Effects of 4-nonylphenol on oxidant/antioxidant balance system inducing hepatic steatosis in male rat

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Chemical compounds studied in this article:
4-Nonylphenol (PubChem CID: 1752)
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Aprotinin (PubChem CID: 22833874)
Bouin’s fluid (PubChem CID: 124013)
Hematoxylin Eosin (PubChem CID: 86598188)
Trizol (PubChem CID: 378478)
Superoxide (PubChem CID: 5359597)
Malondialdehyde (PubChem CID: 10964)
Hydrogen peroxide (PubChem CID: 784)
Diaminobenzidine Tetrahydrochloride (PubChem CID: 23892)
Collagenase (PubChem CID: 5046512)
Tromethamine (Tris) (PubChem CID: 6503)
Sodium chloride (PubChem CID: 5234)
Phenylmethylsulfonyl fluoride (PubChem CID: 4784)
Nitrotetrazolium Blue chloride (PubChem CID: 9281)
Thiobarbituric Acid (PubChem CID: 2723628)

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A B S T R A C T

An emerging literature suggests that early life exposure to 4-nonylphenol (4-NP), a widespread endocrine disrupting chemical, may increase the risk of metabolic syndrome. In this study, we investigated the hypothesis that intraperitoneal administration of 4-NP induces hepatic steatosis in rat. 24 male Sprague-Dawley rats were administered with 4-NP (0, 2, 10 and 50 mg/kg b.wt) in corn oil 30 days. Liver histology, biochemical analysis and gene expression profiling were examined. After treatment, abnormal liver morphology and function were observed in the 4-NP-treated rat, and significant changes in gene expression an indicator of hepatic steatosis and apoptosis were observed compared with controls. Up-regulated genes involved in apoptosis, hepatotoxicity and oxidative stress, increased ROS and decrease of antioxidant enzyme were observed in the 4-NP exposed rat. Extensive fatty accumulation in liver section and elevated serum GOT, GPT, LDH and γ-GT were also observed. Incidence and severity of liver steatosis was scored and taken into consideration (steatosis, ballooning and lobular inflammation). Hepatocytes apoptosis could promote NAFLD progression; Fas/Fasl, TNF-α and Caspase-9 mRNA activation were important contributing factors to hepatic steatosis. These findings provide the first evidence that 4-NP affects the gene expression related to liver hepatotoxicity, which is correlated with hepatic steatosis.

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Abbreviations: APNEs, alkylphenol polyethoxylates; 4-NP, 4-nonylphenol; FAO, fatty acid oxidation; ROS, reactive oxygen species; Cytc, cytochrome c; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic hepatic steatosis; OS, oxidative stress; GOT, glutamic-oxalacetic transaminase; GPT, glutamate pyruvate transaminase; γ-GT, gamma glutamyltransferase; LDH, lactate dehydrogenase; AhR, aril hydrocarbon receptor; PPAR, peroxisome proliferation-activated receptor; TAG, triacylglycerol; FFA, free fatty acid; HSC, hepatic stellate cell; IR, insulin resistance.

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1. Introduction

4-Nonylphenol (4-NP) is the final product of alkylphenol polyethoxylates (APNPs), which is widely used in the preparation of lubricating oil additives, resins, plasticizers, surface-active agents, detergents, paints, cosmetics and can be found in almost all environmental water matrices [19]. 4-NP is persistent and more stable than Nonylphenol polyethanol (NPE) [41]. Its ability to accumulate in the organs of aquatic species is considered a potential hazard for humans and animals exposed within the food chain. This prevalent situation increases the risk of exposure for men who live in urban areas and have a fish based diet (probably from contaminated waters) [24].

To date, studies based on NP compounds toxicity focused mainly on estrogenic effects and related alteration to development of reproductive system in mammalian models (e.g., rat, mouse), and in aquatic organisms (e.g., fish, crustaceans) [42]. Hepatic tissue impairment was observed in different species exposed to NP [57]. In any event, the liver was proved the major organ of accumulation, biotransformation and degradation of environmental pollutants such as 4-NP [32,10]. Specific estrogen receptor exists in the liver, and cellular response involving estrogen interactions have been identified. In rat, NP is extensively glucuronidated by liver microsomes and the glucuronidation is mediated by UGT2B1, an isomeric of UDP-glucuronosyltransferase [59,12] a rainbow trout liver after injection [49]. After glucuronidation, the resultant glucuronide is excreted mainly into the bile in rats. The major metabolites conjugate are NP-glucuronide and p-nonylcarboxyl glucuronide, these were detected in the rat liver and serum after oral administration [12]. Moreover, evaluation of blood biochemistry was considered a useful tool for the diagnosis of liver diseases and assessing the histopathological studies. Laurenzana et al. [60] have reported that NP decreased hepatic testosterone hydroxylation and CYP2C expression level after oral administration, and also inhibit in vitro CYP1A1 activity in rat liver microsomes [25]. Suggesting, potential inhibition of cyt P450s and ER functions by NP, which may delay excretion from the liver.

However, there is limited information concerning the effects of 4-NP based on the liver tissues damage. On the other hand, only a few studies reported the effects of 4-NP at low doses. Based on the above undertaken, there is an urgent need for quality toxicological studies to help understand the underlying molecular mechanism in order to identify biomarkers when 4-NP-induced hepatic steatosis. 4-NP inducing non-alcoholic fatty liver disease (NAFLD) in rat is not yet widely studied and elucidated. We recall that, the mechanisms underlying the development of this disease remain unclear; hepatic steatosis is considered as the manifestation of the metabolic syndrome. In view of this, hepatic steatosis can lead to development of obesity, insulin resistance and type 2 diabetes [36,37]. In addition, excessive weight gain affects more than 60% of diabetic and obese patients [35]. Moreover, below disorders of IR, the ability of insulin to repress hormone sensitive lipase level is reduced, leading to an increased incidence of TAG lipolysis and release of increased FFA in the liver [33]. On the other hand, it has been demonstrated that mitochondrial dysfunction is a key mechanism of drug-induced liver damage, that involves the parent chemical or a reactive metabolite generated through cyt P450 (CYP450) enzymes. 4-NP has toxicological effects that can affect multiple pathways, such as aril hydrocarbon receptor (AhR) and peroxisome proliferation-activated receptor (PPAR) [10]. Previous studies demonstrated the involvement of PPAR isoforms α and β in the regulation of important biological processes, including lipidic and glucidic metabolism [54,50]. Thus, several xenobiotes can induce mitochondrial dysfunction or reactive metabolite generation through cyt P450-mediated metabolism, also involved in the process of β-oxidation of the free fatty acids [30,23]. These mitochondrial disturbances can lead to a variety of deleterious consequences such as oxidative stress, energy shortage, accumulation of triglycerides (steatosis), and cell death [4].

The liver lesion is commonly referred to as macrovacuolar steatosis (MS), which is also observed in large number of obese and diabetic patients, even in those that do not drink alcohol. MS is relatively a benign [19], liver lesion in the short term. Furthermore, studies has also confirmed lipid droplets in mouse fibroblasts (3T3-L1 cells) exposed to 4-NP [31]. Liver injury progression leads to non-alcoholic hepatic steatosis (NASH) and apoptosis, which may end in hepatocellular carcinoma [13]. Bernabò et al. [6] study based on amphibian reported large lipid droplets in liver histology and ultra-structure after acute exposure to NPE. There is evidence that liver apoptosis, cell proliferation, and related genes, Fas/Fasl, Bax/Bcl-2, and Caspase-8 play an important role in the genesis and development of NASH [26]. However, currently there is a paucity of information about the action and mechanism of 4-NP induced hepatic steatosis in rats. Consequently, in this original research, we aimed to profile the gene expression of liver tissues and also gain insight into the mechanisms responsible for 4-NP-induced hepatic steatosis after treatment with 4-NP at various doses during 30 days period in male rats.

2. Materials and methods

2.1. Chemicals and reagents

4-Nonylphenol (NP) was purchased from DR Co. (Augsburg, Germany, purity: 98%). Corn oil was obtained from Sigma–Aldrich (St. Louis, MO, USA). Sigma Chemical Co. (St Louis, MO) USA, Collagenen, Trypsin–EDTA were obtained from GIBCO (Grand Island, NY, USA). Sodium lauryl sulfate from SRL, Eosin stain and Hema-toxilin stain were obtained from HiMedia (Mumbai). LDH, GGT, GP, γ-GT, CAT, GSH-Px, SOD, H2O2 and MDA assay kit (Jiancheng Bioengineering Ltd., Nanjing, China).

2.2. Animals and experimental design

Twenty-four male Sprague-Dawley rats (70–80 g) were obtained from the Experimental Animal Center of Tongji Medical College Animal Laboratory (Wuhan, China). The rats were kept at a controlled temperature (24 ± 3 °C) under 12 h light–dark cycles, humidity (50 ± 5%) environment, and fed libitum. All protocols were approved by the institutional ethics committee for animal research of Ministry of Health, People’s Republic of China and received the certificate number (2011–2456). The rats were randomly divided into four groups, each group containing six rats. Each group (labeled as: control group, low dose group, middle dose group and high dose group) were fed different doses of 4-nonylphenol A 0, 2, 10, 50 mg/kg body weight (b. wt) respectively in corn oil every forty-eight hours by intra-peritoneal injection for 30 days. The doses and time used for the present study were derived from published data [56,55] and the result of our preliminary experiment. After 30-days of treatment, the rats were sacrificed. Livers were excised, blotted and weighed, and either fixed for histopathological studies, or stored at −80 °C respectively.

2.3. Assessment of serum hepatic marker enzymes

Serum was separated from blood by centrifugation at 3000 rpm for 15 min at 4 °C and kept in a freezer. The activities of serum glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), γ-Glutamyl transferase (γ-GT) and lactate dehydrogenase (LDH) were measured using commercially assay
2.4. Evaluation of hepatic antioxidant enzyme and non-enzymatic assays

2.4.1. Enzyme extraction and assay

The liver was homogenized using lysis buffer (containing 1 mM Na₂EDTA, 150 mM NaCl, 10 mM PMSF, 10 mM Tris, 1 mM Aprotinin) to evaluate oxidative stress following the protocol of assay kit (Jiancheng Bioengineering Ltd., Nanjing, China). All operations were done at 4 °C. Protein concentrations were determined using a BCA kit (Beyotime Biotech Inc., China) that employed serum albumin as a standard.

2.4.2. Estimation of liver antioxidant parameters

Measurements of catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) activities, as well as hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) levels were performed.

CAT can decompose H₂O₂, the absorbance at 240 nm decreasing in time with advancing reaction, CAT activity was calculated according to the rate of change in absorbance: one unit is defined as the degradation of 1 nmol H₂O₂ in the reaction system per mg protein per minute.

GSH-Px activities were assayed by quantifying the rate of oxidation of reduced glutathione to glutathione disulfide by H₂O₂. One unit of GSH-Px was defined as the amount that reduced the level of GSH by 1 μM in 1 min/mg protein as determined spectrophotometrically at 412 nm.

SOD activity in supernatants was determined by measuring the reduction of nitro blue tetrazolium (NBT) by O²⁻ produced from the xanthine–xanthine oxidase system. One unit of SOD was defined as the amount protein that inhibits the rate of NBT reduction by 50%. Results were defined as U/mg protein.

Fig. 1. Effect of 4-NP on liver biomarkers. (A) Effect of 4-NP on GOT activities (U/g of protein) in the liver of rats. (B) Effect of 4-NP on GPT activities (U/g of protein) in the liver of rats. (C) Effect of 4-NP on LDH activities (U/mg of protein) in the liver of rats. (D) Effect of 4-NP on γ-GT activities (U/mg of protein) in the liver of rats. Values are expressed as mean ± SD, n = 6. The symbol represents statistical significant (ANOVA) from control: *p < 0.05, **p < 0.01, ***p < 0.001.
2.4.3. Reactive oxygen species

Hydrogen peroxide (H$_2$O$_2$) and titanium dioxide over sulfuric acid were used to form a yellow complex, which has a characteristic absorption in 415 nm. Results were defined as µmol/mg protein. The level were assessed to determine the concentration of H$_2$O$_2$ as a marker for reactive oxygen species (ROS).

2.4.4. Assessment of lipid peroxidation

The liver homogenates was assessed to determine the concentration of malondialdehyde (MDA) as a marker for lipid peroxidation (LPO) and measuring thiobarbituric-acid (TBA) reacting substances at 532 nm. The level of MDA was expressed as nmol MDA per milligram protein.

2.5. Histological and immunohistological examination

2.5.1. Hematoxylin eosin

The liver tissues fixed in Bouin’s solution were transferred to 70% ethanol and embedded in paraffin. They were cut at 4 µm thickness and stained (hematoxylin and eosin (H&E)) based on standard pro-
Table 1
Grading and staging of histopathological of liver steatosis.

| Grade     | Histological characteristics                                                                 |
|-----------|-----------------------------------------------------------------------------------------------|
| 1-Mild    | Steatosis: predominantly macrovesicular                                                            |
|           | Ballooning: occasionally observed                                                                |
|           | Lobular inflammation: scattered and mild acute inflammation                                       |
| 2-Moderate| Steatosis: any degree usually mixed macrovesicular and microvesicular                            |
|           | Ballooning: obvious and present in centrilobular zone                                            |
|           | Lobular inflammation: associated with ballooned hepatocytes                                       |
| 3-Severe  | Steatosis: typically involves >66% of lobules                                                   |
|           | Ballooning: predominantly, marked in centrilobular zone                                           |
|           | Lobular inflammation: scattered acute and chronic inflammation                                   |

According to Brunt et al. grading and staging system for NASH [7].

![Hepatic histology](image)

**Fig. 3.** Hepatic histology of rats livers sections, showing cellular changes, particularly vacuoles (arrows), indicating steatosis. (D) Show several balloon cells that are much larger than the surrounding steatotic hepatocytes but with the same cytoplasmic characteristic as more obvious balloons, such as those seen in (B and C). Observations done at 200× magnification (H&E). (E) Grading for steatohepatitis, three grades are summarized (steatosis, ballooning and lobular inflammation).
cedures. Six slides were prepared from each liver. All sections were evaluated for the degree of liver injury. All specimens were examined using a light microscope (IX-71, Olympus, Tokyo, Japan) with high-power magnification 200×. The extent of hepatocytes steato-
sis was graded (grades 1–3) according to the Brunt et al. grading
and staging system for NASH [7] (Table 1). We considered only
H&E necessary to perform the evaluation and the whole area of
the section of each stained slides was scanned and analyzed in triplicate
for each rat.

2.5.2. In situ nick-end labeling (TUNEL)
To detect apoptotic cell death, paraffin embedded sections were
stained by the TUNEL technique using an in situ apoptosis detec-
tion kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China)
according to the instructions provided by the manufacturer. The
sections were deparaffinized with xylene, rehydrated and treated
with 200 μg/ml proteinase K for 15 min at room temperature.
Endogenous peroxidase was inactivated by covering the sections
with 3% H2O2 in H2O for 5 min at room temperature. End-labeling
was obtained through catalytically adding residues of digoxigenin-
labeled 11-dUTP and dATP to the 3′-hydroxyl ends of DNA with
the enzyme TdT. The reaction buffer containing: dATP, dUTP and
TdT, was performed for 60 min at 37 °C in a humid atmosphere.
The digoxigenin was identified immunohistochemically with a
digoxigenin-specific peroxidase-conjugated antibody (30 min in a
humid atmosphere at room temperature). For the color reac-
tion, metal-enhanced diaminobenzidine was used as substrate.
The sections were counterstained with hemato-xilin. Positive and
negative control sections were included in each sample. The apo-
ptotic cells were identified based on intense brown nuclear staining
observed under light microscope (IX-71, Olympus, Tokyo, Japan).
The data were expressed as the average of apoptotic cell numbers
per sample. Apoptotic cells were identified by their brownish stain-
ing. The whole area of the section was scanned with the high-power
magnification (400×). The apoptotic index was then calculated as
follows: AI = (number of apoptotic cells per section/ total number
of cells per section) x 100%.

2.5.3. PCNA immunohistology
Proliferating cell nuclear antigen (PCNA) immunohistochemi-
cal staining was achieved to evaluate hepatocytes proliferation.
Liver tissues were fixed for 24 h in neutral buffered formalin,
processed routinely and embedded in wax. Immunohistochemi-
cal staining was performed as previously described Kalinichenko
et al., 2003. The liver tissues were sectioned and stained utilizing
a mouse monoclonal antibody against PCNA and the SABC Staining
Kit (Wuhan Boster Biological Technology, Wuhan, China) according
to manufacturer’s protocol, then subjected to photomicroscopic
observation (IX-71, Olympus, Tokyo, Japan) with high-power mag-
nification 200×. PCNA positive cells in the liver sections were
measured. The tissue sections were counted for each rat. The data
were expressed as the average of PCNA positive cell numbers per
sample.

2.6. RNA extraction and RT-PCR
Total RNA was extracted with Trizol reagents (Invitrogen, Carls-
bad, CA, USA) and the purity was determined by the Eppendorf
BioPhotometer (Eppendorf, Germany), which showed an optical
density ratio (OD260/280) between 1.8 and 2.0. Total RNA of 2 μg
was reversely transcribed to the complementary DNA by Revert
Aid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania).
The quantitative RT-PCR was performed with an ABIPRISM®
7900HT Sequence Detection System (Applied Biosystems, USA)
using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX
(Invitrogen, USA). The β-actin was used in parallel for each run as
an internal control. A 10 μl PCR reaction system was used, includ-
ing 2 μl cDNA, 3 μl primers, and 5 μl Super Mix-Rox. A 4-step
experimental run protocol was carried out and the amplification
conditions were as follows: 50 °C for 2 min (UDG incubation); 95 °C
for 2 min (initial denaturation); 40 cycles of 15s at 95 °C (denatu-
ration), and 1 min at 60 °C (elongation). The relative expression
of target genes was calculated using 2ΔΔCT method [38]. The primer
sequences were designed according to the cDNA sequences from
the GenBank. All primers were synthesized by the Biosia Corp
(Shanghai, China). The sequences of primers used as follows:
SOD 1 forward: 5′-ACACAAAGCTGACCACCTGC-3′; reverse:
5′-CCACATTGCCCAGGTCTCC-3′
GPx forward: 5′-GCTCAAGGTTAGGCTCTCC-3′; reverse:
5′-TCACCTGATGCGAACTGTAGG-3′
HSP 70 forward: 5′-ATCTCCTTGGCTTACCTAAAC-3′;
reverse: 5′-CACCAATCTCTCTCAATCTAGA-3′
TFN-α forward: 5′-TATTGGCCACAGCCCTACA-3′;
reverse: 5′-GGAATACAGAAGTGAACACCCATC-3′
Fas forward: 5′-ACATGGCAAAACCATCTTACGA-3′;
reverse: 5′-CTGGTTTCACCTTGACCTTGTA-3′
FasL forward: 5′-CATGGACAGCCATGAAATTAC-3′;
reverse: 5′-CTCTAGGGCCACAAAGTGGACAG-3′
Bcl-2 forward: 5′-TGAAGGGTCCGGTGGATA-3′;
reverse: 5′-CAGGATTTCGCAAGGCTCTTGA-3′
Bax forward: 5′-CAGATGGTCGCCAGACACAAAGA-3′;
reverse: 5′-CGTTCACTGGCAATGAAATCTA-3′
Caspase-9 forward: 5′-TGACACTCTCTCTCAAGGCCGACG-3′;
reverse: 5′-TCAAGGCTCCATGTACCTGAGCC-3′
β-actin forward: 5′-ACTATCGGCAATGAGCCGTC-3′;
reverse: 5′-CTGTGGTTCACATAGGGCTTAC-3′

2.7. Statistical analysis
All values expressed as (mean ± SD) were compared by one way
analysis of variance (ANOVA) followed by the Tukey–Kramer mul-
tiple comparison test. All statistical analyses were carried out using
SPSS statistical package 12.0 (SPSS Inc, Chicago, IL, USA) to deter-
mine whether the treatments of results were significant (*p < 0.05)
or extremely significant (**p < 0.01 and ***p < 0.001) different from
the control groups without 4-NP.

3. Results

3.1. Body and liver weight
At the beginning of the experiment, rats from different groups
showed approximately similar body weight values. The treatment
with 4-NP promoted an increase in the body weight gained when
compared to the initial weight of rats. The final body weight (b.
w) of animals from these groups was not statistically different
in values from untreated animals (Table 2). However, significant
changes were observed between absolute and relative weight of
4-NP–treated groups rat liver.

3.2. Effect of treatments on liver function
The activities of GOT, GPT, LDH and γ-GT in blood are commonly
used to evaluate the liver function. Fig. 1 shows that the activities
of GOT, GPT, LDH (p < 0.01, p < 0.001) and γ-GT (p < 0.05) were sig-
nificantly increased in response to 4-NP-treated group compared
to control.

3.3. Effect of treatments on hepatic ROS and LPO
H2O2 and MDA assay were routinely used to measure the extent
of lipid peroxidation. Results of Table 3 shows that the levels of

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H₂O₂ (p < 0.01) at 50 mg/kg b. wt and MDA (p<0.01) at 10 and 50 mg/kg b. wt were significantly increased in response to 4-NP-treated group compared to control.

3.4. Effect of treatments on antioxidant enzyme activity

Three antioxidant enzymes (SOD, CAT and GSH-Px) on rat liver were determined and the results are shown in Table 3. All the antioxidant enzyme activities were decreased significantly (p < 0.05, p < 0.01, p < 0.001) in response to 4-NP-treated group compared to control.

3.5. Effect of expression of oxidative-stress-related genes in liver

The levels of SOD1, GPx and HSP70 in the livers (Fig. 2A–C) showed significant decrease in 4-NP-treated group compared to control (p < 0.05, p < 0.01). While the level of HSP70 increased significantly (p < 0.05).

3.6. Effect of expression of apoptosis-related genes in liver

The levels of Casp-9, Bax and Bcl-2 in the livers (Fig. 2DF) showed significant increases in 4-NP-treated group compared to control (p < 0.05). While the level of Bcl-2, which decreased significantly (p < 0.05) (Fig. 2E).

3.7. Effect of expression of hepatotoxicity-related genes in liver

The levels of Fas, Fasl and TNF-α in the livers (Fig. 2H–I) showed significant increase in 4-NP-treated group compared to control (p < 0.05, p < 0.01).

3.8. Histological and immunohistological examination

3.8.1. Hematoxylin eosin

Taking into consideration the alterations observed in plasma biomarkers, oxidative stress markers, and the microscopic aspect of the liver, no change was observed in the rats given corn oil. Thus changes refer especially to the 4-NP treated group. The accumulation of lipids revealed by H&E technique (vacuoles in white) (Fig. 3B–D) was confirmed by TUNEL (lipids in gray) (Fig. 4B–D).

Fig. 3E. Show the liver Steatosis, ballooning and lobular inflammation scores according to Brunt et al. [7] The liver ballooning scores of D group were higher than those of the B and C group at each point. While lobular inflammation scores were higher than those of the B and D group at each point.

3.8.2. In-situ nick-end labeling (TUNEL)

The TUNEL outcomes of all treatment groups are presented in (Fig. 3). After evaluation with the light microscopic has revealed a characteristic appearance of the normal hepatic tissue for the control group (Fig. 4A). However, TUNEL-positive cells increased significantly in response to 4-NP treatment at 10 and 50 mg/kg b. wt. In contrast, no TUNEL positive cells were seen in control group. Thus, despite similar metabolic abnormalities, an equivalent degree of steatosis has also been observed in 4-NP treated group. Observed histological changes in liver tissues have shown a dose-dependent increase (Fig. 4B–D).

3.8.3. PCNA immunohistology

The PCNA outcomes of all treatment groups are presented in (Fig. 5). PCNA is an auxiliary protein of DNA polymerase-delta and higher level of its expression is correlated cell proliferation, suggesting PCNA is an excellent marker of cellular proliferation. After evaluation with the light microscopic has revealed characteristic appearance of normal hepatic tissue of control group (Fig. 2A). Conversely, in 4-NP-treated were markedly increased PCNA positive cell (4.64%, 8.87% and 9.07%) respectively in response to 4-NP (2, 10 and 50 mg/kg b. wt) in the liver tissues compared with corresponding control group. However, no significant PCNA positive cells were observed in corresponding control group. Whereas significant histological alterations in liver tissues have observed a dose-dependent manner (Fig. 5CD).

4. Discussion

NAFLD has become the leading cause of chronic liver injury in developed countries. Although the exact cause of NAFLD is still unknown, there are emerging experimental and epidemiological studies proposing that some environmental contaminants may exert effects on promoting metabolic diseases and associated NAFLD [17]. In this study, we sought to investigate whether admin-

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Table 2
Body weight (g) and selected absolute (g) and relative organ weight (mg/g) of male rats in control and treatment groups. Doses of 4-NP (mg/kg b.wt.).

|                  | Initial body weight (g) | Final body weight (g) | Liver weight Absolute (g) | Relative-to-body (mg) |
|------------------|-------------------------|-----------------------|--------------------------|-----------------------|
| Control          | 78.93 ± 5.96            | 246.63 ± 14.89        | 7.63 ± 1.54              | 30.82 ± 5.27          |
| 2 mg             | 75.25 ± 4.72            | 243.40 ± 11.76        | 7.94 ± 1.13              | 32.72 ± 5.08          |
| 10 mg            | 73.37 ± 6.61            | 260.30 ± 16.63        | 9.23 ± 0.78              | 35.69 ± 4.20          |
| 50 mg            | 76.33 ± 5.97            | 267.91 ± 14.91        | 9.44 ± 0.67              | 35.26 ± 2.54          |

Values are expressed as mean ± SD, n = 6. The symbol represents statistical significant (ANOVA) from control.

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Table 3
Effect of 4-NP on liver oxidative stress and antioxidant enzyme activity in all experimental groups. Doses of 4-NP (mg/kg b.wt.).

|                  | CAT (U/mg prot) | GSH-Px (U/mg prot) | SOD (U/mg prot) | H₂O₂ (μmol/mg prot) | MDA (nmol/mg prot) |
|------------------|----------------|-------------------|----------------|--------------------|-------------------|
| Control          | 14.045 ± 2.08  | 1.932 ± 0.15      | 14.502 ± 2.79  | 2.045 ± 0.41       | 1.461 ± 0.49      |
| 2 mg             | 10.23 ± 1.91   | 1.280 ± 0.45      | 14.156 ± 2.18  | 2.428 ± 1.00       | 1.881 ± 0.55      |
| 10 mg            | 5.833 ± 2.67   | 0.632 ± 0.39      | 12.359 ± 1.82  | 2.451 ± 0.28       | 3.013 ± 0.46      |
| 50 mg            | 4.318 ± 2.32   | 0.471 ± 0.29      | 10.414 ± 2.20  | 3.066 ± 0.47       | 3.199 ± 0.81      |

Effect of 4-NP on liver antioxidant enzymes and reactive oxygen species. Values are expressed as mean ± SD, n = 6. The symbol represents statistical significant (ANOVA) from control.

*p < 0.05.
**p < 0.01.
***p < 0.001.
Administration of 4-NP induces steatosis in rats. Plasma GOT, GPT, LHD and \( \gamma \)-GT are the most sensitive markers in the diagnosis of hepatic injury. The results showed that the levels of GOT, GPT, LHD and \( \gamma \)-GT were significantly increased in response to 4-NP-treated group compared to control. GOT and GPT are normally localized in the hepatic cytoplasm, while increase in serum GOT and GPT levels by 4-NP suggests hepatic damage with their subsequent release into circulation. This has also been confirmed by [39]. It is important to note that, some studies have shown no changes in the serum SGOT and SGPT even at high doses of 4-NP [11,53]. This contradiction could be due to the degree of toxicity depending on the dose, time, frequency, and strains.

Through hepatic histopathological examination and serum assays has also confirmed, microvesicular steatosis was observed in 4-NP-treated group. As illustrated in result section the hepatocytes ballooning is the most characteristic feature of steatosis-hepatitis; which is typically associated with formation of Mallory’s hyaline. The severity of steatosis appears to be important in determining the extent of apoptosis. For this purpose, we carried out the grading and staging system for NASH according to [7].

To investigate the mechanism of intraperitoneal exposure to 4-NP, which lead to increased susceptibility of the development of NAFLD in male rats, a massively parallel deep-sequencing was carried out to identify the genome-wide profiles of the liver tissues after treatment. We observed that the expression of Fas/Fasl, TNF-

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**Fig. 4.** Detection of apoptosis TUNEL assay in the liver tissue of rats. The presence of apoptosis after treatment with (2, 10 and 50 mg/kg b.wt) of 4-NP by intraperitoneal injection for 30 days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) (B–D) DNA fragmentation, characteristic of apoptosis (blacks arrows), (red arrows) indicates ballooning. (E) The graph representing average number of TUNEL-positive cells in each group. Observations done at 200× magnification. Apoptotic index of liver (%). The tissue sections were counted for each rat. The symbol represents statistical significant (ANOVA) from control: *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 5.** 4-NP promotes hepatic cell proliferation in acute liver injury. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was carried out as previously described. (A) PCNA staining liver sections in the rats without 4-NP administration. (B–D) Low middle and high dose respectively, arrows indicate PCNA+ cells in the liver sections. (E) Quantitative expression of PCNA was significantly higher in the liver cells at dose 10 and 50 mg/kg b. wt. The graph representing average number of PCNA+ cells in each group. Observations done at 200× magnification. PCNA+ index of liver (%). The tissue sections were counted for each rat. The symbol represents statistical significant (ANOVA) from control: *p < 0.05, **p < 0.01, ***p < 0.001.
α and Casp-9 mRNA activation in 4-NP-treated group increased with hepatic steatosis. The expression level increased gradually showing a positive correlation with NASH levels and the liver apoptosis percentage, suggesting that the incidence and development of up regulation of Fas/FasL, TNF-α and Casp-9 activation at dose 10 and 50 mg/kg b.wt. respectively thus indicating that 4-NP induces extrinsic apoptosis; as echoed by Jubendradass et al. [19]. In addition to this, TNF-α acts as a simulator of NAPDH-dependent H₂O₂ generation [22]. Increase of TNF-α and lipid peroxidation were observed at 4-NP-treated group and particularly significant at dose 50 mg/kg b.wt. This is the most important factor causing and maintaining insulin resistance [48], and closely related to the development of NAFLD and other metabolic disorders [16]. Despite the existing correlation between steatosis and insulin resistance, below disorders of IR, the ability of insulin to repress hormone sensitive lipase level is reduced, leading to an increased incidence of TAG lipolysis and release of increased FFA into the liver [47]. This process is an independent hazard factor for NAFLD severity.

Several genes associated with liver function were significantly changed. Alterations in the expression of Bcl-2 and Bax genes indicate the involvement of an intrinsic apoptotic mechanism. Whereas caspases point towards the role of an alternative pathway [18]. In our present findings, dysregulation of Casp-9 and Bax mRNA (apoptotic), and also Bcl-2 expression (pro-survival) results in the release of cyt C; cyt C, a pro-apoptotic factor released from outer mitochondrial membrane to cyt C, form a complex with Apaf-1 and procaspase-9, resulting in activation of Casp-9. Activated Casp-9 activates the effector Casp-3 leading to degradation of DNA and cellular constituents [1]. Nevertheless, in response to DNA damage, protein p53 will be activated [34]. This protein, directly or indirectly, modulates the expression of some proteins that control mitochondrial membrane permeability (MMP), resulting in the release of cyt C [14]. Mitochondrial cyt C together with the Apaf-1 (apoptotic protease activating factor 1) and dATP (nucleotide precursors) form apoptosis that activates Casp-9 (initiator caspase). Furthermore, this initiator caspase begins the apoptosis process through the activation of executor caspase [45]. DNA fragmentation was confirmed by TUNEL assay. These observations further validate the theory that mitochondria dysfunction induced by 4-NP can be a cause, effect or concurrent feature in the development of NAFLD. This assertion has been confirmed by [40]. In addition, mitochondrial dysfunction involved in the process of β-oxidation of FFA, then impairs fat homeostasis in the liver, but also leads to an overproduction of oxidative stress, resulting in the generation of ROS that trigger LPO, cytokine overproduction and cell death [46]. On the other hand, ROS produced mitochondria FAO in liver cells will further simultaneously release cyt C, which eventually triggers hepatic apoptosis through the mitochondria-dependent pathways [27].

A wide range of injurious stresses relevant to liver disease lead to MMP pore opening [29]. It can therefore be proved that, ROS overproduction or antioxidant reductions are the direct causes of oxidative stress. Previous research have reported that endocrine disruptor like bisphenol A exhibit adverse effects at very low doses. However, only a few studies have reported the effects of NP at low doses. Administration of NP at a dose level of 50 μg/kg b.wt/day for 30 days has been shown to increase oxidative stress in blood of adult male rats. Many factors may cause the production of ROS, including 4-NP exposure. The change in antioxidant enzyme activities is relevant to the ability of the liver to fight against oxidative stress during 4-NP exposure. This study showed that SOD, CAT, GSH-Px levels were significantly decreased in the liver after the treatment of 4-NP compared with the control group. In order to support this hypothesis we have considered in our work that enhance of H₂O₂ and MDA as marker for LPO and as indicator of oxidative liver injury [9]. All these indicated that 4-NP promotes antioxidant activity to induce liver damage. SOD presents in three isoforms, copper-zinc-containing superoxide dismutase (Cu–Zn–SOD, SOD1), manganese containing superoxide dismutase (Mn–Zn–SOD, SOD2) and extracellular superoxide dismutase (Cu–Zn–SOD, SOD3). The SOD1 isoenzyme is the most abundant one in the cytoplasm [5]. Reduction of SOD1 activity in 4-NP-treated group in this study might be due to the enhanced production of superoxide radical anions. GPX is an antioxidant enzyme, which modifies the peroxide anion to a non-toxic hydroxyl compound in order to protect the membrane structure and function. The decrease of these antioxidants enzymes may be suggestive for the process of LPO. These processes are situated to be responsible for initiating necroinflammation, and ROS, which are generated during FFA metabolism in microsomes, peroxisomes and mitochondria. These cytotoxic ROS and LPO products are able to disperse within the extracellular interspaces worrying HSC and Kupffer cells [47], that may play substantial roles in the evolution of chronic liver inflammation and fibrosis development [43].

HSP70 is one of the most abundantly induced proteins under a variety of stress conditions and the extensive oxidative stress marker at present [51], and is often correlated with the emphasis on the process of apoptosis [3] by inhibiting the formation of apoptosome [8]. This can also suppress the apoptotic process through other pathway by blocking the activation of stress induced kinases such as apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinases (JNK) [15]. The increase in the levels of HSP70 mRNA may be due to the presence of higher event of OS and cell damage. This can be confirmed by the increase in the number of positive PCNA cell staining of hepatocytes, which is the central molecule responsible for taking decisions of life and death of the cell. Its increase is associated with cell death [28]. This observation may be due to ROS induction and disruption of the balance between ROS and antioxidant defense system. This data concurs with previous findings [21]. On the other hand, Excess FA accumulation in hepatocytes induces oxidative stress in mitochondria but also microsomes and peroxisomes. This study also demonstrated increase in body weight gain and absolute and relative liver weight of 4-NP treatment rats. The augmentation of the size of the liver is commonly observed in patients with several Hepatomegalgy [2] and also might be a crucial mechanism for its developmental toxicity in male rat hepatocytes.

In addition, FAO and oxidative stress occurs in the mitochondria, and are considered to play a critical role in the pathogenesis of NAFLD [58]. Hepatocytes apoptosis is commonly manifested in several liver diseases. In previous years, large progress has been made with regard to elucidate the role of apoptosis in the occurrence and development of NAFLD (e.g., [44,52] and its subsequent progression to NASH, liver fibrosis, cirrhosis and liver cancer.

5. Conclusion

Intraportal administration of 4-NP promotes an increase in the susceptibility to hepatic steatosis, which is associated with apoptosis, positive proliferation of hepatocytes and subsequent oxidative stress prior to the development of NAFLD. However, this present study may merit discussion and it would be important to research new mechanism-specific biomarkers of hepatocellular injury, which would be a suitable opportunity to assess the impacts of variations in 4-NP concentrations on liver function and damages as well as protein measurements by immunoblot analysis to support this conclusion. However, our present research demonstrated the first evidence that 4-NP promote hepatic steatosis in male rats, disturbing hepatotoxicity-related genes expression in liver; as well as up-regulation of the expression of Fas/FasL ratio.
and TNF-α, which are a positive correlation of NALD and also have been associated with many pathologic states in humans.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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