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Genotoxic, cytotoxic, and cytopathological effects in rats exposed for 18 months to a mixture of 13 chemicals in doses below NOAEL levels

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Highlights

- Long term exposure to very low doses of chemicals mixture lead to genotoxic and cytotoxic effects
- Monotonic genotoxic effect observed only in females rat
- Dose-dependent cytotoxic effects in testis in males
- Dose-dependent cytotoxic effects in liver, stomach, kidney, lung, brain- both sexes

Abstract

The present study investigates the genotoxic and cytotoxic effects of long term exposure to low doses of a mixture consisting of methomyl, triadimefon, dimethoate, glyphosate, carbaryl, methyl parathion, aspartame, sodium benzoate, EDTA, ethylparaben,

buthylparaben, bisphenol A and acacia gum in rats. Four groups of ten Sprangue Dawley rats (5 males and 5 females per group) were exposed for 18 months to the mixture in doses of 0xNOAEL, 0.0025xNOAEL, 0.01xNOAEL and 0.05xNOAEL (mg/kg bw/day). After 18 months of exposure, the rats were sacrificed and their organs were harvested. Micronuclei frequency was evaluated in bone marrow erythrocytes whereas the organs were cytopathologically examined by the touch preparation technique. The exposure to the mixture caused a genotoxic effect identified only in females. Cytopathological examination showed specific alterations of tissue organization in a tissue-type dependent manner. The observed effects were dose-dependent and correlated to various tissue parameters. Specifically, testes samples revealed degenerative and cellularity disorders, liver hepatocytes exhibited decreased glycogen deposition whereas degenerative changes were present in gastric cells. Lung tissue presented increased inflammatory cells infiltration and alveolar macrophages with enhanced phagocytic activity, whereas brain tissue exhibited changes in glial and astrocyte cells' numbers. In conclusion, exposure to very low doses of the tested mixture for 18 months induces genotoxic effects as well as monotonic cytotoxic effects in a tissue-dependent manner.

Keywords: mixtures, pesticides, food additives, genotoxicity, cytotoxicity

1. Introduction

Chronic diseases or non-communicable diseases are responsible, according to the World Health Organisation (WHO) for the death of almost 41 million people each year, which means approximately 71% of all deaths registered globally. Among the chronic diseases the cardiovascular pathologies, are on the top of death causes, followed by neoplasms, respiratory diseases and diabetes. Indeed, these four disease categories represent 80% of all premature chronic disease deaths (GBD 2015 Risk Factors Collaborators, 2016). Modern

lifestyle is increasingly mentioned among the risk factors involved in the increased incidence of chronic diseases evidenced in the last years. The causality of chronic diseases is a complex entity that encompasses several risk factors that are influenced by various drivers or determinants acting at different levels. Several activity levels have been proposed including proximal ('cause of the risk factor'), medial ('cause of the cause') as well as distal ('cause of the cause of the cause') levels (Egger and Dixon, 2014). The progress in the study of chronic disease has lead to the definition of meta-inflammation, a term used for the 'low-grade, chronic and systemic inflammation' that was observed first in obesity and then associated and linked with other chronic pathologies (Hotamisligil et al., 1993; Medzhitov, 2008). Meta-inflammation is induced by various factors named generically 'anthropogens' that comprise all the 'man-made" environments, their by-products and/or lifestyles facilitated by latter, some of which may be detrimental to human health' (Egger, 2012). Importantly, the effects caused by anthropogens become evident when the exposure is chronic and as the response is not specific, the effects are mainly systemic. The susceptibility of individuals to anthropogens is associated with a genetic predisposition to chronic diseases and/or with epigenetic changes that can influence the susceptibility to chronic diseases.

Safe levels are set by regulatory agencies, for all range of chemicals that the general population is exposed to and are characterized as ADI (acceptable daily intake) or TDI (tolerable daily intake) (Pisanello, 2014). Even so, the biomonitoring studies continue to associate exposure to several chemicals in doses below the regulatory limits to certain diseases or health problems (Docea et al., 2017; Fenga et al., 2017). Therefore, neurological diseases (Baltazar et al., 2014; Dardiotis et al., 2013; Vinceti et al., 2014, 2017; Zaganas et al., 2013), obesity (Petrakis et al., 2017; Vassilopoulou et al., 2017), cardiovascular diseases (Bulka et al., 2019; Georgiadis et al., 2018; Philips et al., 2017), respiratory diseases (Veremchuk et al., 2018), infertility (Mehrpour et al., 2014), diabetes (Gangler et al., 2019;

Howard, 2019), cancers (Antwi et al., 2015; Marotta et al., 2019; Patel and Sangeeta, 2019) have been reported as correlated to chronic low dose exposure. One explanation for the observed association is that the safety levels are set using studies that evaluate the exposure to a single chemical and follow one critical health effect and do not take in consideration the real-life exposure scenario where the individual is exposed to a combination of chemicals from different sources (Kostoff et al., 2018). These combined exposures could initiate several types of interactions some of them leading to additive or synergistic effects, process that was demonstrated for the mixtures of endocrine disruptors or for pesticides (Bergman et al., 2013; Colosio et al., 2012; McCance and Huether, 2013).

These findings attract and focus the attention of scientists in the domain of toxicology and associated areas of research on the need of including the real-life risk simulation (RLRS) scenario in standard toxicological testing in order to set appropriate regulatory levels for combined chemical exposure. This endeavour involves evolving testing approaches from standard "one chemical-one critical effect" testing to combinatory toxicants experiments (Docea et al., 2016; Hernandez and Tsatsakis, 2017; Tsatsakis and Lash, 2017; Tsatsakis et al. 2016, 2017, 2018). Indeed, recently new approaches have been proposed for real-life risk simulation (Tsatsakis et al., 2017) that intend to address important unresolved issues. The matter in question is that while it is possible to obtain satisfactory values for safe levels of chemicals, that take in consideration the exposure to one chemical and the evaluation of one critical effect through current standard animal experiments, this approach may miss the evaluation of non-predictive effects or non-linear dose-response effects that could appear during combined exposure.

The present study examines the effects of the long term exposure (18 months) to very low doses, below NOAEL levels, of a cocktail of 13 chemicals to which humans are often in contact, on several biological parameters in an animal model. We have used a mixture

containing carbaryl, dimethoate, glyphosate, methomyl, methyl parathion and triadimefon, pesticides found as residues in food samples according to European Union report (EFSA, 2015c) and sodium benzoate, calcium disodium ethylene diamine tetraacetate (EDTA), ethylparaben, butylparaben, bisphenol A, aspartame and acacia gum, food and lifestyle additives found in everyday used products (EFSA, 2013b; EFSA, 2017; Mantovani, 2016; Nishihama et al., 2016 Tzatzarakis et al., 2017). The above mentioned chemicals were chosen as common components of normal modern lifestyle having as source food, water and lifestyle products,

2. Material and Methods

2.1.Animal study

In the study were included 40 eight-week-old Sprague Dawley (CD-SD) rats (20 males and 20 females), initially obtained from Charles River Laboratories (Charles River, Wilmington, MA) further reproduced under standard conditions in the University of Medicine and Pharmacy of Craiova Animal Husbandry. Before starting the experiment, the animals were divided into four groups of ten, five males and five females per group, and were acclimatized to the new housing conditions for two weeks before the study began. The animals were housed in specific animal cages with two or three animals of the same sex per cage in controlled climate conditions: 19-23°C room temperature, 35-55% humidity and 12 hours light/dark cycle. The animals were exposed for 18 months to a mixture of 13 chemicals in four dose ranges as follows: 0xNOAEL (mg/kg bw/day), 0.0025xNOAEL (mg/kg bw/day), 0.01xNOAEL (mg/kg bw/day) and 0.05xNOAEL (mg/kg bw/day). All the chemicals used in the mixture with their corresponding toxicological relevance values and negative outcomes are presented in Table 1.

The details of the methodology used regarding the animal treatment were based on the method proposed by Tsatsakis et al. (Tsatsakis et al., 2017) and detailed in the study of Docea et al. (Docea et al., 2018, 2019; Tsatsakis et al., 2019).

The animal study was approved by the Ethical Committee of the University of Medicine and Pharmacy of Craiova and all the procedures utilized were ain conformance to the European directive for the animal experiments EU Directive 2010/63/EU (European Commission Directive 2010/63/EU).

2.2.Organ collection

At the end of the experiment, the animals were sacrificed by exsanguination from the abdominal aorta under isoflurane anaesthesia after 12 h of fasting. The following organs were collected from each rat for further analysis: liver, stomach, kidney, lung, brain, heart, spleen, pancreas, muscle, testis from males and uterus from females.

2.3.Micronuclei assay in bone marrow

The micronucleus test used to establish genotoxicity and cytotoxicity was performed according to the OECD regulations for micronucleus test in mammalians' erythrocytes (OECD TG 474). Briefly, thigh bone marrow prints were done in triplicates for each rat, prints were stained with Giemsa and the micronuclei counted under a light microscope (Nikon E200) with 100X objective in immersion oil. The samples were examined by researchers who were not aware of the sample identity. Micronuclei counting was performed in a blind manner where 4000 cells were counted for each simple and the percentage of cells (%) presenting micronuclei were shown as mean of the triplicates.

2.4.Touch preparation technique (TPT) for cytological examination of tissue samples

Touch preparation technique (TPT) was used for cytological examination of the tissue samples. Specifically, after the organ retrieval, the tissue was cross-sectioned and imprinted on 3 glass slides. In continuation, the slides were fixed with alcohol 60%, stained with the pap method and analyzed under a light microscope (Nikon ECLIPSE E 400) (20x) (Diamantis et al., 2013).

The cytological examination of tissue samples was performed using the following score of criteria as presented in Table 2.

2.5.Statistical analysis

Data were analyzed using STATA software version 13.1 (StataCorp, USA). The data for micronuclei analysis were assessed using paired t-test. The data for the score of injury were expressed as mean. Count data were expressed as the ratio between the number of rats for which the effect was positive and the total number of rats in the group.

One-way ANOVA followed by Dunnet's post-hoc test were applied to explore differences in the whole groups (ANOVA) and for the bivariate comparisons with control group (Dunnet). A significant difference was considered at $P<0.05$.

3. Results

3.1. Micronuclei frequency in bone marrow

Micronuclei frequency exhibited a dose-response increase for both sexes as proved by the one-way ANOVA and Dunnets' t test (Tables 3A, 3B). However, only the high dose female group presented a statistically significant increase (12.4 vs 6.2 for the control, see Table 3C). Based on the proposed interpretation from the OECD TG 474 these observations lead to a positive test for females and an equivocal for males. Cytotoxicity was evaluated by the percentage ratio of the number of immature erythrocytes to the total number of

erythrocytes (PCEs/(PCEs+NCEs) %) (Table 3D). Decrease of the ratio was observed for all treatment groups when compared with the control group, indicating a mild cytotoxicity and without exceeding the limit of 20% of decrease. As proved by the one-way ANOVA test, there is a statistically significant increase of the PCEs/(PCEs+NCEs) % and MNPCEs ‰. Similar results were observed even when the distribution of the values was considered as non-canonical. Thus, we can conclude that the exposure to the chemical mixture can indeed result to a net increase of the above mentioned values.

Table 3A. One-way ANOVA test was applied to explore differences in the whole groups. 95%CI (95% confidence interval), 95% LB (95% lower bound), 95%UB (95% upper bound)

Table 3B. Dunnet's post-hoc test for the bivariate comparisons with control group

Table 3C: Mean micronuclei frequency for each exposure group compared to the control group is presented. P value is calculated using paired t-test. Statistical significance is set at $P<0.05$.

Table 3D: Erythrocytes subgroup ratio expressed as a percentage of the number of micronucleated PCEs over the total number of PCEs and NCEs.

3.2.Cytopathological examination of tissue samples by the touch preparation technique

The examination of the testis tissue by TPT showed a dose dependent negative effect as regarding the degree of testis tissue cellularity and concomitant degenerative changes. Specifically, in the 0.0025 x NOAEL group the treatment induced a score of injury for organ cellularity of 1.2 that corresponds to approximately 10% reduction in number of cells in

tissue whereas no tissue degeneration was evident when compared with the control group (score 0.2) ($P<0.05$). However, the score of injury for the degrees of cellularity was 1.8 and 2.8 in 0.01xNOAEL and 0.05xNOAEL groups respectively and furthermore an identification of degenerative changes of the cells was determined with a score of 1.2 and 2.8 for the 0.01xNOAEL and 0.05xNOAEL groups respectively ($P<0.05$) (Table 4,5 and Figure 2).

Liver cytological evaluation showed no modification as regarding the number of cells in tissues in any of the treatment groups. However, a hepatocyte glycogen loss, was determined with a score of relevance for the 0.01xNOAEL and 0.05xNOAEL treatment groups of 1.8 and 2.8 espectively ($P<0.05$), with no differences between male and female animals (Table 4, 5 and Figure 3,4).

Evaluation of the stomach tissue of the 0.0025xNOAEL group showed no alteration of cell morphology and degree of cellularity as well as no indice of cell degeneration (P=NS). However, dose dependent degenerative changes of epithelial cells were observed in 0.01xNOAEL and 0.05xNOAEL groups, with an injury score of 1.8 and 2.8 respectively ($P<0.05$), with no diferences between males and females evident. Moreover, an increase of tubular cell aggregates was observed in a dose dependent manner, with no differences between males and females Table 4 and 5, Figure 5).

In kidneys, a dose dependent decrease of the level of celularity was observed for the 3 treatment groups with a higher score in females compared to males in 0.0025xNOAEL and 0.001xNOAEL groups. Regarding the growth in tubular aggregates, the control, 0.0025xNOAEL and 0.01xNOAEL group showed no effect, but in 0.05xNOAEL group a

score of 2.8 was observed which is traslated to an intense growth in tubular cell aggregates with no differences between males and females (Table 4,5 and Figure 6, 7).

In lung tissue, a dose dependent increase of inflammatory cells infiltration as well as enhanced phagocytic activity by alveolar macrophages was observed for both male and female rats. Specifically, the increase in infiltration of inflammatory cells was assessed with an injury score of 0.8, 1.8 and 2.8 for 0.0025xNOAEL, 0.01xNOAEL and 0.05xNOAEL treamant groups, as regarding female rats. Indexes of injury increased from 0 (control group) to 1.2, 1.8 and 3 for the 0.0025xNOAEL, 0.01xNOAEL and 0.05xNOAEL treament groups respectively ($P<0.05$), as regarding male rats (Table 4, 5 and Figure 8, 9). Likewise, an enhanced phagocytic activity by alveolar macrophages was detected in 0.0025xNOAEL, 0.01xNOAEL and 0.05xNOAEL female treatment groups with index of activation score of 0.8, 2 and 2.8, respectively. Similiar effects were identified in 0.0025xNOAEL, 0.01xNOAEL and 0.05xNOAEL male treatment groups with index of activation score of 1.2, 1.8 and 2.8 in males, respectively ($P<0.05$) (Table 4,5 and Figure 8, 9).

Exposure to the chemical mix induced morphological changes of the brain tissue, treatment. Thus, a dose-dependent dependent decrease in glial and astrocyte cells was evident with an injury score for females being increased from 0 (control group) to 0.8, 1.8 and 3 for the 0.0025xNOAEL, 0.01xNOAEL and 0.05xNOAEL groups respectively. The same trend was evident for male rat brain tissue as the exposure to the chemical mix initiated modification of the injury score from 0 (control group) to 1.2, 2.2 and 2.8 for the 0.0025xNOAEL, 0.01xNOAEL and 0.05xNOAEL groups, respectively ($P<0.05$) (Table 4,5 and Figure 10, 11).

Histopathological examination of heart, spleen, pancreas, muscle and uterus tissue of treated with the chemical mix rats did not identify changes in any of the traement groups.

4. Discussion

During the last three decades, public health awareness has found tremendous resonance across various government organizations and scientific communities worldwide. Under this scope, strict legislations for chemical use have been enforced and numerous chemicals have been banned from use. However, the public is yet to be protected. Search of the epidemiologic data for various diseases and syndromes (of both malignant and non-malignant etiology brings to the forefront the conclusion that even though the constellation of the acute disease incidence has been partially controlled, other chronic or more benign pathologies have emerged. It is indicative that, the incidence of neurodegenerative disorders, cardiovascular diseases, obesity and metabolic syndrome, infertility (especially in men) and inflammatory disorders of any nature have dramatically increased (Inhorn and Patrizio, 2015). Even though this phenomenon can be partially explained from the improvements in diagnosing and recording, it is difficult to accept that this is the only reason. In fact, during the last decade, increasing evidence highlights the toxic hazard of various chemicals for public health even at levels that were, until then, considered to be safe for human use (Yang et al., 2017). This means that the general population does not suffer only from a delayed wash-out from the harmful chemicals used in the past, but also suffers from the chronic exposure to low doses of less harmful but far from innocent substances. Indeed, it is suggested that, this chronic exposure to low concentrations of various chemicals allows the incriminated substances to distort the protective mechanisms and homoeostasis and thus, lead to chronic diseases. A drawback of, most of the research protocols addressing the issue of exposure to low doses of chemicals, treated their subjects to a single or a small mix of chemicals for a relatively small period of time. Due to the limitations of the study protocols,

no firm conclusions can be drawn regarding the actual effect of this type of exposure on human health. Thus, the need to obtain data mimicking chronic exposure to a larger mix of substances that correlates better with real life conditions was the driving force of this study. In fact, long term exposure to low dose of chemical mixtures [approximately at the level of the acceptable daily intake (ADI)] is proposed as a more efficient simulator of real life conditions able to highlight the true hazards of everyday use of domestic, agricultural, industrial and chemical warfare products and compounds (Tsatsakis et al, 2017). In the present study the effects of exposure to 13 compounds [six pesticides (carbaryl, dimethoate, glyphosate, methomyl, methyl parathion and triadimefon) and seven food and lifestyle additives (sodium benzoate, calcium disodium ethylene diamine tetraacetate (EDTA), ethylparaben, butylparaben, bisphenol A (BPA), aspartame and acacia gum] for a period of 18 months were determined in adult male and female rats in order to address the issue of initiating an additive effect (to our knowledge, there is no other published study that uses such a great number of substances and especially for such a prolonged period). Indeed, it has earlier been shown that a combination of low number of compounds does not necessarily induce additive effects (Bianchi-Santamaria et al., 1997). Furthermore, we have used doses that range from 0.05 to 0.0025 of NOAEL that and may trigger more subtle effects. The long expose may induce unknown compensatory mechanisms and hence, allows us to obtain a more objective model of real life long term exposure.

We have to point out that in terms of the rat's life span and established extrapolation models, the utilized settings of exposure would correlate to 45 years of exposure to the mentioned low doses of compounds, and starting from the age of 4 (Sengupta, 2013).

4.1.Micronuclei frequency in bone marrow

When estimating possible genotoxic and cytotoxic effects from chronic exposure to various substances, micronuclei frequency can prove to be a reliable marker. In fact, previous studies have demonstrated that micronuclei frequency accurately correlates the degree of DNA damage (when present) with the exposure to genetoxic substances (Fenech et al., 2016). It is also well documented that micronuclei frequency can serve as a potential biomarker for health risk estimation in real life (Bonassi et al., 2007). However, a literature review on the topic of micronuclei frequency fluctuation after chemical exposure proved that not only there can be conflicting data on the same substance but also there is no other study using such a great mixture and for so long. Thus, our results should be interpreted with care given their uniqueness. In detail, as proved both from the ratio of PCE to total erythrocytes ratio, the clear cytotoxic effect exhibited across all high-dose groups (0.05xNOAEL) suggests that the chronic exposure to the mixture negatively affected the animals in an irreversible manner. This result can be attributed mostly to synergistic effects of the substances that injured key organs both in metabolizing and excreting these substances (namely the liver and the kidneys). Interestingly, a mild predominance of the females was evidenced without any obvious explanation. On the contrary, the same values for the medium-dose groups (0.01xNOAEL) exhibited a boarder-line cytotoxicity that could indicate a mild and possibly reversible injury of the organism that could potentially be overwhelmed if the animals were left untreated for a certain period of time. Lastly, no cytotoxic effects were exhibited from the low-dose groups (0.025 x NOAEL) using the micronuclei assay, suggesting that the organism of most animals was able to manage the adverse effects exerted from the chemicals, if there were any at all. Regarding the genotoxic effects of the mixture, the mean number of MNPCEs revealed a strong genotoxic effect only on the female high-dose group (0.05xNOAEL). This result could be interpreted as another explanation for the cytotoxic effects that were shown. When cellular DNA is damaged to a certain degree, various cellular

mechanisms (DNA repair, energy use, lipid transportation, cell-to-cell communication) are either seized or deregulated resulting to cellular death (and thus cytotoxicity). However, rather interesting is that no genotoxic effects were evidenced across the rest of the groups (0.01 and 0.0025xNOAEL) even though a tendency towards this result can be seen from the results of the medium-dose groups (0.01xNOAEL) and the total high-dose group (0.05xNOAEL).

Previously, in a mouse model a significant increase of MNPCE in bone marrow cells was found after sub-chronic administration of organophosphate insecticide dimethoate (Ayed-Boussema et al., 2012). A more recent study with glyphosate-based herbicides showed that only males exhibited significant responses, whereas; no effects on female rats were evident (Ghisi et al., 2016). Furthermore, methomyl, a carbamate insecticide, when introduced intra-peritoneally in Swiss CD1 mice induced genotoxic effects, probably through formation of active oxygen species (Bolognesi et al., 1994). Likewise, testing the combination of three carbamate insecticides (propoxur, methomyl, and aldicarb) in BALB/c mice a significant increase in MNPCE in peripheral blood was reported (Wei et al, 1997)

In earlier studies the effects of several pesticides on MNPCE emergence were tested in animal models. Specifically, the genotoxic effects of 4 organophosphorous pesticides, were tested in rat bone marrow cells and while methylparathion and phorate induced alterations in MNPCE, no such effect was observed upon fenitrothion treatment (Grover and Malhi, 1985; Vijayaraghavan and Nagarajan, 1994). In a study, published over 25 years ago, fifteen pesticides were tested by gavage in Wistar rats at various doses during 24 hr with no changes in the frequency of micronuclei determined (Dolara et al., 1993). On the other hand, Bisphenol A when orally administered in adult rats of both sexes induced MNPCE, probably correlated to simultaneous detection of oxidative stress (Tiwari et al., 2012). Moreover, Pzh-

Sfis male mice treated with sub-chronic bisphenol A presented micronuclei induction in both peripheral blood and bone marrow reticulocytes (Radzikowska et al, 2012)

In the present study we have used a mixture composed of the highest number of compounds reported so far in order to address the issue of initiating an additive effect. Indeed, it has earlier been shown that a combination of low number of compounds does not necessarily induce additive effects (Bianchi-Santamaria et al., 1997). Furthermore, we have used doses that range in the 0.05-0.0025xNOAEL that and may trigger more subtle effects. Another, seminal difference from other previously published studies is that the duration of exposure in our model system spans the longest reported period of testing. The long expose may induce unknown compensatory mechanisms and hence, allows us to obtain a more objective model of real life long term exposure

4.2.Cytopathological examination of tissue samples by touch preparation technique

Cytopathological examination of tissue samples by touch preparation technique revealed a dose-related response as regarding cell degeneration with the higher doses being correlated with worse outcomes. However, pathological findings were not established for all organs.Thus, heart, spleen, pancreas, muscle and uterus tissue did not exhibit differences in comparison to control tissue. In contrast, testes presented a great diminish of the cellular population (suggesting male infertility and testosterone misbalance), liver had increased cellular damage and sinusoidal dilation, which are precursors to cirrhosis and biliary obstruction, stomach had marked gastritis, kidneys (depending on the dose) suffered from glomerulonephritis to extended renal injury and renal failure, lungs (again depending on the dose) exhibited pattern of diffuse bronchiolitis/alveolitis and pneumonia while brain suffered from a tremendous decrease of neural cells counts suggesting the presence of an established neurodegenerative disorder. A detailed interpretation of the results is presented below and in

figure 12 is represent a summary of the literature regarding the groups of chemicals that affects each organ.

Testes

The study of the male gonad, revealed that the chemical mix significantly deregulated in a proportionate manner the cellularity of all testicular cells (seminiferous tubules almost disappeared). On the other hand, only the 0.01xNOAEL and 0.05xNOAEL groups suffered from cellular degenerative changes. Even though, an appreciation of the motility of sperm cells and the levels of testosterone are lacking, it is obvious that all groups suffer from testicular toxicity. Given the lack of degenerative changes in the 0.0025xNOAEL group, these toxic effects can be considered to be reversible. However, for the other two groups the injury is rather irreversible (especially for the 0.05xNOAEL group). Based on the composition of the chemical mix, one would blame EDTA, ethylparaben, butylparaben and BPA for these results given their well-known effects on male reproductive system. However, going through the literature of the other chemicals as well, it is interesting that almost all of them can affect, even at low doses, the male reproductive system. Methomyl, a carbamate insecticide, is found to induce testicular lesions characterized by moderate to severe degenerative changes (Mahgoub and El-Medany, 2001; Shalaby et al., 2010). Triadimefon, a triazole fungicide, was found to disrupt testosterone homeostasis which is a key hormone for the male reproductive system. In fact, it was found to increase serum testosterone levels and affect hepatic response probably via the alteration of CAR and PXR signaling pathways for steroid metabolism in the male rat liver (Goetz et al., 2007; Goetz and Dix, 2009). Dimethoate, an organophosphate insecticide, was also found to negatively affect sperm cells and spermatogenesis (Sayim, 2007). Glyphosate, the active substance in the major herbicide Roundup®, administrated in low levels was found to induce testosterone decrease while in high levels necrosis of various classes of testicular cells (mainly Sertoli) was reported

(Cassault-Meyer et al., 2014; Clair et al., 2012; de Liz Oliveira Cavalli et al., 2013). Carbaryl, a carbamate insecticide, was found to mainly affect sperm motility and morphology via interruption of pre-meiotic (eg. gamma-glutamyl transpeptidase is increased while glucose-6-phosphate dehydrogenase is decreased) and post-meiotic (eg. sorbitol dehydrogenase is decreased, lactate dehydrogenase is increased) spermatogenic cell enzymes (Pant et al., 1995). Methyl parathion, an organophosphate pesticide, was reported to induce biochemical changes in testes and thus affecting sperm cells (Narayana, 2007). While aspartame (an artificial non-saccharide sweetener), sodium benzoate (a food preservative) and acacia gum (a natural mixture of polysaccharides and glycoproteins used as an additive in food and chemical industry) were not found to affect testicular physiology or morphology, EDTA (an additive used as a preservative or stabilizer), ethylparaben, butylparaben (preservatives in cosmetic and pharmaceutical products) and BPA (a starting material for the synthesis of plastics) were found to affect male fertility. EDTA administration caused a significant decrease in biochemical parameters and antioxidant enzymes activity (Khalil et al., 2008) while ethyl- and butyl-parabens and BPA via their estrogen mimetic action suppressed mitochondrial function and impaired testicular structure and function (Dere et al., 2018; Oishi, 2001; Tavares et al., 2009; Ullah et al., 2018; Zhang et al., 2014). Overall, it is obvious that the overall effects of a dose-related impaired testicular morphology that we present herein is indeed the result of the synergistic effect of the majority of administered chemical compounds.

Liver

Liver is the site where most of the substances, especially those taken orally, undergo first-pass metabolism. For this reason, studying this organ becomes a crucial step towards the in-depth understanding of the toxic profile of the chemical mix. As presented in Tables 4 and 5, liver cellularity was not affected in any group. However, glycogen loss (another determinant

of hepatic injury) exhibited a significant and proportionate increase in the 0.01xNOAEL and 0.05xNOAEL groups for both sexes. In fact, these findings suggest that, as the liver is the main detoxification organ has functional reserves in terms of withstanding low dose intake chemicals. Nonetheless, its protective mechanisms can be overwhelmed if the dose increases. Generally, depending on the impaired pathway, different diseases and syndromes can be proclaimed. For example, if the pathways of lipid acid metabolism are affected, then fatty liver disease and steatosis would be the primary end-result. On the other hand, if a member of the CYP superfamily is affected then the intoxicating mechanisms may be impaired leading to conditions with focal necrosis and possibly cirrhosis. All of the administered substances are not connected with hepatotoxicity per se. However, a close look to the literature proves the opposite. Most of the six pesticides can indeed induce liver toxicity. Methomyl was able to reduce the levels of cytochrome P450 and cause an increase of the hepatocyte injury biomarkers (Garg et al., 2009; Patil et al., 2008). Triadimefon, was also found to cause a decrease of the number of several cytochrome P 450 enzymes (Cyp1a1, Cyp2b1, Cyp3a2), transporters (Abcb1a, Abcc3) and genes encoding for enzymes involved in fatty acid or phospholipid metabolism (Pparγc1a, Sc4 mol) (Al-Eryani et al., 2015; Heise et al., 2018). Demethoate administration was found to cause fatty changes, reduced number of hepatocytes, necrosis, and tissue degeneration induced mainly via the oxidative stress thus the high rate of lipid peroxidation in hepatocytes causes severe damage to them (Kwape et al., 2013). Glyphosate, was proved to significantly disturb major hepatic proteins involved in organonitrogen metabolism and fatty acid β-oxidation leading to peroxisomal proliferation, steatosis and necrosis (Mesnage et al., 2018). Carbaryl and methyl parathion were also found to cause hepatocellular injury as evidenced by the increase of relative biomarkers (ALT/AST) with a concomitant increase of the connective tissue (Du et al., 2012; Mahajan et al., Mesnage et al., 2018). On the same wavelength, most of the food or pharmaceutical additives

were found to cause liver damage. Aspartame, being metabolized to phenylalanine, aspartic acid, and methanol, was detrimental for the normal liver function since notable hepatocellular injury biomarkers were elevated and fatty acid accumulation was evident probably via severely reduced levels of hepatic glutathione (GSH) as a result of down-regulated catalytic subunit of glutamate cysteine ligase (GCLc) (Finamor et al., 2017). Moreover, dose-dependent necrotic and cirrhotic effects of sodium benzoate are also proved (Oyewole, 2012). Both ethyl- and butyl-parabens have been associated with liver injury with the main pathogenetic model being that of increased mitochondrial membrane permeability (González-Cuevas et al., 2011). BPA is found to be able of inducing an oxidative stress via down-regulation of most hepatic anti-oxidant enzymes (glutathione, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and catalase) and decreased gene expression of them (Hassan et al., 2012). On the contrary, EDTA administration has been related with antioxidant effects as it is able to induce the activity of antioxidant enzymes, to decrease lipid peroxidation, hepatic inflammation and fibrosis in cirrhotic rat models (González-Cuevas et al., 2011). Finally, acacia gum has demonstrated equally hepatoprotective effects thanks to its anti-oxidant value since it was found to be able to ameliorate cirrhotic effects in rats (Hamid et al., 2018).

Stomach

Examination of the stomach revealed that even though the level of cellularity was not affected at any group, gastric cells of the 0.01xNOAEL and 0.05xNOAEL groups suffered from degenerative changes which contributed substantially to the alteration of tubular cell architecture. Acknowledging that it can be considered as a precursor for altered gastric acid production and gastric lesions (such as gastritis) thereafter. Unfortunately, the current literature lacks evidence for the potential adverse effects of the most chemicals we administered though there are some helpful data for a few of them. Glyphosate is described to

degenerate gastric epithelial cells affecting the gastric glands as well (Tizhe et al., 2014). Aspartame, maybe through its metabolite methanol, can also affect gastric cells but either high doses are needed or the existence of a distorted mucus layer created by an inflammatory micro-environment (Abd Elfatah et al., 2012). Interestingly it is described that in vagotomized and adrenalectomized rats, EDTA was able to significantly reduce basal gastric acid production, an effect that could persist for at least 2 hours after its administration (Glavin and Szabo, 1993). Taking all these into consideration, one can suppose that a synergistic effect of a more alkali environment that EDTA could create, may allowed the rest of the chemicals (such as glyphosate) to act with minimal alteration of their chemical structure. Thus, the distortion of gastric tubular architecture can be considered as an effect of more than one substances.

Kidney

Kidneys due to their function as one of the main excreting routes for xenobiotic substances are exposed to a greater amount of toxic substances in relation to the other organs. Thus, it is logical to assume that subjects exposed to a chemical cocktail would exhibit pronounced renal damage. Indeed, this is proved from the renal examination of our specimens. In more detail, a significant decrease of cellularity was found in all treated groups in a dose-depended manner. This decrease affected all cell types of the renal parenchyma (glomerulus, tubules) along with diffuse hemorrhagic regions and dilated tubular lumen. However, tubular cell aggregates were visible only in the 0.05xNOAEL group. This means that a pronounced nephrotoxic effect of the mix that can also be described as renal failure, lead to glomerulonephritis and renal injury as proved by the aggregates. This type of injury is described for the most of the chemicals administered. Methomyl is described to cause renal injury with accompanying elevated renal biomarkers (Djeffal et al., 2015; Sakr et al., 2018). Dimethoate is proved to create glomerular and tubular degeneration, hemorrhage, tubular

widened lumen and glomerular shrinkage (Alarami, 2015). On the other hand, even though there are numerous studies advocating the nephrotoxic effect of Roundup ®, glyphosate is not connected with it (Dedeke et al., 2018; Wunnapuk et al., 2014). However, this is not the case for methyl parathion which is proved to cause distinguished renal damage in every renal subunit via oxidative stress (Fuentes-Delgado et al., 2018; Kalender et al., 2007). Moreover, the same oxidative nephrotoxic effect with renal cell apoptosis is exhibited by aspartame (A. Al-Eisa et al., 2018). Additionally, bisphenol A was found capable of acting directly on the kidney mitochondria, causing mitochondrial oxidative stress, dysfunction, and subsequently, leading to whole organ damage (Jalal et al., 2018; Kobroob et al., 2018). On the contrary, the nephroprotective effects of acacia gum were brought to light when it was found to ameliorate the nephrotoxic damage from cisplatin administration (Al-Majed et al., 2003). Overall, it is logical to attribute the renal injury and renal failure for the case of the high dose group to the chemical mix.

Lung

The main finding from lung examination was the presence of inflammatory responses. In detail, all treated groups exhibited advanced inflammatory infiltration in their alveoli in a dose-response manner. Moreover, phagocytic activity was also present in all subjects (except from the female low dose group where the score is not significantly different from the control group) following the same pattern as before. In fact, these results are consistent with mild, moderate and severe bronchopneumonia and bronchoalveolitis that worsened according to the dose increase. This finding suggests that the chemical mix not only triggers an inflammatory reaction of the host but also negatively affects the immune system (Zhao et al., 2014) given the worse presentation of the 0.05xNOAEL group. Thus, these subjects are prone to lower tract respiratory infections. Unfortunately, since touch preparation did not contain specimen from the upper respiratory tract, no appreciation of the integrity of cilia can be made.

Nonetheless, it seems possible that middle and high dose groups would suffer from damaged ciliated cells and or goblet cells, making the production of the mucus or its clearance insufficient. Even though for almost half of the administered chemicals there are no sufficient data regarding their potential toxic effect on lung, there are some interesting prior published data supporting the above findings. Dimethoate is proved to cause histopathological changes in lung tissue such as emphysema and hemorrhages due to an increased oxidative damage (Amara et al., 2012). In the same view, methyl parathion is found to be able to cause lung injury via its oxidative and pro-inflammatory action (Du et al., 2011). Glyphosate was also found to cause lung hemorrhages and lung epithelial cell damage without knowing the underlying mechanism (Adam et al., 1997; Khot et al., 2014). However, carbaryl can only cause mild architectural damages to the alveoli (Tośś-Luty et al., 2001). Finally, BPA can also cause inflammation and oxidative stress responses with increased levels of malondialdehyde (MDA), reduced concentrations of superoxide dismutase (SOD), and upregulation of Interleukin-18 (IL-18) expression in lung tissue (Abedelhaffez et al., 2017).

Brain

Despite the fact that brain is partially protected by the blood brain barrier against numerous substances, our findings prove that our mixture induces post-blood brain barrier effects. In fact, a dose-related decrease of glial and astrocyte counts was evident across all treated groups. While a relative male predominance on the 0.0025xNOAEL and 0.01xNOAEL groups was exhibited, this pattern reversed for the 0.05xNOAEL group where females had relatively higher rates of decreased cell counts. From a clinical point of view, these findings can be interpreted as histological evidence of neurodegenerative disorders of oxidative stress origin that rats may had suffered from. Interestingly, current literature highlights the potential neurotoxic effects for the most compounds administered and further relates them with distinct brain and cognitive disorders. Methomyl is found to induce

oxidative stress on the cortex and hippocampus vital areas for the conscious and subconscious processing (Abedelhaffez et al., 2017; Adam et al., 1997). Triadimefon was also related with disturbances of retinoic acid metabolism (a molecule that is critical in the development and maintenance of spatial memory) in hippocampus. In addition, triadimefon has been linked with increased locomotion and stereotypic behavior in rodents through pathways similar to those produced by indirect-acting dopamine agonists (mainly in striatum) (Freeborn et al., 2015; Walker et al., 1990; Xi et al., 2012). Moreover, dimethoate is proved to cause significant oxidative stress in various brain regions (Sharma et al., 2005). Glyphosate, linked with Parkinson's disease, is also found to cause oxidative stress. The main mechanisms that seem to induce this action are NMDA receptor activation, impairment of cholinergic transmission, astrocyte dysfunction, ERK1/2 over-activation, decreased p65 NF-κB phosphorylation (which are associated with oxidative stress and glutamate excitotoxicity). Interestingly, rats that were exposed to glyphosate for a long period exhibited depressive-like behavior, highlighting the effects in hippocampus and striatum (Cattani et al., 2017; Gallegos et al., 2018; Hernández-Plata et al., 2015; Rebai et al., 2017). Carbaryl and methyl parathion can also induce neurotoxicity mainly through the inhibition of AChE and down-regulation of muscarinic receptors in various regions including striatum, hippocampus, frontal cortex, thalamus and midbrain (Herr et al., 2010; Sun et al., 2003). Chronic exposure to aspartame is proved to alter electrolyte homeostasis, monoamine neurotransmitter synthesis and induction of oxidative status in brain following a dose-depended pattern which may prove to be dangerous for various cognitive functions (Abhilash et al., 2014, 2013; Ashok and Sheeladevi, 2014). Lastly, bisphenol A is linked with marked lesions in cortex, hypothalamus and hippocampus where it can induce marked oxidative stress and significant increase in the excitatory and inhibitory amino acid neurotransmitters (Arambula et al., 2016; Khadrawy et

al., 2016).Overall, it seems that most of the chemicals contained in the chemical mix we administered are able to cause significant neurotoxicity in a synergistic manner.

5. Conclusions

The exposure to very low doses of a mixture of 13 chemicals induced genotoxic effects only in females. In testis, the level of cellularity and degenerative changes were affected in a dose-dependent manner. The exposure to the chemicals mixture affects the level of glycogen from the liver in a dose-dependent manner in both sexes. In stomach there were observed degenerative changes of the cells and changes in tubular cell architecture that increase with the doses tested in both sexes. The level of cellularity and tubular cell aggregates in the kidney were dose-dependent affected by the mixture. In lungs, the level of inflammatory cells and phagocytic activity by alveolar macrophages increased with the dose of the mixture administered in both sexes. In the brain the mixture induced changes in glial and astrocyte cells in both sexes. No cytotoxic effects were observed at heart, spleen, pancreas, muscles and uterus level.

In summary, this study supports the hypothesis that the combined every-day life exposure, even in very low doses that are considered safe can lead to adverse effects after long term exposure. The risk assessment for the determination of the safety levels of chemicals should be updated in order to take into account the real-life exposure scenarios and to pass from the single-chemical approach to real life risk simulation approach.

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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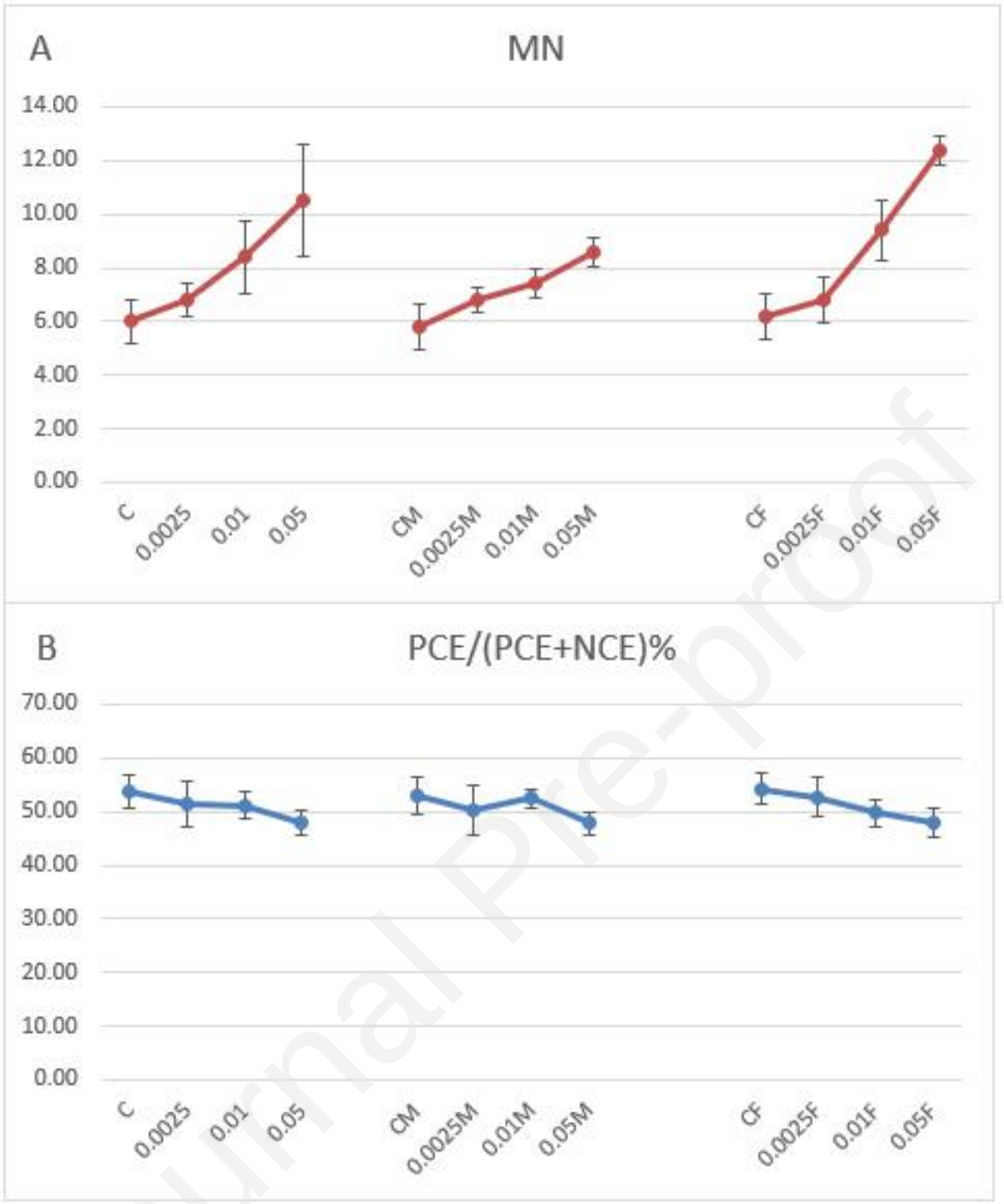

Figure 1 A) The effect of exposure to chemical mixture on mean micronuclei frequency in rat bone marrow cells. Horizontal axis: doses x NOAEL, vertical axis: mean MN frequency B) The effect of exposure to chemical mixture on erythrocytes ratio [PCE/(PCE+NCE) %] in rat bone marrow cells. Horizontal axis: doses x NOAEL,

vertical axis: erythrocytes ratio fluctuation. C: control, CM: control males, CF: control females

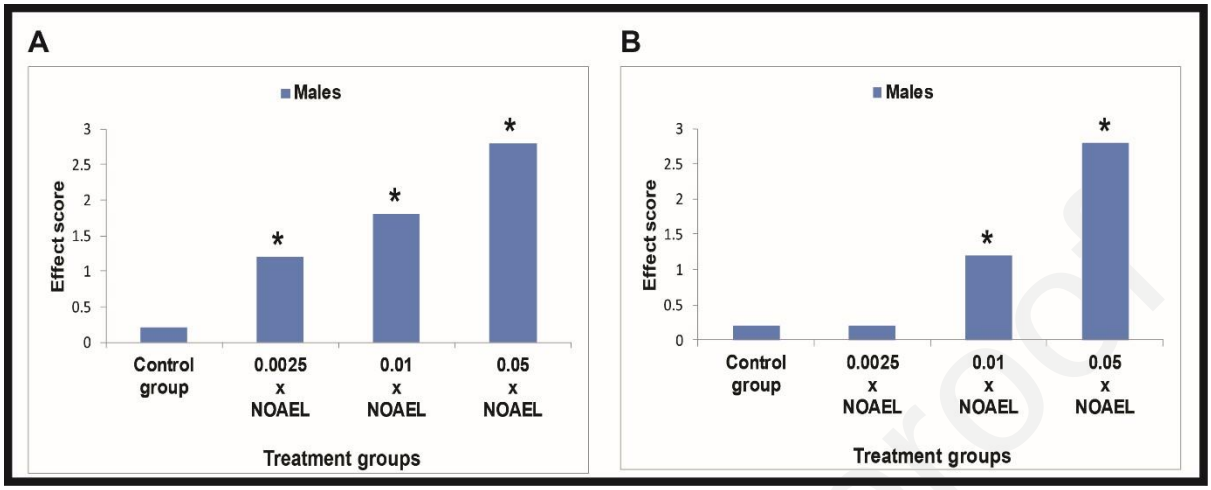

Figure 2. Effect of exposure to chemical mixture on testis tissue morphology. Testis cytopathological changes as determined by TPT. A. Degree of cellularity. B. Degenerative changes of the cells

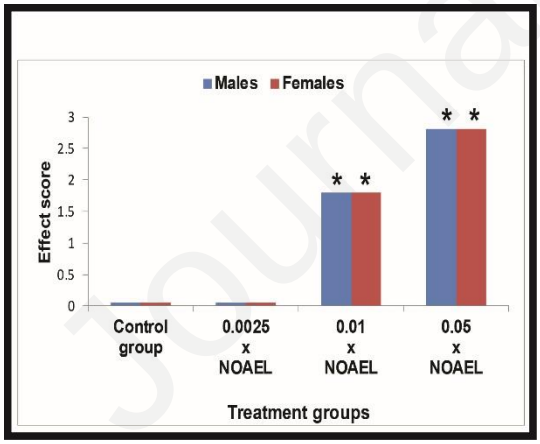

Figure 3. Effect of exposure to chemical mixture on liver tissue morphology. Liver cytopathological changes as determined by TPT. Liver hepatocyte glycogen content

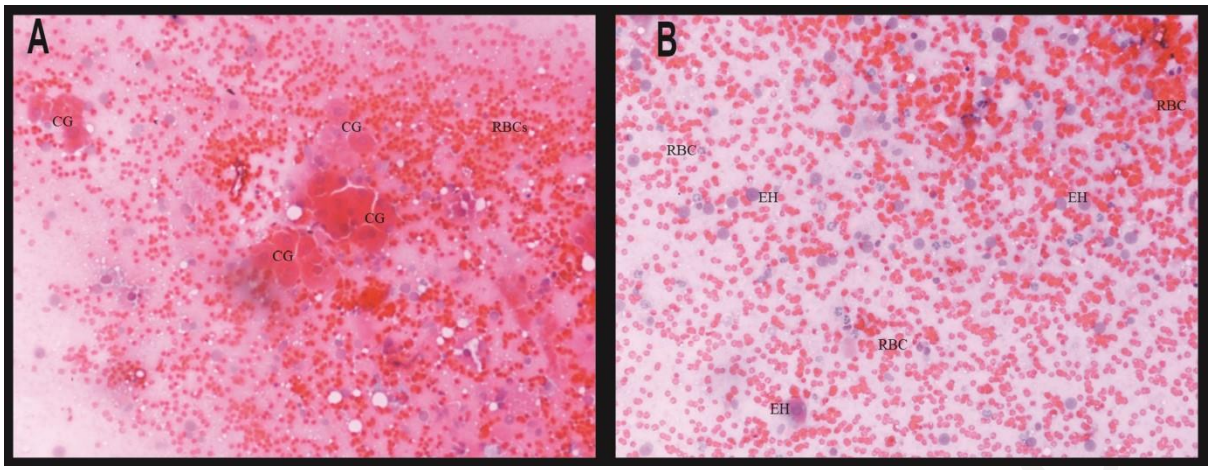

Figure 4. Cytopathological examination of liver tissue. A. Control group x200: CG= Hepatocytes with intact glycogen content, RBCs= red blood cells.; B: 0.05x NOAEL group x200. EH= empty hepatocytes (Hepatocytes with lost glycogen deposition), RBC= Red blood cells.

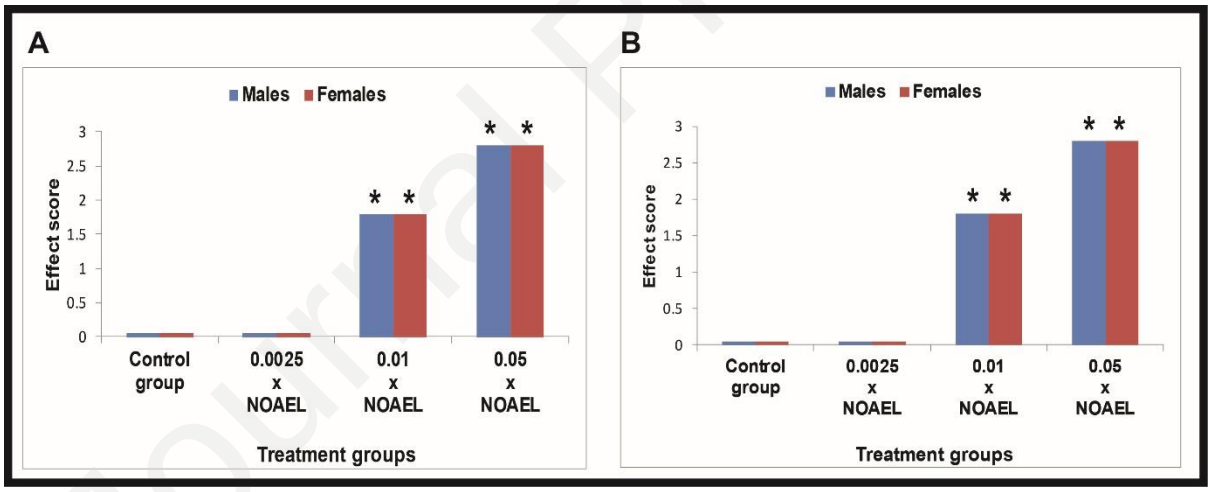

Figure 5.Effect of exposure to chemical mixture on stomach tissue morphology. Stomach cytopathological changes as determined by TPT. A. Degenerative changes of the cells. B. Tubular cell architecture changes.

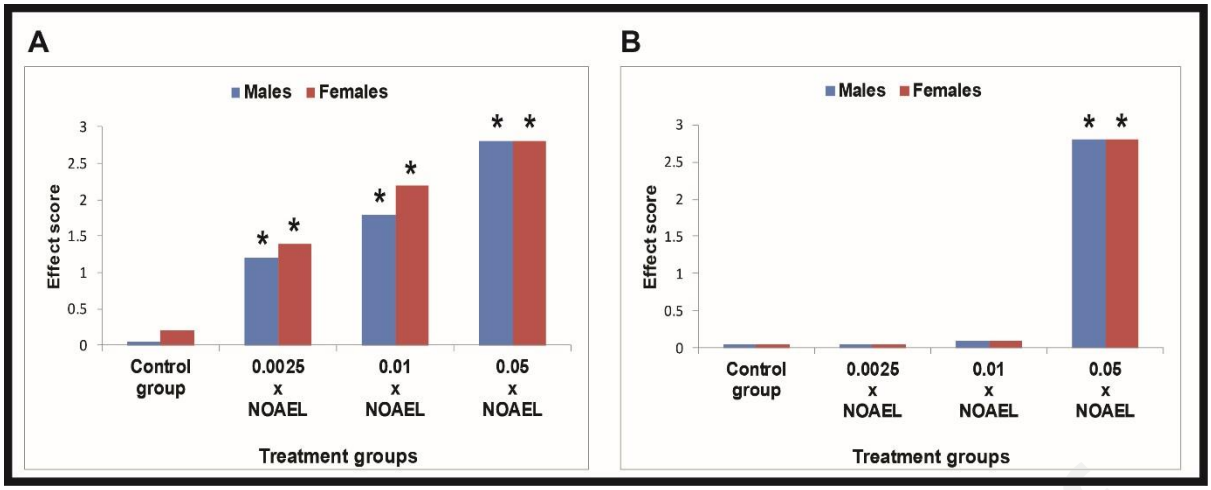

Figure 6. Effect of exposure to chemical mixture on kidney tissue morphology. Kidney cytopathological changes as determined by TPT. A. Level of cellularity. B.Tubular cell aggregates.

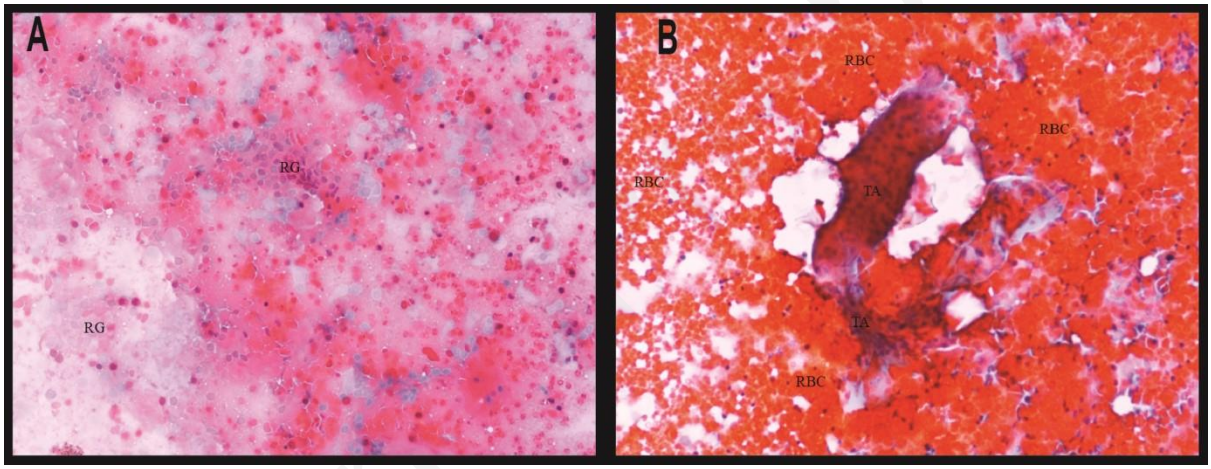

Figure 7. Cytopathological examination of kidney tissue. A. Control group x 200. RG=Renal glomerulus. Normal shape and number of renal glomeruli with normal cellularity level. B. 0.05x NOAEL group x200. TA= Tubular cell aggregates, RBC=Red blood cells. Severe numerical reduction in cellularity with intense growth in tubular cell aggregates forming renal tubules molds with diffuse hemorrhagic lesions and destruction of the tissue

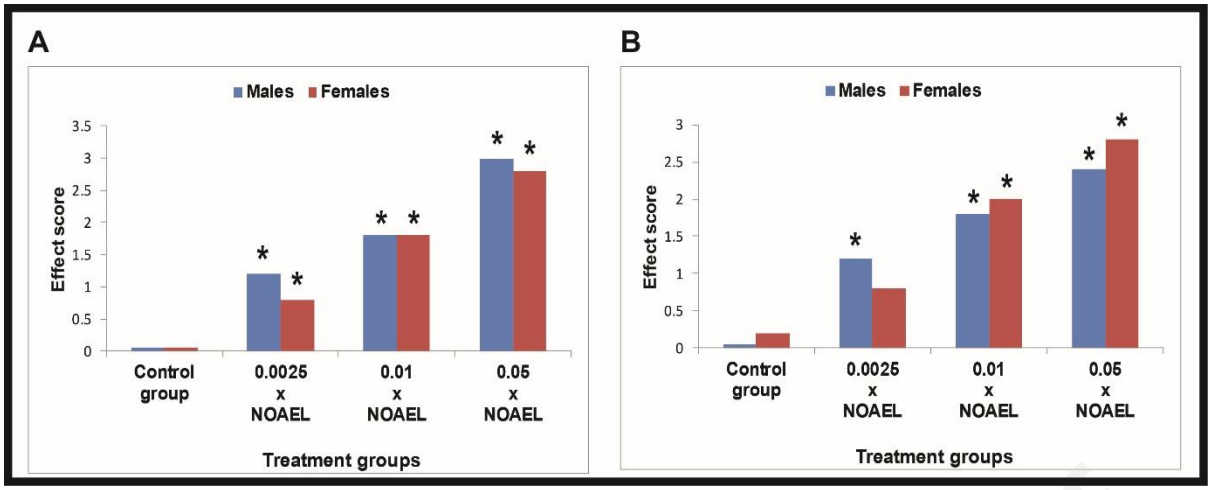

Figure 8. Effect of exposure to chemical mixture on lung tissue morphology. Lung Cytopathological changes as determined by TPT. A. Inflammatory cells. Phagocytic activity by alveolar macrophages.

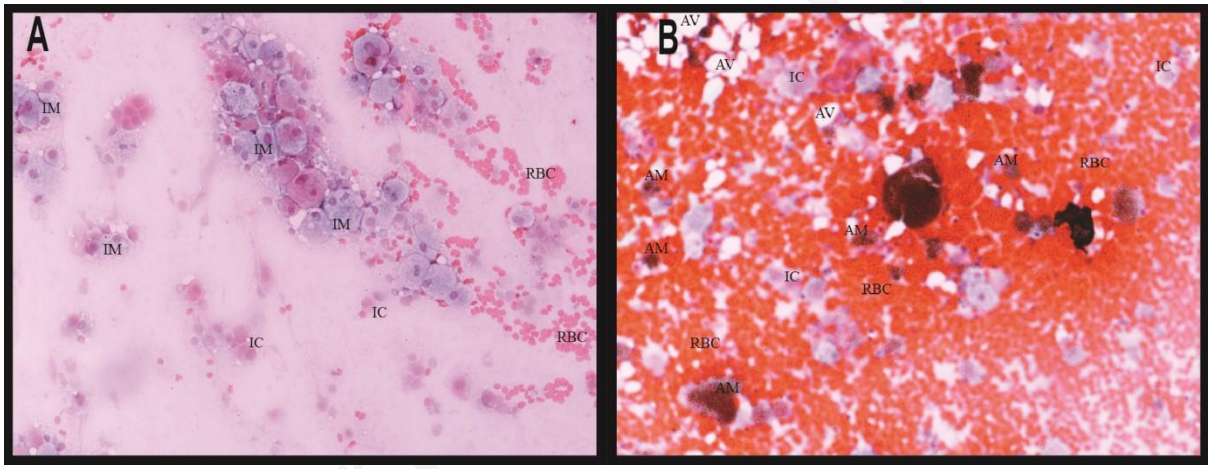

Figure 9. Cytopatological examination of lung tissue. A. Control group x200. AV= alveolus, IM= inactive macrophages, IC= Inflammatory cells, RBC= Red blood cells. No phagocytic activity by alveolar macrophages with mild inflammatory cells infiltration and low presence of red blood cells. B. 0.05x NOAEL group x200. AV= alveolus, AM= activated macrophages, IC= Inflammatory cells, RBC= Red blood cells. Marked phagocytic activity by alveolar macrophages with inflammatory cells infiltration and diffuse hemorrhagic infiltration

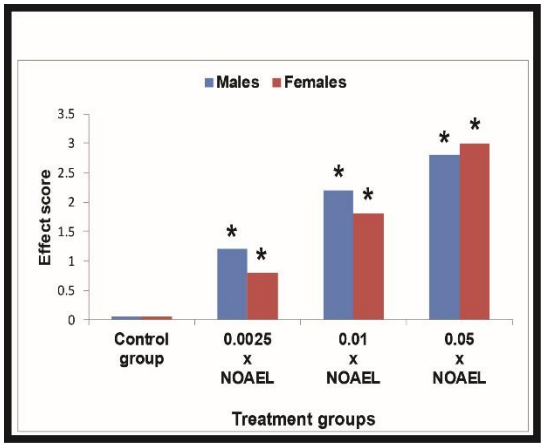

Figure 10. Effect of exposure to chemical mixture on brain tissue morphology. Brain Cytopathological changes as determined by TPT Glial and astrocyte cells.

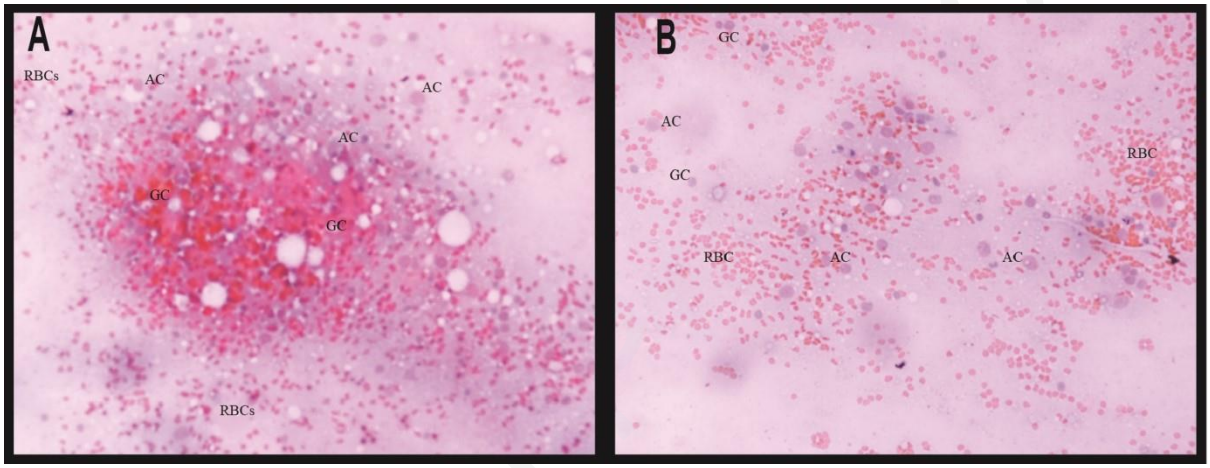

Figure 11. Cytopatological examination of brain tissue. A. Control group x200, AC= astrocytes, GC=glial cells RBC= Red blood cells. Normal number of glial cells and astrocytes with red blood cells presence. B. 0.05x NOAEL group x200, AC= astrocytes, GC= glial cells, RBCs=Red blood cells. Severe numerical reduction of glial and astrocyte cells with red blood cells presence

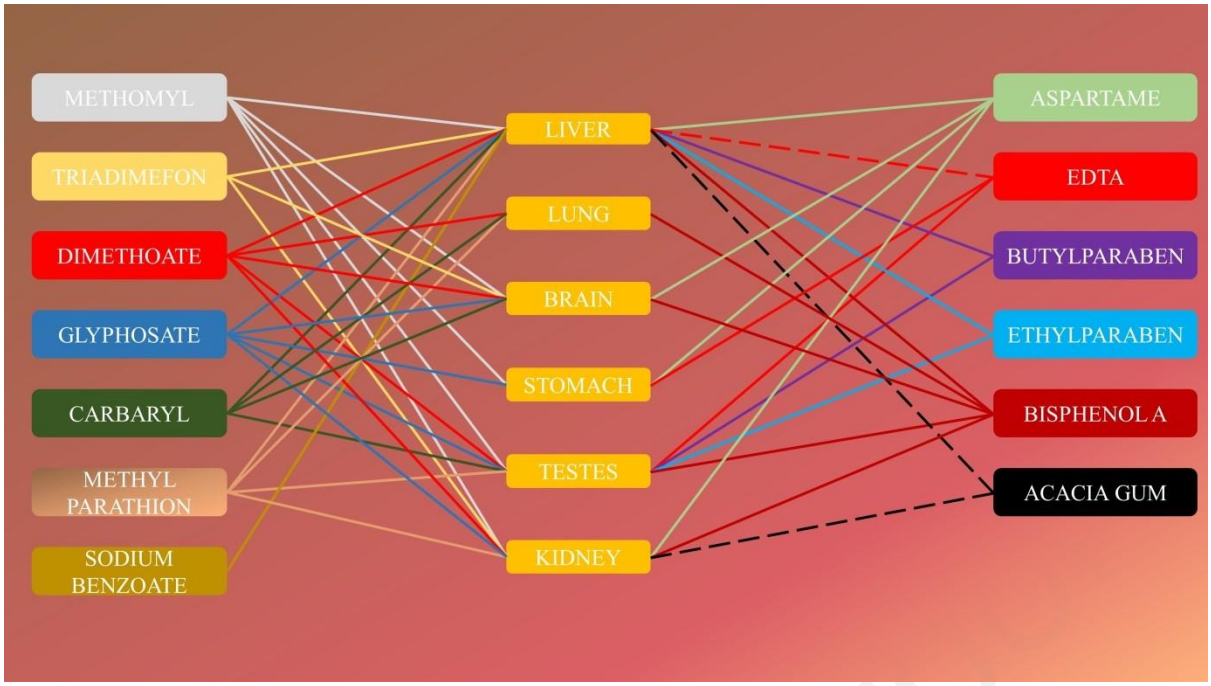

Figure 12. Graphical representation of a literature summary regarding the groups of chemicals that affect each organ. Solid lines represent a negative effect while dashed lines represent a protective effect

Table 1. The toxicological reference values for the individual chemicals used in the mixture and the critical effect

Chemical	ADI/TDI (mg/kg bw/day)	NOAEL rats (mg/kg bw/day)	Negative outcome
Methomyl	0.0025 (EFSA, 2009a)	0.25 (EFSA, 2009a)	Neurotoxicity
Triadimefon (Bayleton)	0.03 (EFSA, 2009b)	3.4 (EFSA, 2009b)	Hepatotoxicity
Dimethoate	0.001 (EFSA, 2013a)	0.1 (EFSA, 2013a)	Reproduction, neurotoxicity and developmental neurotoxicity
Glyphosate	0.5 (EFSA, 2015a)	50 (EFSA, 2015a)	Developmental toxicity
Carbaryl	0.0075 (EFSA, 2006)	1 (EFSA, 2006)	Neurotoxicity
Methyl parathion	0.003 (EFSA, 2011)	0.3 (EFSA, 2011)	Neurotoxicity
Aspartame	40 (EFSA, 2013b)	4000 (EFSA, 2013)	Developmental and maternal toxicity, carcinogenicity
Sodium benzoate	5 (EFSA, 2016)	500 (EFSA, 2016)	Developmental toxicity
EDTA	2.5 (European Commission, Joint Research Centre, 2004)	250 (European Commission, Joint Research Centre, 2004)	Reproductive toxicity
Ethylparaben	10 (EFSA, 2004)	1000	Male reproductive system
Butylparaben	0.5 (EFSA, 2004)	500	Male reproductive system
Bisphenol A	0.004 (EFSA, 2015b)	0.4	Kidney and liver toxicity
Acacia gum	34 (EFSA, 2010)	3411 (EFSA, 2010)	No observed toxicity

Table 2. The scoring system used for the quantification of the injury of the organs analysed by touch preparation technique (comparison is made with controls)

Organ	Effect	Grade of injury	Score	Effect description
Testis	Cellularity	*No*	0	Normal cell number
		Mild	1	≤10% reduction in cellularity
		Moderate	2	≤30% reduction in cellularity
		Severe	3	>30% reduction in cellularity
	Degenerative changes of the cells	*No*	0	Normal cellular structure
		Mild	1	≤10% cellular/nuclear deformities
		Moderate	2	≤30% cellular/nuclear deformities
		Severe	3	>30% cellular/nuclear deformities
Liver	Cellularity	*No*	0	Normal cell number
		Mild	1	≤10% reduction in cellularity
		Moderate	2	≤30% reduction in cellularity
		Severe	3	>30% reduction in cellularity
	Glycogen loss	*No*	0	Normal cellular glycogen content
		Mild	1	≤10% cellular glycogen lost
		Moderate	2	≤30% cellular glycogen lost

		Severe	3	>30% cellular glycogen lost
Stomach	Degenerative changes of the cells	*No*	0	Normal cellular structure
		Mild	1	≤10% cellular/nuclear deformities
		Moderate	2	≤30% cellular/nuclear deformities
		Severe	3	>30% cellular/nuclear deformities
	Cellularity	*No*	0	Normal cell number
		Mild	1	≤10% reduction in cellularity
		Moderate	2	≤30% reduction in cellularity
		Severe	3	>30% reduction in cellularity
	Tubular cell architecture changes	*No*	0	Growth in tubular cell aggregates is absent
		Mild	1	≤10% increase of tubular cell aggregates
		Moderate	2	≤30% increase of tubular cell aggregates
		Severe	3	>30% increase of tubular cell aggregates
Kidney	Cellularity	*No*	0	Normal cell number
		Mild	1	≤10% reduction in cellularity
		Moderate	2	≤30% reduction in cellularity
		Severe	3	>30% reduction in cellularity
	Level of growth in	*No*	0	Growth in tubular cell aggregates is absent
		Mild	1	≤10% increase of tubular cell aggregates

	tubular cell aggregates	*Moderate*	2	≤30% increase of tubular cell aggregates
		Severe	3	>30% increase of tubular cell aggregates
Lung	Inflammatory Cells	*No*	0	No inflammatory cell infiltration
		Mild	1	≤10% increase of inflammatory cells
		Moderate	2	≤30% increase of inflammatory cells
		Severe	3	>30% increase of inflammatory cells
	Phagocytic activity by alveolar macrophages	*No*	0	No alveolar macrophages present
		Mild	1	≤10% increase of phagocytic activity (presence of phagosomes)
		Moderate	2	≤30% increase of phagocytic activity (presence of phagosomes)
		Severe	3	>30% increase of phagocytic activity (presence of phagosomes)
Brain	Glial and astrocyte cells number	*No*	0	Normal cell number
		Mild	1	≤10% numerical reduction
		Moderate	2	≤30% numerical reduction
		Severe	3	>30% numerical reduction
Heart	Degenerative changes of	*No*	0	Normal cellular structure
		Mild	1	≤10% cellular/nuclear deformities
		Moderate	2	≤30% cellular/nuclear deformities

	the cells	*Severe*	3	>30% cellular/nuclear deformities
Spleen	Degenerative changes of the cells	*No*	0	Normal cellular structure
		Mild	1	≤10% cellular/nuclear deformities
		Moderate	2	≤30% cellular/nuclear deformities
		Severe	3	>30% cellular/nuclear deformities
Pancreas	Degenerative changes of the cells	*No*	0	Normal cellular structure
		Mild	1	≤10% cellular/nuclear deformities
		Moderate	2	≤30% cellular/nuclear deformities
		Severe	3	>30% cellular/nuclear deformities
Muscle	Degenerative changes of the cells	*No*	0	Normal cellular structure
		Mild	1	≤10% cellular/nuclear deformities
		Moderate	2	≤30% cellular/nuclear deformities
		Severe	3	>30% cellular/nuclear deformities
Uterus	Degenerative changes of the cells	*No*	0	Normal cellular structure
		Mild	1	≤10% cellular/nuclear deformities
		Moderate	2	≤30% cellular/nuclear deformities
		Severe	3	>30% cellular/nuclear deformities

Table 3. The effect of exposure to chemical mixture on micronuclei frequency in rat bone marrow cells

Descriptives	Group	Mean	SD	95%CI		Minimum	Maximum		
				95%LB	95%UB				
PCEs/(PCEs+NCEs) %	Control	53,6	3,1	51,4	55,8	49,6	58,2	F	5,957
	0,0025xNOAEL	51,5	4,2	48,5	54,5	46,2	57,4	df1, df2	3, 36
	0.01xNOAEL	51,1	2,5	49,3	52,9	46,4	54,4	p	**0,002**
	0.05xNOAEL	47,8	2,3	46,1	49,5	44,8	52,2		
MNPCEs ‰	Control	6	0,8	5,4	6,6	5	7	F	22,005
	0,0025xNOAEL	6,8	0,6	6,3	7,3	6	8	df1, df2	3, 36
	0.01xNOAEL	8,4	1,3	7,4	9,4	7	11	p	**<0,001**
	0.05xNOAEL	10,5	2,1	9	12	8	13		

(A)

Multiple Comparisons						
Dependent Variable			(I) Dose			
			Control	0,0025xNOAEL	0.01xNOAEL	0.05xNOAEL
			Sig.	Sig.	Sig.	Sig.
PCEs/(PCEs+NCEs) %	LSD	Control		0,136	0,083	**0,000**
		0,0025xNOAEL	0,136		0,797	**0,012**
		0.01xNOAEL	0,083	0,797		**0,022**
		0.05xNOAEL	0,000	0,012	0,022	
	Dunnett t (2-sided)[b]	Control		0,307	0,197	**0,001**
MNPCEs ‰	LSD	Control		0,190	**0,000**	**0,000**
		0,0025xNOAEL	0,190		**0,011**	**0,000**
		0.01xNOAEL	0,000	0,011		**0,001**
		0.05xNOAEL	0,000	0,000	0,001	
	Dunnett t (2-sided)[b]	Control		0,410	**0,001**	**0,000**
b. Dunnett t-tests treat one group as a control, and compare all other groups against it.						

(B)

Micronuclei in PCEs		
Male Subjects	**MN ± SD**	**p**

Control	5.8 ± 0.84	
0.0025 x NOAEL	6.8 ± 0.45	0.69
0.01 x NOAEL	7.4 ± 0.55	0.52
0.05 x NOAEL	8.6 ± 0.55	0.28
Female Subjects	**MN ± SD**	**p**
Control	6.2 ± 0.84	
0.0025 x NOAEL	6.8 ± 0.84	0.81
0.01 x NOAEL	9.4 ± 1.14	0.23
0.05 x NOAEL	12.4 ± 0.55	**0.03**
All subjects	**MN ± SD**	**p**
Control	6.0 ± 0.81	
0.0025 x NOAEL	6.8 ± 0.63	0.75
0.01 x NOAEL	8.4 ± 1.35	0.35
0.05 x NOAEL	10.5 ± 2.07	0.09

(C)

Male Subjects	**PCEs/(PCEs+NCEs) %**
Control	52.96 ± 3.48
0.0025xNOAEL	50.20 ± 4.58
0.01 x NOAEL	52.44 ± 1.68
0.05 x NOAEL	47.76 ± 2.18
Female Subjects	**PCEs/(PCEs+NCEs) %**
Control	54.24 ± 2.93
0.0025xNOAEL	52.76 ± 3.73
0.01 x NOAEL	49.80 ± 2.57
0.05 x NOAEL	47.84 ± 2.74
All subjects	**PCEs/(PCEs+NCEs) %**
Control	53.60 ± 3.11
0.0025 x NOAEL	51.48 ± 4.16
0.01 x NOAEL	51.12 ± 2.48
0.05 x NOAEL	47.80 ± 2.34

(D)

Table 4. Results of cytopathological examination of tissue samples by the touch preparation technique

Organ	Effect	Grade of injury	MALES				FEMALES			
			Control	0.0025 x NOAEL	0.01 x NOAEL	0.05 x NOAEL	Control	0.0025 x NOAEL	0.01 x NOAEL	0.05 x NOAEL
Testis	Cellularity	*No*	4/5	-	-	-	-	-	-	-
		Mild	1/5	4/5	-	-	-	-	-	-
		Moderate	-	1/5	4/5	1/5	-	-	-	-
		Severe	-	-	1/5	4/5	-	-	-	-
	Degenerative changes of the cells	*No*	4/5	4/5	-	-	-	-	-	-
		Mild	1/5	1/5	4/5	-	-	-	-	-
		Moderate	-	-	1/5	1/5	-	-	-	-
		Severe	-	-	-	4/5	-	-	-	-
Liver	Cellularity	*No*	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
		Mild		-	-	-	-	-	-	-
		Moderate	-	-	-	-	-	-	-	-
		Severe	-	-	-	-	-	-	-	-
	Glycogen loss	*No*	5/5	5/5	-	-	5/5	5/5	-	-
		Mild	-	-	1/5	-	-	-	1/5	-
		Moderate	-	-	4/5	1/5	-	-	4/5	1/5
		Severe	-	-	-	4/5	-	-	-	4/5
Stomach	Degenerative changes of the cells	*No*	5/5	5/5	-	-	5/5	5/5	-	-
		Mild	-	-	1/5	-	-	-	1/5	-
		Moderate	-	-	4/5	1/5	-	-	4/5	1/5
		Severe	-	-	-	4/5	-	-	-	4/5
	Cellularity	*No*	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
		Mild	-	-	-	-	-	-	-	-
		Moderate	-	-	-	-	-	-	-	-
		Severe	-	-	-	-	-	-	-	-
	Tubular cell architecture changes	*No*	5/5	5/5	-	-	5/5	5/5	-	-
		Mild	-	-	1/5	-	-	-	1/5	-
		Moderate	-	-	4/5	1/5	-	-	4/5	1/5
		Severe	-	-	-	4/5	-	-	-	4/5
Kidneys	Cellularity	*No*	5/5	-	-	-	4/5	-	-	-
		Mild	-	4/5	1/5	-	1/5	4/5	-	-
		Moderate	-	-	4/5	1/5	-	1/5	4/5	1/5
		Severe	-	1/5	-	4/5	-	-	1/5	4/5
	Level of growth in	*No*	5/5	5/5	5/5	-	5/5	5/5	5/5	-
		Mild	-	-	-	-	-	-	-	-

	tubular cell aggregates	*Moderate*	-	-	-	1/5		-	-	-	1/5
		Severe	-	-	-	4/5		-	-	-	4/5
Lungs	**Inflammatory Cells**	*No*	5/5	-	-	-		5/5	1/5	-	-
		Mild	-	4/5	1/5	-		-	4/5	1/5	-
		Moderate	-	1/5	4/5	-		-	-	4/5	1/5
		Severe	-	-	-	5/5		-	-	-	4/5
	Phagocytic activity by alveolar macrophages	*No*	5/5	-	-	-		4/5	1/5	-	-
		Mild	-	4/5	-	1/5		1/5	4/5	1/5	-
		Moderate	-	1/5	5/5	1/5		-	-	4/5	1/5
		Severe	-	-	-	3/5		-	-	-	4/5
Brain	**Glial and astrocyte cells**	*No*	5/5	-	-	-		5/5	1/5	-	-
		Mild	-	4/5	-	-		-	4/5	1/5	-
		Moderate	-	1/5	4/5	1/5-		-	-	4/5	-
		Severe	-	-	1/5	4/5		-	-	-	5/5
Heart	**Degenerative changes of the cells**	*No*	5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
		Mild	-	-	-	-		-	-	-	-
		Moderate	-	-	-	-		-	-	-	-
		Severe	-	-	-	-		-	-	-	-
Spleen	**Degenerative changes of the cells**	*No*	5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
		Mild	-	-	-	-		-	-	-	-
		Moderate	-	-	-	-		-	-	-	-
		Severe	-	-	-	-		-	-	-	-
Pancreas	**Degenerative changes of the cells**	*No*	5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
		Mild	-	-	-	-		-	-	-	-
		Moderate	-	-	-	-		-	-	-	-
		Severe	-	-	-	-		-	-	-	-
Muscles	**Degenerative changes of the cells**	*No*	5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
		Mild	-	-	-	-		-	-	-	-
		Moderate	-	-	-	-		-	-	-	-
		Severe	-	-	-	-		-	-	-	-
Uterus	**Degenerative changes of the cells**	*No*	-	-	-	-		5/5	5/5	5/5	5/5
		Mild	-	-	-	-		-	-	-	-
		Moderate	-	-	-	-		-	-	-	-
		Severe	-	-	-	-		-	-	-	-

Table 5. The effect of exposure to chemical mixture on rat tissues morphology as determined by the touch preparation technique and expressed as the average score of injury

Organ	Effect		MALES					FEMALES			
			Control	0.0025 x NOAEL	0.01 x NOAEL	0.05 x NOAEL		Control	0.0025 x NOAEL	0.01 x NOAEL	0.05 x NOAEL
Testis	Cellularity		0.2	1.2*	1.8*	2.8*		-	-	-	-
	Degenerative changes of the cells		0.2	0.2	1.2*	2.8*		-	-	-	-
Liver	Cellularity		0	0	0	0		0	0	0	0
	Glycogen loss		0	0	1.8*	2.8*		0	0	1.8*	2.8*
Stomach	Degenerative changes of the cells		0	0	1.8*	2.8*		0	0	1.8*	2.8*
	Cellularity		0	0	0	0		0	0	0	0
	Tubular cell architecture changes		0	0	1.8*	2.8*		0	0	1.8*	2.8*
Kidneys	Cellularity		0	1.2*	1.8*	2.8*		0.2	1.4*	2.2*	2.8*
	Level of growth in tubular cell aggregates		0	0	0.1	2.8*		0	0	0.1	2.8*
Lungs	Inflammatory Cells		0	1.2*	1.8*	3*		0	0.8*	1.8*	2.8*
	Phagocytic activity by alveolar macrophages		0	1.2*	1.8*	2.8*		0.2	0.8	2*	2.8*
Brain	Glial and astrocyte cells		0	1.2*	2.2*	2.8*		0	0.8*	1.8*	3*
Heart	Degenerative		0	0	0	0		0	0	0	0

	changes of the cells										
Spleen	**Degenerative changes of the cells**		0	0	0	0		0	0	0	0
Pancreas	**Degenerative changes of the cells**		0	0	0	0		0	0	0	0
Muscles	**Degenerative changes of the cells**		0	0	0	0		0	0	0	0
Uterus	**Degenerative changes of the cells**		-	-	-	-		0	0	0	0

P value for the difference between the four groups was calculated using ANOVA test and the contrasts were performed with Dunnet post-hoc test adjusted for multiple comparisons;

*P<0.05