Research article

Influence of triazines and lipopolysaccharide coexposure on inflammatory response and histopathological changes in the testis and liver of BalB/c mice

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ABSTRACT

Background: Triazines are environmental active chemicals that have been reported to alter the inflammatory status of the gonads. We tested the anti-inflammatory effect of the triazines (atrazine; ATZ, simazine; SMZ and cyanazine; CYZ) on the testis and compared it with the more classical liver model that has substantial populations of resident macrophages comparable to the testis.

Methods: BalB/c mice were treated with 25 mg/kg ATZ, SMZ and CYZ for 30 days and injected with lipopolysaccharide (0.5 mg/kg *i.p.*) 6 h before sacrifice. Myeloperoxidase activity and nitric oxide level in the testis and liver homogenates were determined by spectrophotometry whereas tumor necrosis factor-alpha and interleukin-6 concentrations were evaluated by immunoassay. Haematoxylin and eosin stained sections of the tissues were observed using a light microscope.

Results: Myeloperoxidase activity, nitric oxide, tumor necrosis factor-alpha, and interleukin-6 levels were decreased in the liver and testis of the triazines co-treated animals. SMZ has the most potent inhibitory effect and ATZ the least effect on inflammatory mediators in both tissues. Microscopic evaluation showed loss of inflammatory cells in the inter-tubular areas of the testis and few patchy masses of infiltrating inflammatory cells around the central vein of the liver.

Conclusion: Triazines inhibit the levels of inflammatory mediators in the testis and liver of mice. The anti-inflammatory effect of triazines in a lipopolysaccharide-induced inflammation model was established in this study.

1. Introduction

Environmental endocrine disrupting chemicals can lead to a broad range of disorders including endocrine dysfunction, infertility, increased rates of inflammatory infections, and autoimmune diseases [1]. Widespread use of these chemicals also increases the chance for, and rate of exposure to other foreign chemicals that the immune system must contend with. Therefore, it is possible that endocrine disrupting chemicals (EDCs) may worsen the effects of infectious diseases [2–4].

The triazine family of herbicides, cyanazine (CYZ), atrazine (ATZ) and simazine (SMZ) are widely used agrochemicals that are

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commonly detected in surface and groundwater, where they persist for several months [5]. They are extensively used to control broad-leave and grassy weeds in farms during the cultivation of sorghum, maize, wheat, sugar cane crops, vegetables; peas, chickpeas, beans, lentils, onions, potatoes, and sweet corn [6–8]. Triazine chemicals are recognised as environmental endocrine and immune disruptors in several experimental models [9]. For instance, ATZ alters immune function and disease resistance in animals [10,11] raising the possibility that it potentiates the susceptibility to clinical and infectious diseases [2,9]. Studies have also shown that mixtures of chlorotriazine and its metabolites influence the severity of inflammation in the prostrate of Long-Evans rats [12]. Long-term exposure to ATZ can induce the dysregulation of pro-/anti-inflammatory cytokine expression in the common carp [13]. Furthermore, SMZ has been reported to inhibit the expression of inducible nitric oxide synthase (iNOS) in the testes of mice, and the level of nitric oxide in rat's testicular Leydig cells *in vitro* [8]. Simazine was found to inhibit lipopolysaccharide (LPS)-induced production of nitric oxide and tumor necrosis factor-alpha (TNF-α) in murine macrophages [14], influence immune functions of mice [15], interfere with the production of interleukin-1 and interferon [16], and inhibit the activity of macrophages against tumors and viruses [15]. Although the roles of CYZ and SMZ on inflammation in tissues have been less well studied than ATZ, some studies have reported their toxicities in hormone-dependent tissues [17]. Thus, it is believed that triazine chemicals may act on the inflammatory and immune responses for host defence and potentially influence infectious- and inflammation-related diseases [2,4,9,11,18].

When pathogens or product of pathogens e.g. LPS encounter innate immune cells, the innate immune system provide alarm signals in the form of inflammatory cytokines, that degrade the pathogens and present the pathogen-derived peptides to adaptive immune cells. The primary function of the adaptive immune cells is to recognize and kill infected cells [19]. Inflammatory cytokines regulate the antigen sensitivity, proliferation and trafficking of both effector and established memory T-cell populations, giving the host an advantage over the invading pathogen [20,21]. Lipopolysaccharide has been used as an important model for inducing local inflammation in several tissues. For instance, intraperitoneal injection of LPS induces the generation of pro-inflammatory mediators, such as nitric oxide, interleukin 1β, and iNOS [22–25]. There is also increasing evidence that TNF-α and IL-6, and nitric oxide signalling are key factors in various aspects of liver diseases; because of their capacities to mediate hepatic inflammation, necrosis and apoptosis of the cells of the liver [26,27]. Therefore the aim of this study is to investigate the effect of triazines on the histological features and inflammatory cytokines (TNF-α and IL-6), myeloperoxidase activity and nitric oxide concentration in the testes of mice, and to ascertain whether the response of these inflammatory mediators is different in other tissues such as the liver, which has a stronger pro-inflammatory response to LPS [28].

2. Materials and methods

Mice specific ELISA kits for TNF-α and IL-6were obtained from U-CyTech biosciences (Belgium), ATZ (2-chloro-4,6-diamino-1,3,5-triazine), SMZ (2,4-bis (ethylamino)-6-chloro-1,3,5-triazine), CYZ, tetramethylbenzidine, sulphanilamide, N-(1-naphthylethylenediamine dichloride were obtained from Sigma-Aldrich, Chemical Co. (St. Louis, MO USA). All other chemicals used for this research were of analytical grade and commercially available.

2.1. Animals, treatments and sample collections

Adult male albino mice, weighing 25–30 g were obtained from the animal facility of the Department of Anatomy, Delta State University, Abraka in Nigeria and housed in the animal facility of the Department of Biochemistry, University of Port Harcourt, Choba. They were allowed a week to acclimatize in the animal facility before used in the experiment. Food and water were provided *ad libitum* throughout the experiment. All animal studies were conducted in accordance with the principles and procedures outlined in *A Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science and published by the National Institute of Health [29] and were approved by the Department of Biochemistry Research Ethics Committee (UPH/BCHREC/2018/014), University of Port Harcourt, Nigeria.

Eighty animals were randomly divided into eight groups of ten animals each, group 1: Control animals, group 2: LPS alone (0.5 mg/kg) (Koga et al., 1994), group 3: ATZ alone (25 mg/kg b.w.), group 4: CYZ alone (25 mg/kg b.w), group 5: SMZ (25 mg/kg b.w), group 6: ATZ (25 mg/kg b.w) + LPS (0.5 mg/kg b.w), group 7: CYZ (25 mg/kg b.w) + LPS (0.5 mg/kg b.w), group 8: SMZ (25 mg/kg b.w) + LPS (0.5 mg/kg b.w). The animals were administered triazines by oral gavage every other day for 30 days and intraperitoneal injection of LPS was done 6 h prior to sacrifice on the 30th day of triazine treatment. In our preliminary study, the 25 mg/kg b.w dose of the tested triazines selected for this study was the least dose that inhibited serum and testicular inflammatory mediators' e.g. nitric oxide and myeloperoxidase activity on co-treatment with LPS (0.5 mg/kg b.w) (Unpublished observations). Furthermore, the animals co-treated with these chemicals survived until end of pilot studies (30 days) and had no overt changes in appearance and behaviour along with absent of effect on body weight (Table 1). It has also been previously reported that this dose, for example, with ATZ is safe [30], and was the least dose that altered inflammation-related cytokines in mice [11]. Although this dose of triazine (25 mg/kg b.w) is

Table 1
Body weights (g) of treated mice at the end of study.

Control	LPS	ATZ	ATZ + LPS	CYZ	CYZ + LPS	SMZ	SMZ + LPS
22.37 ± 1.48[a]	22.68 ± 3.32[a]	24.28 ± 3.23[a]	24.95 ± 4.06[a]	22.25 ± 1.64[a]	24.70 ± 4.78[a]	24.49 ± 3.50[a]	24.7 ± 3.09[a]

Data are mean ± SD (N = 10); Triazines: ATZ = atrazine; SMZ = simazine; CYZ = cyanazine; [a]same superscript on the same row are not significantly different (*p* > 0.05).

higher than environmental levels of triazines, range of doses of triazines similar to the selected dose have consistently been reported in many laboratory studies to have adverse effects on gonadal functions and immune suppressive properties [5,11,31]. Additionally, triazines e.g. ATZ can be found in the environment at concentrations up to 224 ppb in ground water and drinking water supplies [32]. However, humans are exposed to ATZ at a thousand fold higher concentration in the occupational set-up than seen in residential exposures [5].

After sacrifice of animals, the testes and liver were removed aseptically, rinsed in ice-cold 1.15 % potassium chloride. The animals of triazines and LPS groups received solution of the tested chemicals diluted in dimethyl sulfoxide (not more than 0.5 %) while the animals of the control group not treated with triazines or LPS received 0.5 % dimethyl sulfoxide vehicle by oral gavage (2 μL/g b.w). The dosage of LPS (0.5 mg/kg b.w) was used to induce tissue inflammation in the present study as previously reported [33]. At this dose, LPS hepatoxic effects and induced levels of TNF-α was reported at 6 h post-injection in animals. The hepatotoxicity was also characterized by high level of TNF-α beginning at 2 h as well as severe hepatic injury at 6 h after the LPS challenge [33]. Furthermore, over a time frame (3–6 h) similar to control for liver and testis, LPS post-injection was able to raise the expressions of TNF-α and IL-6 both in the liver and testis extracts. Within this time period, TNF-α was maximally stimulated in the testis by LPS, and the concentration of other inflammatory cytokines including IL-1β as early as 3 h was raised 8-fold [28]. Furthermore, at 6 h post-LPS injection, elevated TNF-α expression was also observed in the rat testis [34]. Other inflammatory markers, including nitric oxide were also noted to be upregulated by LPS post-injection after 6 h, a time point that approximated with a maximum inhibition of testosterone production and testicular damage [22]. Thus, the 6 h was carefully chosen in this study based on these previous findings, and was also validated in our preliminary studies to correspond to a time point we observed high levels of the tested cytokines and testicular injury and mild systemic inflammation [28]. The right testis and fragments of the liver were fixed for histological evaluations, whereas the left testis and other portions of the liver were stored at −25 °C for biochemical assays.

2.2. Tissue processing for biochemical assays

Tissue sample homogenates (1: 4) that were used for myeloperoxidase and Griess assays were prepared in 0.1 M sodium phosphate buffer (pH 7.4), and those used for cytokine assays (TNF-α and IL-6) were prepared in ice-cold lysis buffer (100 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 % Triton X-100, 0.5 % sodium deoxycholate and 1 % protease inhibitor cocktail). After centrifugation at 10, 000 revolutions per minute for 20 min at 4 °C, the isolated supernatants were used for the assay of the various biochemical parameters.

2.3. Immunoassay for TNF-α and IL-6

The cytokines, TNF-α and IL-6 were determined by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's manual (U-CyTech biosciences, Utrecht, Netherlands). The sample supernatants were mixed with PBS containing 2 % BSA (1: 2) before they were subjected to ELISA. After washing off the excess and unbound materials, the bound analyte is allowed to bind to a biotinylated detection antibody. The detection antibody allows the binding of the antigen-antibody complex with a streptavidin horse radish peroxidase (SPP) conjugate which forms blue-coloured product after addition of a chromogenic substrate. The colour reaction is directly proportional to the amount of cytokine bound, and is determined by comparison with a standard curve of known cytokine concentration (IL-6: 4–256 pg/ml; TNF-alpha: 2–128 pg/ml). The absorbance was measured at 450 nm. The sensitivity of the TNF-α and IL-6 immunoassay was 2 and 4 pg/ml respectively.

2.4. Determination of myeloperoxidase activity

Myeloperoxidase activity was determined as previously reported by Andrews and Krinsky [35] using tetramethylbenzidine (TMB), as substrate. In brief, a small portion of the sample (10 μl) was mixed with 110 μl of TMB (2.9 mM) and 80 μl of H_2O_2 (0.75 mM). After incubation for 5 min at 37 °C, the reaction was stopped with 60 μl of the stop reagent (2 M H_2SO_4) and optical density was read at 450 nm.

2.5. Estimation of nitric oxide concentration by the Griess assay

Measurement of nitric oxide production was measured using the Griess reagent (N-1-naphthylethylenediamine dichloride and sulphanilamide) as reported previously [36]. Briefly, samples were deproteinated with 5 % zinc sulphate by centrifugation at 5000*g* for 20 min. The supernatant collected was incubated with equal volume of the Griess reagent (0.1 % N-1-naphthylethylenediamine dichloride and 1 % sulphanilamide) at room temperature for 20 min. The optical density was recorded at 540 nm and nitrite concentration was calculated by comparing the absorbance recorded with a nitrite standard reference curve (0–100 μM).

2.6. Histological procedures for light microscopy

Testes were cross sectioned at the equatorial plane and fixed in Bouin's solution whereas the liver was fixed in 10 % buffered formalin. The tissues were fixed over night before they were processed for histological examination. After fixation, the samples were dehydrated in graded concentrations of ethanol and subsequently embedded in paraffin. The five slides prepared from each mouse, with tissue sections (5 μm thick) were stained with haematoxylin and eosin for light microscopy.

2.7. Quantification of histopathology

To quantify the histological features of the testes, 10 randomly chosen circular seminiferous tubules were selected, totalling 50 tubules per animal. In each tubular section, the number of tubules with desquamated germ cells in the lumen, degenerated epithelium, and clump of giant cells in the epithelium and inflammatory cells in the intertubular spaces were quantified and the numbers of these atypical seminiferous tubules and inflammatory cells were recorded and the mean calculated [37]. To assess the incidence of histo-pathologies in the liver, 50 digital images from 10 animals were analysed at $\times$ 400 magnification. Using the grid function in Image J soft-ware, with 84-intersection points overlapped in histological images, the following histological features were recorded: foci of inflammatory cells, sinusoidal dilatations, von Kupffer cells hyperplasia and cytoplasmic vacuolisations were quantified per animal and the mean calculated.

2.8. Statistical analysis

Data are presented as mean $\pm$ standard deviation. All statistical analyses were done using GraphPad Prism version 6 software (GraphPad Software, Inc., San Diego, CA, USA). Datasets were found to be normally distributed using the Shapiro–Wilk test and satisfies the homogeneity of variance assumption when tested with the Levene's test. All statistical analyses were performed by one-way analysis of variance and Tukey's post hoc multiple comparison tests. p values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. General state of mice

All animals survived until the end of the experiment (30 days). There were no overt changes in appearance and behaviour, as well as no obvious changes in body weight (Table 1). The mice move around freely during the first few hours of LPS injection, but at 6 h post-treatment of LPS, the animals started to assume a hunched posture and the lack of movement was obvious.

3.2. Testis and liver weights of mice at the end of study

At the end of study, LPS treatment of mice or their exposure to triazines did not affect liver weight ($p > 0.05$). Absolute testis weight was not affected by ATZ, but CYZ and SMZ exposures of mice were observed to decrease testis weight (Table 2). Exposures of the animals to triazines after LPS treatment (ATZ + LPS; CYZ + LPS; SMZ + LPS) could also decrease testis weight relative to the LPS effect alone ($p < 0.05$) (Table 2). There was a trend for the relative testis weight to decrease after ATZ treatment, but the change did not reach a statistical level of significance when compared to the control values ($p = 0.0511$) (Table 3). When the weight of the liver was expressed as organ weight index, it was decreased significantly when compared to the control values after exposure to the triazines ($p < 0.05$) (Table 3). Furthermore, exposure of mice to ATZ or SMZ after LPS treatment (ATZ + LPS; SMZ + LPS) was found to increase relative liver weight ($P < 0.05$), and those of CYZ + LPS did not reach significance statistical level ($p = 0.075$) when compared to the LPS values (Table 3).

3.3. TNF-alpha concentrations in the liver and testis of mice

As expected LPS challenged mice alone had higher levels of TNF-α both in the testes and liver compared to untreated control animals ($p < 0.05$). Furthermore, animals that were exposed to triazines (ATZ, CYZ and SMZ) alone had significantly decreased levels of TNF-α compared to the untreated control mice and the effect was most severe in the liver and testes of SMZ exposed mice and least in mice exposed to ATZ. However, treatment with LPS following triazine exposure was observed to decrease TNF-α concentration in both tissues relative to the LPS-treated mice ($p < 0.05$). Treatment with SMZ either alone or co-treated with LPS had higher inhibitory effect on TNF-α level in both the testes and liver (Fig. 1a and b).

3.4. IL-6 concentrations in the liver and testis of mice

The levels of IL-6 were increased in the liver and testes of LPS-treated mice and decreased in triazines (ATZ, CYZ and SMZ) exposed

Table 2
Absolute Testis and liver weights of treated mice at the end of study.

Tissue	Control	LPS	ATZ	ATZ + LPS	CYZ	CYZ + LPS	SMZ	SMZ + LPS
Testis	0.131 ± 0.014	0.110 ± 0.021	0.090 ± 0.041	0.062 ± 0.012*	0.080 ± 0.035**	0.057 ± 0.010*	0.071 ± 0.019**	0.054 ± 0.012*
Liver	0.771 ± 0.229[a]	0.714 ± 0.222[a]	0.569 ± 0.186[a]	0.788 ± 0.096[a]	0.552 ± 0.169[a]	0.78 ± 0.100[a]	0.528 ± 0.175[a]	0.738 ± 0.093[a]

Data are mean ± SD (N = 10); Triazines: ATZ = atrazine; SMZ = simazine; CYZ = cyanazine; *Versus LPS; **Versus control ($p < 0.05$); [a]same superscript on the same row are not significantly different ($p > 0.05$).

Table 3

Relative testis and liver weights of treated mice at the end of study.

Tissue	Control	LPS	ATZ	ATZ + LPS	CYZ	CYZ + LPS	SMZ	SMZ + LPS
Testis	0.585 ± 0.064	0.478 ± 0.091	0.351 ± 0.168	0.247 ± 0.049*	0.361 ± 0.156**	0.231 ± 0.042*	0.289 ± 0.076**	0.220 ± 0.049*
Liver	3.83 ± 0.550	3.496 ± 0.601	2.343 ± 0.768**	3.161 ± 0.386[†]	2.481 ± 0.764**	3.158 ± 0.405	2.155 ± 0.716**	2.986 ± 0.377[†]

Data are mean ± SD (N = 10); Triazines: ATZ = atrazine; SMZ = simazine; CYZ = cyanazine; *Versus LPS; **Versus control ($p < 0.05$); [†]Versus corresponding triazine value ($p < 0.05$).
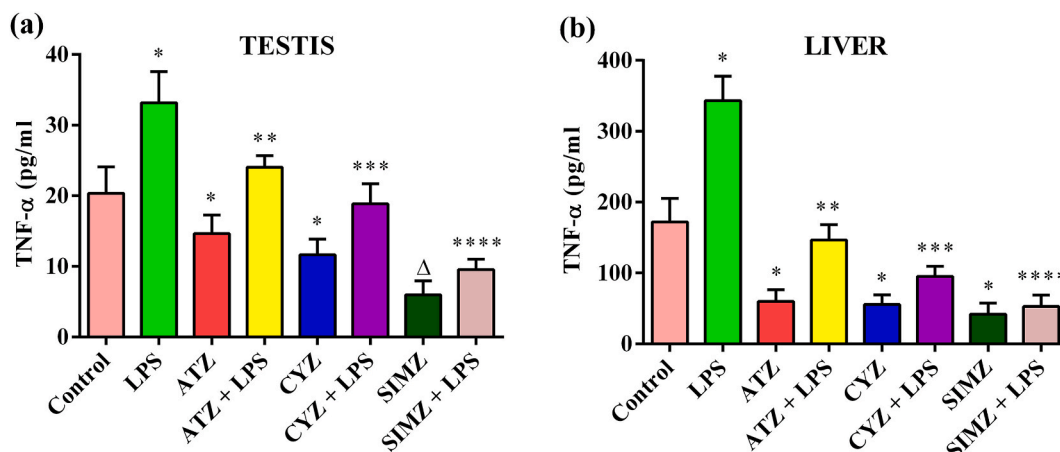

Fig. 1. Triazines modulate the secretion of tumor necrosis factor-alpha (TNF-alpha) in the testis (a) and liver (b) of LPS-stimulated mice. Data are mean ± standard deviation (N = 10). *Versus control, **Versus LPS, ***Versus ATZ + LPS, ****Versus CYZ + LPS, Δ Versus ATZ & CYZ (p < 0.05). ATZ = Atrazine; CYZ = Cyanazine; SMZ = Simazine; LPS = Lipopolysaccharide.

animals relative to the control values ($p < 0.05$). Treatment with LPS following triazine exposure was also observed to decrease IL-6 concentration in both tissues relative to the LPS alone treated mice ($p < 0.05$). Furthermore, SMZ exposure either alone or co-treated with LPS had higher inhibitory effect on IL-6 concentration in both the testes and liver (Fig. 2a and b).

3.5. Nitrite concentrations in the testes and liver of mice

Treatment of mice with LPS was observed to increase nitric oxide concentration in the testes of mice relative to control values ($p > 0.05$). However, LPS challenged mice following triazine exposure (ATZ, CYZ and SMZ) showed decreased levels of nitric oxide relative LPS values ($p < 0.05$). The inhibitory effect on testicular nitric oxide by the triazines was higher in SMZ + LPS relative to the ATZ + LPS
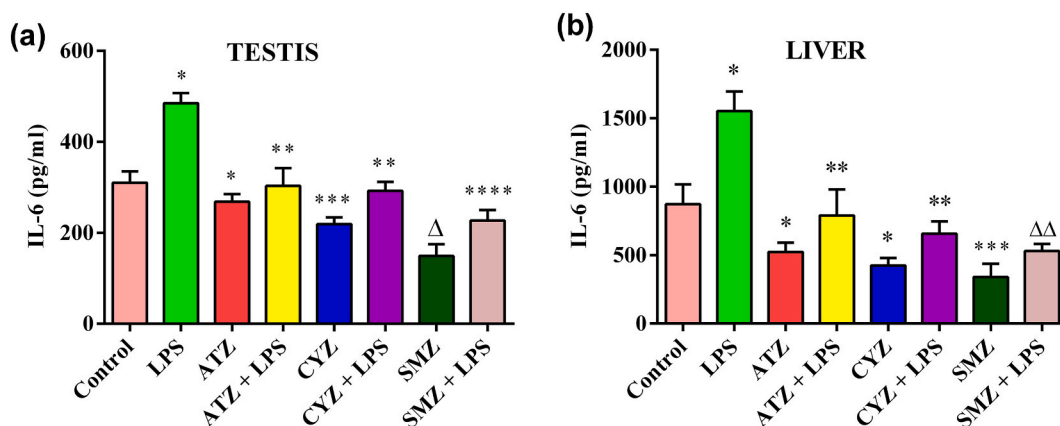

Fig. 2. Triazines modulate the secretion of interleukin-6 (IL-6) in the testis (a) and liver (b) of LPS-stimulated mice. Data are mean ± standard deviation (N = 10). *Versus Control, **Versus LPS, ***Versus ATZ, Δ Versus CYZ, ****Versus CYZ + LPS, ΔΔVersus ATZ + LPS (p < 0.05). ATZ = Atrazine; CYZ = Cyanazine; SMZ = Simazine; LPS = Lipopolysaccharide.

challenged mice which had the least effect. Furthermore, mice that were exposed to the triazines (ATZ, CYZ and SMZ) alone were also observed to have decreased level of nitric oxide relative to control values ($p < 0.05$), and ATZ had the least inhibitory effect whereas SMZ had the highest inhibitory effect on nitric oxide level. In the liver of these animals, LPS treatment was found to also significantly increase nitric oxide concentration ($p < 0.05$) whereas the triazines were found to decrease it significantly relative to control values ($p > 0.05$). Furthermore, SMZ treatment alone was found to have higher inhibitory effect on hepatic nitric oxide level than CYZ effect whereas ATZ had the least effect relative to the SMZ values ($p < 0.05$). Furthermore, the liver of LPS challenged mice following triazine exposure (ATZ, CYZ and SMZ) showed decreased concentrations of nitric oxide relative LPS values ($p < 0.05$). The nitric oxide level inhibitory effect of the triazines was also highest in the SMZ + LPS treated mice and lowest in the ATZ + LPS challenged mice (Fig. 3a and b).

3.6. Myeloperoxidase activities in the liver and testis of mice

Treatment of mice with LPS did not alter myeloperoxidase activity in the testes of mice relative to control values ($p > 0.05$). However, LPS challenged mice following triazine exposure (ATZ, CYZ and SMZ) showed decreased activity of myeloperoxidase activity relative LPS values ($p < 0.05$). The myeloperoxidase inhibitory effect of the triazines was higher in SMZ + LPS and CYZ + LPS relative to the ATZ + LPS challenged mice. Furthermore, mice that were exposed to the triazines (ATZ, CYZ and SMZ) alone for 30 days were also observed to have decreased activity of myeloperoxidase enzyme relative to control values ($p < 0.05$). In the liver of these animals, LPS treatment was found to significantly increase myeloperoxidase activity ($p < 0.05$) whereas the triazines were found not to change it significantly relative to control values ($p > 0.05$). Although, there was tendency for SMZ alone to decrease myeloperoxidase enzyme activity, the effect did not reach statistical significant level relative to control value ($p > 0.05$). Furthermore, the liver of LPS challenged mice following triazine exposure (ATZ, CYZ and SMZ) showed decreased activity of myeloperoxidase activity relative LPS values ($p < 0.05$). The myeloperoxidase inhibitory effect of the triazines was also highest in the SMZ + LPS treated mice and lowest in the ATZ + LPS challenged mice (Fig. 4a and b).

3.7. Histopathology of the testis and liver of mice

Histopathological evaluation of the testis with light microscopy showed that the control animal had well defined seminiferous tubules and germinal epithelium with germ cells at different levels of maturation. The interstitial space appeared normal with presence of interstitial cells including androgen secreting Leydig cell and inflammatory cells including neutrophils (Fig. 5). The basement membrane is intact and Sertoli cells could be seen there together with Leydig cells at the interstitial space. After treatment with LPS, germ cells were found to have defoliated in to the lumen of the seminiferous tubules, the epithelium were thin and has few giant cells which appear to be clumps of round spermatids. Some of the giant cells in the epithelium are dead cells and there are vascular spaces and vacuoles which are more accentuated towards the epithelium. The interstitial areas appear intact and contain interstitial cells including Leydig cells as well as the presence of inflammatory cells (Fig. 5).

The testes of mice exposed to triazines showed various pathological changes. For the ATZ-treated mice, dead cells were seen in the epithelium, the tubules appears degenerated and have desquamated germ cells mostly round spermatids in the lumen and few in-flammatory cells in the interstitial areas. However, LPS (0.5 mg/kg) challenge mice following ATZ exposure showed fewer germ cells in the tubules (ST) that appeared degenerated. Necrotic germ cells were seen in some areas along with few inflammatory cells in the interstitial areas near the basement membrane (Fig. 6). Many areas of the epithelium of the seminiferous tubules of tissues from SMZ-exposed mice are severely damaged and don't have germ cells and some of the tubules have no noticeable lumen. Desquamated germ
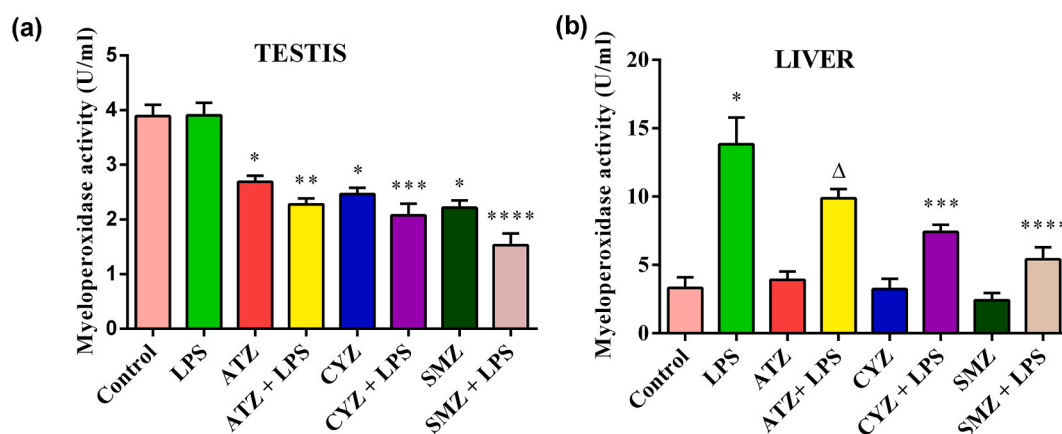

Fig. 3. Triazines modulate nitric oxide concentrations in the testis and liver of LPS-stimulated mice. Data are mean ± standard deviation (N = 10). *Versus Control, ᐃVersus LPS, **Versus ATZ, ᐃᐃVersus CYZ, ***Versus CYZ + LPS (p < 0.05). ATZ = Atrazine; CYZ = Cyanazine; SMZ = Simazine; LPS = Lipopolysaccharide.
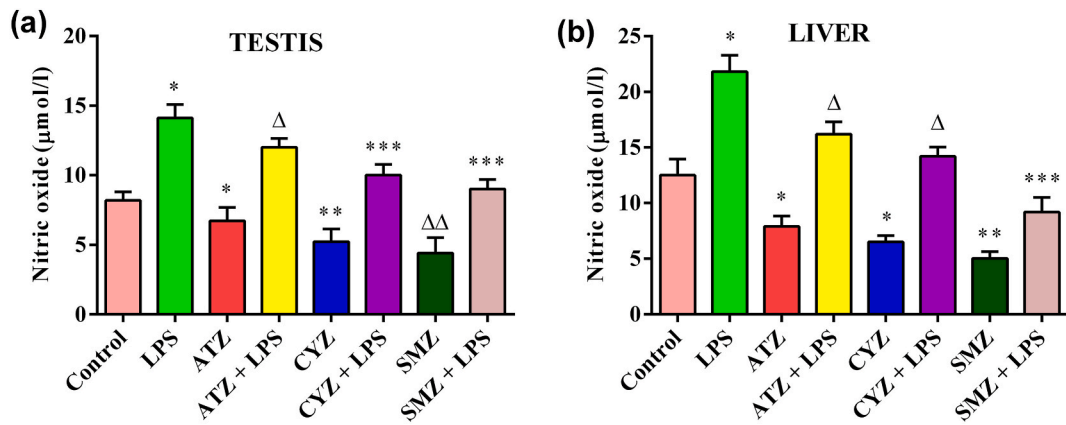
Fig. 4. Triazines modulate myeloperoxidase enzyme activity in the testis and liver of LPS-stimulated mice. Data are mean ± standard deviation (N = 10). *Versus Control, $^\Delta$Versus LPS, **Versus ATZ, ***Versus ATZ + LPS, ****Versus CYZ + LPS (p < 0.05). ATZ = Atrazine; CYZ = Cyanazine; SMZ = Simazine; LPS = Lipopolysaccharide.
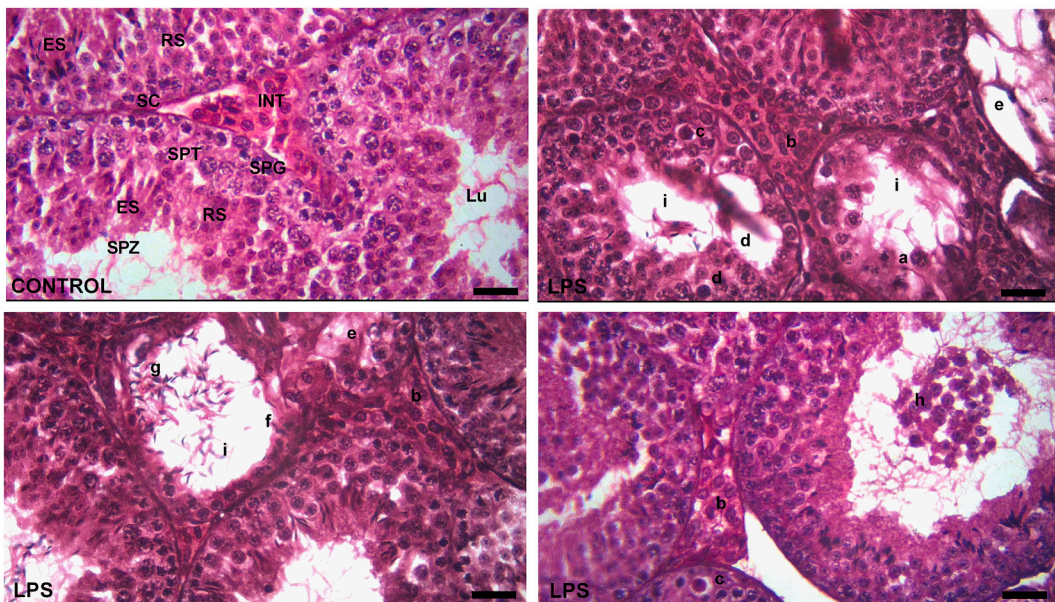

Fig. 5. Cross section of the seminiferous tubules of control and LPS-injected animals. As expected, the histological features of the control mice showed normal arrangement of germ cells at different stages of maturation in the intact germinal epithelium. The basement membrane is intact and Sertoli cells could be seen there together with Leydig cells at the interstitial space. The histological features of the seminiferous tubules of the lipopolysaccharide (LPS) challenge mice include clumps of giant cells (a), inflammatory cells in the interstitial areas (b), dead cells (c), large vacuolar spaces (d), degenerated tubules (e), thin epithelium (f), defoliated elongated spermatids (g), defoliated round spermatids (h), abnormal luminal space (i). SPG = Spermatogonia; RS = Round Spermatids; ES = Elongated Spermatids; SPZ = Tuffs of spermatozoa; SC = Sertoli Cells; Lu = Lumen; INT = Interstitial Space; SPT = Spermatocytes. LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).

cells in the lumen, clump of dead giant cells and cellular debris were also seen in the lumen and many areas of the epithelium. Some tubules were lined with single-layer of germ cells on the basement membrane especially with spermatogonia and Sertoli cells. There are scanty cellular contents in the interstitial areas including the androgen secreting Leydig cells and infiltrating neutrophils and macrophages (Fig. 7). The features in the seminiferous tubules of SMZ-treated mice testes following LPS challenge include degenerated epithelium, disorganized tubules and few interstitial cells including inflammatory cells in the interstitial areas (Fig. 7). After CYZ exposure the seminiferous tubular lumen were seen to be occluded with germ cells mostly of round spermatids and spermatocytes. Some apoptotic giant cells could be seen in some areas within the epithelium. Other features include missing germ cells in some tubules and few interstitial cells including Leydig cells and some inflammatory cells including neutrophils near the basement membrane in the interstitial areas. The features of seminiferous tubules of mice exposed to CYZ following LPS treatment includes few germ cells in the tubules, giant dead cells in the epithelium, many abnormal round spermatids and the presence of few interstitial cells in the interstitial
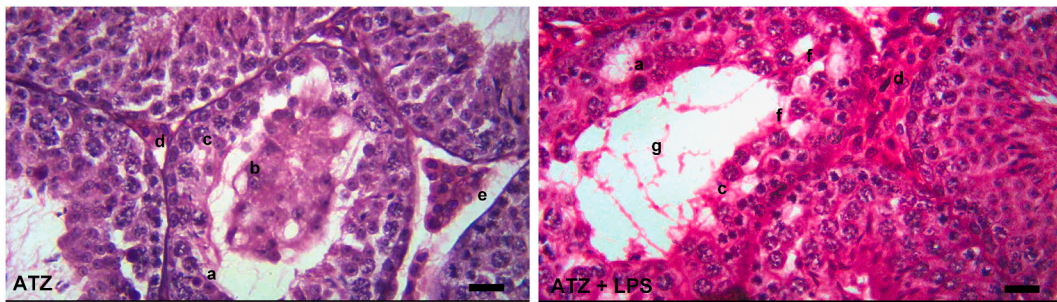
Fig. 6. Cross section of the seminiferous tubules of ATZ exposed mice and those co-treated with LPS showing: dead giant cells (a), degenerated tubules with defoliated germ cells in the lumen (b), thin epithelium with few germ cells (c), few inflammatory cells in the interstitial areas near basement membrane (d), interstitial space (e), small size vascular spaces (f), wide luminal space (g). ATZ = Atrazine; LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).
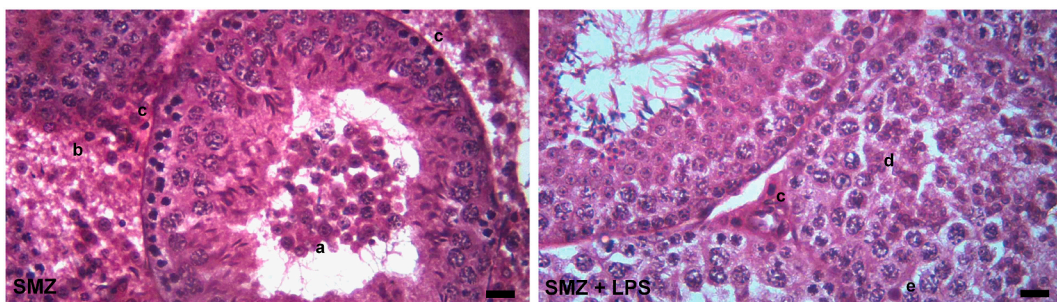

Fig. 7. Cross section of the seminiferous tubules of simazine-treated mice and those co-treated with LPS showing: desquamated epithelial germ cells in the lumen (a), atrophied tubules with few spermatogonia and Sertoli cells lining the basement membrane (b), interstitial areas with few infiltrating neutrophils and macrophages near the basement membrane (c), degenerated tubules (d), dead germ cells. SMZ = Simazine; LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).

areas (Fig. 8). Quantification of the histological features revealed that the numbers of aberrant tubules were increased in the triazines groups compared with the values after LPS treatment ($p < 0.05$), and were highest in the SIMZ exposed animals that were injected with LPS compared with the ATZ + LPS and CYZ + LPS groups. As expected, LPS injection significantly increased the numbers of inflammatory cells, whereas those mice that were exposed to triazines were found to have lower numbers of inflammatory cells in the interstitial areas compared with the control values ($p < 0.05$). Furthermore, the numbers of inflammatory cells were lowest in the interstitial areas of those animals exposed to SIMZ alone and highest in those exposed to ATZ alone ($p < 0.05$). The animals that were exposed to SIMZ and injected with LPS (SIMZ + LPS) were found to have lower population of inflammatory cells in the inter-tubular space compared with those in the ATZ + LPS and CYZ + LPS groups of animals (Fig. 9a and b).

As expected, histopathological examination of the liver with light microscopy showed that the control mice have normal hepatocytes with hepatic sinusoids separating the interconnecting cords of hepatocytes. Von Kupffer cells were also found inside the
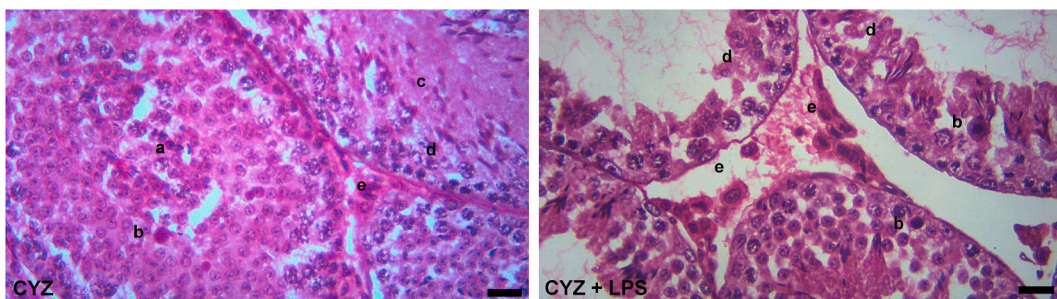

Fig. 8. Cross section of the seminiferous tubules of cyanazine-treated mice and those co-treated with LPS showing: lumen occluded with germ cells especially round spermatids and spermatocytes (a), dead cells (b), abnormal round spermatids in degenerated tubules (c), tubules with few germ cell layers (d), interstitial spaces with few cellular compositions including inflammatory infiltrating cells (e). CYZ = Cyanazine; LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).

sinusoids. After LPS treatment the liver showed many aggregate distributions of infiltrating macrophages surrounding the hepatocytes and central vein including kupffer cell hyperplasia. Many of these inflammatory cells were also found inside the hepatic sinusoids (Fig. 10). For the ATZ-treated mice, the hepatocytes are normal and few inflammatory cells could be seen in some dilated hepatic sinusoids and around the central vein. After LPS (0.5 mg/kg) treatment following ATZ exposure, the liver also showed normal hepatocytes and slightly congested central vein and a decrease aggregate of mass of inflammatory cells around the central vein compared to the LPS challenged mice (Fig. 11). The liver section of SMZ-exposed mice also showed normal hepatocytes and few inflammatory cells around the central vein whereas those co-treated with LPS have sparse mass of inflammatory cells around the central vein and surrounding the hepatocytes. Other features include hepatic sinusoidal dilatation and the sinusoids have few Kupffer cells inside (Fig. 12). Similarly, the hepatocytes of the liver of mice exposed to CYZ have normal hepatocytes and few isolated inflammatory cells around the central vein (Fig. 13). After LPS (0.5 mg/kg) treatment following CYZ exposure, the liver has sparse mass of accumulated inflammatory cells around the central vein and hepatic sinusoidal dilatations (Fig. 13).

Quantification of histopathologies in the liver revealed that the LPS treatment increased the foci of inflammatory cells compared with the control, but it was decreased by the triazines cotreatment when compared with the LPS values ($p < 0.05$). While ATZ or CYZ exposure significantly decreased the foci of inflammatory cells compared with the control, SIMZ exposure ($p < 0.05$) decreased these values when compared with the ATZ values ($p < 0.05$) (Fig. 14a). Additionally, von Kupffer cells hyperplasia was significantly increased by LPS administration compared with the control values, but it was decreased by the triazines cotreatment (Fig. 14b). The effects of triazines on von Kupffer cells hyperplasia were higher in the SIMZ + LPS and CYZ + LPS groups compared with the ATZ + LPS values ($p < 0.05$). Furthermore, exposure to ATZ, CYZ or SIMZ decreased von Kupffer cells hyperplasia compared with the control ($p < 0.05$). Hepatic sinusoidal dilations were not affected by LPS treatment compared with the control, but it was increased in the triazines group when compared with the control. It was also increased in the triazines groups following LPS administration when compared with the values after LPS treatment alone ($p < 0.05$). Furthermore, SIMZ exposure following LPS injection was found to significantly increase sinusoidal dilations when compared with the ATZ + LPS values (Fig. 14c). Vacuolization in hepatocytes were not detected after LPS injection when compared with the control, but it was increased by triazines co-treatment and when the animals were exposed to triazines alone (Fig. 14d). Cytoplasmic vacuolization in hepatocytes also differed significantly between the triazines cotreated groups, being higher in the CYZ + LPS and SIMZ + LPS compared with the ATZ + LPS values ($p < 0.05$).

4. Discussion

The data in the present study demonstrated that the testes of triazines-treated mice showed much lower levels of the cytokines: TNF-α and IL-6 compared to the values in the liver. As expected, LPS stimulated mice had higher levels of both cytokines in the liver and testes which were significantly decreased compared to the LPS values after the triazines exposure. The increase of TNF-α concentration in the liver following LPS injection was about 10-fold compared to the testis. It is possible that longer treatment time would yield higher increase in the cytokine concentration [38]. However, what is important to note was that the liver produced more cytokines than the testis within the time frame tested. This could account for the higher vulnerability of hepatic tissues to inflammatory stimuli than the testis. Among the triazines, SMZ had the most potent effect on the cytokines either when administered separately or concurrently with LPS, suggesting that the testis has diminished capacity compared to the liver for production of the pro-inflammatory cytokines [28].

In this study, ATZ, SMZ, and CYZ showed anti-inflammatory effects by suppressing LPS-induced increase in TNF-α and IL-6 levels,
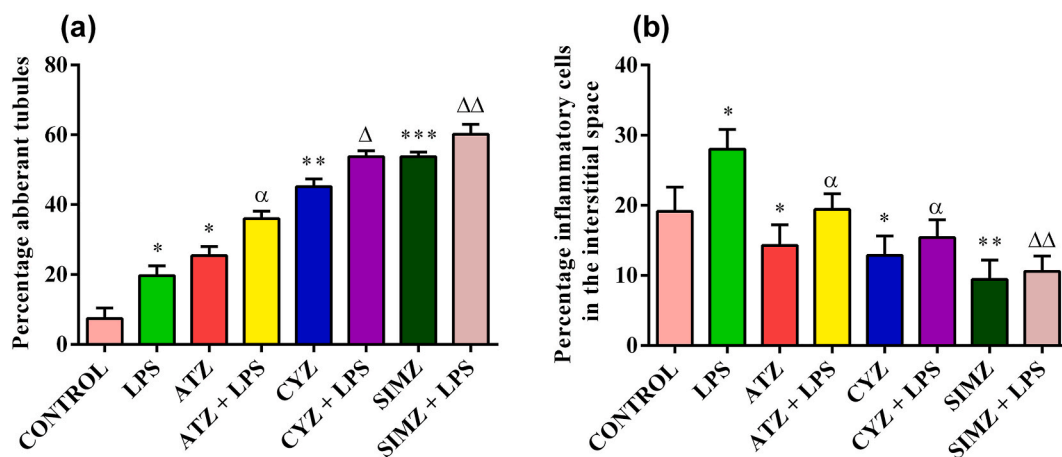

Fig. 9. Quantification of histological features detected in the testes of triazines and LPS co-treated mice. (a) Average numbers of aberrant tubules (b) mean numbers of inflammatory cells per inter-tubular space. Data are mean ± standard deviation (N = 10). *Versus Control, ᵅVersus LPS, **Versus ATZ, ᐃVersus ATZ + LPS, ᐃᐃVersus CYZ + LPS, ***Versus CYZ (P < 0.05; ANOVA and Tukey's *post hoc* multiple comparisons test); CYZ = Cyanazine; LPS = Lipopolysaccharide; SMZ = Simazine; ATZ = Atrazine; N = sample size in each group that was used for analysis; Aberrant tubules = total numbers of tubules with desquamated germ cells in the lumen, degenerated epithelium, and clump of giant cells in the epithelium.
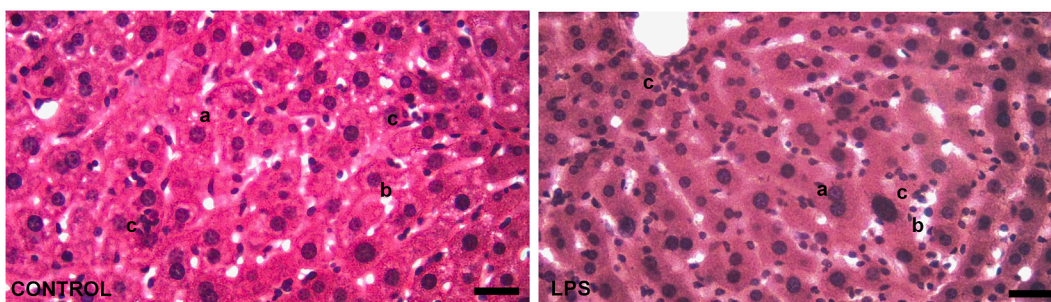
Fig. 10. Cross section of the liver of control and LPS-stimulated mice showing normal hepatic lobule consisting of hepatocytes (a) and sinusoids (b) separating the interconnecting cords of hepatocytes. Note that many aggregate distributions of inflammatory cells (c) surrounding the slightly congested central vein and increased prominent von Kupffer cells in the hepatic sinusoids were found in the LPS-challenged mice. LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).
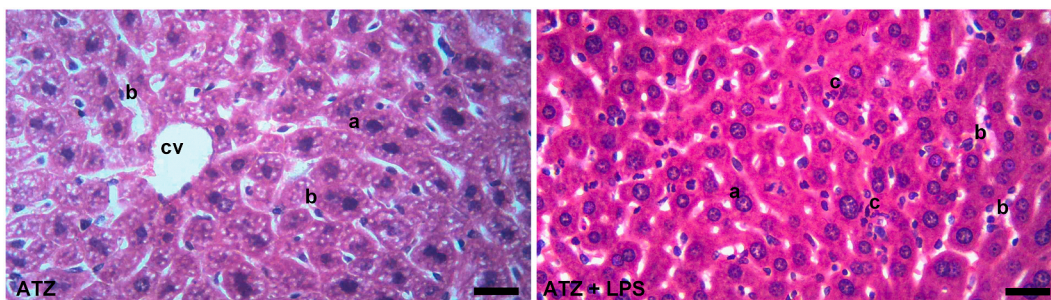

Fig. 11. Cross section of the liver of ATZ exposed mice and those co-treated with LPS showing normal hepatocytes (a), dilated sinusoids with few inflammatory cells in the endothelium (b) and sparse mass of inflammatory cells around the central vein (cv) in the ATZ-treated mice. Note the decrease aggregate of mass of inflammatory cells around hepatocytes (c) in the ATZ + LPS co-treated mice. ATZ = Atrazine; LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).
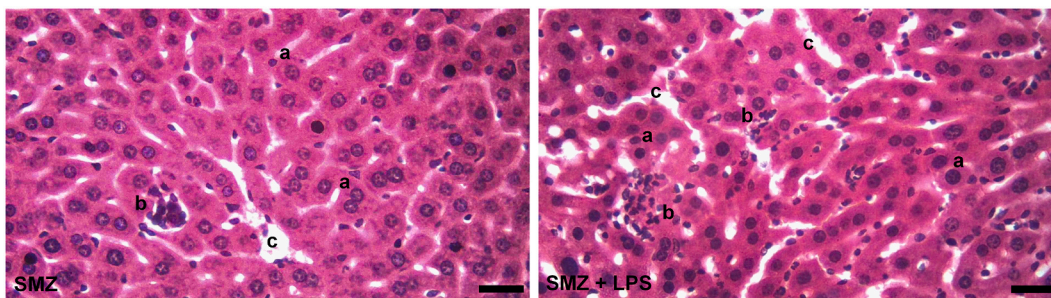

Fig. 12. Cross section of the liver of SMZ exposed mice and those co-treated with LPS showing normal hepatocytes (a), inflammatory cells around the central vein (b) and hepatic sinusoids (c). Note the few patches of inflammatory areas around the central vein and hepatic sinusoidal dilatation with few von Kpuffer cells in the endothelium of the sinusoids. SMZ = Simazine; LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).

hence inhibiting gonadal inflammation. It is expected for LPS to induce a variety of inflammatory responses by inducing the expressions of inflammation-related genes [39]. Hence, our present results on the increased concentrations of IL-6 and TNF-α are in agreement with what is reported in the literature on LPS and testicular inflammation [23,28,40]. Triazine chemicals have previously been reported to induce immunotoxic effects on the liver [41,42] and testis of rodents [43]. Because the levels of IL-6 and TNF-α are lower in the testis than the liver, even in the control animals without LPS stimulation, indicated that the testis is resistance to LPS-induced experimental inflammation, and supports the concept of the immune-privilege phenotype of the testis [44]. Previous studies have reported the inhibition of TNF-α by ATZ and SMZ in murine macrophage [16], human leukocytes [45] and in zebra fish [46], thereby supporting our findings. Furthermore, the decreased IL-6 levels in the liver and testis homogenates that was higher in the SMZ treated group, either in the presence or absence of LPS stimulation, also confirmed a more potent anti-inflammatory effect of SMZ compared to the other triazines.

The present study also showed that LPS stimulation failed to induce myeloperoxidase activity in the testis but not in the liver,
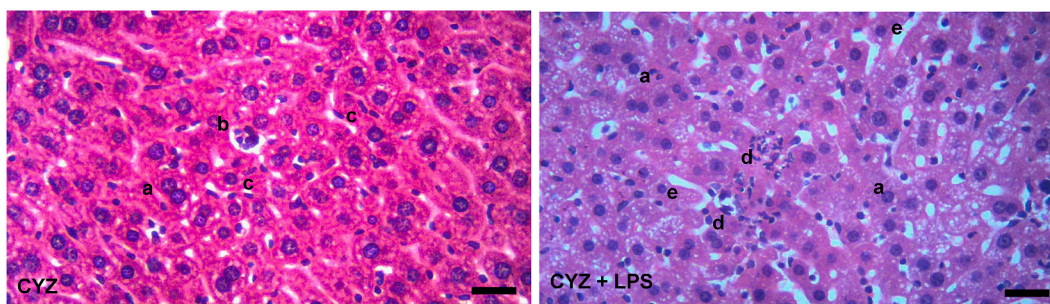
Fig. 13. Cross section of the liver of CYZ exposed mice and those co-treated with LPS (CYZ + LPS) showing normal hepatocytes (a). Note the few infiltrating cells in the central vein (b) and the sparse distribution of inflammatory cells (c) in the liver of CYZ-treated mice. Note the few patches of inflammatory cells (d) and dilated hepatic sinusoids containing very few Kpuffer cells (e). CYZ = Cyanazine; LPS = Lipopolysaccharide. (Mag. 400 × ., Scale bar = 50 μm).
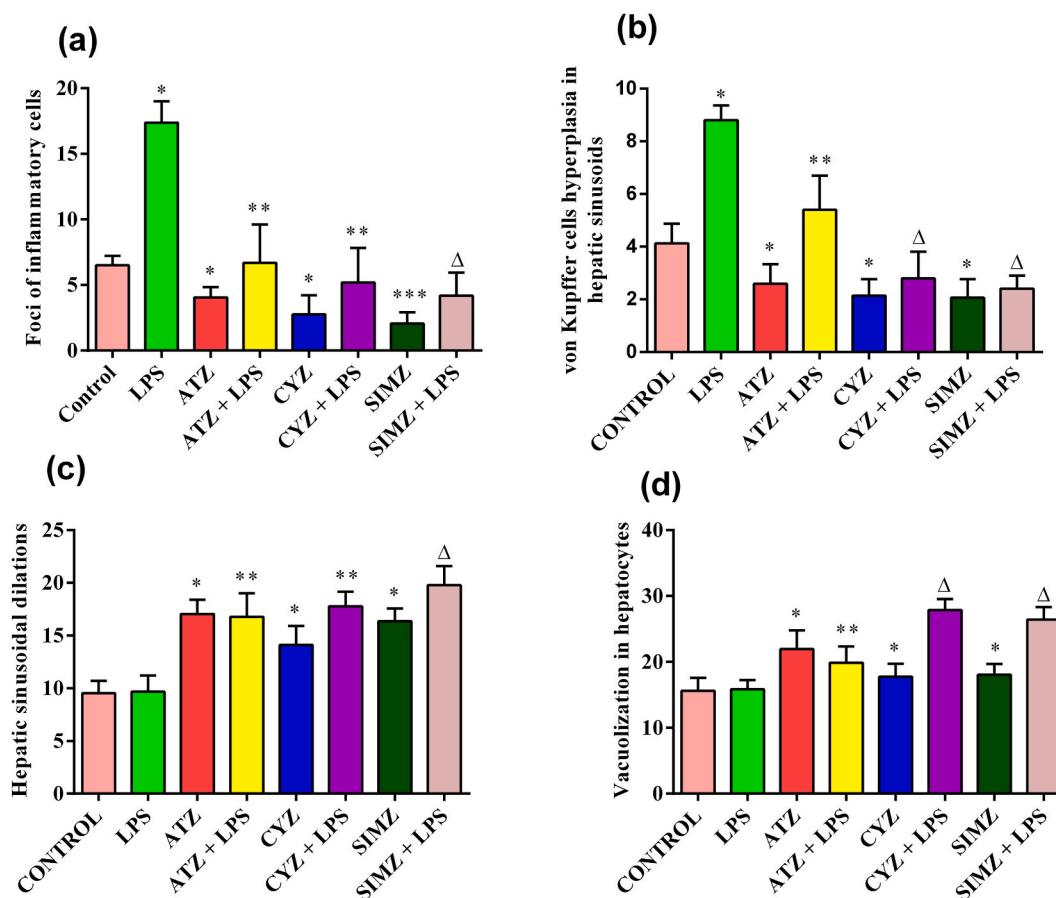

Fig. 14. Quantification of the histological features detected in the liver of triazines and LPS co-treated mice. (a) Foci of inflammatory cells; (b) von Kupffer cells hyperplasia; (c) dilated hepatic sinusoids; (d) vacuolization in hepatocytes; Data are mean ± standard deviation (N = 10). *Versus Control, **Versus LPS, ***Versus ATZ, $^\Delta$Versus ATZ + LPS, $^\alpha$Versus CYZ + LPS ($P < 0.05$; ANOVA and Tukey's *post hoc* multiple comparisons test); CYZ = Cyanazine; LPS = Lipopolysaccharide; SMZ = Simazine; ATZ = Atrazine; N = sample size in each group that was used for analysis.

further confirming the resistance of the testis to LPS-induced inflammation [28]. The triazines treatments separately or during concurrent administration with LPS were found to decrease myeloperoxidase activity in the liver and testis and with SMZ being more potent than the other triazines. Myeloperoxidase is usually released from activated immune system, probably from the azurophilic granules of neutrophils after LPS injection, tissue damage, and inflammation. Thus, the high activity of myeloperoxidase in the liver of LPS stimulated animals is an inflammatory response to hepatic toxicity [18], whereas its low activity in the testis and liver of triazines cotreated LPS mice is associated with the immunosuppressive effects of triazines [18,47,48].

Nitric oxide plays important bio-regulatory roles in several physiological processes, e.g. testicular inflammation, and its decrease by triazines and on co-treatment with LPS in this study indicates the anti-inflammatory effects of the triazines [22,35,49], and with SMZ being more potent than the other triazines. Several studies have established that the testis is immunoprotective because some of its components e.g. Sertoli cells have immunosuppressive properties [28,44,50]. This is thought to be the reason why the levels of pro-inflammatory cytokines and nitric oxide and myeloperoxidase activity were lower in the testis than the liver in the present study. Thus, the detectable basal nitric oxide is suggestive of its role in the normal function of both tissues, for instance, regulatory processes during spermatogenesis and normal testosterone production, besides having inflammatory-regulatory effects on the testis [22], anti-inflammatory and antithrombotic effects in the hepatic system [51]. The inhibitory effect of SMZ on nitric oxide release in mice is known to alter the normal development and reproductive activity of mice [8], indicating that the anti-inflammatory effects of triazines are associated with their anti-fertility effects. Since nitric oxide forms part of the innate immune system, the decrease in their testicular levels by triazines are expected to drive male infertility problems in men with impaired testicular functions due to inflammatory disorders [52–54].

As expected, the testes of control animals showed well defined seminiferous tubules with germ cells at different developmental levels of maturation, and intact interstitial areas with normal population of interstitial cells. After LPS stimulation, immature germ cells were found in the lumen of the seminiferous tubules, the epithelium appeared thin, and with few clumps of giant round spermatids. Furthermore, the vacuoles in the tubules were more accentuated towards the epithelium, and the interstitial areas, although intact, showed the presence of inflammatory cells, including neutrophils and macrophages. Other studies have also reported that systemic inflammation induced by LPS compromises spermatogenesis and caused testicular damage [22,23,34,39]. Our findings that LPS induces degenerative changes in the seminiferous tubules are thus consistent with most of these studies, and confirm that tissue damage is a more sensitive marker than fluctuations in nitric oxide concentrations [38]. The fact that the local levels of TNF-α and IL-6 were increased in the testes of the LPS-stimulated animals in this study confirms that the LPS-induced testicular toxicity was mediated by pro-inflammatory cytokines [39]. The testes of mice exposed to ATZ also appeared degenerated and have mostly desquamated round spermatids in the lumen, and few inflammatory cells in the interstitial areas as reported previously in many other studies [30,31, 54,55]. Similarly, the major histological features including fewer inflammatory cells, scanty cellular contents in the interstitial areas, including the androgen secreting Leydig cells and infiltrating neutrophils and macrophages, damaged tubules and degenerated epithelium in the testes of CYZ and SMZ exposed mice and on co-treatment with LPS, support the testicular toxicity of triazines [5,8, 30]. The current findings are in agreement with the hypothesis that triazines have anti-inflammatory effects in mice that could have contributed to the observed testicular injury.

The major changes observed in the liver after LPS treatment were many aggregates of infiltrating macrophages surrounding the hepatocytes and central vein including kupffer cells hyperplasia. Many of these inflammatory cells were also found inside the hepatic sinusoids, confirming the pro-inflammatory effects of LPS [56]. For the triazines treated mice or those co-treated with LPS, a sparse mass inflammatory cells could be seen in some dilated hepatic sinusoids and around the central vein, suggesting that the anti-inflammatory effects of triazines were responsible for the decreased aggregate mass of inflammatory cells around the central vein. The fact that ATZ decreases the level of the anti-inflammatory cytokine, IL-4 in mice splenic cells and induces myelosuppressive effects in the hepatoma cell lines and liver [11,57] further support the immunosuppressive and anti-inflammatory effects of triazines in mammalian model systems [2,8]. The present findings also support the fact that LPS causes tissue injury by mechanisms involving chemical mediators such as nitric oxide, TNF-α and other cytokines [58,59]. It is also important to note that inflammatory cells e.g. macrophages in the testes are deficient in production of cytokines with relatively normal inflammatory functions unlike those of the liver and other tissues such as the kidney, lung, and skin that possesses normal inflammatory activity, and can react to LPS with a strong pro-inflammatory response [28,60]. Although there are wide structural and functional variations between the testis and liver, the use of the liver in comparison to the testis in this study is justifiable based on the fact that both organs have a large resident macrophage population [28] and the same volume densities of inflammatory cells [61,62].

It is believed that the anti-inflammatory effects of ATZ is due to its interference on dopamine signalling leading to the blunting of their immunomodulatory effects and consequently, the inactivation of the immune system [63,64]. One major drawback of this study is that it did not measure dopamine-responsive pathways in the testis and hepatic tissues, and because triazines act as estrogen-mimetics to decrease dopamine level that may block the feedback regulation that controls immune responses [64], the findings of the present study do not account for the direct involvement of endocrine effects.

5. Conclusions

The present study evaluated the anti-inflammatory effects of the triazine chemicals and their ability to inhibit LPS-induced pro-inflammatory responses in the liver and testis of adult mice. We found that triazines decreased the concentrations of pro-inflammatory cytokines, nitric oxide level, and myeloperoxidase activity in both the liver and testis, and also inhibited LPS-induced increase in the levels of these inflammatory markers, except for myeloperoxidase activity which remained unchanged in the testis after LPS injection but were decreased on co-treatment with the triazines. Because the changes in the inflammatory variables where to a greater extent in the liver than the testis confirm that the liver is more vulnerable to inflammatory toxicity than the testis, and exerts a stronger pro-inflammatory response. These differential responses play a role in the susceptibility to, and progression of infections in the liver and testis. However, SMZ demonstrated higher anti-inflammatory effect than the other triazines (ATZ, CYZ) in both tissues, since it showed higher inhibitory effects on TNF-α and IL-6 levels, and on testis weight. This raises the concern that triazine chemicals may influence the development of infectious-and inflammatory-related diseases including inflammation-mediated male infertility and hepatic inflammation.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Sunny O. Abarikwu: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chidimma J. Mgbudom-Okah:** Writing – original draft, Investigation. **Lauritta C. Ndufeiya-Kumasi:** Writing – original draft, Investigation. **Vivian E. Monye:** Investigation. **Oke Aruoren:** Investigation. **Ogechukwu E. Ezim:** Supervision. **Stephen I. Omeodu:** Supervision. **Iniobong A. Charles:** Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] M. Kumar, D.K. Sarma, S. Shubham, M. Kumawat, V. Verma, A. Prakash, et al., Environmental endocrine-disrupting chemical exposure: role in non-communicable diseases, Front. Public Health 8 (2020) 553850.

[2] T. Ohnishi, T. Yoshida, A. Igarashi, M. Muroi, K.-I. Tanamoto, Effects of possible endocrine disruptors on MyD88-independent TLR4 signalling, FEMS Immunol. Med. Microbiol. 52 (2008) 293–295.

[3] B. Winans, M.C. Humble, B.P. Lawrence, Environmental toxicants and the developing immune system: a missing link in the global battle against infectious disease? Reprod. Toxicol. 31 (2011) 327–336.

[4] C.-H. Kuo, S.-N. Yang, P.-L. Kuo, C.-H. Hung, Immunomodulatory effect of environmental disrupting chemicals, Kaohsiung J. Med. Sci. 28 (2012) 537–542.

[5] S.O. Abarikwu, O.E. Ezim, C.N. Ikeji, E.O. Farombi, Atrazine: cytotoxicity, oxidative stress, apoptosis, testicular effects and chemopreventive Interventions, Front. Toxicol. 5 (2023) 1246708.

[6] K.R. Solomon, D.B. Baker, P. Richards, K.R. Dixon, S.J. Klaine, T.W. La Point, et al., Ecological risk assessment of atrazine in North American surface waters, Environ. Toxicol. Chem. 15 (1996) 31–76.

[7] S.O. Abarikwu, A.C. Adesiyan, T.O. Oyeloja, M.O. Oyeyemi, E.O. Farombi, Changes in sperm characteristics and induction of oxidative stress in the testis and epididymis of experimental rats by a herbicide, atrazine, Arch. Environ. Contam. Toxicol. 58 (2010) 874–882.

[8] H.-O. Park, J. Bae, Disturbed relaxin signaling pathway and testicular dysfunction in mouse offspring upon maternal exposure to simazine, PLoS One 7 (2012) e44856.

[9] P.A. Thompson, M. Khatami, C.J. Baglole, J. Sun, S.A. Harris, E.Y. Moon, et al., Environmental immune disruptors, inflammation and cancer risk, Carcinogenesis 36 (2015) S232–S253.

[10] N.A. Karrow, J.A. McCay, R.D. Brown, D.L. Musgrove, T.L. Guo, D.R. Germolee, Oral exposure to atrazine modulates cell-mediated immune function and decreases host resistance to the B16F10 tumor model in B6C3F1 mice, Toxicology 209 (2005) 15–28.

[11] S. Zhao, J. Liu, F. Zhao, F. Wu, N. Li, Q. Suo, et al., Sub-acute exposure to the herbicide atrazine suppresses cell immune functions in adolescent mice, BioSci. Trends. 7 (2013) 193–201.

[12] J.P. Stanko, R.R. Enoch, J.L. Rayner, C. C Davis, D.C. Wolf, D.E. Malarkey, et al., Effects of prenatal exposure to a low dose atrazine metabolite mixture on pubertal timing and prostate development of male Long-Evans rats, Reprod. Toxicol. 30 (2010) 540–549.

[13] D. Chen, Z. Zhang, H. Yao, Y. Cao, H. Xing, S. Xu, Pro- and anti-inflammatory cytokine expression in immune organs of the common carp exposed to atrazine and chlorpyrifos, Pestic. Biochem. Physiol. 114 (2014) 8–15.

[14] K.R. Kim, E.W. Son, D. K Rhee, S. Pyo, The immunomodu- latory effects of the herbicide simazine on murine macrophage functions in vitro, Toxicol. Vitro 16 (2002) 517–523.

[15] R. Ren, D.J. Sun, H. Yan, Y.P. Wu, Y. Zhang, Oral exposure to the herbicide simazine induces mouse spleen immunotoxicity and immune cell apoptosis, Toxicol. Pathol. 41 (2013) 63–72.

[16] K.R. Kim, E.W. Son, S. Hee-Um, B.O. Kim, D.K. Rhee, S. Pyo, Immune alterations in mice exposed to the herbicide simazine, J. Toxicol. Environ. Health 66 (2003) 1159–1173.

[17] C.P. Florian, S.R. Mansfield, J.R. Schroeder, Differences in GPR30 regulation by chlorotriazine herbicides in human breast cells, Biochem. Res. Int. 2016 (2016) 2984081.

[18] S.O. Abarikwu, A.L. Oleribe, C.J. Mgbudom-Okah, C.L. Onuah, C.S. Chikwendu, E.N. Onyeike, The protective effect of fluted pumpkin seeds against atrazine-induced testicular injury, Drug Chem. Toxicol. 45 (2022) 799–809.

[19] S.A. Condotta, M.J. Richer, The immune battlefield: the impact of inflammatory cytokines on CD8+ T-cell immunity, PLoS Pathog. 13 (2017) e1006618.

[20] G.R. Starbeck-Miller, H.H. Xue, J.T. Harty, IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo, J. Exp. Med. 211 (2014) 105–120.

[21] M.J. Richer, L.L. Pewe, L.S. Hancox, S.M. Hartwig, S.M. Varga, J.T. Harty, Inflammatory IL-15 is required for optimal memory T cell responses, J. Clin. Invest. 125 (2015) 3477–3490.
[22] M.K. O'Bryan, S. Schlatt, O. Gerdprasert, D.J. Phillips, D.M. de Kretser, M.P. Hedger, Inducible nitric oxide synthase in the rat testis: evidence for potential roles in both normal function and inflammation-mediated infertility, Biol. Reprod. 63 (2000) 1285–1293.
[23] M.M. Reddy, S.V. Mahipal, J. Subhashini, M.C. Reddy, K.R. Roy, G.V. Reddy, et al., Bacterial lipopolysacharide-induced oxidative stress in the impairement of steroidogenesis and spermatogenesis in rats, Reprod. Toxicol. 22 (2006) 493–500.
[24] C.Y. Lin, W.H. Wang, S.H. Chen, Y.W. Chang, L.C. Hung, C.Y. Chen, et al., Lipopolysaccharide-Induced nitric oxide, prostaglandin e2, and cytokine production of mouse and human macrophages are suppressed by pheophytin-b, Int. J. Mol. Sci. 18 (2017) 2637.
[25] F. Rao, H. Tian, W. Li, H. Hung, F. Sun, Potential role of punicalagin against oxidative stress induced testicular damage, Asian J. Androl. 18 (2016) 627–632.
[26] A. Morikawa, Y. Kato, T. Sugiyama, N. Koide, D. Chakravortly, T. Yoshida, et al., Role of nitric oxide in LPS-induced hepatic injury in D-galactosamine-sensitised mice as an experimental endotoxic shock model, Infect. Immun. 67 (1999) 1018–1024.
[27] M.W. Robinson, C. Harmon, C. O'Farrelly, Liver immunology and its role in inflammation and homeostasis, Cell. Mol. Immunol. 13 (2016) 267–276.
[28] M.K. O'Bryan, O. Gerdprasert, D.J. Nikolic-Paterson, A. Meinhardt, J.A. Muir, L.M. Foulds, et al., Cytokine profiles in the testes of rats treated with lipopolysaccharide reveal localized suppression of inflammatory responses, Am. J. Physiol. Regul. Integr. Comp. Physiol. 288 (2005) R1744–R1755.
[29] PHS (Public Health Service), Public Health Service Policy on Humane Care and Use of Laboratory Animals. US Department of Health and Human Services, Health Research Extension Act, Washington, DC, 1996 [PL 99-158.
[30] S.O. Abarikwu, G.M.J. Costa, N. de Lima E Martins Lara, S.M.S.N. Lacerda, L.R. de França, Atrazine impairs testicular function in BalB/c mice by affecting Leydig cells, Toxicology 455 (2021) 152761.
[31] A.B. Victor-Costa, S.M. Bandeira, A.G. Oliveira, G.A. Mahecha, C.A. Oliveira, Changes in testicular morphology and steroidogenesis in adult rats exposed to atrazine, Reprod. Toxicol. 29 (2010) 323–331.
[32] T. Roh, P.S. K Knappett, D. Han, G. Ludewig, K.M. Kelly, K. Wang, et al., Characterization of arsenic and atrazine contaminations in drinking water in Iowa: a public health concern, Int. J. Environ. Res. Publ. Health 20 (2023) 5397.
[33] K. Koga, M. Ogata, I. Takenaka, T. Matsumoto, A. Shigematsu, Ketamine suppresses tumor necrosis fator-alpha activity and mortality in carrageenan-sensitized endotoxin shock model, Circ. Shock 44 (1994) 160–168.
[34] L. Li, P. Ma, Y. Liu, C.O.W.S. Huang, F. Tang, J.V. Zhang, Intermedin attenuates LPS-induced inflammation in the rat testis, PLoS One 8 (2013) e65278.
[35] P.C. Andrews, N.I. Krinsky, Quantitative determination of myeloperoxidase using tetramethylbenzidine as substrate, Anal. Biochem. 127 (1982) 346–350.
[36] C.Y. Ezirim, S.O. Abarikwu, A.A. Uwakwe, C. Mgbudom-Okah, Protective effects of *Anthocleista djalonensis* A. Chev root extracts against induced testicular inflammation and impaired spermatogenesis in adult rats, Mol. Biol. Rep. 46 (2019) 5983–5994.
[37] O.E. Ezim, S.O. Abarikwu, Fluted pumpkin seeds protect the spermatogenesis score index and testicular histology of caffeine treated rats, Andrologia 54 (2022) e14578.
[38] A.A. Halawa, M.A. El-Adl, M.F. Hamed, A.Z. Balboula, M.A. Elmetwally, Lipopolysaccharide prompts oxidative stress and apoptosis in rats' testicular tissue, J. Vet. Healthcare. 1 (2018) 20–31.
[39] F. Wang, W. Liu, Q. Jiang, M. Gong, R. Chen, H. Wu, et al., Lipopolysaccharide-induced testicular dysfunction and epididymitis in mice: a critical role of tumor necrosis factor alpha, Biol. Reprod. 100 (2019) 849–861.
[40] M.R. Metukuri, C.M. Reddy, P.R. Reddy, P. Reddanna, Bacterial LPS-mediated acute inflammation-induced spermatogenic failure in rats: role of stress response proteins and mitochondrial dysfunction, Inflammation 33 (2010) 235–243.
[41] N.M. Filipov, L.M. Pinchuk, B.L. Boyd, P.L. Crittenden, Immunotoxic effects of short-term atrazine exposure in young male C57BL/6 mice, Toxicol. Sci. 86 (2005) 324–332.
[42] I. Holásková, M. Elliott, K. Brundage, E. Lukomska, R. Schafer, J.B. Barnett, Long-term immunotoxic effects of oral prenatal and neonatal atrazine exposure, Toxicol. Sci. 168 (2019) 497–507.
[43] A.A. Rooney, R.A. Matulka, R.W. Luebke, Developmental atrazine exposure suppresses immune function in male, but not female Sprague-Dawley rats, Toxicol. Sci. 76 (2003) 366–375.
[44] A. Meinhardt, M.P. Hedger, Immunological, paracrine and endocrine aspects of testicular immune privilege, Mol. Cell. Endocrinol. 335 (2011) 60–68.
[45] R.J. Hooghe, S. Devos, E.L. Hooghe-Peters, Effects of selected herbicides on cytokine production in vitro, Life Sci. 66 (2000) 2519–2525.
[46] Z. Liu, Z. Fu, Y. Jin, Immunotoxic effects of atrazine and its main metabolites at environmental relevant concentrations on larval zebrafish (Danio rerio), Chemosphere 166 (2017) 212–220, 2017.
[47] B. Descampiaux, V. Herno, A. Brice, F. Erb, Application of the kinetic of RNA synthesis on Hep 38 cells. Study of the medium-term cytotoxicity of atrazine, Toxicol. Lett. 74 (1994) 121–128.
[48] S. Soltanian, Effect of atrazine on immunocompetence of red-eared slider turtle (Trachemys scripta), J. Immunot. 13 (2016) 804–809.
[49] Y. Luo, Y. Zhu, W. Basang, X. Wang, C. Li, X. Zhou, Roles of nitric oxide in the regulation of reproduction: a review, Front. Endocrinol. 12 (2021) 752410.
[50] P. Mital, G. Kaur, J.M. Dufour, Immunoprotective Sertoli cells: making allogenic and xenogeneic transplantation feasible, Reproduction 139 (2010) 495–504.
[51] M.G. Clemens, Nitric oxide in liver injury, Hepatology 30 (1999) 1–5.
[52] W.R. Winnal, J.A. Muir, S. Liew, J.J. Hirst, S.J. Meachem, M.P. Hedger, Effects of chronic celecoxib on testicular function in normal and lipopolysaccharide-treated rats, Int. J. Androl. 32 (2009) 542–555.
[53] C. Bogdan, Nitric oxide synthase in innate and adaptive immunity. An update, Trends Immunol. 36 (2015) 161–178.
[54] L.C. Ndufeiya-Kumasi, S.O. Abarikwu, R. Ohanador, E.S. Omoregie, Curcumin improves the protective effects of quercetin against atrazine-induced testicular injury in adult Wistar rats, Andrologia 54 (2022) e14445.
[55] E. Martins-Santos, C.G. Pimenta, P.R.N. Campos, M.B. Franco, D.A. Gomes, G.A.B. Mahecha, et al., Persistent testicular structural and functional alterations after exposure of adult rats to atrazine, Reprod. Toxicol. 73 (2017) 201–213.
[56] K. Hamesch, E. Borkham-Kamphorst, P. Strnad, R. Weiskirchen, Lipopolysaccharide-induced inflammatory liver injury in mice, Lab. Anim. 49 (2015) 37–46.
[57] B. Sawicki, G. Durand, J.D. Dewitte, D. Ratanasavanh, C. Richi, M.C. Leglise, Myelosuppressive activity of two herbicides, atrazine and dinoterb, on human haematopoietic progenitor cells: an in vitro assay to evaluate the effects of intermediate or long-term exposure, Toxicol. Vitro 12 (1998) 183–190.
[58] Y. Lu, X. Wang, A.I. Cederbaum, Lipopolysaccharide-induced liver injury in rats treated with the CYP2E1 inducer pyrazole, Am. J. Physiol. Gastrointest. Liver Physiol. 289 (2005) G308–G319.
[59] J. Zhong, I.V. Deaciuc, R. Burikhanov, W.J.S. de Villiers, Lipopolysaccharide-induced liver apoptosis is increased in interleukin-10 knockout mice, Biochim. Biophys. Acta 1762 (2006) 468–477.
[60] M.I. Luster, D.R. Germolec, T. Yoshida, F. Kayama, M. Thompson, Endotoxin-induced cytokine gene expression and excretion in the liver, Hepatology 19 (1994) 480–488.
[61] L. Bouwens, M. Baekeland, R. De Zanger, E. Wisse, Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver, Hepatology 6 (1986) 718–722.
[62] J. Wang, N.G. Wreford, H.Y. Lan, R. Atkins, M.P. Hedger, Leukocyte populations of the adult rat testis following removal of the Leydig cells by treatment with ethane dimethane sulfonate and subcutaneous testosterone implants, Biol. Reprod. 51 (1994) 551–561.
[63] S. Thomas Broome, K. Louangaphay, K.A. Keay, G.M. Leggio, G. Musumeci, A. Castorina, Dopamine: an immune transmitter, Neural. Regen. Res. 15 (2020) 2173–2185.
[64] V. Galbiati, E. Buoso, R. d'Emmanuele di Villa Bianca, R.D. Paola, F. Morroni, G. Nocentini, et al., Immune and nervous systems interaction in endocrine disruptors toxicity: the case of atrazine, Front. Toxicol. 3 (2021) 649024.