In vivo genotoxicity evaluation of Cylindrospermopsin in rats using a combined micronucleus and comet assay

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Abstract

Cylindrospermopsin (CYN) is a potent cyanotoxin recognized as an emerging human threat due to its cytotoxicity and potential carcinogenicity. Although the genotoxicity of CYN has been extensively studied *in vitro*, limited data are available on its *in vivo* genotoxicity. The aim of this study was to evaluate the *in vivo* genotoxicity of pure CYN (7.5-75 µg/kg body weight) after oral exposure of rats through a combined assay of the micronucleus test (MN) in bone marrow, and the standard and modified comet assay in stomach, liver and blood. Also, histopathological changes in stomach and liver were evaluated. Positive results in the MN test were observed in bone marrow in the exposed rats at all the tested concentrations. However, the comet assay revealed that CYN did not induce DNA strand breaks nor oxidative DNA damage in any of the tissues investigated. Finally, histopathological changes were observed in stomach and liver (7.5-75 µg/kg) in intoxicated rats. These results could indicate that CYN is able to induce irritation in stomach before its biotransformation in rats orally exposed, and genotoxicity in bone marrow.

Keywords: Cylindrospermopsin, genotoxicity, rats, Micronucleus, Comet assay, oxidative DNA damage

1. Introduction

The cyanobacterial polyketide-derived alkaloid toxin - cylindrospermopsin (CYN) - is one of the most active cyanotoxins which can adversely affect humans by different pathways. Dermal contact with CYN may occur during showering, bathing or during recreational activities (Pichardo et al., 2017); moreover, the main human exposure route is through ingestion of contaminated drinking water or dietary intake via consumption of cyanotoxins in contaminated food as fish, vegetables and algal dietary supplements (Testai et al., 2016). It has general cytotoxic activity, and in mammals can cause liver, kidney, thymus, and hearth damage (Pearson et al., 2010; Moreira et al., 2012). Moreover, CYN is known to induce neurotoxic (Hinojosa et al., 2019) and pro-genotoxic effects (Zegura et al., 2011; Puerto et al., 2018). It is considered a potential carcinogen (Falconer and Humpage et al., 2001). This cyanotoxin is gaining importance as its occurrence and expansion are increasing, in line with the CYN-producing species. Thus, CYN is considered as an emerging toxin (Poniedzialek et al., 2012; Pichardo et al., 2017; Buratti et al., 2017). This cyanotoxin is produced by several cyanobacterial genera, such as *Cylindrospermopsis, Aphanizomenon, Umezakia, Chrisosporum,* and *Anabaena,* with *C. raciborskii* and *A. ovalisporum* being considered as the main CYN producers (Kokocinski et al., 2017).

Cylindrospermopsin is a stable tricyclic alkaloid (415 Da) with a central functional guanidine moiety linked at C7 to a hydroxymethyluracil through a hydroxyl bridge (Ohtani et al., 1992). Some mechanisms of toxicity are linked to the presence of the uracil moiety (Banker et al., 2001; Shen et al., 2002). Although the genotoxicity of CYN is still controversial, the presence of the reactive guanidine and sulphate groups (Bain et al., 2008) suggested the possible interaction of CYN or its metabolites with nucleic acids. Actually, the mechanisms potentially involved in its genotoxicity are still under investigation (Testai et al., 2016; Hercog et al., 2017; Puerto et al., 2018).

Although no response on bacteria (Ames test) was detected in *in vitro* genotoxicity studies (Sieroslawaska, 2013; Puerto et al., 2018), several studies using various assays (reviewed by Zegura et al., 2011; Testai et al., 2016; Pichardo et al., 2017) indicated that CYN is pro-genotoxic and needs to be metabolically activated by cytochrome P-450 enzymes to become genotoxic (Bazin et al., 2010; Humpage et al., 2005; Straser et al., 2011). Straser et al. (2011) provided the first evidence that exposure to CYN induced transcription of *CYP1A1* and *1A2* isoforms, supporting the assumption that they are involved in CYN metabolic activation to electrophilic genotoxic intermediates. However, contradictory results from studies carried out to elucidate if CYN is a genotoxic or a pro-genotoxic compound were reported, depending on the experimental model (e.g. CYN-metabolizing capabilities of the cell lines or the model employed), the CYN concentrations assayed, and the type of assay used. Recently, Puerto et al. (2018) applied a battery of different *in vitro* assays following the recommendations of the European Food Safety Authority guidelines (EFSA, 2011), this is, the bacterial reverse mutation test (OECD 471) and the micronucleus (MN) test (OECD 487) to investigate the genotoxicity of pure CYN. Negative response was reported in the Ames test, whereas positive results were found only when the metabolic fraction S9 was employed in the MN test, in agreement with the global consideration of the pro-genotoxic activity of CYN in *in vitro* metabolically active mammalian cells (Zegura et al., 2011; Puerto et al., 2018). Further *in vitro* test tests were carried out, such as the mouse lymphoma thymidine-kinase assay (OECD 490) on L5178YT$k^{+/-}$ cells; and the comet assay on Caco-2 cells , including the standard and enzyme-modified versions (Puerto et al., 2018). Negative results were found in both assays (with and without metabolic activation) (Puerto et al., 218), whereas some *in vitro* standard comet assay studies indicated that CYN required metabolic activation to exhibit its genotoxic activity (Humpage et al., 2005; Straser et al., 2011, 2013; Zegura et al., 2011).

Globally, the genotoxicity of CYN is still controversial, and EFSA recommended new studies to elucidate the mechanisms involved (Testai et al., 2016). In addition, the previous *in vivo* studies are very scarce. In mice, CYN or its metabolites were able to bind to proteins and

DNA forming adducts in the liver (Shaw et al., 2000; Norris et al., 2001). Increased DNA fragmentation was also reported in the liver of mice treated by intraperitoneal injection (i.p.) (Shen et al., 2002), Bazin et al. (2012) demonstrated in mice that CYN administered either i.p. or orally induced primary DNA damage in the colon as well as cell death in the liver and kidneys. After oral dosing, it was also able to induce damage in bone marrow. However, the treatment doses were quite high and limited the validity of the study (Testai et al., 2016). Genotoxic effects were reported after the application of the comet assay and MN test on the liver and blood of mice treated i.p. with water contaminated with cellular content of *C. raciborskii* and with the products resulted from its chlorination (Fonseca et al., 2014). Recently, DNA damage in the liver of rats was reported using the alkaline comet assay after a single i.p. administration of *C. raciborskii* extract at different doses, and pure toxin (Dordevic et al., 2017).The combination of the comet assay with bacterial repair enzymes recognizing specific DNA damage, such as endonuclease III (Endo III, which responds to oxidized pyrimidines), and formamidopyrimidine DNA glycosylase (Fpg, which recognizes altered purine bases including 8-oxo-guanine) increases both the sensitivity of the assay in terms of the ability to detect a wider range of damage, and also its specificity (Azqueta et al., 2009). Despite the oxidative stress is involved in the toxicity mechanisms of CYN (Guzmán-Guillén et al., 2013) it has been not elucidated whether this mechanism contributes to CYN genotoxicity, as negative results have been obtained when the enzyme-modified comet assay has been applied *in vitro* (Straser et al., 2013; Puerto et al., 2018), although this assay has not been performed *in vivo* to date. Thus, new *in vivo* genotoxicity studies of CYN should be performed following the recommendations of EFSA because of the inconclusive and contradictory results obtained previously (EFSA, 2011; Testai et al., 2016). Furthermore, as far as we know, no *in vivo* studies on pure CYN genotoxicity were performed in rats exposed by oral route. The oral genotoxic potential of CYN is a topic worth of research because oral ingestion is one of the main human exposure routes to cyanotoxins, and the importance of genotoxicity in the risk assessment process.

The mechanisms of CYN genotoxicity and carcinogenicity are not totally elucidated. It seems that oxidative DNA damage could be involved (Sieroslawska and Rymuszka, 2015; Guzmán-Guillen et al., 2013a,b), although in some studies the DNA damage may not be induced by oxidative stress (Humpage et al., 2005; Gutiérrez-Praena et al., 2011; Straser et al., 2013; Puerto et al., 2018). Moreover, CYN modulated the expression of some DNA damage response genes, such as levels of *P53* regulated genes related to cell cycle arrest, DNA damage repair and apoptosis (Zegura et al., 2011). *In vitro*, at molecular level, CYN changed the expression of genes involved in xenobiotic metabolism, in immediate-early response/signalling, and DNA damage response (Hercog et al., 2017).

Considering all these facts and following the recommendations of EFSA, the aim of the present work was to investigate *in vivo*, for the first time in rats as experimental model, the potential genotoxic effects induced after oral administration (gavage) of pure CYN standard, by application of a combined MN-comet assay. Moreover, the comet assay has been combined with Endo III and Fpg enzymes. The selected target tissues were bone marrow for MN test (OECD 474, 2016a), and the liver, stomach (OECD 489, 2016b) and blood cells for the standard and enzyme-modified comet assay (endonuclease III, Endo III, E.C. 3.1.21.5 and formamidopyrimidine DNA glycosylase, Fpg, E.C. 3.2.2.23). The potential histopathological changes were also investigated in stomach and liver.

2. Materials and methods

2.1. *Supplies and chemicals*

Cylindrospermopsin standard (purity 95%) was purchased from Alexis Corporation (Lausen, Switzerland). All chemicals reagents were purchased from Sigma-Aldrich (Madrid, Spain), C-viral S.L. (Seville, Spain), Gibco (Biomol, Seville, Spain) and Moltox (Trinova, Biochem, Germany).

2.2. Animal hosting and nourishing conditions

The *in vivo* experiments were authorized by the Ethics Committee on Animal Experimentation of the University of Sevilla (09/03/2016/028). Moreover, all animals received humane care for the protection of animals utilized for scientific purposes in concordance with the Directive 2010/63/UE.

Seven-week-old male Wistar rats, strain RjHan:WI (type outbred rats) were provided by the Centre for Animal Production and Experimentation from the University of Sevilla (Espartinas, Spain). After arrival, the animals were weighed and accommodated into polycarbonate cages with stainless steel covers. Then, the animals were acclimatised to the environmental conditions (12-h dark/light cycle, controlled temperature (23 ± 1 ºC), relative humidity (55 ± 10%) for 1 week before the experiment. During this time, the animals were fed with standard laboratory diet (Harlan, 2014; Harlan Laboratories, Barcelona, Spain) and water *ad libitum.*

2.3. Experimental design and treatment

The exposure dose was selected according to previous studies (Chernoff et al. 2018) in mice, in which slight signs of damage were observed for 75 µg CYN/kg body weight (bw) after 90 d oral exposure; and in wistar rats, in which genotoxicity was observed for 79.80 µg CYN/kg body weight (bw) after i.p injection (Dordevic et al., 2017). The International Conference on Harmonisation (ICH) S2 guidelines and the MN and Comet assay protocols (OECD 474 and 489, respectively) recommend the use of a maximum dose and 2 additional lower doses (Bowen et al., 2011) appropriately spaced by less than √10 (OECD 489, 2016b). Thus the doses were established at 75, 23.7 and 7.5 µg CYN/kg bw.

Takin into account that the OECD guideline 474 (OECD, 2016a) recommends to use 5 animals per group and the OECD 489 (OECD, 2016b), allows to use 3 animal for positive controls, after acclimation for 1 week, 23 male rats were weighted in order to ensure that weight variation did not exceed ± 20 % and randomly divided into 5 groups: 1 negative control group (C-) (5 male rats) treated with water (vehicle); 1 positive control group (C+) (3 male rats)

exposed to 200 mg/kg bw ethylmethanesulfonate (EMS) and 3 exposed groups (5 male rats per group) treated with 75; 23.7 or 7.5 µg CYN/kg bw. All doses were prepared in water in a final volume of 1 mL. Animals were dosed by gavage using an enteral feeding tube (Vygon, Ecouen, France) at 0; 24 and 45 h in agreement to the recommendations of Bowen et al. (2011). The animals were sacrificed 3 h after the final dose administration for combined comet and MN endpoints, following the recommendations of Bowen et al., (2011). During the treatment period, clinical signs and body weight were recorded.

2.4. Sample collection

Selected tissues were removed, dissected, rinsed with cold saline solution and weighed. After weight, stomach and liver were quickly processed for the comet assay as is described in section 2.6. Moreover, blood samples were collected and maintained in Vacutainer® sodium heparin tubes (Becton Dickinson, Rutherford, NJ). Samples for MN assay were collected from the bone marrow of both femurs of each animal and immediately processed. For the histopathological study, a portion of stomach and liver were collected according to Mellado-Garcia et al., (2016) and Llana-Ruiz-Cabello et al., (2016).

2.5 MN assay

MN test was conducted following the recommendations of OECD guideline 474 (OECD, 2016a) and Corcuera et al. (2015). Two slides, 1 per femur of each animal, were prepared with bone marrow cells re-suspended in a drop of foetal bovine serum. Then, these slides were fixed in absolute methanol, air dried and stained with 10% Giemsa. The polychromatic erythrocytes (PCE) among total erythrocytes (normochromatic erythrocytes (NCE) + (PCE)) ratio and the PCE among NCE ratio were calculated by counting 500 erythrocytes per animal. The incidence of micronucleated immature erythrocytes (MNPCE) was

calculated by counting a total of 5000 PCE per animal and results were expressed as % MN, following Llana-Ruiz-Cabello et al., (2016).

2.6. Isolation of single-cell suspensions for the comet assay

Single cell suspensions from stomach and liver were isolated based on the methods of Corcuera et al. (2015) and Mellado-García et al. (2016). Briefly, tissues were washed with Merchant´s buffer (MB) (0.14 M NaCl, 1.47 nM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 7.4) and discarded. After that a portion of each tissue were homogenized in cold. Finally, the homogenates were centrifuged, filtered and mixed with 5mL buffer until slide preparation. Heparinized blood samples were mixed v/v (1/1) with phosphate buffered saline (PBS) solution and the lymphocytes were isolated with Histopaque® (Sigma-Aldrich, Madrid, Spain) and centrifuged (400 G, 30 min) (Llana-Ruiz-Cabello et al., 2018). Finally, the cells were washed twice with PBS and re-suspended in PBS at a concentration of 2 x 10^5 cells/mL.

2.7. Standard and enzyme-modified comet assay

The standard comet assay has been performed according to the recommendations of OECD guideline 489 (OECD, 2016b). For blood samples, 30 µL of cells suspension was mixed with 140 µL of 0.5% low-melting point agarose. Twelve 5 µL aliquots were placed on a microscope slide (Llana-Ruiz-Cabello et al., 2018). For stomach and liver, the cells suspensions were mixed with 1% low-melting point agarose and the mixtures were placed on a microscope slide similar to blood samples (Mellado-Garcia et al., 2016; Llana-Ruiz-Cabello et al., 2018). Then, the standard and modified comet assays were performed as previously described by Mellado-García et al. (2016). Briefly, after lysis, slides were washed 3 times for 5 min with enzyme buffer (40 mM HEPES; 0.1M KCl; 0.5 mM EDTA; 0.2 mg/mL bovine serum albumin; pH 8). Then, 2 gels in each slide were exposed to 30 µL lysis solution; enzyme buffer alone (buffer F); buffer F containing Fpg or Endo III during 30 min in a metal box at 37 ºC.

Afterwards, nuclei were denatured, and electrophoresis was performed for 20 min, 0.81 V/cm up to 400 mA. DNA was neutralized in PBS, washed with water and fixed with 70% and absolute ethanol before staining. Images of at least 150 randomly selected nuclei per animal were analysed with the image analysis software Comet Assay IV (Perceptive Instruments, UK). The % DNA in tail represents DNA strand breaks and oxidized damage in DNA bases. Endo III and Fpg sensitive sites were calculated by subtracting the % of DNA in tail after enzyme buffer incubation from the % of DNA in tail after repair enzymes incubation.

2.8. Histopathogical analysis

Tissue samples for histological analysis were taken from stomach and liver of control and exposed rats. For light microscopy (LM), samples were fixed in 10% formaldehyde for at least 24 h at 4 ºC and then dehydrated in ethanol, immersed in xylol and embedded in paraffin wax using an automatic processor. Tissue sections of 3-5 mm were deparaffinised followed by rehydration, stained with haematoxylin and eosin (HE), and mounted with Crystal/Mount (Paraplast, Oxford Labware, St. Louis, MO). Then, liver samples were stained for glycogen content assessment with periodic acid Schiff (PAS) (Sigma-Aldrich, Madrid, Spain). For electron microscopy (EM), samples were prefixed in 2% glutaraldehyde at least for 10 h at 4 ºC. Then, postfixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Epon. Sections of 50-60 nm were cut with an LKT microtome and were mounted on a copper grid and stained with uranyl acetate and lead citrate. The tissue sections were examined in a Philips CM10 electron microscope (FEI. Eindhoven, The Netherlands).

2.9. Statistical analyses

The results of MN test are presented as mean ± SD, the analysis of variance (ANOVA) was performed followed by Dunnett's multiple comparison test. For the standard and enzyme modified comet assays, mean ± SD of the medians were calculated for each group. The

distribution of the data was checked for normality using the Kolmogorov-Smirnov test and total scores of the different groups were compared using the non-parametric Kruskal Wallis test followed by Dunn´s multiple comparison test. The Analyses were performed using Graph-Pad InStat software (Graph- PadSoftware Inc., La Jolla, USA).

3. Results

3.1 Micronucleus assay

Significant differences in the PCE/total erythrocytes and PCE/NCE ratios were observed in male rats exposed to the highest concentration assayed of CYN (75 µg/kg bw; $p<0.01$), recorded 3 h after the triple dose of CYN was administrated by gavage (Table 1). The positive control, EMS, induced similar significant decreases. Moreover, all treated groups, at every tested CYN dose, exhibited significant increases in the percentage of MN in immature erythrocytes, when compared with the negative control group. There were no dose-dependent differences in any treatment.

3.2 Standard and enzyme-modified comet assay

Results in the standard comet assay showed that CYN did not induce DNA strand breaks in the liver, stomach or blood cells at any dose assayed (Figure 1). Moreover, no differences in the % of DNA in tail were observed in any tissues exposed to 7.5-75 µg/kg bw CYN and post-treated with Endo-III (Figure 2). In addition, the effects of CYN on liver, stomach and blood were analysed with Fpg post-exposure (Figure 3) and there were no differences observed in the frequency of Fpg-sensitive sites among samples from the exposed groups and negative control group in liver, stomach and blood cells.

Positive control group exposed to EMS exhibited DNA damage in liver ($p<0.001$), stomach ($p<0.001$) and blood ($p<0.001$) cells in all the experiments (Figures 1, 2 and 3, respectively).

3.3 Clinical and Histopathological study

No rats died during the experiment and no signs of toxicity were observed during the treatment period. In the present work, although no clinical signs of toxicity were recorded in any of the groups, significant altered gastric mucus secretion after oral administration of pure CYN was observed. Body weight changes were no observed in any treated animals. Moreover, the relative weight (RW) of stomachs and livers were similar in all the treated animals, and no significant differences were found in comparison to the negative control group (data not shown). Histopathological results from rats orally exposed to CYN displayed significant differences in stomach and liver morphology compared to the negative control group. The morphological study of stomachs from control and exposed rats are shown in Figure 4. In general, under LM and EM normal gastric mucosa was observed in the control group (Fig 4A, B). However, mucosal damage was observed in the CYN treated rats (Fig 4 E-J). Specifically, the gastric mucosa exhibited significant reactive changes in surface foveolar cells with dilated ducts that increased with the administered CYN concentration (Fig 4H, I). Moreover, a partial degranulation of surface and deep mucosa was observed in animals exposed to low doses (7.5 and 23.7 µg/kg bw CYN) (Fig 4J, G), which was total at the highest dose (75 µg/kg bw CYN) and in the positive control (EMS) (Fig 4D, F). The mucous, the gastric defensive element, and mucous cells exhibited degenerative alterations. The mucous content of surface epithelial cells was decreased at 23.7 and 75 µg/kg bw CYN (Fig 4E, G). Finally, rats treated with EMS exhibited an increase in mucosal cells and dilation of the mucosa surface (Fig 4C).

A normal hepatic parenchyma was observed in liver both under LM and EM, for unexposed rats (Fig 5A, B). However, rats exposed to CYN exhibited significant changes. Firstly, some modifications in the nuclei structure were observed in treated groups. An increase in endomitosis with the production of binucleated hepatocytes was seen for all the doses assayed (Fig 5G, I, K, M, N). Moreover, animals exposed to the highest dose (75 µg/kg bw CYN) showed a high mitotic frequency (Fig 5I, J). These alterations were observed in the positive control group as well (Fig 5C, E, F). Secondly, the liver of the rats from the treated groups showed proliferation of smooth endoplasmic reticulum (SER) with dilation of endoplasmic reticulum vesicles (Fig 5H, L) similarly to the positive control group (Fig 5D).

4. Discussion

The interest in the assessment of the genetic toxicity of CYN is increasing due to its potential carcinogenic action. Although globally this cyanotoxin is considered as pro-genotoxic, the *in vitro* toxicological assays performed until now revealed contradictory results of its potential genotoxicity and mechanisms of action. Furthermore, the *in vivo* genotoxicity studies are very scarce; they are carried out mainly in mice, and some are not following the international protocols, such as the OECD guidelines.

It is speculated that CYN or its metabolites may exert toxic effects through DNA and/or RNA reactions because of its structural characteristic (its nucleoside structure and the presence of potentially reactive guanidine and sulphate groups) (Shen et al., 2002). Some authors proposed different mechanisms of action for CYN genotoxicity, such as: induction of DNA strand breaks (Shen et al., 2002; Humpage et al., 2005; Dordevic et al., 2017); formation of covalent bindings between DNA and CYN, or DNA and CYN metabolites (Shaw et al., 2000); and formation of micronuclei containing lagging chromosome fragments or whole chromosomes (Humpage et al., 2000, Sieroslawaska and Rymuszka, 2015).

To improve *in vivo* genotoxicity assessment of xenobiotics in general, it is internationally considered that the combination/integration of the *in vivo* MN and Comet assay into a single study is scientifically justified (Rothfuss et al., 2011). In this sense, this is the first study which evaluated CYN *in vivo* genotoxic response in rats, exposed to CYN by oral route (gavage), combining the comet assay on cells isolated from stomach, liver and blood , and the MN test in bone marrow cells, following the OECD 489 and 474 guidelines (OECD, 2016b, a, respectively) with modifications described below. The combined assay, proposed by Bowen et al. (2011), has the advantage of reducing animal usage according to the 3Rs principles (Replace, Reduce and Refine), as it is also recommended by the OECD protocols (OECD, 2016b). Besides, it improves the sensitivity and specificity of the detection, and decreases the number of false negative results (Mughal et al., 2010; Kirkland et al., 2019). Moreover, the novelty of the current work is the use of DNA repair enzymes (Endo III and Fpg) which increases the

sensitivity of the *in vivo* comet assay by allowing the detection of potential oxidative DNA damage before the DNA break occurs (Azqueta et al., 2009; Llana-Ruiz-Cabello et al., 2016; Mellado-Garcia et al., 2016).

Regarding the experimental procedure, the OECD guidelines 474 (OECD, 2016a) and 489 (OECD, 2016b) recommends to use both sexes when relevant differences in toxicity were described. In this sense, although there are no studies in rats, Chernoff et al. (2018) reported that CYN induce more toxicity in male mice than in females. Therefore, the present study was carried out only in males, the more sensitive sex, in order to simplify and ensure the success of this complicated technique. Moreover, to determine the highest exposure dose employed in the present study, suitable data were studied. Although, the maximum tolerated dose (MTD), which is the highest dose recommended by OECD protocols has been not formerly stablished, previous studies regarding toxicity in rodents have been reported. In mice, Humpage and Falconer (2002) indicated that the no-observed-adverse-effect level (NOAEL) for CYN in mice was 30 µg CYN/kg bw. Some years later, Chernoff et al. (2018) tried to re-evaluate the NOAEL by increasing exposure concentration (75-300 µg CYN/kg bw) and reported toxicity in mice after 90 d oral exposure in all the concentration range assayed. In wistar rats, after i.p injection of 79.80 µg CYN/kg bw genotoxicity was observed (Dordevic et al., 2017). Hence, in order to reduce animal use, the exposure dose was selected according to these previous studies in which slight signs of damage were observed for 75 µg CYN/kg bw after 90 d oral exposure in mice (Chernoff et al. 2018), and genotoxicity in wistar rats after i.p injection of 79.80 µg CYN/kg bw (Dordevic et al., 2017). Once the highest dose was selected taking into account the available data on the scientific literature, the additional lower doses were calculated following the recommendations of OECD (OECD, 2016a, b).

The significant decreases in the PCE/total and PCE/NCE ratios observed in the rats exposed to the highest dose of CYN is a sign of bone marrow toxicity showing the exposure of this tissue (e.g. evidence of the exposure of the bone marrow to a test substance may include a depression of the immature to mature erythrocyte ratio, PCE/NCE (OECD, 2016a)). The induction of MN in bone marrow cells of all CYN-exposed rats by oral route was independent

of treatment concentrations. In this regard, the apparent inverse dose-response observed in the percentage of MN, where the animals exposed to the lowest dose of CYN presented the highest value in percentage of MN, was not statistically significant when the data from all doses assayed were pooled. This positive result in MN induction is in contrast with the negative results reported in colonic cells of mice 24 h after an oral administration of CYN (1-4 mg/kg bw) and in bone marrow after i.p. administration (Bazin et al., 2012). It should be noted that these discrepancies in response could be explained by the differences in the experimental model (mice/rat); the doses applied and time of response; and general differences in the followed protocols. The observed *in vivo* induction of MN by CYN in rats confirms the *in vitro* genotoxic activity reported previously (Humpage et al., 2000, Sieroslawaska and Rymuszka, 2015). Previous studies reported that metabolic transformation of CYN is crucial for induction of toxicity and genotoxicity (Humpage et al., 2005; Fessard and Bernard, 2003; Bain et al., 2008). Moreover, according to Straser et al. (2011) and Hercog et al. (2017) CYN induced the up-regulation of CYP genes *CYP1A1* and *CYP1A2* providing evidence that these enzymes are involved in the metabolic activation of the toxin. The xenobiotic-metabolising system comprises several hundred enzymes, which are usually expressed with high selectivity in varying tissues, especially in the liver of mammals. The CYP enzyme system ranks first of all the xenobiotic-biotransforming enzymes, in catalytic versatility, involvement in the activation of CYN; and levels in liver endoplasmic reticulum (Parkinson et al., 2013). Therefore, it would be responsible of the activation of pure CYN after *in vivo* oral administration in rats. In this regard, no significant variations were found in the frequency of MN in binucleated cells (BNMN) in undifferentiated human hepatoma cell line HepaRG cells (Bazin et al., 2010), nor in absence of exogenous metabolic activation S9 fraction in L5178YTk $^{+/-}$ cells (Puerto et al., 2018). Significant increments of BNMN frequency were observed, however, in differentiated cells (with CYN-metabolising capability) (Bazin et a., 2010), or in the presence of S9 fraction (Puerto et al., 2018).

The comet assay is an acceptable choice to confirm *in vitro* gene mutation activity in terms of general genotoxicity. It is considered an adequate assay to detect potent genotoxicants

in liver and gastro intestinal (GI) tract, with liver, stomach and blood being the most widely studied tissues (Kirkland et al., 2019). In the case of CYN, only 2 *in vivo* studies applied the alkaline comet assay in mice, reporting positive results. Thus, CYN induced DNA strand breakage in the liver after a single i.p. dose of 0.2 mg/kg bw (Shen et al., 2002), and in the colon after i.p. injection of 50-200 µg CYN/kg bw (Bazin et al., 2012). However, this administration is not representative of the usual oral exposure to CYN as the route of administration is one of the most important factors affecting the results of *in vivo* genotoxicity tests (Sasaki et al., 1998). The comet assay was also assayed in mice exposed by gavage to CYN (1-4 mg/kg bw) and damage was observed in colon and bone marrow samples (Bazin et al., 2012).

In the present study, the alkaline comet assay revealed that CYN did not induce DNA strand breaks in any of the tested tissues (liver, stomach, and blood) at the doses and conditions assayed, even when histopathological changes evidenced exposure of these tissues. In contrast, CYN induced simple DNA breaks in the liver of mice exposed i.p. to pure CYN (Shen et al., 2002) or to extracts of *C. raciborskii* (Fonseca et al., 2014) and in rats injected i.p. with CYN (Dordević et al., 2017).

DNA oxidation might be a mechanism of CYN-produced genetic damage in mammals. This damage is mainly induced by reactive oxygen species (ROS) after toxin or xenobiotic exposure when elevated levels of ROS or depressed antioxidant defences may lead to an increase in the steady-state level of unrepaired cellular DNA damage (Azqueta et al., 2009). Oxidative stress is one of the toxic mechanisms proposed responsible for CYN *in vitro* toxicity (as reviewed by Pichardo et al., 2017), and *in vivo* in fish (Puerto et al., 2011; Guzman-Guillén et al., 2013a,b). Increased DNA oxidation was demonstrated *in vivo* in tilapia exposed to subchronic and low doses of CYN-producing *A. ovalisporum* cells (Guzmán-Guillen et al., 2013a), with no changes induced by the pure toxin (Gutierrez-Praena et al., 2011; Guzman-Guillen et al., 2013b, 2015). However, in the present study, the modified comet assay revealed, for the first time, no oxidative damage induced by CYN in pyrimidine or purine bases of the DNA (Endo III or Fpg, respectively) of hepatocytes, stomach and blood cells from rats exposed to any of the concentration assayed (7.5-75 µg CYN/kg bw). In the same way, the scarcely

applied *in vitro* enzyme-modified comet assay did not show CYN-induced oxidative DNA damage (Straser et al., 2013; Puerto et al., 2018). Further *in vivo* studies in mammals are needed to elucidate the role of oxidative stress in the induced DNA damage.

Although histopathology was generally not used to evaluate cytotoxicity in comet studies (Kirkland et al., 2019), the OECD recommends to evaluate histopathological changes associated with genotoxicity such as: cell infiltration; apoptotic or necrotic changes and inflammation (OECD 2016b). In the present work, although no clinical signs of toxicity were recorded in any of the groups, significantly altered gastric mucus secretion after oral administration of pure CYN was observed for the first time to our knowledge. Moreover, other authors reported apoptosis of lymphocytes in the Peyer´s patches in the sections of duodenum and jejunum of mice exposed orally to 2 and 4 mg/kg bw CYN (Bazin et al., 2012). Foveolar cells are mucus-producing cells which cover the inside of the stomach, protecting it from the gastric acid. The lesions observed in the stomach of the exposed rats could be related to a possibly irritant effect of the toxin because the gastric mucus is the first line of defence against luminal irritants (Iwabuchi et al., 2013). In fact, Seawright et al. (1999) reported that freeze-dried cultures of *C. raciborskii* can induce this damage in mice exposed to 2.5 to 8.5 mg/kg bw of CYN equiv. These results and the absence of genotoxicity in stomach cells reinforce the complex metabolism of CYN, which seems to need bioactivation to exhibit genotoxic activity (Zegura et al., 2011; Puerto et al., 2018).

The liver is considered the primary organ in the biotransformation of CYN (Dordevic et al., 2017), and CYP450-derived metabolites of this toxin were responsible for both the acute cytotoxicity and genotoxicity (by comet assay) observed *in vivo* in mice (Humpage et al., 2005). Following this hypothesis, some differences in toxicity should be observed towards other organs where biotransformation has not yet occurred (such as the stomach) by the time CYN reaches the liver. In the present work, some histopathological changes observed in the liver cells of intoxicated rats could be related with its genotoxicity and with tumour development. In this regard, the changes observed in the liver cytoplasmic organoids of rats exposed to CYN (such as proliferation of RER and SER, or vacuolization) are in agreement with previous results

reported for rats exposed i.p. to CYN (Dordević et al., 2017), and to *C. raciborskii* extracts i.p or by gavage (Falconer et al., 1999). In general, the liver changes in the present work are smaller than those reported previously in mice exposed to *C. raciborskii* extracts (Hawkins et al., 1985) or in fish exposed to a single dose of CYN by gavage (200 or 400 µg CYN/kg bw) (Puerto et al., 2014). Humpage and Falconer (2002) showed that hepatocyte injury was more evident in mice exposed orally to CYN concentrations above 120 µg/kg bw/day, with an increase in the presence of massive necrosis or inflammation processes. Hence, some authors reported no histopathological damage after an exposure of up to 3 µg/kg bw CYN in rats (Almeida et al., 2013). In general, the histopathological changes reported in this study could be related with degradation processes induced by xenobiotics (Agellon and Michalak, 2017). The dilation observed in SER could indicate toxicity induced in the smooth surface elements, vulnerable to hepatotoxic agents (Richard and Stenger, 1970), like CYN, whose hepatotoxicity was previously demonstrated in several *in vivo* models, in mammals (Seawright et al., 1999; Falconer et al., 1999, 2001; Humpage and Falconer, 2003; Bazin et al., 2012; Dordevic et al., 2017) and in fish (Gutierrez-Praena et al., 2012; Puerto et al., 2014). In the present work, degenerative changes were observed at perilobular hepatocytes in rats exposed to low doses of CYN (7.5 or 23.7 µg/kg bw), while the highest dose (75 µg/kg bw) induced changes in both perilobular and centrillobular hepatocytes. In some cases, the severity of the lesions increases with the dose. Necrosis or apoptosis of hepatocytes of mice were reported after oral (2.5-8.3 mg CYN equivalents/kg bw) (Seawright et al., 1999), intraperitoneal (50 µg/kg bw CYN) (Chernoff et al., 2010), or oral subchronic exposure to CYN (Chernoff et al., 2018). Moreover, the presence of polyploid cells observed at all doses of CYN could be associated with abnormal cell division because of cellular stress (toxic exposure) or with tumour development (Gentric and Desdouets, 2014). High mitotic frequency was observed in animals exposed to the highest dose (75 µg/kg bw CYN). The proliferation of hepatocytes as a manifestation of toxicity may occur in 2 ways: regeneration to restore the lost liver mass, or induced hyperplasia (Pop and Cattley, 2013).

The present results add significantly to the current state of knowledge on the genotoxic effects of CYN. The number of *in vivo* studies on the topic remains, however, limited and further research is needed to confirm these findings and elucidate the role of oxidative stress in the induced DNA damage.

5. Conclusions

We can conclude that at the concentrations assayed CYN is able to induce genotoxicity in bone marrow, but not in liver, stomach and blood samples of wistar rats orally exposed in a combined MN-Comet assay applying OECD protocols. Moreover, the histopathological changes in stomach and liver suggest that metabolic transformation of CYN is needed to induce toxicity and genotoxicity. Therefore, further research is necessary to elucidate the toxicological profile and mechanisms of action of CYN as the number of *in vivo* studies on CYN genotoxicity evaluated by oral exposure are scarce.

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Figure captions

Figure 1: The level of DNA damage measured in stomach, liver and blood of male rats exposed to CYN as the formation of strand breaks (SBs) by the standard comet assay. The levels of DNA strand breaks are expressed as % DNA in tail. All values are expressed as mean ± SE. ***(P<0.001) Significantly different from control.

Figure 2: The level of DNA damage measured in stomach, liver and blood of male rats exposed to CYN as the formation of oxidative DNA damage as Endo III-sensitive sites. The levels of oxidised pyrimidines are expressed as % DNA in tail. All values are expressed as mean ± SE. *** (P<0.001) Significantly different from control.

Figure 3: The level of DNA damage measured in stomach, liver and blood of male rats exposed to CYN as the formation of oxidative DNA damage as Fpg-sensitive sites. The levels of oxidised purines are expressed as % DNA in tail. All values are expressed as mean ± SE. ***(P<0.001) Significantly different from control.

Figure 4. Histopathological changes in stomach of rats treated with CYN. (A, C, E, G, I) HE stained stomach section. Bars, 100 µm. (B, D, F, H, J) Ultrastructural observations. Bars, 5, 10, 2, 2, 5 µm, respectively. (A, B) Negative control rats. (A) Details of gastric mucosa with normal gastric cells. (B) Details of normal mucosa cell. (C, D) Positive control rats exposed to EMS. (C) Details of the increase of mucosa cells (circle) and dilation of secretory ducts (arrows). (D) Mucosa cells degranulation (circle). (E, F) Rats treated with 75 µg/kg bw CYN. (E) Details of gastric mucosa with a decrease in mucous cells (circle). (F) Hyalinization of mucous cells (circle). (G, H) Rats treated with 23.7 µg/kg bw CYN. (G) Degenerative changes in the superficial mucosa. (H) Dilation of intracellular canaliculi of gastric parietal cells (circle). (I, J) Rats treated with 7.5 µg/kg bw CYN. (I) Excretory ducts dilated and full of mucosal substance. (J) Mucosa cells degranulation (circle).

Figure 5. Histopathological changes in liver of rats treated with CYN. (A, C, E, G, I, K, M) HE stained stomach section. Bars, 50, 50, 50, 100, 100, 100, 100 µm, respectively. (B, D, F,

H, J, L, N) Ultrastructural observations. Bars, 2, 3, 3, 5, 2, 2, 10 µm, respectively. (A, B) Negative control rats. (A) Normal hepatic parenchyma. (B) Details of apparently normal hepatocytes. (C, D, E, F) Positive control rats exposed to EMS. (C) Details of hepatic cordons with binucleated hepatocytes (circle). (D) Details of dilated rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER). (E) Details of polyploids hepatocytes and mitosis (arrow). (F) Details of chromosomes from mitosis (arrow). (G, H, I, J) Rats treated with 75 µg/kg bw CYN. (G) Details of liver parenchyma with polyploids (arrow) and binucleated (circle) hepatocytes. (H) Details of dilated smooth endoplasmic reticulum (circle). (I) Details of liver parenchyma with polyploids and binucleated hepatocytes in mitosis (circle). (J) Details of chromosomes from mitosis (arrow). (K, L) Rats treated with 23.7 µg/kg bw CYN. (K) Details of liver parenchyma with polyploids (arrow) and binucleated (circle) hepatocytes. (L) Details of perilobular hepatocytes with dilated smooth endoplasmic reticulum (circle). (M, N) Rats treated with 7.5 µg/kg bw CYN. (M) Details of liver parenchyma with polyploids (arrow) and binucleated hepatocytes (circle). (N) Hyperplasia of perilobular hepatocytes (circle).

Table caption

Table 1: Micronucleus assay results. Bone marrow cytotoxicity expressed as polychromatic erythrocytes (PCE) among total erythrocytes (normochromatic erythrocytes (NCE) + PCE), ratio PCE among NCE and the micronuclei induction expressed as % MN. All values are expressed as mean ± SD. The significant levels observed are *p<0.05, ** p<0.01, or ***p<0.001.

Figure 1

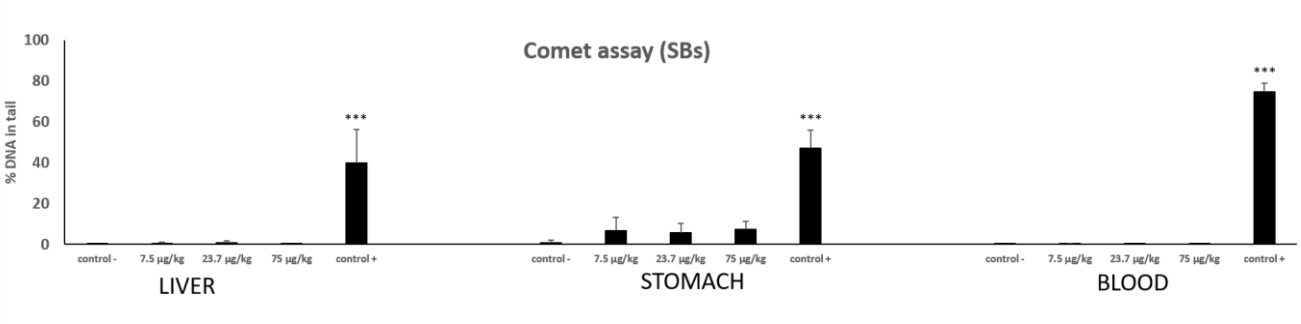

Figure 2

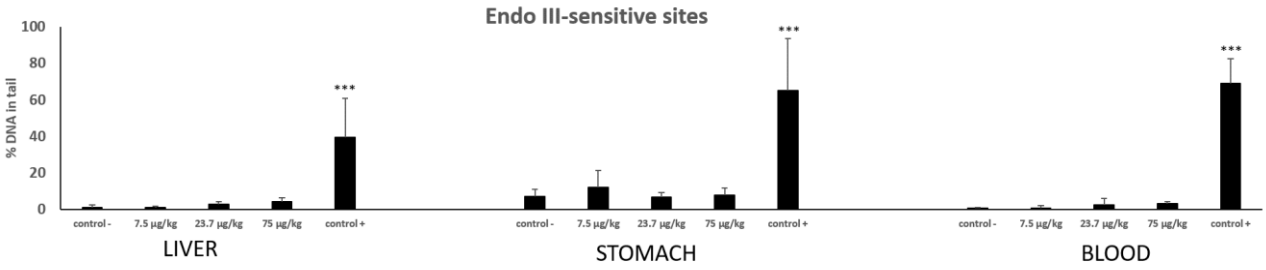

Figure 3

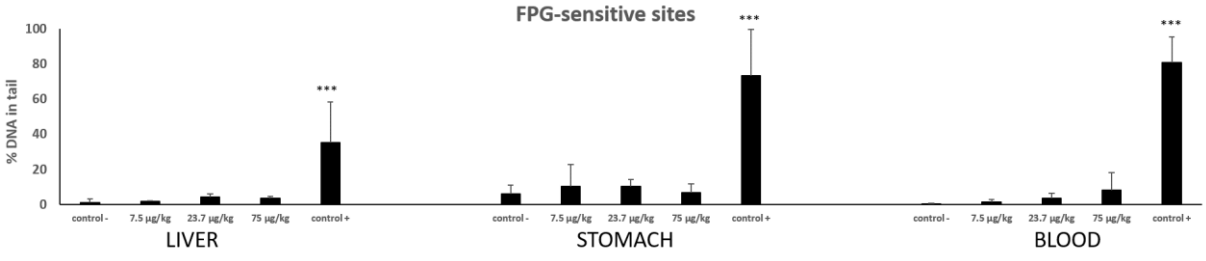

Figure 4

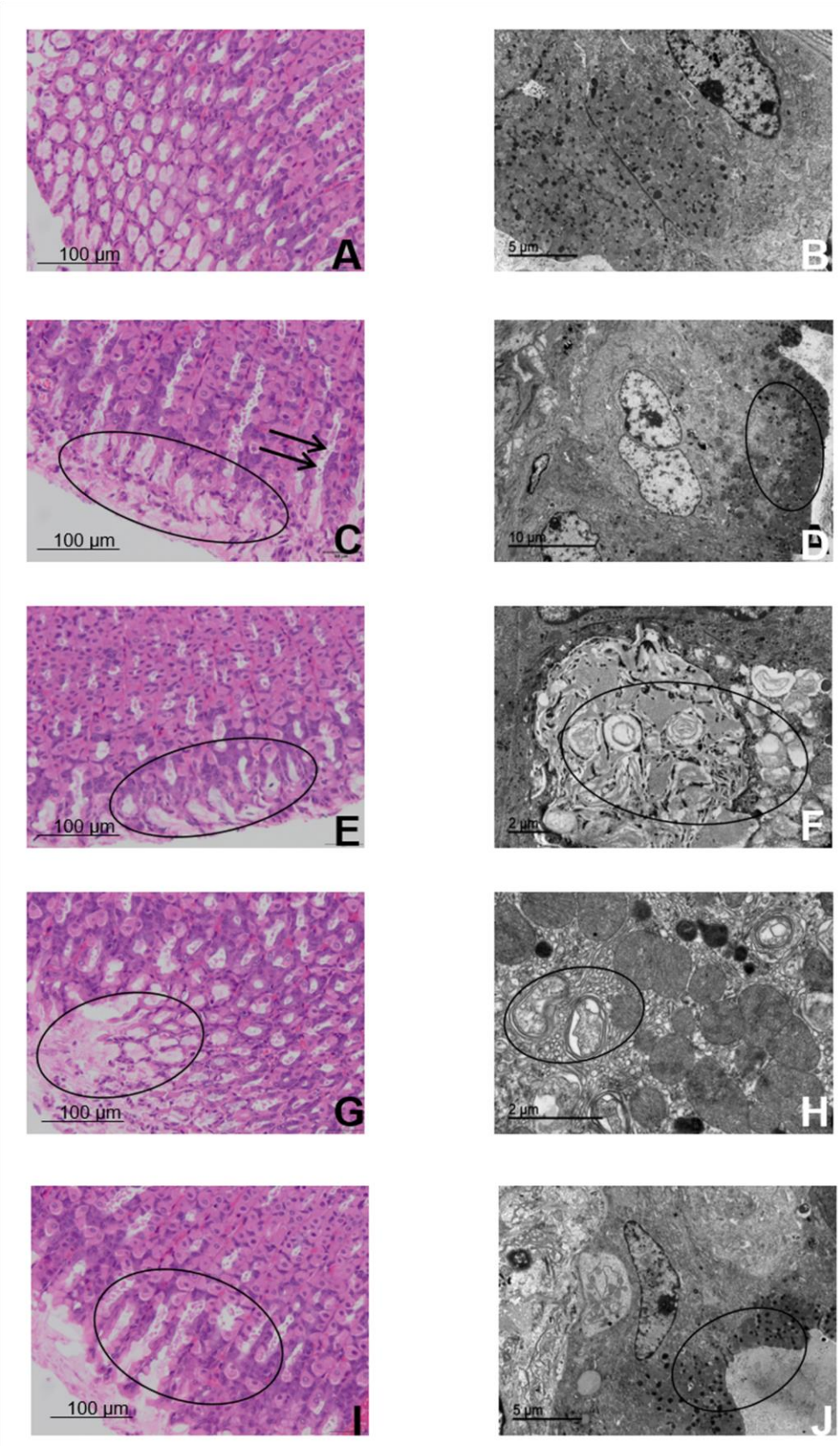
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Figure 5

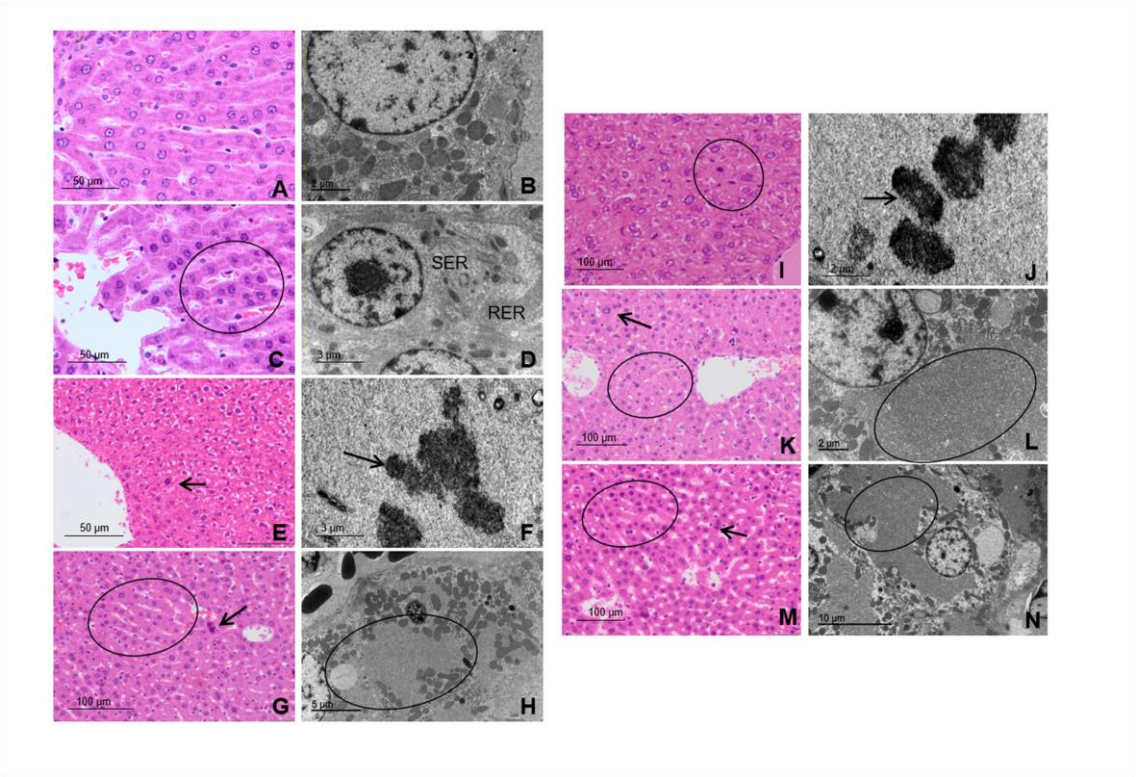

Table 1

Groups	N	Dose	PCE/Total	% MN	PCE/NCE
Negative Control	5	-	0.50 ± 0.02	0.19 ± 0.17	0.98 ±0.01
Positive Control (EMS)	3	200 mg/Kg	0.33 ± 0.03**	2.03 ± 0.05***	0.51 ± 0.08**
CYN	5	7.5 µg/Kg	0.50± 0.04	2.19 ± 0.79***	1.01 ± 0.15
	5	23.7 µg/Kg	0.45 ± 0.03	1.72 ± 0.74**	0.82 ± 0.08
	5	75 µg/Kg	0.36 ± 0.03**	1.65 ± 0.56**	0.56 ± 0.01**