Evaluation of toxic effects induced by repeated exposure to Cylindrospermopsin in rats using a 28-day feeding study.

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Abstract

Cylindrospermopsin (CYN) is a toxin with a world-wide increasing occurrence. It can induce toxic effects both in humans and the environment, and toxicity studies are needed to complete its toxicological profile. In this sense, *in vivo* oral toxicity studies with pure CYN are scarce. The aim of this work was to perform a repeated dose 28-day oral study in rats following the OECD guideline 407 to provide information on health hazard likely to arise from this kind of exposure. Male and female Sprague-Dawley rats were dosed with 18.75, 37.5 and 75 µg CYN/kg b.w./day. After the study period, no clinical signs or mortality and no significant differences in final body weight, body weight gain and total feed intake in both sexes were observed. Only in females some biochemical parameters (triglycerides (TRIG) levels and aspartate aminotransferase (AST) activity) as well as changes in the weight of organs (absolute liver weight values, relative kidney/body weight ratios or relative liver weight/brain weight ratios) were altered, but without toxicological relevance. Histopathological analysis revealed a very mild affectation of liver and kidney in rats. These results suggest the need to perform longer oral toxicity studies to define the potential consequences of long term CYN exposure.

Keywords: Cylindrospermopsin, cyanotoxins, 28-Day exposure, rats, oral toxicity, histopathology

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1. Introduction

Cylindrospermopsin (CYN) is at present one of the most important cyanotoxins worldwide as a consequence of its broad distribution. This cyanobacterial toxin can be produced by a variety of cyanobacterial species being its main producers *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* (Kokocinski et al., 2017). CYN is a stable tricyclic alkaloid with a guanidine group connected to a hydroxymethyluracil by a hydroxyl bridge (Ohtani et al., 1992). Due to its zwitterionic character CYN is a highly water soluble compound (Chiswell et al., 1999). It has been described that the dissolved concentrations of CYN in the water are usually higher than the intracellular concentrations, which can constitute even more than 99% of total CYN available, due to up to 90% of total CYN is identified outside the cells (Rücker et al., 2007). For this reason, the hazard and intensity of the effects to both human health and the environment after CYN exposure could be greater than for others cyanotoxins. Actually, it was associated to human illnesses developed after ingestion of conventionally-treated municipal drinking water (De la Cruz et al., 2013).

Regarding to humans, they can be exposed to CYN by different routes such as dermal contact during showering, bathing or recreational activities (Pichardo et al., 2017). However, the main route of human exposure is by the ingestion of drinking water or the intake of cyanotoxins contaminated food such as fish, mollusks, vegetables and crops (Gutierrez-Praena et al., 2013; Testai et al., 2016; Machado et al., 2017; Buratti et al., 2017). The primarily target organ of CYN activity is the liver (Hawkins et al., 1985; Bernard et al., 2003; Saker et al., 2003). Moreover, other organs such as kidneys, lungs, thymus, heart, stomach, and intestine, can also be affected (Hawkins et al., 1997; Falconer et al., 1999; Seawright et al., 1999; Bernard et al., 2003; Humpage and Falconer, 2003; Falconer and Humpage, 2006; Guzmán-Guillén et al., 2017). Furthermore, the progenotoxic effects of CYN has been shown *in vitro* and *in vivo* (Štraser et al., 2011; Puerto et al., 2018; Diez-Quijada et al., 2019).

The best-known CYN mechanisms of action are the irreversible inhibition of proteins (Terao et al., 1994; Froscio et al., 2003) and glutathione synthesis (Runnegar et al., 1995) Besides, some authors have pointed out that oxidative stress is involved in the toxicity of CYN (Puerto et al., 2011; Guzmán-Guillén et al., 2013). Cytochrome P450

enzymes are required for the metabolic activation of CYN, playing an important role in the development of its toxicity (Humpage et al., 2005; Bazin et al., 2010; Štraser et al., 2011; Puerto et al., 2018). But mechanisms responsible of its genotoxicity and potential carcinogenicity (Žegura et al., 2011; Diez-Quijada et al., 2019) are not totally elucidated so far.

The fact that CYN can be accumulated in food encourages the efforts performed to understand its potent toxicity and to define acceptable thresholds of exposure. To protect consumers from the adverse effects of CYN, a provisional Tolerable Daily Intake (TDI) of 0.03 μg/kg body weight (b.w.) has been established and a guideline safety value of 1 μg/L in drinking water proposed by Humpage and Falconer, (2003). In this line, the European Food Safety Authority (EFSA) considers that there are also data gaps regarding the characterization of the toxicological profile of several cyanotoxins other than Microcystins (MCs), as is the case of CYN (Testai et al., 2016). More recently, the World Health Organization (WHO) has proposed a provisional lifetime drinking-water guideline value for CYN of 0.7 μg/L (WHO, 2020), this is lower than the one established for MC-LR (1 μg/L). However, in the revised Drinking Water Directive (Directive (EU) 2020/2184), CYN is not considered and MC-LR maintains its guidance value.

In a risk evaluation framework of dietary contaminants oral toxicity studies are of key importance to establish safety criteria for human exposure. Some *in vivo* studies have been already carried out in rodents to clarify CYN toxicity. Most of them use extracts of CYN-producing cyanobacteria, such as *Cylindrospermopsis raciborskii*, as test item. These studies reported different toxic effects depending on the dose range and exposure way selected. However, although experiments with cyanobacterial extracts can provide valuable hints of the effects of the cyanotoxins they contain, the presence of many other different compounds in the extracts can also derive in misleading conclusions (Falconer, 2007). Moreover, most of the studies used mice as experimental model (Hawkins et al., 1985; Harada et al., 1994; Terao et al., 1994; Hawkins et al., 1997; Falconer et al., 1999; Seawright et al., 1999; Underdal et al., 1999; Shaw et al., 2000; Bernard et al., 2003). This is also the case in subchronic toxicity studies (Humpage and Falconer 2003; Reisner et al., 2004; Sukenik et al., 2006; Chernoff et al., 2018), the ones preferred to derive health guidance values.

Despite the rat being the preferred experimental model in toxicology studies by international organizations, such as the Organisation for Economic and Cooperation Development (OECD) guidelines, as far as we know, only a single study has been performed in (only) male albino Wistar rats (Đorđević et al., 2017). The authors used a single i.p. administration of an extract of *C. raciborskii* at different doses, and pure CYN, and observed the effects 24 or 72h after the treatment. However, the route of exposure used is not relevant for food contaminants.

Thus, taking into account the scarcity of toxicological studies with pure CYN, the aim of the present study was to assess for the first time the subchronic toxicity of pure CYN in male and female Sprague-Dawley rats orally exposed to different doses of this cyanotoxin for 28 days following mainly the OECD 407 guideline (OECD, 2008). Body weight, body weight gain, food and water consumption were recorded. Furthermore, biochemistry and haematological parameters were evaluated, and after necropsy, organ weights and histopathological changes were investigated in order to complete the toxicological evaluation of this toxin.

2. Materials and Methods

2.1. Supplies and chemicals

For the experiment, commercial powder neutral gelatine from pork protein (Jesús Navarro S.A., Alicante, Spain) was used as vehicle in the exposed and control groups. All chemical reagents were obtained from Sigma-Aldrich (Madrid, Spain).

2.2. Test compound

Cylindrospermopsin (CYN) standard (95% purity) was obtained from Alexis Corporation (Lausen, Switzerland). For the 28-day study, different formulations were made every day during all the experiment (4 weeks) and on Fridays they were also prepared for the weekend. To prepare individual dietary dose formulations, the toxin was incorporated in 3 mL of neutral gelatine. The amount of toxin included (µL of pure CYN) was calculated in order to provide the doses selected to the treated groups and were later solidified at 4 ºC overnight. The use of gelatine allows a better control of dosage and it has been used previously with good results (Mantecón et al., 2019; Cascajosa et al., 2020).

2.3. Animal housing and nourishing conditions

The 28-day toxicity study was carried out at the Central Service of Experimental Animals of the University of Córdoba (SAE, Córdoba, Spain) under the code 20-CAM-01 in agreement with the OECD Guideline 407 (OECD, 2008). All animals received human care in conformity with the Directive for the protection of animals utilized for scientific purposes (Directive 2010/63/UE, Decision 2020/569/UE and RD 1386/2018). All methods have been authorized by the Ethical Animal Experimentation Committee of the University of Córdoba and by the Junta de Andalucía (project no. 20-03-2017/047).

For the 28-day study, 24 male and 24 female Sprague-Dawley rats strain RjHan:SD were supplied by Janvier Labs (Le Genest-Saint-Isle, France). Animals were acclimatised during 1 week to the environmental conditions before the experiment (room temperature 22 ± 3 ºC, relative humidity 50-60%, 12 hours light/dark cycle). During this time rats were fed with standard laboratory diet (Harlan Laboratories, Barcelona, Spain) and water *ad libitum*, and were examined by a veterinary surgeon. At the beginning of the dosing, the animals were 5 weeks old, and the average body weights (b.w.) were 173.5 ± 6.24 g and 155.3 ± 3.10 g, for males and females, respectively. Rats were individually accommodated in cages and were randomly assigned to the control and dose groups (6 rats/sex/group).

2.4. Study design and treatment

The doses for the 28-day toxicity study were chosen in agreement with a previous study of 90-day oral exposure to CYN in mice (Chernoff et al., 2018). In this work, an increase in organ body weight ratios (liver and kidney), reduction in blood urea nitrogen (BUN), increase in albumin level and lymphocyte and monocyte count, as well as histopathological lesions in liver and kidney were reported after exposure to 75 µg CYN/kg b.w. Consequently, 75 µg CYN/kg b.w. was chosen as the highest dose to be tested in the study, and following the recommendations of the OECD Guideline 407 (OECD, 2008), a descending dose levels were chosen using a factor of two. For this reason, the doses selected were 75, 37.5 and 18.75 µg CYN/kg b.w./day.

2.5. Body weight, food and water consumption

Animals were observed every day, and body weights and measurements of food and water consumption were recorded weekly. The mean body weights per group and sex were determined weekly from the individual animals. The registered data are depicted in the figures (figures 1-4) for the 4 weeks of the assay. The feed conversion efficiency (FCE) was established by the ratio of food intake (g)/ weight gained (g), in accordance with Escobar et al. (2015).

2.6. Haematology and blood chemistry

Blood samples were obtained from the heart of animals by intracardiac injection (animals were anesthetized with isoflurane, total and deep anaesthesia was used). Prior blood collection animals were fasted overnight at the end of the experimental period (week 4). The haematological parameters assessed were: red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MHCH), platelet blood count (PLT), red cell distribution width blood (RDW), prothrombin time (PT), partial thromboplastin time (PPT), white blood cell count (WBC), and different leucocyte cells count: neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO) and basophils (BA). These parameters were determined on the automatic haematology analyser Cell-Dyn 3700 (Abbot, GMI, MI, USA).

The standard serum biochemistry parameters were evaluated with an automatic chemistry analyser Cobas 6000 (Roche Diagnostics, IN, USA). The following parameters were assessed: glucose (GLUC), blood urea nitrogen (UREA), creatinine (CREAT), total bilirubin (BILI-T), total cholesterol (CHOL), triglycerides (TRIGL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), albumin (ALB), total protein (TOT PROT), sodium (Na^+), potassium (K^+) and calcium (Ca^{++}) ions.

2.7. Gross necropsy, organ weights and histopathology

At the end of the study, animals were fasted overnight before the sacrifice. Animals were profoundly anesthetized with isoflurane, later exsanguinated by intracardiac injection and subsequently euthanized with CO_2 by inhalation to proceed to the necropsy. All rats were macroscopically analysed as well as the organs after extraction. Samples of the following tissues and organs were gathered, and weight wet

without delay following dissection: brain, liver, heart, kidneys, testicles and epididymis (males), and uterus with cervix and ovaries (females), and adrenals.

According to the recommendations of the OECD Guideline 407 (OECD, 2008) full histopathology was performed on the preserved organs and tissues of all animals in the control and the highest dose group (75 µg/kg b.w./day CYN). For the histopathological study light microscopy examination of liver, heart, kidneys, small and large intestines, mesenteric lymph node, pancreas, stomach, trachea, lungs, gonads (testicle/ovary), skeletal muscle, skin and brain (including cerebrum, cerebellum and pons) was carried out. For the analysis, tissue samples were first fixed in 10% phosphate-buffered formalin for 24h at 4 ºC, and later immediately dehydrated in ethanol, immersed in xylol, and embedded in paraffin wax using an automatic processor. Sections of 4 µm were stained with hematoxylin and eosin and the microscopical examination was performed in a Modular Microscopy BX43 with a camera XC50 (Olympus, Shinjuku, Tokyo, Japan). The histopathogical evaluation was done under the following scores according to the severity of the lesions: 0, no significant lesions (0%); 1, very mild (<10%); 2, mild (11-25%); 3, moderate (26-50%); 4, severe (51-75%); and 5: very severe (>75%) (Diez-Quijada et al., 2020).

2.8. Statistical analysis

Different variables, including body weight, body weight gain, food and water consumption, haematology, clinical chemistry, and organ weights were determined by sex and dose group, statistically analysed, and summarized using mean and standard deviation (SD). One-way Analysis of Variance (ANOVA) was carried out to evaluate potential differences in the different variables studied. Normality assumption was tested using Kolmogorov-Smirnov test. If those tests were statistically significant, multiple comparisons were made using Tukey-Kramer Multiple Comparisons Test. If non-normality, comparison was made with Kruskal-Wallis test followed by Dunn's multiple Comparison Tests. Differences were considered significant from * $p < 0.05$. All the statistical analysis were conducted using GraphPad InStat software (GraphPad Prism 9 Software Inc., La Jolla, CA, USA).

3. Results

3.1. Survival and Clinical observations

All rats survived the 4 weeks of the study. Clinical and visual observations during the study period did not show abnormalities in the groups studied. In general, there were no changes in behaviour or locomotor activity during the period of study due to CYN repeated exposure.

3.2. Body weight, body weight gain, food and water consumption, and feed conversion efficiency

No statistically significant differences were observed in body weight between treated and control groups in males (Fig.1a) and females (Fig.1b). In both sexes, the body weight increased during the study period with a normal behavioural pattern for this species. In the same way, no significant changes were noticed in % body weight gain in males (Fig.2a) and females (Fig.2b).

There were no significant changes in food consumption in males (Fig.3a) and females (Fig.3b) exposed to CYN for 28 days at any dose assayed (18.75, 37.5 or 75.0 µg/kg b.w.) in comparison to control groups and between exposed groups. However, water consumption was significantly different in males exposed to 37.5 µg/kg CYN in the third week (Fig.4a) when compared with the control group. In the case of females, no differences were recorded (Fig. 4b) at any dose group in comparison to the control group.

Globally, the performance of Sprague-Dawley rats exposed to CYN by oral route (diet) at the doses assayed (18.75, 37.5 and 75 µg/kg CYN) after 28 days of treatment is shown in Table 1. No significant differences were reported in initial and final body weight, body weight gain and total feed intake in both sexes in any treatment group in comparison to their control groups. Also, the feed conversion ratio neither showed any noteworthy change in the animals treated with the different doses of CYN. The body weight gain and total feed intakes were higher for males in comparison to females, although the feed conversion ratio for females was higher at all the doses studied.

3.3. Haematology and blood chemistry.

Haematology and blood coagulation parameters measured after repeated oral administration of pure CYN in male and female rats are shown in Table 2. Most of them remain unaltered after CYN exposure, only prothrombin time (PT) was increased in males at the medium dose of 37.5 µg/kg CYN, and in females at the lower dose of 18.75 µg/kg CYN. These changes were not considered to be of biological relevance, because they were sporadic, not related to CYN concentration. Moreover, all the values remained within the normal range for the strain (Cascajosa et al., 2020). There were no significant alterations in the differential white blood cell counts of rats exposed to CYN in comparison to the untreated groups (Table 3).

Clinical biochemistry data of rats exposed to CYN are summarized in Table 4. Most of these parameters did not change after repeated exposure to the toxin. Male rats were unaltered. In females, a significant increase of GLUC levels was observed in rats exposed to the lowest and medium doses (18.75 and 37.5 µg/kg CYN) in comparison to the control group ($p<0.001$), whereas the rats exposed to the highest dose (75 µg/kg) were not affected; in any case, all GLUC values could be considered within the normal range (Delwatta et al., 2018). Moreover, a significant decrease in UREA concentrations occurred in rats receiving the same experimental doses (lowest and medium doses of CYN) ($p<0.05$), but all values were within historical/expected values (Delwatta et al., 2018). In addition, females exhibited a significant reduction in serum CHOL concentrations at the three doses assayed ($p<0.01$ and $p<0.001$), being all the values obtained considered normal for this strain (Mantecón et al., 2019; Cascajosa et al., 2020). Significant increases in TRIGL levels at the lowest and medium dose groups were also observed ($p<0.001$), although they lowered at the highest dose of CYN. The AST activity was also elevated after oral exposure at the intermediate and highest dose groups (37.5 and 75 µg/kg CYN) with respect to the control group ($p<0.001$). Levels of Na^+ ions were increased in the lowest and medium dose groups (18.75 and 37.5 µg/kg CYN) in comparison to the control group ($p<0.001$), being all these values considered normal for this strain (Matsukawa et al., 1993; Mantecón et al., 2019; Cascajosa et al., 2020).

3.4. Necropsy, organ weights and histopathology

Macroscopic examination of organs and tissues from rats exposed to CYN did not revealed unusual damage. Data for absolute organ weight-related effects after repeated oral exposure to CYN are shown in Table 5. No significant differences were found in males at any toxin concentration. In females, absolute total body weight (after food deprivation) and liver weights were significantly increased at the lowest and medium CYN doses administered (18.75 or 37.5 µg/kg CYN) in comparison to the control group.

Relative organ weight/body weight of rats fed with CYN were summarized in Table 6. Male rats were unaltered, and in female rats relative liver weights were significantly elevated at the medium dose of 37.5 µg/kg CYN ($p<0.001$) when compared with the control group. Moreover, a slight but significant decrease ($p<0.05$) was observed in the relative kidney weight/body weight of females exposed to the lowest dose of 18.75 µg/kg CYN, in comparison to the control group. This change has no apparent relation to CYN doses, and it was not considered toxicologically relevant (Mellado et al., 2016; Mantecón et al., 2019; Cascajosa et al, 2020). Relative organ weight/brain weight of rats treated are shown in Table 7. In general, no significant changes were observed in males, and in females only the relative liver weight/brain weight ratio were increased at the lowest and medium concentrations of CYN (18.75 and 37.5 µg/kg CYN) ($p<0.001$) when compared to the control group.

Regarding the histopathological examination, tissues of male and female rats were assessed for pathology related to the CYN exposure. From the tissues studied, only livers and kidneys showed some slight microscopical differences between the control group and the rats administered with the highest CYN dose (75 µg/kg) although these alterations were minimal. Moreover, no differences were visualised between males and females. Livers displayed a very mild hepatic sinusoidal ectasia (Fig. 5A) and a few numbers of hepatocytes with apoptotic features (Fig. 5B) or spotty necrosis (Fig. 5D) mainly localised in the surroundings of the centrilobular vein. Occasionally, hepatocytes presented eosinophilic cytoplasmic inclusions (Fig. 5C). Kidneys from rats administered with 75 µg/kg CYN presented slight hyaline casts within the proximal renal tubules corresponding with an eosinophilic and homogeneous proteinaceous substance associated with increased glomerular permeability and mild degeneration of the renal tubules characterized by cell swelling with cytoplasmic vacuolation and pale staining

and fragmented cytoplasm (Fig. 6). The rest of organs examined did not display any microscopical feature compatible with toxicity by the CYN exposure, not even in the 75 µg/kg/day CYN group.

Discussion

In the present work we show for the first time the effects induced by pure CYN in male and female rats orally exposed to repeated doses of 18.75, 37.5 or 75.0 µg CYN/kg b.w./day by the diet during 28 days following the OECD 407 guideline (OECD, 2008). Globally, no differences were found in body weight, food and water consumption and feed conversion efficiency, at any dose assayed in both sexes. Only one of the haematological parameters evaluated (PT) showed changes in both sexes, but it deemed not to be related to CYN concentrations and without toxicological significance. Some biochemical parameters were significantly different only in female rats in comparison to the control group, although most of them were not dose-dependent and their values were within their respective normal ranges for this strain, with the exception of TRIG values and AST activities. Only livers and kidneys showed slight microscopical differences between the control group and the rats administered with the highest CYN dose (75 µg/kg), but no histopathological changes were found in any other organ studied.

The comparison of our results with those obtained in other *in vivo* studies in mammals is difficult due to the scarcity of CYN studies in contrast to other cyanotoxins. Most of them were performed in mice, using the purified toxin or extracts from cyanobacterial species (*C. raciborskii*) containing CYN (and other toxins and substances). In some cases the i.p. injection was the route of administration (Hawkins et al., 1985; Terao et al., 1994; Underdal et al., 1999; Bernard et al., 2003; Đorđević et al., 2017), although numerous studies have used the oral administration (Seawright et al., 1999; Falconer and Humpage, 2001; Humpage and Falconer, 2003; Reisner et al., 2004; Sukenik et al., 2006; Chernoff et al., 2018) as this is the most representative for human exposure. In addition, some studies employed both, oral and i.p injection, to identify differences in toxicity linked to the exposure route (Falconer et al., 1999; Shaw et al., 2000; Bazin et al., 2012). The LD_{50} of CYN (i.p.) in mice has been established in 2 mg/kg after 24 h and decreased to 0.2 mg/kg after 5 days (Terao et al., 1994), and by

oral route was approximately 6 mg/kg (Shaw et al., 2000), being the LD_{50} a toxicological endpoint representative of acute toxicity. Among the subchronic studies carried out in mice, Humpage and Falconer (2003) performed a series of experiments to determine a no-observed-adverse-effect level (NOAEL) for CYN. They observed liver and kidney damage in male Swiss albino mice after treatment with a cyanobacterial extract containing CYN in their drinking water (0–657 µg CYN/kg/day) for 10 weeks and after daily oral exposure to purified CYN (0–240 µg CYN/kg/day) for 11 weeks. In this study, changes in organs and body weights, and modifications in several biochemical parameters (serum bilirubin and bile acids levels and urine protein/creatinine concentrations) were reported, being in this case the kidney the most sensitive organ to CYN. The NOAEL obtained was 30 µg CYN/kg/day on the basis or urine analysis, which provided the proposed Tolerable Daily Intake (TDI) of 0.03 µg CYN/kg/day, and the first proposed safety value of 1 µg/L in drinking water. Some years later, Chernoff et al. (2018) tried to re-evaluate the NOAEL by increasing exposure concentration (75–300 µg CYN/kg b.w.). They reported toxicity in mice after 90 days oral exposure at all the concentrations assayed, and consequently, they could not determine a NOAEL for any dose level.

In relation to the material tested, there are considerable differences in the toxicity exhibited by several *C. raciborskii* strains and pure CYN in different animal models and among CYN-producing cyanobacteria from different localities (Seawright et al., 1999; Bernard et al., 2003; Kinnear, 2010; Pichardo et al., 2017). Thus, Bernard et al. (2003) tested *in vivo* the toxicity of cell extracts of different *C. raciborskii* strains in mice and found fatal acute neurotoxicity, hepatotoxicity but also absence of toxicity, depending on the toxins and the toxicological activity of unknown compounds detected. Moreover, a higher toxicity of *C. raciborskii* lyophilised extracts than the equivalent amount of pure CYN has been demonstrated in several studies (Falconer et al., 1999; Hawkins et al., 2001; Metcalf et al., 2002; Đorđević et al., 2017), which may be due to the presence of further toxins in the extracts or synergistic effects. Similarly, previous studies have also reported differences in genotoxic effects in several models depending on various compounds present in the extracts (Bouaïcha et al., 2005; Žegura et al., 2011). All these facts confirm the limitations indicated by EFSA in relation to the toxicity studies performed with poorly characterised cyanobacterial extracts (with a number of confounding factors) and therefore they are not useful for risk assessment

purposes (Testai et al., 2016). Consequently, EFSA highlights the particular need of the toxicological research of the pure standard toxin. However, the scarcity of toxicological studies with cyanotoxins, especially when repeated doses are required, is mainly due to the limited availability and the high cost of commercial pure standards as it is the case of CYN. All these considerations justify to perform the present work.

The findings of the present study could be compared with the only *in vivo* work carried out in rats (experimental model recommended by several OECD guidelines for toxicity studies), specifically in males albino Wistar rats exposed acutely with a single i.p. administration of a methanolic extract of *C. raciborskii* (from Serbia) at different doses of CYN (1500-12,000 µg CYN/kg b.w.) and an amount of pure CYN (79.80 µg CYN/kg b.w.) for 24 or 72h (Đorđević et al., 2017). The data obtained showed that the alterations in serum biochemical parameters did not produce meaningful liver damage in rats. The authors suggested that the toxicity in the liver could be considered reversible if the animals are no longer in contact with the toxin. Regarding the experimental procedure, the OECD Guideline 407 (OECD, 2008) recommends to use both sexes when relevant differences in toxicity were described, and some authors suggested that long –term oral toxicity studies are needed with both sexes (Falconer and Humpage, 2006). The present work, with male and female rats exposed to repeated doses of CYN/kg b.w., for 28 days, is pioneer using both sexes, in order to know potential differences linked to gender. Our results indicate differences between the control and CYN-treated groups in only one haematological biomarker (TP) in both sexes, and in some biochemical parameters (such as GLUC, UREA, CHOL, AST activities, Na^+ levels) in females, which reached statistical significance. But most of them were generally sporadic, not related to CYN dose and not considered of toxicological significance. These results are in agreement with those obtained by Đorđević et al. (2017) with male rats. In general, in the present study males were unaltered after exposure to the different doses of CYN, whereas females were more sensitive, showing higher TRIG values at the lowest and medium doses (18.75 and 37.50 µg CYN/kg b.w.) but not at the highest dose of CYN. Moreover, increased AST activity was found in females at all doses assayed. By contrast, in mice, the toxin administered i.p induced more toxicity in male than in females (Chernoff et al., 2018).

In relation to the results obtained at the necropsy, males were unaltered and females showed statistically increased absolute liver weight, increased relative liver

weight/body weight and relative liver weight/brain weight ratios. In all cases, no correlation to CYN treatment doses could be established, because they appeared at the lowest or medium doses, whereas at the highest dose, these values remained similar to the control groups and were considered as normal values. Moreover, in the organs examined in this study, only livers and kidneys showed some slight microscopical differences at 75.0 µg CYN/kg compared with the controls, which could be interpreted as certain degree of toxicity at this dose. In addition, although renal effects seemed to be important after chronic low dose exposure in mice (Humpage and Falconer, 2003), in this study, very mild renal microscopical changes including intratubular hyaline casts and degeneration of renal tubules were observed in the rats administered with the highest dose.

Regarding the dose range assayed in the present work, the highest dose of 75.0 µg CYN/kg b.w./day was chosen taking into account the previous toxic effects induced by purified CYN in mice orally exposed (75-300 µg/kg/d) for 90 days (Chernoff et al., 2018). Slight signs of damage were observed from 75 µg CYN/kg b.w., such as increases in liver and kidney/body weight ratios, decreased levels of blood urea nitrogen (BUN), signs of hepatic inflammation, and histopathological damage to hepatic and renal tissues. Moreover, in Wistar rats, after a single i.p injection of 79.80 µg CYN/kg b.w., genotoxicity was observed (Đorđević et al., 2017). In addition, in a previous experiment from our lab, rats exposed by gavage to pure CYN (7.5-75.0 µg/kg b.w.) at 0, 24 and 45 h, showed irritation in the stomach and changes in hepatocytes (Diez-Quijada et al., 2019). By contrast, in the present study, at the same dose of 75.0 µg/kg b.w, only scarce microscopical changes were observed in the livers of rats. This discrepancy could be due to the route of exposure: i.p or gavage versus oral exposure through the diet (gelatine). Usually, oral gavage administration result in higher peak concentration and higher area under concentration-time curve of xenobiotics in plasma and higher toxic effects (Kapetanovic et al., 2006). In fact, in the case of MC-LR, when the toxin was administered orally (by gavage) for 90 days a NOEL of 40 µg/kg b.w./day was observed (Fawell et al., 1994), whereas after administration of MC-LR-containing extracts through the diet, the NOAEL value was higher (333 µg/kg b.w./day) (Schaeffer et al., 1999). Gavage corresponds to a bolus dose, resulting in tissue concentrations higher than those attained after the more gradual introduction of a dietary treatment,

giving time to the detoxification/excretion systems to be efficient (Funari and Testai, 2008).

In comparison to other *in vivo* studies carried out with CYN, some researchers have reported that the toxin induced more several biochemical/histological effects than in the present work. Thus, Chernoff et al. (2018) observed in rats at all doses assayed (75-300 µg/kg/d for 90 d) hepatic and renal damage with histopathological alterations, liver and kidneys body weights ratio increased, increased serum ALT activity, decreased blood urea nitrogen (BUN) and CHOL in males. These results are in agreement with those found in another study, with male mice exposed to CYN through drinking water with a daily intake of 66 µg CYN/kg b.w. for 3 weeks (Reisner et al., 2004). These authors found increases in the relative weights of liver, testicles and kidneys, as well as increases in the CHOL level in red blood cells (RBC) and in the plasma after CYN exposure, concomitant with a reduction in CHOL level in the liver. In addition, it was reported augmented haematocrit levels as response to exposure to low levels of CYN and morphological changes in the RBC of mice, which were gradually developed into an acanthocyte-like form, cytoplasmic irregularly spaced projections, etc. (Reisner et al., 2004). Similarly, elevated haematocrit levels in male and female mice after 16 weeks of exposure to variable concentrations of CYN in their drinking water (10-55 µg/kg/day during 42 weeks) was also found as the most important effect observed (Sukenik et al., 2006). By contrast, no haematological effects were found in the present study, and regarding biochemical parameters: 1) CHOL levels were statistically reduced in serum of female rats, although all the values were considered normal, and without toxicological relevance; and 2) the significant increases in TRIGL levels and in the AST activity (although not differences were found in ALT activities, another hepatic biomarker), could be indicative of the potential damage induced in the liver by CYN, although only minimal microscopical changes were described in the livers. The protein synthesis inhibition in the liver by CYN (Terao et al., 1994) may in some degree account for the TRIGL accumulation in the organ, due to the suppression of lipoprotein synthesis and secretion (Seawright et al., 1999).

In a wide sense, it should be noted that discrepancies in responses to CYN exposure found in the scientific literature, when compared with this work, could be explained by several reasons: the experimental model (rats/mice), variability of the experimental conditions, such as doses, time of exposure, time of response and exposure

route (oral through the diet, drinking water or gavage/i.p. injection). In this sense, for food contaminants oral exposure is the preferred one. Among them, the administration of the doses by gelatine tablets have been reported to eliminate pain and distress in experimental animals in comparison to gavage (Dhawan et al., 2018), and also allows a better control of dosage. Additionally, as it has been stated before, the origin and purity of the cyanobacterial extract/standard of CYN assayed can have a role. Our results indicate the need to perform longer *in vivo* studies with pure CYN in rats to confirm these divergences, by administration of the toxin for 90 days, to stablish the NOAEL under experimental conditions following the OECD guidelines.

Conclusion

The present study reports for the first time that after 28-day oral repeated exposure to pure CYN at doses of 18.75, 37.5 or 75.0 µg/kg b.w., male and female rats did not show any clinical signs of toxicity, and any change of individual body weight, relative gain weight, food and water consumption and feed conversion efficiency in both sexes. Moreover, there were no statistically significant differences in haematological and biochemical parameters between treated males and control rats. For females, changes in several biomarkers (TRIG levels and AST activity) as well in the weight of organs (absolute liver weight values, relative kidney/body weight ratios or relative liver weight/brain weight ratios) were found, but were considered as not toxicologically relevant as they were not associated with relevant histopathological signs.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1. Mean body weights (g) of a) male and b) female rats orally exposed to 18.75, 37.5 and 75 µg/kg b.w./day CYN and control rats for 28 days.

Fig. 2. Mean body weight gain (%) of a) male and b) female rats orally exposed to 18.75, 37.5 and 75 µg/kg b.w./day CYN and control rats for 28 days.

Fig. 3. Food consumption (g/week) of a) male and b) female rats orally exposed to 18.75, 37.5 and 75 µg/kg b.w./day CYN and control rats for 28 days

Fig. 4. Water consumption (g/week) of a) male and b) female rats orally exposed to 18.75, 37.5 and 75 µg/kg b.w./day CYN and control rats for 28 days. The significance level observed is # respect to group 2 (37.5 µg/kg/day) in week 3 when $p<0.05$.

Fig. 5. Microscopical changes in livers from rats administered with 75 µg/kg b.w./day CYN. A) Hepatic sinusoidal ectasia; B) Hepatocytes apoptosis (head arrows); C) Eosinophilic cytoplasmic inclusions (arrow); Hepatocytes necrosis (head arrows) in association with lymphocytes. Haematoxylin and eosin stain.

Fig. 6. Microscopical changes in kidneys from rats administered with 75 µg/kg b.w./day CYN. A-D) Intratubular hyaline casts (*) and degeneration (arrows) of the renal tubules. Haematoxylin and eosin stain.

Table captions

Table 1. Effect of 28 days oral exposure to CYN on body weight and food consumption in male and female rats. Values represent the mean ± SD of 6 rats/sex/group. Differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.).

Table 2. Haematology parameters of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). * and ** Significantly different in comparison to control group values when $p<0.05$ and $p<0.01$, respectively.

Table 3. Differential White blood cells count data of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.).

Table 4. Clinical biochemistry of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed are *$p<0.05$, **$p<0.01$ and ***$p<0.001$ in comparison to control group values

Table 5. Absolute organ weight of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated

by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed are $^{**}p<0.01$ and $^{***}p<0.001$ in comparison to control group values.

Table 6. Relative organ weight/body weight of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed are $^{*}p<0.05$ and $^{***}p<0.001$ in comparison to control group values.

Table 7. Relative organ weight/brain weight of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed is $^{***}p<0.001$ in comparison to control group values.

Figure 1

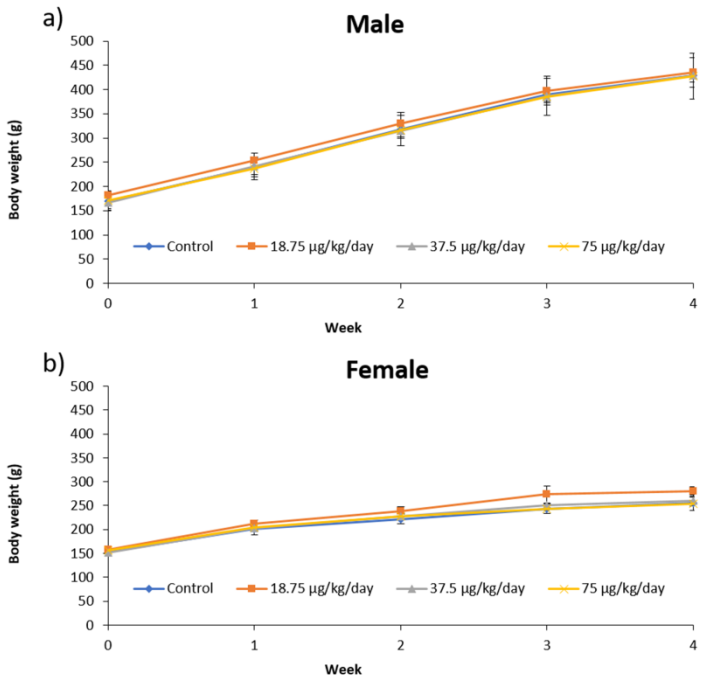

Figure 2

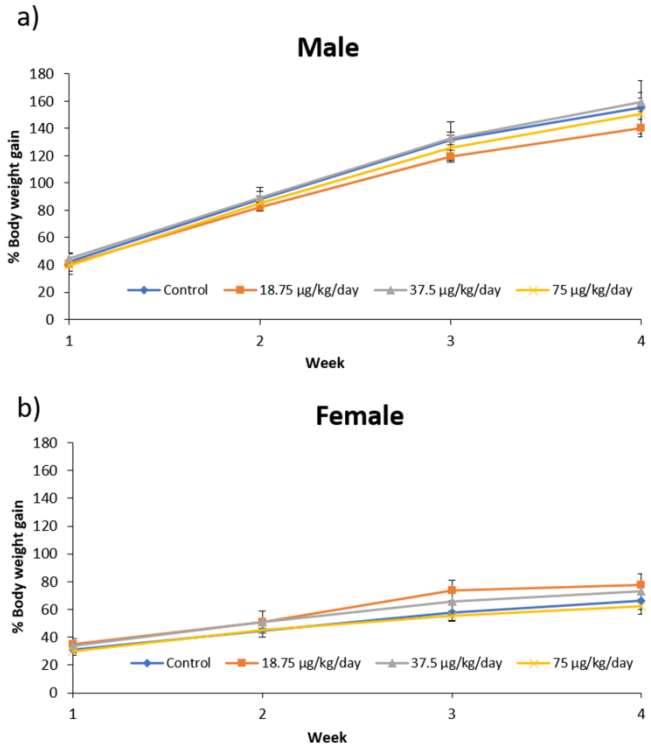

Figure 3

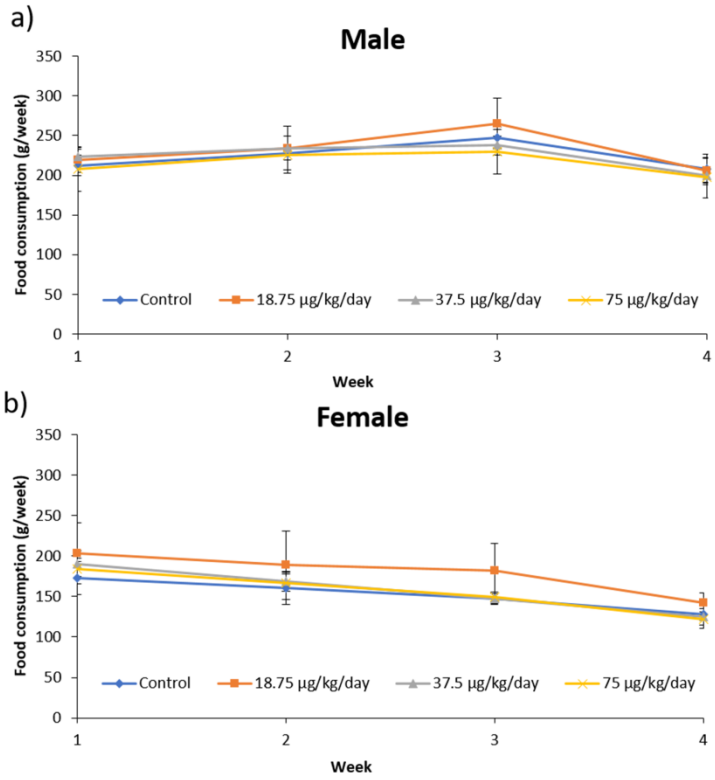

Figure 4

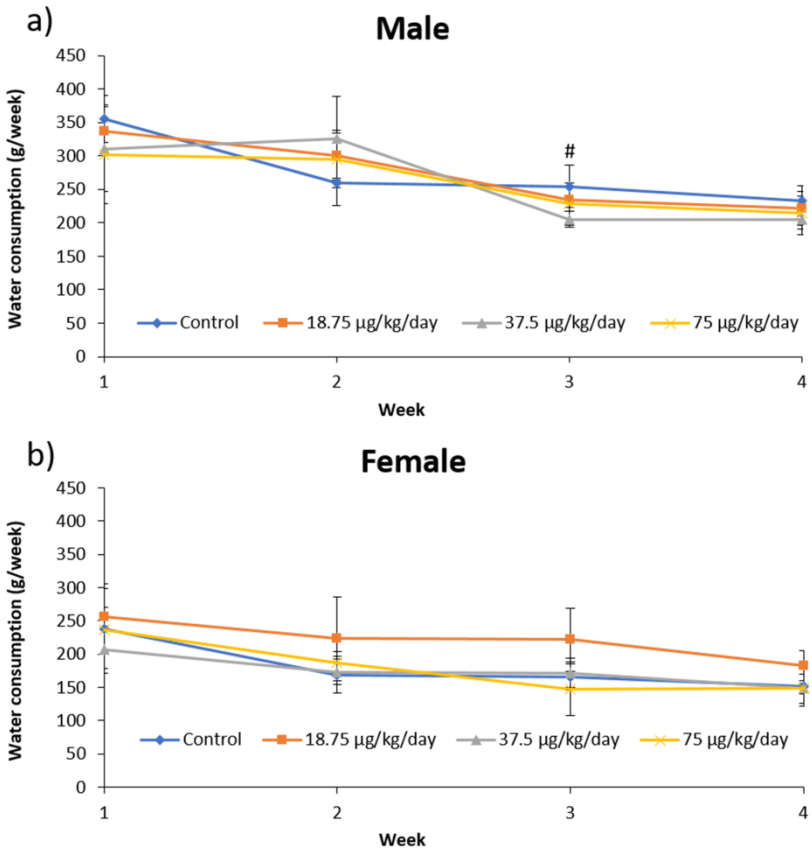

Figure 5

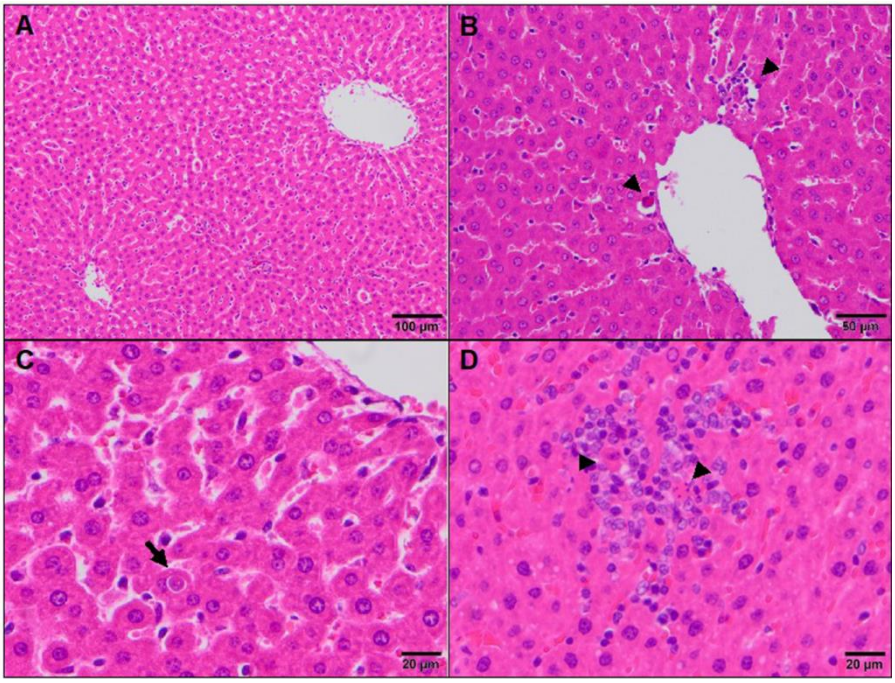

Figure 6

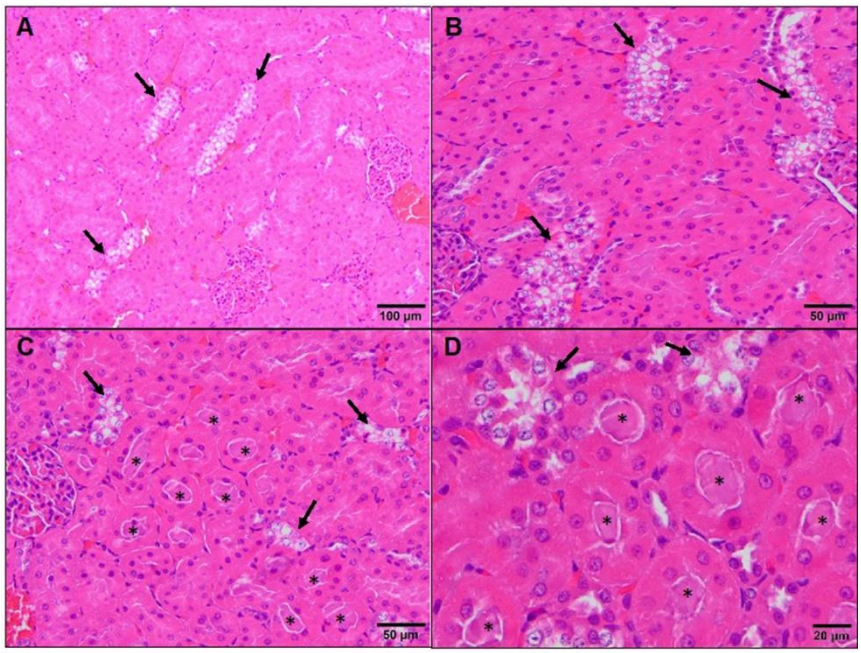

Table 1. Effect of 28 days oral exposure to CYN on body weight and food consumption in male and female rats. Values represent the mean ± SD of 6 rats/sex/group. Differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.).

	MALE				**FEMALE**			
PARAMETERS	**Group 1 (0 µg/kg/day) N=6**	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**	**Group 1 (0 µg/kg/day) N=6**	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**
Initial body weight (g)	167±12.0	181±7.6	170±13.6	176±18.0	155±6.0	157±6.5	151±6.7	158±4.2
	F(20.3)=1.294 p=0.30; N.S.				**F(20.3)= 1.694 p=0.2; N.S.**			
Final body weight (g)	430±12.2	432±24.7	428±7.7	424±39.8	261±14.7	276±10.0	265±8.2	257±5.7
	F(20.3)=0.10 p=0.96; N.S.				**F(20.3)=3.875 p=0.24; N.S.**			
Body weight gain (g)	263±13.0	251±18.3	259±13.8	249±28.1	106±10.2	119±8.6	114±12.4	99±5.3
	F(20.3)=0.67 p=0.58; N.S.				**K.W.=10.76 p=0.05; N.S.**			
Total feed intake (g)	895.3±36.1	911.7±70.2	895.3±21.9	875.2±84.9	619.3±50.8	678.7±109.6	633.8±23.6	625±15.4
	F(20.3)=0.38 p=0.77; N.S.				**K.W.=0.36 p=0.95; N.S.**			
Feed conversion ratio	3.4±0.2	3.6±0.1	3.5±0.2	3.5±0.3	5.9±0.35	5.7±0.8	5.6±0.7	6.4±0.3
	K.W.=4.99 p=0.17; N.S.				**K.W.=3.96 p=0.14; N.S.**			

Values are mean ± SD for 6 rats/sex/group. F: Statistics ANOVA test; K.W: Kruskal-Wallis test; N.S.: Not Significant.

Table 2. Haematology parameters of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). * and ** Significantly different in comparison to control group values when $p<0.05$ and $p<0.01$, respectively.

HAEMATOLOGY DATA SUMMARY									
		MALE				FEMALE			
		Group 1 (0 µg/kg/day) N=6	Group 2 (18.75 µg/kg/day) N=6	Group 3 (37.5 µg/kg/day) N=6	Group 4 (75 µg/kg/day) N=6	Group 1 (0 µg/kg/day) N=6	Group 2 (18.75 µg/kg/day) N=6	Group 3 (37.5 µg/kg/day) N=6	Group 4 (75 µg/kg/day) N=6
RBC 10^6/µL	MEAN ± SD	7.6 ± 0.3	8.0 ± 0.8	8.1 ± 0.2	7.3 ± 0.4	8.4 ± 0.4	8.2 ± 0.4	7.6 ± 0.8	7.7 ± 0.9
			F(20.3)=3.14 p=0.05; N.S.				K.W.=6.478 p=0.09; N.S.		
HGB g/dL	MEAN ± SD	14.2 ± 0.4	14.5 ± 1.3	14.8 ± 0.5	13.8 ± 0.4	15.7 ± 0.5	15.1 ± 0.9	14.4 ± 1.9	14.1 ± 1.4
			F(20.3)= 2.088 p=0.13; N.S.				F(20.3)= 1.832 p=0.17; N.S.		
HCT %	MEAN ± SD	68.5 ± 3.0	67.3 ± 6.4	69.2 ± 2.8	65.2 ± 3.2	74.5 ± 2.6	72.6 ± 4.6	67.4 ± 6.7	67.0 ± 6.4
			F(20.3)=1.068 p=0.38; N.S.				F(20.3)=2.953 p=0.06; N.S.		
MCV fL	MEAN ± SD	89.1 ± 3.5	86.5 ± 2.3	86.1 ± 2.9	88.4 ±1.3	87.2 ± 1.4	87.2 ± 1.4	87.8 ± 1.9	87.6 ± 3.1
			F(20.3)=1.787 p=0.18; N.S.				F(20.3)=0.1139 p=0.95; N.S.		
MCH pg	MEAN ± SD	18.9 ± 0.6	18.5 ± 0.4	18.4 ± 0.4	18.8 ± 0.4	18.1 ± 0.3	18.2 ± 0.3	18.0 ± 0.4	18.4 ± 0.6
			F(20.3)= 1.791 p=0.18; N.S.				F(20.3)=1.026 p=0.40; N.S.		
MCHC g/dL	MEAN ± SD	21.1 ± 0.2	21.4 ± 0.3	21.4 ± 0.5	21.5 ± 0.4	20.8 ± 0.3	25.8 ± 12.4	20.7 ± 0.3	21.0 ± 0.4
			F(20.3)= 1.170 p=0.35; N.S.				K.W.= 1.732 p=0.63 ; N.S.		
PLT 10^3/µL	MEAN ± SD	850.7 ± 123.6	835.3 ± 120.7	915.7 ± 127.9	963.0 ± 78.9	798.7 ± 274.9	839.2 ± 109.2	538.4 ± 322.7	719.0 ± 281.3
			F(20.3)=1.605 p=0.22; N.S.				F(20.3)=1.575 p=0.23; N.S.		
RDW %	MEAN ± SD	15.4 ± 1.1	15.4 ± 0.8	15.8 ± 0.7	16.7 ± 0.9	15.6 ± 0.4	16.4 ± 0.6	15.8 ± 0.9	15.1 ± 0.8
			F(20.3)=2.776 p=0.07; N.S.				F(20.3)=3.610 p=0.05; N.S.		
PT seg	MEAN ± SD	19.3 ± 0.3	20.3 ± 0.5	22.1± 1.7*	19.0 ± 0.9	20.9 ± 0.4	24.1 ± 0.2**	23.3 ± 0.7	20.4 ± 1.8
			K.W.=14.492; *p<0.05				K.W.=18.311; **p<0.01		
PTT seg	MEAN ± SD	25.9 ± 0.4	27.5 ± 5.5	28.8 ± 5.6	29.3 ± 2.6	32.1 ± 0.4	33.9 ± 2.0	32.1 ± 4.4	30.2 ± 4.7
			K.W.=4.121 p=0.25; N.S.				F(20.3)=1.200 p=0.34; N.S.		

RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet (thrombocyte) count; RDW: red cell distribution width blood; PT: prothrombin time; PTT: partial thromboplastin time; F: Statistics ANOVA test; Kruskal-Wallis test (K.W.); N.S.: Not Significant; Significantly different from control when *p<0.05 and **p<0.01.

Table 3. Differential White blood cells count data of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.).

DIFFERENTIAL WHITE BLOOD CELLS COUNT DATA SUMMARY									
		MALE				**FEMALE**			
		Group 1 (0 µg/kg/day) N=6	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**	**Group 1 (0 µg/kg/day) N=6**	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**
WBC 10^3/µL	**MEAN ± SD**	13.1 ± 3.5	12.1 ± 3.6	18.4 ± 1.0	15.3 ± 2.3	13.8 ± 2.2	11.9 ± 1.2	10.4 ± 3.6	10.6 ± 2.4
			K.W.=10.979 p=0.05; N.S.				**K.W.=5.667 p=0.13; N.S.**		
NE %	**MEAN ± SD**	21.6 ± 3.2	25.9 ± 12.4	23.2 ± 8.8	28.6 ± 11.5	18.5 ± 10.1	19.4 ± 4.9	29.5 ± 9.2	23.7 ± 8.3
			F(20.3)=0.6021 p=0.62; N.S.				**F(20.3)=2.177 p=0.12;N.S.**		
LY %	**MEAN ± SD**	67.4 ± 2.8	72.8 ± 11.2	68.7 ± 1.8	64.6 ± 3.9	72.1 ± 8.4	67.3 ± 8.6	61.4 ± 4.3	69.4 ± 8.0
			F(20.3)=1.813 p=0.18; N.S.				**F(20.3)=2.164 p=0.12; N.S.**		
MO %	**MEAN ± SD**	3.9 ± 1.5	1.5 ± 1.2	2.4 ± 1.5	4.7 ± 2.2	2.3 ±1.3	3.8 ± 4.2	4.3 ± 1.7	1.8 ± 1.2
			F(20.3)=4.631 p=0.59; N.S.				**K.W.=9.480 p=0.05; N.S.**		
EO %	**MEAN ± SD**	5.5 ± 1.9	2.3 ± 0.9	4.4 ± 3.6	9.3 ± 3.0	6.2 ± 2.7	7.0 ± 2.2	4.8 ± 1.8	6.6 ± 4.6
			F(20.3)= 8.068 p=0.05; N.S.				**F(20.3)= 0.6037 p=0.62; N.S.**		
BA %	**MEAN ± SD**	0.5 ± 0.4	0.4 ± 0.3	0.5 ± 0.5	0.8 ± 0.2	0.4 ± 0.4	0.4 ± 0.3	0.96 ± 1.1	0.4 ± 0.3
			F(20.3)=1.233 p=0.32; N.S.				**K.W.=3.065 p=0.38; N.S.**		

WBC: white blood cell count; NE: neutrophils; LY: lymphocytes; MO: monocytes; EO: eosinophils; BA: basophils; F: Statistics ANOVA test; Kruskal-Wallis test (K.W.); N.S.: Not Significant.

Table 4. Clinical biochemistry of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed are *p<0.05, **p<0.01 and ***p<0.001 in comparison to control group values.

GLUC: glucose; CREAT: creatinine; Bili-T: total bilirubin; CHOL: total cholesterol; TRIGL: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALKP: alkaline phosphatase; ALB: albumin; TOT PROT: total protein; Na^+: sodium; K^+: potassium; Ca^{++}: calcium. F: Statistics ANOVA test; Kruskal-Wallis test (K.W.); N.S.: Not Significant; The significance levels observed are *p<0.05, **p<0.01 and ***p<0.001 in comparison to control group values.

CLINICAL BIOCHEMISTRY DATA SUMMARY									
		MALE				FEMALE			
		Group 1 (0 µg/kg/day) N=6	Group 2 (18.75 µg/kg/day) N=6	Group 3 (37.5 µg/kg/day) N=6	Group 4 (75 µg/kg/day) N=6	Group 1 (0 µg/kg/day) N=6	Group 2 (18.75 µg/kg/day) N=6	Group 3 (37.5 µg/kg/day) N=6	Group 4 (75 µg/kg/day) N=6
GLUC mg/dL	**MEAN ± SD**	111.7 ± 9.5	120.5 ± 19.6	115.3 ± 3.3	106.7 ± 7.7	109.3 ± 16.7	169.0 ± 5.7***	179.2 ±25.8***	110.2 ± 17.6
		F(20.3)=1.507 p=0.24; N.S.				**F(20.3)=26.148 ***p<0.001**			
UREA mg/dL	**MEAN ± SD**	40.8 ±3.1	43.9 ±4.7	40.6 ±1.4	39.0 ±2.6	36.9 ±3.1	27.9 ± 6.4*	26.6 ± 4.3*	38.7 ± 6.0
		K.W.=4.301 p=0.23; N.S.				**F(20.3)=8.649 *p<0.05**			
CREAT mg/dL	**MEAN ± SD**	0.36 ± 0.0	0.34 ± 0.0	0.33 ± 0.0	0.34 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.30 ± 0.0	0.3 ± 0.0
		F(20.3)=0.7309 p= 0.55; N.S.				**F(20.3)=2.591 p=0.08; N.S.**			
BILI-T mg/dL	**MEAN ± SD**	0.2 ± 0.0	0.2 ± 0.0	0.21 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
		F(20.3)=0.5171 p=0.68 ;N.S.				**F(20.3)=5.488 p=0.74; N.S.**			
CHOL mg/dL	**MEAN ± SD**	95.0 ± 9.8	87.0 ± 8.6	89.5 ± 8.1	93.3 ± 12.3	101.7 ± 10.3	82.7 ± 6.4**	79.3 ± 2.6***	86.0 ± 8.7**
		F(20.3)=0.8213 p=0.50; N.S.				**F(20.3)=10.155 **p<0.01;***p<0.001**			
TRIGL mg/dL	**MEAN ± SD**	97.2 ± 19.8	101.5 ± 16.7	137.5 ± 33.5	116 ± 38.8	56.0 ± 7.6	112.2 ±16.3***	150.7 ±18.3***	51.3 ± 4.2
		F(20.3)=2.408 p=0.09; N.S.				**F(20.3)=80.498 ;***p<0.001**			
AST U/L	**MEAN ± SD**	171.9 ± 19.4	162.0 ± 26.4	126.9 ± 39.3	137.5 ± 22.5	182.7 ± 32.2	195.0 ± 10.7	592.0 ±37.7***	315.4 ±60.5***
		F(20.3)=3.365 p=0.43; N.S.				**F(20.3)=139.18 ;***p<0.001**			
ALT U/L	**MEAN ± SD**	42.5 ± 5.9	35.0 ± 6.2	37.4 ± 4.5	36.4 ± 2.8	36.8 ± 10.0	64.2 ± 31.8	71.0 ± 26.7	64.1 ± 24.2
		F(20.3)=2.534 p= 0.09; N.S.				**K.W.=7.840 p=0.05;N.S.**			
ALKP U/L	**MEAN ± SD**	271.2 ± 34.5	250.8 ± 16.9	237.3 ± 31.2	222.2 ± 37.3	174.8 ± 24.5	188.2 ± 14.3	202.2 ± 52.4	178.3 ± 31.4
		F(20.3)=2.699 p=0.07; N.S.				**F(20.3)=0.7834 p=0.52; N.S.**			
ALB g/dL	**MEAN ± SD**	4.3 ± 0.2	4.1 ± 0.2	4.1 ± 0.2	4.2 ± 0.1	4.2 ± 0.2	4.1 ± 0.2	4.0 ± 0.2	4.2 ± 0.2
		F(20.3)=2.134 p=0.13; N.S.				**F(20.3)=1.190 p=0.34; N.S.**			
TOT PROT g/dL	**MEAN ± SD**	5.8 ± 0.3	5.7 ± 0.2	5.6 ± 0.3	5.7 ± 0.3	5.6 ± 0.1	5.5 ± 0.2	5.3 ± 0.2	5.5 ± 0.2
		F(20.3)=0.7462 p=0.54; N.S.				**F(20.3)=2.370 p=0.10; N.S.**			
Na^+ mmol/L	**MEAN ± SD**	138.3 ± 3.7	139.7 ± 2.2	140.3 ± 3.0	138.2 ± 2.9	136.8 ± 1.5	142.5 ±1.9***	141.7 ±2.2***	137.2 ± 1.5
		F(20.3)=0.7296 p=0.55;N.S.				**F(20.3)=16.796 ***p<0.001**			
K^+ mmol/L	**MEAN ± SD**	5.7 ± 0.4	5.4 ± 0.2	5.4 ± 0.2	5.4 ± 0.3	4.2 ± 0.3	5.2 ± 1.0	5.4 ± 2.1	4.7 ± 0.3
		F(20.3)=1.729 p=0.19; N.S.				**K.W.=6.189 p=0.10; N.S.**			
Ca^{++} mmol/L	**MEAN ± SD**	13.5 ± 1.5	13.3 ± 1.3	12.9 ± 1.1	13.6 ± 0.9	12.6 ± 0.4	11.8 ± 0.6	12.1 ± 0.9	13.1 ± 0.8
		F(20.3)=0.4942 p=0.69; N.S.				**F(20.3)=4.064 p=0.47; N.S.**			

Table 5. Absolute organ weight of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed are $^{**}p<0.01$ and $^{***}p<0.001$ in comparison to control group values.

ORGAN WEIGHT DATA SUMMARY										
MALE						FEMALE				
		Group 1 (0 µg/kg/day) N=6	Group 2 (18.75 µg/kg/day) N=6	Group 3 (37.5 µg/kg/day) N=6	Group 4 (75 µg/kg/day) N=6			Group 1 (0 µg/kg/day) N=6	Group 2 (18.75 µg/kg/day) N=6	Group 3 (37.5 µg/kg/day) N=6	Group 4 (75 µg/kg/day) N=6
BODY W.(g)	MEAN ± SD	409.5 ± 11.6	417.3 ± 23.8	410.0 ± 7.4	402.2 ± 31.2	BODY W.(g)	MEAN ± SD	238.7 ± 10.7	271.3 ±10.3***	260.3 ± 10.9**	240.0 ± 3.4
		K.W.=0.9086 p=0.82; N.S.						F(20.3)=17.355 **p<0.01;***p<0.001			
BRAIN (g)	MEAN ± SD	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	BRAIN (g)	MEAN ± SD	1.9 ± 0.2	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
		K.W.=4.374 p=0.22; N.S.						F(20.3)=0.7325 p=0.54; N.S.			
LIVER (g)	MEAN ± SD	14.4 ± 0.7	14.8 ± 0.8	13.2 ± 0.3	14.8 ±1.8	LIVER (g)	MEAN ± SD	7.8 ± 0.9	11.4 ± 0.3***	12.0 ± 0.5***	8.3 ± 0.8
		F(20.3)=3.228 p=0.05; N.S.						F(20.3)=59.738 ***p<0.001			
HEART (g)	MEAN ± SD	1.6 ± 0.1	1.6 ± 0.1	2.0 ± 1.2	1.7 ± 0.3	HEART (g)	MEAN ± SD	1.0±0.0	1.0±0.1	1.0±0.1	1.1±0.1
		K.W.=1.097 p=0.78; N.S.						F(20.3)=3.269 p=0.10; N.S.			
KIDNEYS (g)	MEAN ± SD	2.8 ± 0.0	2.9 ± 0.2	2.9 ± 0.3	3.0 ± 0.3	KIDNEYS (g)	MEAN ± SD	2.0±0.1	2.0±0.1	2.0±0.1	2.0±0.1
		F(20.3)=0.9421 p=0.44; N.S.						K.W.=0.2036 p=0.98; N.S.			
TESTICLES (g)	MEAN ± SD	3.1 ± 0.2	3.5 ± 0.3	3.3 ± 0.3	3.3 ± 0.1	UTE./CERV (g)	MEAN ± SD	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.3	0.8 ± 0.3
		F(20.3)=2.142 p=0.13; N.S.						F(20.3)=0.3804 p=0.77; N.S			
EPIDIDYMIS (g)	MEAN ± SD	1.0 ±0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	OVARIES (g)	MEAN ± SD	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
		F(20.3)=1.070 p=0.38; N.S.						F(20.3)=1.021 p=0.40; N.S			
ADRENALS (g)	MEAN ± SD	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ADRENALS (g)	MEAN ± SD	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
		F(20.3)=0.2368 p=0.87; N.S.						F(20.3)=0.4815 p=0.70; N.S			

F: Statistics ANOVA test; K.W: Kruskal-Wallis test; N.S.: Not Significant. The significance level observed are $^{**}p<0.01$ and $^{***}p<0.001$ in comparison to control group values.

Table 6. Relative organ weight/body weight of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed are *p<0.05 and ***p<0.001 in comparison to control group values.

F: Statistics ANOVA test; K.W: Kruskal-Wallis test; N.S.: Not Significant. The significance levels observed are *p<0.05 and ***p<0.001 in comparison to control group values.

ORGAN WEIGHT/BODY WEIGHT RATIO DATA SUMMARY										
MALE						**FEMALE**				
		Group 1 (0 µg/kg/day) N=6	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**			**Group 1 (0 µg/kg/day) N=6**	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**
BRAIN (%)	**MEAN ± SD**	0.50 ± 0.02	0.48 ± 0.01	0.52 ± 0.02	0.51 ± 0.05	**BRAIN (%)**	**MEAN ± SD**	0.79 ± 0.08	0.72 ± 0.02	0.75 ± 0.04	0.79 ± 0.03
		F(20.3)=2.198 p=0.12; N.S.						**F(20.3)=2.564 p=0.08; N.S.**			
LIVER (%)	**MEAN ± SD**	3.53 ± 0.16	3.55 ± 0.34	3.21 ± 0.10	3.68 ± 0.30	**LIVER (%)**	**MEAN ± SD**	3.27 ± 0.31	4.19 ± 0.19	4.60 ±0.34***	3.46 ± 0.36
		F(20.3)=3.895 p=0.07; N.S.						**K.W.=18.820 ***p<0.001**			
HEART (%)	**MEAN ± SD**	0.38 ± 0.03	0.38 ± 0.03	0.49 ± 0.28	0.43 ± 0.06	**HEART (%)**	**MEAN ± SD**	0.40 ± 0.02	0.38 ± 0.02	0.37 ± 0.02	0.44 ± 0.04
		K.W.=1.767 p=0.62; N.S.						**F(20.3)=8.310 p=0.14; N.S.**			
KIDNEYS (%)	**MEAN ± SD**	0.68 ± 0.02	0.70 ± 0.08	0.70 ± 0.06	0.75 ± 0.03	**KIDNEYS (%)**	**MEAN ± SD**	0.83 ± 0.05	0.73 ± 0.04*	0.76 ± 0.05	0.83 ± 0.07
		F(20.3)=1.672 p=0.21; N.S.						**F(20.3)=4.546 *p<0.05**			
TESTICLES (%)	**MEAN ± SD**	0.77 ± 0.06	0.83 ± 0.09	0.80 ± 0.06	0.83 ± 0.05	**UTE./CERV. (%)**	**MEAN ± SD**	0.34 ± 0.08	0.27 ± 0.03	0.34 ± 0.12	0.34 ± 0.11
		K.W.=3.247 p=0.36; N.S.						**F(20.3)=0.8116 p=0.50; N.S.**			
EPIDIDYMIS (%)	**MEAN ± SD**	0.24 ± 0.03	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.02	**OVARIES (%)**	**MEAN ± SD**	0.12 ± 0.05	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.04
		F(20.3)=1.436 p=0.26; N.S.						**F(20.3)=1.985 p=0.15; N.S.**			
ADRENALS (%)	**MEAN ± SD**	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	**ADRENALS (%)**	**MEAN ± SD**	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
		F(20.3)=0.3140 p=0.82; N.S.						**F(20.3)=0.3936 p=0.76; N.S.**			

Table 7. Relative organ weight/brain weight of male and female rats fed with 18.75, 37.5 and 75 µg/kg b.w./day CYN for 28 days. Values are mean ± SD for 6 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA test (F values) or by Kruskal-Wallis test (K.W.). The significance levels observed is ***p<0.001 in comparison to control group values.

ORGAN WEIGHT/BRAIN WEIGHT RATIO DATA SUMMARY										
MALE						**FEMALE**				
		Group 1 (0 µg/kg/day) N=6	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**			**Group 1 (0 µg/kg/day) N=6**	**Group 2 (18.75 µg/kg/day) N=6**	**Group 3 (37.5 µg/kg/day) N=6**	**Group 4 (75 µg/kg/day) N=6**
LIVER (%)	**MEAN ± SD**	704.9 ± 42.3	744.4 ± 58.6	623.6 ± 20.7	731.0 ± 109.0	**LIVER (%)**	**MEAN ± SD**	420.5 ± 77.9	579.5 ±30.3***	617.1 ±26.7***	441.3 ± 50.6
		F(20.3)=4.012 p=0.05; N.S.						**F(20.3)=22.566 ***p<0.001**			
HEART (%)	**MEAN ± SD**	76.4 ± 6.3	78.8 ± 4.8	95.9 ± 54.6	85.0 ± 17.2	**HEART (%)**	**MEAN ± SD**	51.6 ± 5.5	52.3 ± 2.8	48.9 ± 1.4	56.1 ± 6.0
		K.W.=1.013 p=0.80; N.S.						**F(20.3)=2.772 p=0.07; N.S.**			
KIDNEYS (%)	**MEAN ± SD**	136.4 ± 9.1	146.6 ± 13.8	135.7 ± 13.8	148.2 ± 19.1	**KIDNEYS (%)**	**MEAN ± SD**	105.4 ± 10.2	101.7 ± 6.7	102.7 ± 8.2	105.5 ± 7.7
		F(20.3)=1.268 p=0.31; N.S.						**F(20.3)=0.3198 p=0.81; N.S.**			
TESTICLES (%)	**MEAN ± SD**	153.7 ± 14.0	174.5 ± 17.2	155.6 ± 11.6	164.4 ± 13.1	**UTE./CERV. (%)**	**MEAN ± SD**	42.9 ± 9.9	37.3 ± 3.7	45.5 ± 17.4	42.9 ± 13.5
		F(20.3)=2.725 p=0.07; N.S.						**F(20.3)=0.4840 p=0.70; N.S.**			
EPIDIDYMIS (%)	**MEAN ± SD**	48.8 ± 5.9	56.5 ± 5.3	51.8 ± 6.1	53.7 ± 7.5	**OVARIES (%)**	**MEAN ± SD**	15.1 ± 7.9	10.6 ± 1.2	13.0 ± 2.2	14.5 ± 4.5
		K.W.=2.536 p=0.47; N.S.						**F(20.3)=1.080 p=0.38; N.S.**			
ADRENALS (%)	**MEAN ± SD**	4.3 ± 1.3	4.0 ± 0.7	3.9 ± 0.7	4.0 ± 0.8	**ADRENALS (%)**	**MEAN ± SD**	5.3 ± 0.7	5.3 ± 0.9	5.8 ± 2.0	5.2 ± 0.8
		F(20.3)=0.2124 p=0.89; N.S.						**F(20.3)=0.3390 p=0.80; N.S.**			

F: Statistics ANOVA test; K.W: Kruskal-Wallis test; N.S.: Not Significant. The significance levels observed is ***p<0.001 in comparison to control group values.