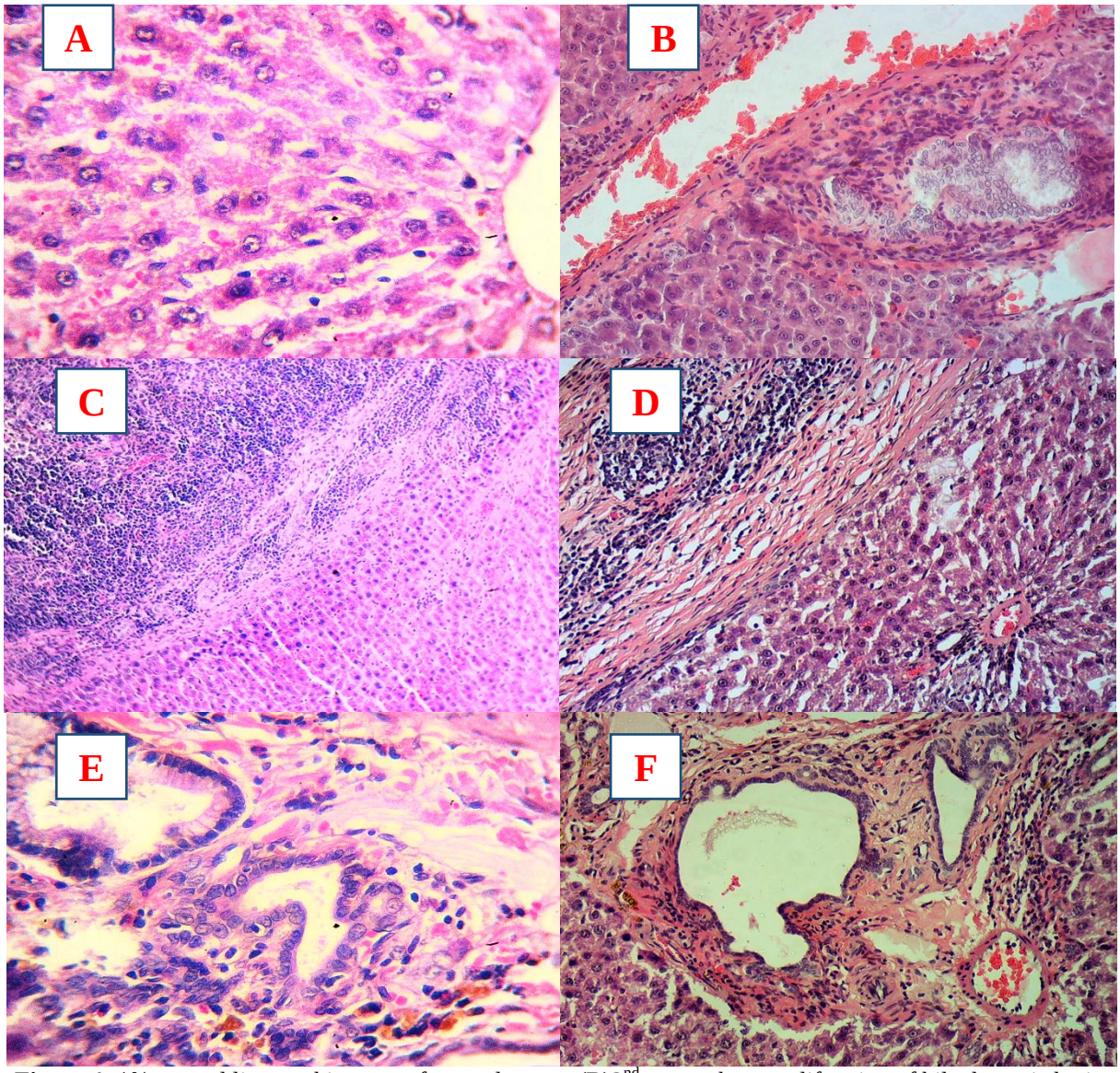

Figure 4: (A) normal liver architecture of control group. **(B)**2nd group show proliferation of bile duct, cirrhotic liver lobules, hepatic necrosis with hemorrhage.**(C)** 2nd group show granulomatous inflammatory reaction. **(D)** 2nd group show area of hepatocyte atrophy, necrosis, vascular congestion and heavy infiltration of inflammatory cells. **(E)** 3rd group show bile duct hyperplasia, presence of bile pigment with edema and inflammatory cells infiltration.**(F)** 3rd group show biliary cirrhosis, congestion of blood vessels and hepatocyte damage. **H&E stain.**

Additionally, Masson trichome stain reflect the toxic effect of NDMA on liver tissue as presence of thick bands of fibrous connective tissue in 2nd and 3rd groups separating atrophic hepatic lobules along with heavy proliferation of bile ducts and accumulation of inflammatory cells as granulomatous inflammatory reaction as presented in figure (5A-D).

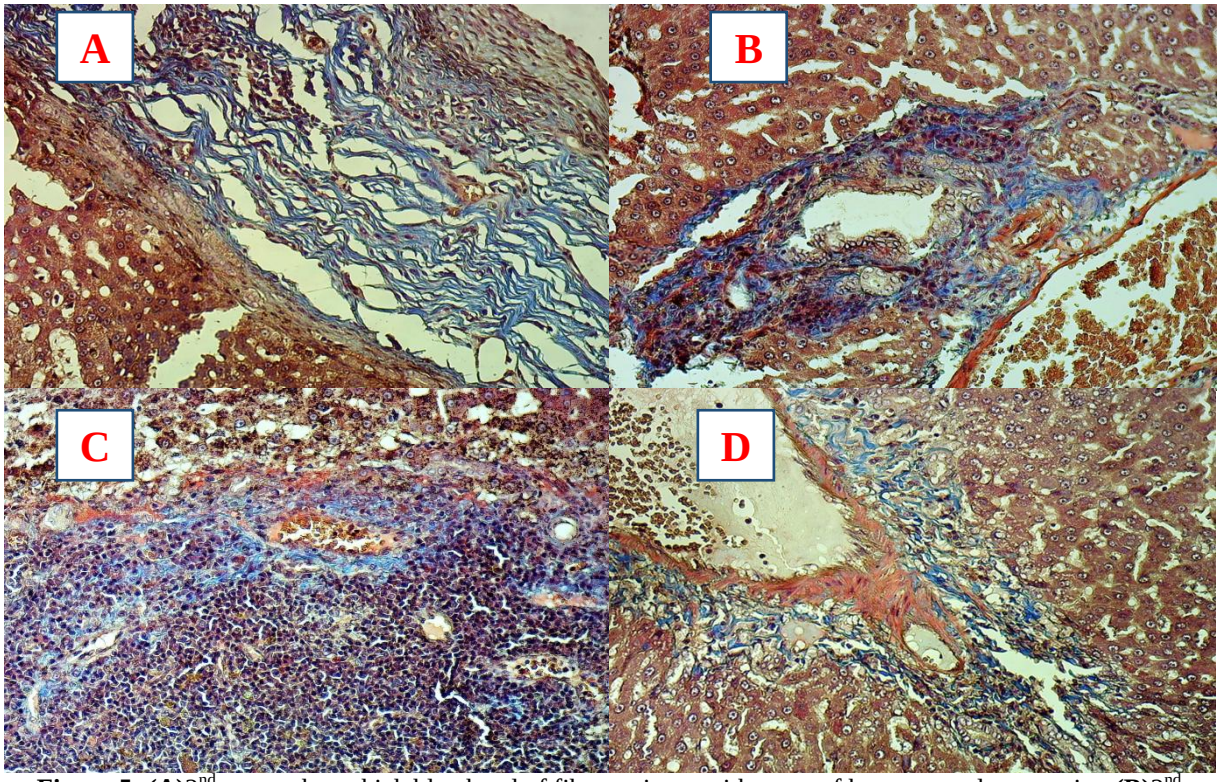

Figure 5: (A)2nd group show thick blue band of fibrous tissue with areas of hepatocyte degeneration.**(B)**2nd group show fibrous tissue separating cirrhotic lobules with atrophic hepatocyte.**(C)** 3rd group show granulomatous inflammatory reaction with presence of blue fibrous tissue and infiltration of inflammatory cells.**(D)**3rd group show vacuolar degeneration of hepatocyte, atrophy, vascular congestion and connective tissue within hepatic parenchyma**. Masson tri-chrome stain.**

DISCUSSION

NDMA found to cause DNA damage of hepatocyte through HPLCG analysis and presented as peaks with a specific retention time. Our result reveals a significant reduction in retention time of 2nd and 3rd group as showed in table (1), this result is in line with (Yotani et al.,2018). This damage to hepatocyte DNA related to the reactive metabolite of NDMA metabolism. However, addition of methyl group usually result in formation of organic molecules; DNA methylation with NDMA result in appearance of damaged DNA peaks at earlier retention time as compared to control. This result may be related to the changing in stereochemistry of DNA structure and formation of more polar compounds that appear at earlier time, this result is in similarity with (Al-Wafi,2018).

It is important to note that presence of peaks overlapping in chromatogram of 2nd group and formation of several peaks along with the main peaks of 3rd group, these changes may be related to DNA fragmentation produced through toxicity of reactive methyl diazonium ion that result from biotransformation of NDMA(Brambilla et al.,1992).

Because the hepatocyte is responsible for elimination the toxic effect of NDMA reactive metabolites, so there will be a reduction in performing it function properly (Al-musawi et al.,2020). Several research mentioned that liver damage is usually associated with increase in serum liver transaminase enzyme and explain this increment with damage of hepatic membrane and release it is enzyme to the serum (AL-Dleamy and Jawad,2022; Abbas and Jawad,2023).As noted in table (2), we observed a significant reduction in serum albumin level in treated groups as compared to control, this result is corresponding with (Cheng et al.,2017). On the other hand, we also noted a significant increase in serum total bilirubin in groups treated with NDMA when compared to control group. These alterations in liver biomarker gives an indication of hepatocyte damage and overwhelming of liver capacity to overcome the toxic effect produced through reactive metabolites and their DNA damage effect (Lee et al.,2016), an oxidative stress condition and generation of reactive oxygen species that target different parts of hepatic cells.

Furthermore, the coagulation process also maintained with liver tissue and we observed a significant increase in prothrombin time in 2nd and 3rd treated groups, this result is in line with (Zermatten et al.,2020) who reported that prothrombin time is increased in cirrhotic patient. This result may related to the impairment of liver tissue to synthesis of essential factor and proteins that participate in coagulation process; ultimately increased prothrombin time as compared to normal homeostatic state (Lv et al.,2023).

Histopathological changes of liver tissue represented with vacuolar degeneration, hepatic atrophy and even necrosisassociated with infiltration of inflammatory cells and congestion of central vein. This result is in line with previous research (Rani et al., 2018 and Lynch et al., 2024) they mentioned that hepatocyte suffer from different degree of histological alteration, impaired function and elevation of their serum markers due to exposure to NDMA; confirm the pathological changes experienced by hepatic tissue.

The hepatic tissue also show varying degree of hepatocyte proliferation along with severe proliferation of bile duct.These changes may be related to the reactive diazonium ion resulting from NDMA metabolism or from reactive oxygen species; both of which adversely affect DNA of liver tissue causing a state of transforming hepatocyte having a potential to form cancer cells (Somade et al.,2020). This result is in similarity with (Kuo et al.,2024) who found an increase in bile duct size and their number following exposure to NDMA along with inflammatory condition within the liver parenchyma (Tang et al.,2018).

The exhaustion of hepatocyte with different adaptation condition to overcome the continuous damage and because the proliferative ability of hepatocyte with induction of NDMA, there will be formation of fibrous connective tissue separating the hepatic lobules and losing their function. This result is confirmed through Masson tri-chrome stain and is matching with (Chein et al.,2014 and Chen et al.,2018), both reported that NDMA cause a network of collagen fiber through the hepatic tissue and suppress their ability to function properly.

CONCLUSION

Based on present findings, NDMA induce different pathological changes in liver tissue extending from degeneration, necrosis to hepatic fibrosis. In addition, the DNA damaging ability of NDMA have the potential of forming initiating cells that could developed to cancerous cells.

Conflict of interest

The authors declare no conflict of interest.

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