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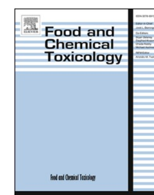

Detrimental effects of 6 months exposure to very low doses of a mixture of six pesticides associated with chronic vitamin deficiency on rats

Aristidis Tsatsakis [a,***], Nadezhda V. Tyshko [b], Marina Goumenou [a,c,**], Svetlana I. Shestakova [b], El'vira O. Sadykova [b], Valentin M. Zhminchenko [b], Ovidiu Zlatian [d], Daniela Calina [e], Valentina A. Pashorina [b], Nikolaj S. Nikitin [b], Marina D. Trebukh [b], Maria S. Loginova [b], Eleanora N. Trushina [b], Oksana K. Mustafina [b], Ludmila I. Avrenyeva [b], Galina V. Guseva [b], Nikita V. Trusov [b], Lidiya V. Kravchenko [b], Antonio F. Hernández [f,g], Anca Oana Docea [h,*]

[a] *Center of Toxicology Science & Research, Medical School, University of Crete, Heraklion, Crete, Greece*
[b] *Federal Research Centre of Nutrition, Biotechnology and Food Safety, Ustinsky proyezd 2/14, 109240, Moscow, Russia*
[c] *General Chemical State Laboratory of Greek Republic, 71202, Heraklion, Greece*
[d] *Department of Microbiology, University of Medicine and Pharmacy, Faculty of Pharmacy, Craiova, 200349, Romania*
[e] *Department of Clinical Pharmacy, University of Medicine and Pharmacy of Craiova, 200349, Craiova, Romania*
[f] *Department of Legal Medicine and Toxicology, School of Medicine, University of Granada, Granada, Spain*
[g] *Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain*
[h] *Department of Toxicology, University of Medicine and Pharmacy, Faculty of Pharmacy, 200349, Craiova, Romania*

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ABSTRACT

This study aimed to evaluate the long-term low-dose effects of exposure to a mixture of 6 pesticide active substances (diquat, imazamox, imazethapyr, tepraloxydin, bentazone, acifluorfen) and to elucidate if chronic vitamin deficiency can influence their toxicity. Two hundred Wistar rats were divided in 4 groups: a vitamin-sufficiency control group, a vitamin-deficiency control group, a vitamin sufficiency test group and a vitamin-deficiency test group. The test groups were treated with the aforementioned pesticides at doses 100 times lower than the corresponding NOAEL. After 6 months, ten rats from each group were sacrificed and a complete evaluation of blood and urine biochemistry, biomarkers of oxidative stress, xenobiotic detoxification enzymes and lysosomal enzymes and organ histopathology was performed. The pesticides mixture and vitamin deficiency determined an increase in alkaline phosphatase levels and urinary calcium levels, abnormal serum lipid profile, and a decrease of total blood proteins levels, red blood cells, haematocrit and haemoglobin. The combination of the two stressors up-regulated CYP1A1, CYP1A2, CYP2B1 and GST levels. This study provides a new proof for the need to move forward from single chemical testing to a more complex approach to account for the multitude of stressors that can challenge the setting of real safety levels.

1. Introduction

In the last years, the industrialization and modernization of the production processes in all sectors entails omnipresent exposure to a cocktail of chemicals. Everything that we eat, drink, breath or use as hygiene and lifestyle products contains mixtures of chemicals. From early intrauterine life till elderly, the individual is continuously exposed to chemicals with beneficial or detrimental effects depending on the doses, windows of exposure and combinations. Many of these exposures are considered risk factors for many diseases, and where associated with genetic patterns can contribute to the occurrence of diseases (Docea et al., 2017). The organism can respond differently to these stressors with time-dependent variations that can vary from changes intended to maintain the homeostasis of the organism to pathological changes

* Corresponding author.
** Corresponding author. General Chemical State Laboratory of Greek Republic, 71202, Heraklion, Greece.
*** Corresponding author.
E-mail addresses: tsatsaka@uoc.gr (A. Tsatsakis), marina.goumenou@gmail.com (M. Goumenou), ovidiu.zlatian@gmail.com (O. Zlatian), ajerez@ugr.es (A.F. Hernández), ancadocea@gmail.com (A.O. Docea).

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associated with different shapes of dose-response curves (Agathokleous, 2018; Agathokleous and Calabrese, 2020). Till now the toxicological evaluation of chemicals has been focused mainly on testing high doses of single chemicals in experimental animals for setting safety limits and to identify target organ(s) (Tsatsakis et al., 2016). Unfortunately, this approach does not mirror the real-life exposure scenario in which individuals are exposed simultaneously to low doses of multiple stressors (Tsatsakis et al., 2016). The safety evaluation of mixtures has been done till now only for commercial mixtures and for mixtures of chemicals that share the same mode of action, the same phenomenological effect or target organ, but they don't incorporate the dose-response spectrum and the temporal variations that can better predict the time-dependent dose responses to environmental relevant mixtures. Studies have shown that mixtures of xenobiotics can lead not only to predictable additive effects but also to unpredictable synergistic, or antagonistic effects (Lukowicz et al., 2018; Mesnage et al., 2020) (Hernández et al., 2013; Docea et al., 2018, 2019; Tsatsakis et al., 2019a, 2019b, 2019c; Fountoucidou et al., 2019; Sergievich et al., 2020). These effects cannot be predicted by standard single chemical toxicological evaluations and need a more complex approach for protecting the population. Starting from these limitations inherent to single-chemical testing, new methodologies have been proposed for simulating the real-life exposure scenario in which individuals are exposed to multiple stressors from different sources on a long term regimen (Docea et al., 2016; Goumenou and Tsatsakis, 2019; Hernández and Tsatsakis, 2017; Tsatsakis et al., 2017, 2019d; Tsatsakis and Lash, 2017).

Environmental or occupational exposure to plant protection products has been associated with a full range of chronic disorders, such as hepatic diseases (Freire et al., 2015), haematological diseases (Freire et al., 2015; García-García et al., 2016), renal diseases (Georgiadis et al., 2018a; Năstăsescu et al., 2020), cardiovascular diseases (Georgiadis et al., 2018b), obesity and metabolic syndrome (Petrakis et al., 2017), neurological diseases (Aloizou et al., 2020), infertility (Sifakis et al., 2017) and even cancers (Vakonaki et al., 2013; Dolapsakis et al., 2001). These findings can be explained by the fact that the so-called safety levels are set based on single compound experiments that follow one critical effect, while in reality humans are exposed to a cocktail of chemicals that, depending on the dose, can modify the effects or lead to different target organs effects (Hernández et al., 2013).

B vitamins and folate are implicated in a lot of functions of the organism and their deficiency has been associated with an increased risk for cardiovascular disorders, degenerative diseases, immune dysfunctions and inflammatory diseases (Mikkelsen and Apostolopoulos, 2018). Vitamin B2 or riboflavin deficiency can alter iron absorption and has been implicated in metabolic disorders associated with impaired tryptophan metabolism and mitochondrial function (Thakur et al., 2017). Vitamin B3 or niacin is an indispensable vitamin that plays a role in more than 400 NAD(P) (nicotinamide adenine dinucleotide phosphate)-dependent reactions in all areas of human metabolism. The deficiency of this vitamin is associated with genome instability, apoptosis, chromosomal breakage, telomere erosion and cancer development (Kirkland, 2012). Vitamin K or menadione is a cofactor of γ-glutamyl carboxylase involved in the activation of a family of vitamin K dependent-proteins. These proteins have several functions in the organism related to reproduction, bone and vascular function and metabolism (Fusaro et al., 2020). Studies have shown that vitamin K deficiency is followed by a decrease of matrix Gla protein (a vitamin K-dependent protein that inhibits vascular calcification), thus increasing the risk of cardiovascular diseases (Piscaer et al., 2017).

Starting from the new methodology for the toxicological testing of chemical mixtures under the real-life risk simulation approach proposed by Tsatsakis et al. (2017), this study aimed to evaluate the long-term effects of exposure to a mixture of 6 frequently used pesticides (diquat, imazamox, imazethapyr, tepraloxydin, bentazone, and acifluorfen) in low doses (100 times below their corresponding critical NOAELs). These chemicals were selected as they are herbicides usually found as residues in food and drinking water (A)A: Overview of Sod (2002); Authority (2017); Agency (2011). Diquat mechanism of toxicity in humans is associated with generation of oxidative stresss eventually leading to hepatotoxicity, neurotoxicity and nephrotoxicity (Magalhães et al., 2018a). Imazamox is considered safe to non-target species, showing low toxicity in animal studies with the critical effect being decrease in bodyweight gain and the decrease in food consumption (Authority, 2016). The long-term effects of imazethapyr are not completely known, even if the general population can be exposed through food or water route (Koutros et al., 2009; Imazethapyr; Pesticide Tolerance, 2002). The main target organs for tepraloxydim toxicity were the liver, the spleen/hematopoietic system and reproductive system (Federal Register/Vol. 7, 2011) Bentazone has been associated with impairment of blood coagulation and liver and kidney effects in regulatory studies (Authority, 2015a) Acifluorfen is associated with liver and kidney toxicity as well as teratogenicity and carcinogenicity in non-target organisms (Kenyon and Duke, 1985) and the proposed mechanisms underlying cytotoxicity in humans include activation of nuclear receptors (CAR and PPARα) whereas inhibition of protoporphyrinogen oxidase inhibition each may play some role in rodent liver changes (Kuwata et al., 2016) In order to simulate more the real-life exposure scenario, plant protection products were used instead of pesticide active substances. This study also addressed whether chronic vitamin deficiency can influence the toxicity of the pesticides mixture.

2. Materials and methods

2.1. Animal experiment

This article presents part of the results of a larger study whose methodology was previously described by Tsatsakis et al. (2019b). Briefly, 200 male Wistar rats, 30 days old, from Affiliated Unit "Stolbovaya" of Scientific Center for Biomedical Technology of the Federal Medical and Biological Agency, Moscow, Russia, were divided into 4 experimental groups (50 rats each):

- C-100 group – or the control group that received the standard rat diet (AIN-93) that contained 100% of the dose of water-soluble vitamins requested for the normal developing of the rats and water *at libitum* for 6 months.
- C-25% - or the vitamin-deficiency control group that received a modified AIN-93 diet with only 25% of the dose of water-soluble vitamins and water *at libitum* for 6 months.
- T-100 group – or the vitamin-sufficiency test group that received the standard rat diet that contained 100% of the dose of water-soluble vitamins requested for the normal developing of the rats and water *at libitum* and a mixture of 6 pesticides in the form of plant protection products (diquat, imazamox, imazethapyr, tepraloxydin, bentazone and acifluorfen) at a concentration 100 times lower than the NOAEL (corresponding to the acceptable daily intake –ADI– for individual pesticides) included in the diet for 6 months (Table 1).
- T-25 group – or the vitamin-deficiency test group that received a modified AIN-93 diet with only 25% of the dose of water-soluble vitamins and water *at libitum* and a mixture of 6 pesticides in the

Table 1
Composition of the pesticide mixture and the corresponding critical NOAEL levels.

Pesticide	NOAEL (mg/kg bw)	References
Acifluorfen	1.25	(A)A: Overview of Sod (2002)
Bentazone	9	Authority (2015b)
Diquat	0.19	(WHO) WHO, 1998)
Imazamox	1068	Memorandum (2001)
Imazethapyr	55	Authority (2017)
Tepraloxydim	5	Agency (2011)

form of plant protection products (diquat, imazamox, imazethapyr, tepraloxydin, bentazone and acifluorfen) at a concentration 100 times lower than the NOAEL (corresponding to the ADI for individual pesticides) included in the diet for 6 months (Table 1).

The doses of water-soluble vitamins necessary for the normal rat development contained in the standard AIN-93 diet are: 0.6 g/kg diet thiamine (vitamin B1), 0.6 g/kg diet riboflavin (vitamin B2), 0.7 g/kg diet pyridoxine (vitamin B6), 3 g/kg diet niacin (vitamin B3), 1.6 g/kg diet calcium pantothenate (vitamin B5), 0.2 g/kg diet folic acid (vitamin B9), 0.0025 g/kg diet cyanocobalamin (vitamin B12) and 0.075 g/kg diet menadione (vitamin K) (Tyshko and Shestakova, 2018).

The animals were kept in special animal cages, 2 animals per cage at constant temperature (22 ± 1 °C) and humidity (50 ± 10%) and 12 h dark/light cycle. Each animal was observed daily for the occurrence of signs of toxicity and body weight was monitored weekly over the whole study period. The feed intake was measured every two days and the concentration of the pesticide mixture in feed was adjusted according to the consumption for accurate exposure of the animals to the selected doses. At 6 months after starting the experiment, 10 rats from each group were sacrificed by isoflurane overdose followed by decapitation according to American Veterinary Medical Association guidelines (Leary, 2013) and Rules of laboratory practice approved by Order of the Ministry of Health of the Russian Federation No. 193n of January 01, 2016. Rat blood was collected and analyzed for complete blood count (CBC), clinical biochemistry parameters, biomarkers of oxidative stress and antioxidant enzymes evaluation. The activity of xenobiotic detoxification enzymes and lysosomal enzymes was determined in liver microsomal fraction and cytoplasmic fraction, respectively. The thymus, heart, lungs, liver, kidneys, adrenals, spleen, small intestine, testicles, and prostate gland were collected for histological evaluation.

2.2. Chemicals

The pesticides used in the study were in the form of plant protection products bought from BASF SE, Germany: Galaxy Top, VRK (160 g/L acifluorfen, CAS number: 50594-66-6), Bazagran, BP (480 g/L bentazone, CAS number: 25057-89-0), Sukhovey, BP (150 g/L diquat, CAS number 85-00-7), Pulsar (40 g/L imazamox, CAS number:114311-32-9), Pivot, VK (100 g/L imazethapyr, CAS number: 81335-77-5), Aramo 45, CE (45 g/L, CAS number: 149979-41-9).

The vitamins used in the study were bought from Merck KGaA, Germany: thiamine (CAS number: 67-03-8), riboflavin (CAS Number: 83-88-5), pyridoxine (CAS Number: 65-23-6), nicotinic acid (CAS Number: 59-67-6), calcium pantothenate (CAS Number:137-08-6), folic acid (CAS Number: 59-30-3), cyanocobalamin (CAS Number: 68-19-9), menadione (CAS Number: 58-27-5).

2.3. Serum biochemical tests

Blood was collected from the heart in 8 mL Vacuette® Tube Cat Serum Separator Clot Activator. After serum separation, the serum samples were kept at −20 °C till further analysis. Konelab 20i automatic biochemical analyzer (Thermo Fisher Scientific, USA) with corresponding Thermo Fisher Scientific, USA reagents were used for the determination of GLU: glucose; ALT:alanine aminotransferase; ALB: albumin; AMY:amylase; TP: total protein; BILT: total bilirubin; BILD: direct bilirubin; GLOB: globulin; CPK: creatine phosphokinase; Ca2+: calcium; CRE: creatinine; LDH: lactate dehydrogenase; Mg^{2+}: magnesium; BUN: blood urea nitrogen; TRYG: tryglyceride; CHOL: cholesterol; ALKP: alkaline phosphatase; AST:aspartate aminotransferase; K+: potassium; Na+: sodium; Cl-: chloride.

2.4. Urine biochemical tests

Twenty-four-hour urine samples were collected using metabolic cages (Tecniplast, Italy) and aliquots were transferred into Eppendorf Safe-Lock Tubes of 1.5 mL. Urine samples were kept at +18 °C for less than 2 h until analysis. Konelab 20i automatic biochemical analyzer (Thermo Fisher Scientific, USA) with corresponding Thermo Fisher Scientific, USA reagents were used for Glucose, Relative density, Uric Acid, Ca^{2+}, Creatinine, Mg^{2+}, Urea, Phosphorus determination. Qubit® Fluorometer (Invitrogen, USA) with Molecular Probes™ life technologies reagents were used for protein determination. Urine pH was measured with a pH meter MP220 Basic (Mettler-Toledo GmbH", Switzerland).

2.5. Blood haematological parameters

Blood samples were collected from the heart and immediately aliquoted into 2 mL Vacuette® Tube with K3-EDTA as an anticoagulant. RBC (red blood cells), HGB (haemoglobin), HCT (haematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin), MCHC (mean corpuscular haemoglobin concentration), WBC (white blood cells), NEU (neutrophil granulocytes), EOS (eosinophil granulocytes), BAS (basophil granulocytes), LYM (lymphocytes), MON (monocytes), PLT (platelets), MPV (mean platelet volume) and PCT (plateletcrit) were determined using an Ac•T 5diff CP (Cap Pierce) haematology analyzer (Beckman Coulter, Inc., USA) using the corresponding reagent bought from Beckman Coulter, Inc., USA.

2.6. Oxidative stress parameters in blood and liver

All antioxidant enzymes described below were determined in erythrocytes using a spectrophotometer GENESYS 2 (Thermo, USA). Glutathione reductase activity was determined by the method of Tillotson (Tillotson and Sauberlich, 1971) modified by Mal'tsev (Mal'tsev and Vasil'ev, 1994) by measuring the rate of decrease in optical density at 340 nm caused by oxidation of NADPH at 37 °C. Glutathione peroxidase activity was determined according to the method of Mills (1959) at 340 nm and 37 °C. Catalase activity was determined by the method of Oshino (Oshino et al., 1973) modified by Mal'tsev (Mal'tsev and Vasil'ev, 1994), by measuring the decrease in optical density at 340 nm caused by the oxidation of NADH at 37 °C. Superoxide dismutase activity was determined by the method of Niashikimi et al. (Nishikimi et al., 1972) modified by Maltsev (Mal'tsev and Vasil'ev, 1994) (see below).

Total antioxidant activity was determined as the FRAP (Ferric Reducing Antioxidant Power) assay (Benzie and Strain, 1996). A volume of 0.6 mL of the test material (blood, plasma and liver cytoplasmic fraction) was added to 1.86 mL of the reaction medium containing 0.83 mM 2,4,6-tripyridyl-s-triazine and 1.67 mM $FeCl_3 \cdot 6H_2O$ in 0.25 M acetate buffer, pH 3.6, and the change in optical density was measured at 593 nm.

Ethoxyresorufin (CYP1A1) was determined in liver microsomal fraction by means of the ethoxyresorufin dealkylase enzyme activity assay (Nakajima et al., 1999). The fluorescence intensity was measured at λex = 574 nm and λem = 596 nm using a Fluorescence spectrophotometer Cary Eclipse (Agilent, USA). Methoxyresorufin (CYP1A2) and pentoxyresorufin (CYP2B1) were also determined in liver microsomal fraction by means of the methoxy- and pentoxyresorufin dealkylase activity, respectively (Burke and Mayer, 1983). The rate of resorufin formation was determined by the fluorescence intensity at λex = 530 nm and λem = 585 nm at 24 °C using the same Fluorescence spectrophotometer.

Glutathione S-transferase (GST) activity was determined in the cytoplasmic fraction of the liver by the method of Habig et al. (1974) by monitoring the change in optical density at 340 nm at 25 °C. The quantitative calculation was carried out taking into account the coefficient of molar extinction of 1 chloro 2,4 dinitrobenzene ε = 9.6 mM-1cm-1.

The activity of arylsulfatases A and B, β-galactosidase, and β

glucuronidase was determined in homogenates and liver cytosolic fraction using a Spectrophotometer Agilent HP 8453 (Agilent, USA) using the method of Dingle (Reid E: Lysosomes — A lab, 1977). After 30 min of incubation at 37 °C, the optical density was measured at 515 nm for arylsulfatases A and B, and at 420 nm for β-galactosidase and β-glucuronidase. The total protein content in the cytoplasmic and microsomal liver fractions was determined by the method of Lowry et al. (1951) with Folin's reagent using the same spectrophotometer.

The concentration of malondialdehyde (MDA) was determined based on the reaction between MDA and thiobarbituric acid. At high temperature and acidic pH a coloured trimethine complex containing one MDA molecule and 2 molecules of thiobarbituric acid is formed. Optical density of the trimethine complex was measured at 532 nm in a SmartSpec ™ 3000 Spectrophotometer (Bio-Rad Laboratories, USA). MDA in erythrocytes was determined by the method of Ernster and Nordenbrand (1967), the concentration of MDA in serum was determined using the method of Michara (Mihara et al., 1980), and the content of MDA in the liver was determined by the method of Kostiuk (Kostiuk and Potapovich, 1987). Calculations were performed using a molar extinction coefficient of 1.56 × 105 cm-1 x M-1.

2.7. Histopathological evaluation of the organs

Thymus, heart, lungs, liver, kidneys, adrenals, spleen, small intestine, testicles, and the prostate gland were histologically investigated. Organs were fixed in 10% formalin solution, preparations stained with hematoxylin-eosin and van Gieson's stain. Histological preparations were assessed in light microscope AxioImager Zl (Carl Zeiss GmbH, Germany). Morphometry was performed with AxioVision 4.8 (Carl Zeiss Vision GmbH, Germany).

2.8. Statistical methods

Data were collected in Microsoft Excel (Microsoft Corporation, USA) and then transferred to STATA software (StataCorpLLc, USA) for statistical analysis. All data were expressed as mean ± standard deviation.

The groups were compared by ANOVA method with Dunnet's post-hoc adjustment if data were normally distributed (tested by Shapiro-walk test) or by Kruskal-Wallis non parametric test with Mann-Whitney post-hoc tests with Holm Sidak's adjustment if the data were not normally distributed. All P values were considered significant if they were less than 0.05.

The differences between groups were calculated as percentage of means difference divided by the first group mean. The following comparisons were made:

- T-100 vs. C-100: inform on the specific effect of the pesticide mixture.
- T-25 vs. C-100: inform on the combined effect of the pesticide mixture and the vitamin-deficient diet.
- T-25 vs C-25: inform on the combined effect of the pesticide mixture and the vitamin-deficient diet relative to the vitamin-deficient control rats (this comparison is similar to T-100 vs. C-100 but under the stressful conditions of a vitamin-deficient diet).
- C-25 vs C-100: inform on the independent effect of the vitamin-deficient diet in control rats.
- T-25 vs T-100: inform on the effect of the vitamin-deficient diet on animals treated with the pesticide mixture

Longitudinal data analysis (body weight, feed consumption and feed efficiency) was performed using the mean difference. The differences between groups were assessed using Gompertz growth model. The parameters of the models were then tested for significant differences by Student's t tests.

The comparison between the time evolution of feed consumption and feed efficacy was performed by comparing groups two by two using linear regression. If the regression coefficient significantly differed from zero it was meant that the two sets of values differed significantly.

3. Results

3.1. Weight gain of rats over time

All the growth models showed a very good fit (adjusted R-squared of ≥0.992). The pesticide mixture (T100 group vs C100 group) caused a statistically significant decrease in Wmax (maximum weight) and inflection time ($p < 0.001$ for both), and a statistically significant increase in the growth rate (Table 2). Decreased vitamin consumption (C25 group vs C100 group) caused a statistically significant decrease in Wmax ($p < 0.001$) and in the growth rate ($p = 0.020$) but it had no effect on the inflection time (Table 2). The combined effect of pesticides and vitamin deficiency (T25 group vs C100 group) showed a significant decrease in Wmax and inflection time ($p < 0.001$ for both) and a significant increase in the growth rate ($p < 0.001$) (Table 2). For Wmax, a clear additivity between the two effects was observed. The pesticide mixture had an effect on the inflexion time under the vitamin deficiency conditions (Fig. 1). Interestingly, the pesticide mixture seems to inhibit totally the decreasing effect of the vitamin deficiency, leading to an increase of the rate equal to the one observed when non-vitamin deficient animals were exposed to the mixture (Table 2).

3.2. Serum biochemical parameters

Table 3 shows the levels of serum biochemistry parameters for the 4

Table 2
Growth model comparison between rat groups.

	a = Wmax						
	Value	Standard Error	FD	Texp	Tth	P	D
C100	614.3	5.4	52	–	–	–	–
T100 (vs C100)	549.7	2.5	52	10.9	2.007	**<0.001**	−10.5%
C25 (vs C100)	557.5	5.0	52	7.7	2.007	**<0.001**	−9.2%
T25 (vs. C100)	482.7	4.5	52	18.7	2.007	**<0.001**	−21.4%
T25 (vs. C25)	482.7	4.5	52	11.2	2.007	**<0.001**	−13.4%
	xc=Ti						
	Value	Standard Error	FD	Texp	Tth	P	D
C100	4.43	0.11	52	–	–	–	–
T100 (vs C100)	3.80	0.06	52	5	2.007	**<0.001**	−14.3%
C25 (vs C100)	4.48	0.11	52	0.2	2.007	0.7515	+1.2%
T25 (vs. C100)	3.59	0.13	52	4.9	2.007	**<0.001**	−18.9%
T25 (vs. C25)	3.59	0.13	52	5.2	2.007	**<0.001**	−19.9%
	k = growth rate coefficient						
	Value	Standard Error	FD	Texp	Tth	P	D
C100	0.1564	0.0051	52	–	–	–	–
T100 (vs C100)	0.1862	0.0038	52	4.7	2.007	**<0.001**	+19.0%
C25 (vs C100)	0.1405	0.0043	52	2.4	2.007	**<0.05**	−10.2%
T25 (vs. C100)	0.1861	0.0080	52	1.0	2.007	**<0.001**	+19.0%
T25 (vs. C25)	0.1861	0.0080	52	5.0	2.007	**<0.001**	+32.4%

Note: Wmax: maximum weight; Ti: inflection time, k: growth rate coefficient; D: difference.

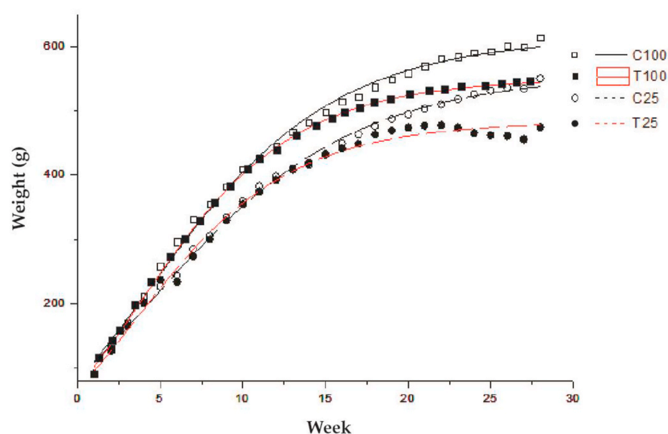

Fig. 1. Weight gain (and 95% prediction limits) for the control and treatment groups fitted using the Gompertz growth curve model.

groups of rats and Table 4 the percent changes for these parameters. A significant increase in alkaline phosphatase (ALKP) levels was observed in vitamin-deficient rats treated with the pesticide mixture as compared to control rats; however, a lower (non-significant) increase in ALKP was observed in relation to vitamin-deficient control rats.

Serum lipid parameters were affected by exposure to the pesticide mixture both in the presence or absence of a vitamin-deficient diet. In this respect, a significant decrease of cholesterol level (CHO) was observed in the group exposed to the pesticide mixture ($P < 0.05$), and a somewhat greater decrease in the treated group with a vitamin-deficiency diet ($P < 0.05$); conversely, the effect of vitamin deficiency in unexposed rats was limited. Triglycerides (TRYG) were slightly decreased in the vitamin-deficient group, but this effect was more pronounced in the group exposed to the pesticide mixture and reached the statistical significance and greater magnitude when rats were exposed to both stressors ($P < 0.05$). A significant increase of lipase enzyme was observed in rats with normal vitamin intake after exposure to the pesticide mixture (T100 group) ($P < 0.05$) and a slightly lower increase in the vitamin-deficient rats (T25 group) ($P < 0.05$); hence, no greater effect was observed for exposure to pesticides in combination with hypovitaminosis (Tables 3 and 4).

Total blood proteins (TP) levels were significantly decreased in vitamin-deficient rats exposed to the pesticide mixture as compared to the control group with a deficient vitamin intake ($P < 0.05$) (Tables 3 and 4).

3.3. Urine biochemical parameters

Urinary calcium levels were significantly increased in the vitamin-deficient group exposed to the pesticide mixture relative to the control group with or without a vitamin-deficient intake ($P < 0.05$), although the effect was greater when compared with the former group. No other significant changes were observed for other urine parameters either in the vitamin-deficient rats or in rats exposed to the pesticide mixture (Tables 5 and 6).

3.4. Haematological parameters (complete blood count)

Exposure to the pesticide mixture failed to have any impact on hematological parameters; however, when associated with vitamin deficiency, a slightly but statistically significant decrease was observed in the number of peripheral erythrocytes (RBC), hematocrit (HCT) and blood haemoglobin levels (HGB) ($P < 0.05$) (Tables 7 and 8).

Table 3
Serum biochemical parameters in the 4 groups of rat expressed as mean ± SD.

Parameter	GROUPS			
	Control 100	Control 25	Test 100	Test 25
GLU (mmol/L)	8.01 ± 0.46	7.87 ± 0.76	7.69 ± 0.64	7.32 ± 0.46
ALT (U/L)	42.85 ± 6.4	41.72 ± 9.29	47.5 ± 19.81	41.19 ± 8.04
ALB (g/L)	28.67 ± 0.7	27.65 ± 1.28	26.37 ± 2.63	26.79 ± 1.27
AMY (U/L)	2602.35 ± 377.43	2531.07 ± 518.69	2545.68 ± 396.92	2466.85 ± 307.17
TP (g/L)	62.89 ± 3.5	61.98 ± 4.06	61.34 ± 5.84	57.34 ± 3.24^{Ŧ}
BILT (μmol/L)	17.66 ± 9.99	15.56 ± 5.22	15.77 ± 12.46	12.43 ± 5.51
BILD (μmol/L)	4.32 ± 2.31	4.12 ± 1.59	3.98 ± 3.1	3.68 ± 1.97
GLOB (g/L)	34.22 ± 3.44	34.33 ± 4.48	34.97 ± 4.91	30.55 ± 2.53
Iron (μ/L)	35.5 ± 8.6	40.66 ± 5.93	37.49 ± 10.84	35.82 ± 4.74
CPK (U/L)	4646.66 ± 605.29	4733.59 ± 591.53	4857.81 ± 510.16	4951.63 ± 454.4
Ca^{2+} (mmol/L)	2.71 ± 0.13	2.68 ± 0.14	2.62 ± 0.24	2.62 ± 0.12
CRE (μmol/L)	61.45 ± 5.35	59.16 ± 1.66	59.35 ± 4.84	60.55 ± 5.66
LDH (U/L)	1297.56 ± 328.92	1217.96 ± 265.41	1255.01 ± 358.32	1195.49 ± 422.8
Lipase (U/L)	20.43 ± 2.24	19.81 ± 1.74	23.98 ± 2.95$^{\#}$	22.97 ± 2.76^{Ŧ}
Mg^{2+} (mmol/L)	0.67 ± 0.06	0.65 ± 0.09	0.6 ± 0.05	0.61 ± 0.05
Uric acid (μmol/L)	115.71 ± 30.18	97.15 ± 23.24	97.43 ± 26.91	97.4 ± 32.01
BUN (mmol/L)	4.12 ± 0.73	4.63 ± 0.73	4.21 ± 0.62	3.98 ± 0.7
TRYG (mmol/L)	2.61 ± 1.07	2.24 ± 0.57	2.02 ± 1.51	1.63 ± 0.84$^{Ŧ,\#}$
Phosphorus (mmol/L)	1.73 ± 0.23	1.87 ± 0.19	1.78 ± 0.23	1.86 ± 0.19
CHOL (mmol/L)	2.83 ± 0.67	3.06 ± 0.55	2.58 ± 0.4	2.14 ± 0.32$^{Ŧ,\#}$
ALKP (U/L)	137.75 ± 67.15	173.57 ± 46.42	134.45 ± 50.06	226.88 ± 60.44$^{\#}$
AST (U/L)	170.25 ± 33.64	128.8 ± 5.81	159.2 ± 37.34	131.8 ± 18.19
K^{+} (mmol/L)	6.2 ± 0.36	6 ± 0.29	5.5 ± 0.43	5.92 ± 0.28
Na^{+} (mmol/L)	147.25 ± 2.22	149 ± 8.92	146 ± 8.69	146.2 ± 3.42
Cl^{-} (mmol/L)	104 ± 1.63	104.6 ± 5.55	103.4 ± 6.84	102.6 ± 2.61

Note: Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests: ^{Ŧ} – $P < 0.05$ when the group is compared with C25; $^{\#}$ - $P < 0.05$ when the group is compared with C100. GLU: glucose; ALT:alanine aminotransferase; ALB: albumin; AMY: amylase; TP: total protein; BILT: total bilirubin; BILD: direct bilirubin; GLOB: globulin; CPK: creatine phosphokinase; Ca^{2+}: calcium; CRE: creatinine; LDH: lactate dehydrogenase; Mg^{2+}: magnesium; BUN: blood urea nitrogen; TRYG: tryglyceride; CHOL: cholesterol; ALKP: alkaline phosphatase; AST: aspartate aminotransferase; K^{+}: potassium; Na^{+}: sodium; Cl^{-}: chloride.

3.5. Oxidative stress parameters, xenobiotic-metabolising enzymes and lysosomal enzymes

A significant increase in ethoxyresorufin (CYP1A1), methoxyresorufin (CYP1A2), pentoxyresorufin (CYP2B1) and GST was observed in vitamin-deficient rats exposed to the pesticide mixture relative to the control group with adequate vitamin intake ($P < 0.05$). The comparison with the vitamin-deficient control group showed significant increased activity only for GST ($P < 0.05$) and notably for CYP1A2 ($P < 0.05$) (Tables 9 and 10). No significant effect was observed in the vitamin-deficiency group alone or in the group treated only with the pesticide mixture when compared with control group with adequate vitamin intake.

Table 4
Percent changes in serum biochemical parameters in the 4 groups of rats.

Parameter	C25 vs. C100	T100 vs. C100	T25 vs. C100	T25 vs. C25	T25 vs. T100
GLU (mmol/L)	↓1.8%	↓4.0%	↓8.6%	↓7.0%	↓4.8%
ALT (U/L)	↓2.6%	↑10.9%	↓3.9%	↓1.3%	↓13.3%
ALB (g/L)	↓3.6%	↓8.0%	↓6.6%	↓3.1%	↑1.6%
AMY (U/L)	↓2.7%	↓2.2%	↓5.2%	↓2.5%	↓3.1%
TP (g/L)	↓1.5%	↓2.5%	↓8.8%	↓7.5%[Ŧ]	↓6.6%
BILT(μmol/L)	↓11.9%	↓10.7%	↓29.6%	↓20.1%	↓21.2%
BILD(μmol/L)	↓4.6%	↓7.9%	↓14.8%	↓10.7%	↓7.5%
GLOB(g/L)	↑0.3%	↑2.2%	↓10.7%	↓11.0%	↓12.6%
Iron(μ/L)	↑14.5%	↑5.6%	↑0.9%	↓11.9%	↓4.5%
CPK (U/L)	↑1.9%	↑4.5%	↑6.6%	↑4.6%	↓1.9%
Ca2+ (mmol/L)	↓1.1%	↓3.3%	↓3.3%	↓2.2%	0%
CRE (μmol/L)	↓3.7%	↓3.4%	↓1.5%	↑2.4%	↑2.0%
LDH(U/L)	↓6.1%	↓3.3%	↓7.9%	↓1.8%	↓4.7%
Lipase (U/L)	↓3.0%	↑17.4%[Ŧ]	↑12.4%	↑16.0%[Ŧ]	↓4.2%
Mg2+ (mmol/L)	↓3.0%	↓10.5%	↓9.0%	↓6.2%	↓1.7%
Uric acid (μmol/L	↓3.0%	↓10.5%	↓9.0%	↓6.2%	↓1.7%
BUN (mmol/L)	↓3.0%	↓10.5%	↓9.0%	↓6.2%	↑1.6%
TRYG (mmol/L)	↓14.2%	↓22.6%	↓37.5%[Ŧ]	↓27.2%[Ŧ]	↓19.3%
Phosphorus (mmol/L)	↑8.1%	↑2.9%	↑7.5%	↓0.5%	↑4.5%
CHOL (mmol/L)	↑8.1%	↓8.8%	↓24.4%[Ŧ]	↓30.1% [Ŧ]	↓17.1% [Ŧ]
ALKP (U/L)	26.0%	↓2.4%	↑64.7% [Ŧ]	↑30.7%	↑68.8%
AST (U/L)	↓24.4%	↓6.5%	↓22.6%	↑2.3%	↓17.2%
K+ (mmol/L)	↓3.2%	↓11.3%	↓4.5%	↓1.3%	↑7.6%
Na+ (mmol/L)	↑1.2%	↓0.9%	↓0.7%	↓1.9%	↑0.1%
Cl⁻(mmol/L)	↑0.6%	↓0.6%	↓1.4%	↓1.9%	↓0.8%

Note: [Ŧ] Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests – $P < 0.05$. GLU: glucose; ALT: alanine aminotransferase; ALB: albumin; AMY: amylase; TP: total protein; BILT: total bilirubin; BILD: direct bilirubin; GLOB: globulin; CPK: creatine phosphokinase; Ca2+: calcium; CRE: creatinine; LDH: lactate dehydrogenase; Mg2+: magnesium; BUN: blood urea nitrogen; TRYG: tryglyceride; CHOL: cholesterol; ALKP: alkaline phosphatase; AST: aspartate aminotransferase; K+: potassium; Na+: sodium; Cl-: chloride.

Table 5
Urine biochemical parameters in the 4 groups of rats expressed as mean ± SD.

Parameter	GROUPS			
	Control 100	Control 25	Test 100	Test 25
Diuresis (mL)	13.05 ± 4.07	11.5 ± 5.13	9.78 ± 3.41	9.76 ± 7.74
pH	6.94 ± 0.42	6.48 ± 0.16[#]	6.86 ± 0.17	6.43 ± 0.4[#]
Protein (g/L)	1.74 ± 0.36	1.75 ± 0.48	1.78 ± 0.47	1.72 ± 0.44
Glucose (mmol/L)	0.04 ± 0.02	0.03 ± 0.02	0.05 ± 0.03	0.03 ± 0.02
Relative density	1.01 ± 0.01	1.02 ± 0.01	1 ± 0.01	1.01 ± 0.01
Uric Acid (μmol/L)	1747.14 ± 482.77	1453.4 ± 669.24	2237.22 ± 912.73	1438.11 ± 740.73
Ca^{2+} (mmol/L)	2.2 ± 1.35	2.67 ± 1.94	1.68 ± 0.94	5.99 ± 4.13 [Ŧ,#]
Creatinine	12904.21 ± 4578.51	11459.56 ± 4998.17	17358.91 ± 7767.68	14250.52 ± 5093.46
Mg^{2+}(mmol/L)	4.89 ± 2.42	5.79 ± 3.54	4.84 ± 0	6.38 ± 2.5
Urea (mmol/L)	369.11 ± 57.04	366.02 ± 85.83	361.69 ± 122.96	378.13 ± 69.93
Phosphorus (mmol/L)	42.79 ± 16.09	42.83 ± 20.04	51.99 ± 22.04	51.18 ± 26.47

Note: Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests: [Ŧ] – $P < 0.05$ when the group is compared with C25; [#] - $P < 0.05$ when the group is compared with C100.

3.6. *Organ weight and histopathology*

A significant increase in kidneys, adrenals, testes, prostate and brain weight was observed in vitamin-deficient rats exposed to the pesticide mixture as compared to control rats with adequate vitamin intake ($P < 0.05$). However, no significant effect was found for the vitamin-deficiency or the pesticide treated groups relative to their respective control groups. In contrast, the thymus weight was significantly decreased in vitamin-deficient rats exposed to the pesticide mixture as compared to the control rats with adequate vitamin intake (Tables 11 and 12). No pathological changes in tissue structure or endemic hemorrhage was observed in thymus, heart, lungs, liver, kidneys, adrenals, spleen, small intestine, testicles, and prostate gland from any the rat groups studied (Figs. 2 and 3).

4. Discussion

This study evaluated the effects of six months exposure of rats to a mixture of six pesticides at doses corresponding to their individual NOAEL/100 (for the selected pesticides it corresponded to their ADI) for many that are considered safe for consumers. In the same time, we sought whether vitamin deficiency may modify the detrimental effects of the pesticide mixture on a rat model. The wide spectrum of biological functions modulated by B vitamins, vitamin K and folates determines that long-term deficiency of these vitamins increases the risk of developing chronic pathologies (Mikkelsen and Apostolopoulos, 2018).

In the real-life scenario, individuals are continuously exposed to a combination of pesticides from different sources, such as food, water or in some circumstances even air. Studies have shown that these mixtures, even at low concentrations of individual pesticides considered as safe for consumers, can have detrimental effects for the organism (Docea et al., 2018, 2019; Tsatsakis et al., 2019a, 2019b; Fountoucidou et al., 2019).

At 6 months of follow-up, the vitamin-deficiency rat group showed a decrease in the growth rate and maximum weight, without modification of the inflexion time. Previous studies have shown that vitamin B6 deficiency determines a decrease in appetite and weight gain in rats compared with animals on a vitamin B6 sufficient diet (Wolfson and Kopple, 1987). Studies that investigated the effect of biotin (vitamin B7) deficiency on glucose homeostasis showed that this deficiency determines an increase of glucagon concentration and a decrease of insulin secretion (Larrieta et al., 2012). Glucagon function is associated with a decrease in food intake and body weight gain (Campbell and Drucker, 2015), which is in accordance with our results. While exposure to the pesticide mixture over 6 months determined a decrease of the maximum weight and inflexion time, an increased in growth rate was observed for the vitamin-deficiency group. When the two stressors acted together, a greater effect was observed on the decrease in weight gain, the effect of the pesticide mixture on the inflexion time decrease was attenuated in

Table 6
Percent changes in urine biochemical parameters in the 4 groups of rats.

Parameter	C25 vs. C100	T100 vs. C100	T25 vs. C100	T25 vs. C25	T25 vs. T100
Diuresis (mL)	↓11.9%	↓25.1%	↓25.2%	↓15.1%	↓0.2%
pH	↓6.6[Ŧ]	↓1.2%	↓7.4%[Ŧ]	↓0.8%	↓6.3%
Protein (g/L)	↑0.6%	↑2.3%	↓1.2%	↓1.7%	↓3.4%
Glucose (mmol/L)	↓25.0%	↑25.0%	↓25.0%	↑0.0%	↓40.0%
Relative density	↑1.0%	↓1.0%	↑0.0%	↓0.98%	↑1.0%
Uric Acid (μmol/L)	↓16.8%	↑28.1%	↓17.7%	↓1.1%	↓35.7%
Ca2+ (mmol/L)	↑21.4%	↓23.6%	↑172.2%[Ŧ]	↑124.3%[Ŧ]	↑256.6% [Ŧ]
Creatinine	↓11.2%	↑34.5%	↑10.4%	↑24.4%	↓17.9%
Mg2+(mmol/L)	↑18.4%	↓1.0%	↑30.5%	↑10.2%	↑31.8%
Urea (mmol/L)	↓0.8%	↓2.0%	↑2.4%	↑3.3%	↑4.6%
Phosphorus (mmol/L)	↑0.1%	↑21.5%	↑19.6%	↑19.5%	↓1.6%

Note: [Ŧ] Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests – $P < 0.05$.

Table 7
Haematological parameters (complete blood count) in the 4 groups of rats expressed as mean ± SD.

Parameter	GROUPS			
	Control 100	Control 25	Test 100	Test 25
RBC (10^12/L)	8.67 ± 0.44	8.63 ± 0.32	8.66 ± 0.45	8.06 ± 0.59 [Ŧ]
HGB (g/L)	152.8 ± 6.65	150.6 ± 3.37	150 ± 7.13	141.4 ± 8 [Ŧ,#]
HCT (%)	46.18 ± 1.76	45.57 ± 1.35	45.44 ± 2.41	42.54 ± 2.62 [Ŧ,#]
MCV (fL)	17.63 ± 0.58	17.9 ± 1.73	17.34 ± 0.58	17.59 ± 0.73
MCH (pg)	17.63 ± 0.58	17.47 ± 0.62	17.59 ± 0.58	17.59 ± 0.73
MCHC (g/L)	330.7 ± 5.5	330.5 ± 3.14	330.2 ± 4.02	332.3 ± 4.74
WBC (10^9/L)	11.09 ± 4.66	12.17 ± 4.77	12 ± 3.7	9.21 ± 2.36
NEU #(10^9/L)	20.41 ± 4.99	21.07 ± 6.54	24.4 ± 11.48	21.64 ± 6.25
EOS #(10^9/L)	3.59 ± 1.4	2.86 ± 1.2	3.3 ± 1.28	3.17 ± 0.88
BAS #(10^9/L)	1.21 ± 0.62	0.76 ± 0.16	1.32 ± 0.81	1.11 ± 0.47
LYM #(10^9/L)	61.24 ± 8.35	63.94 ± 7.97	60.4 ± 11.33	64.04 ± 7.21
MON #(10^9/L)	11.14 ± 2.35	10.83 ± 3.33	10.58 ± 2.08	10.04 ± 3.54
PLT (10^9/L)	618.1 ± 83.93	578.8 ± 70.79	544.5 ± 53.85	628.8 ± 67.09
MPV (fL)	7.04 ± 0.27	7.15 ± 0.56	7.27 ± 0.31	6.61 ± 0.29
PCT (mL/L)	0.44 ± 0.07	0.41 ± 0.06	0.4 ± 0.03	0.41 ± 0.04

Note: Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests: [Ŧ] – $P < 0.05$ when the group is compared with C25; [#] - $P < 0.05$ when the group is compared with C100. RBC: red blood cells; HGB: haemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cells; NEU: neutrophil; EOS: eosinophil granulocytes; BAS: basophil granulocytes; LYM: lymphocytes; MON: monocytes; PLT:platelets; MPV: mean platelet volume and PCT: plateletcrit.

Table 8
Percent changes in haematological parameters (complete blood count) in the 4 groups of rats.

Parameter	C25 vs. C100	T100 vs. C100	T25 vs. C100	T25 vs. C25	T25 vs. T100
RBC (10^12/L)	↓0.5%	↓0.1%	↓7.0%	↓6.6%[Ŧ]	↓6.9%
HGB (g/L)	↓1.4%	↓1.8%	↓7.5% [Ŧ]	↓6.1% [Ŧ]	↓5.7%[Ŧ]
HCT (%)	↓1.3%	↓1.6%	↓7.9%[Ŧ]	↓6.6%[Ŧ]	↓6.4%[Ŧ]
MCV (fL)	↑1.5%	↓1.6%	↓0.2%	↓1.7%	↑1.4%
MCH (pg)	↓0.9%	↓0.2%	↓0.2%	↑0.7%	0%
MCHC (g/L)	↓0.1%	↓0.2%	↑0.5%	↑0.5%	↑0.6%
WBC (10^9/L)	↑9.7%	↑8.2%	↓17.0%	↓24.3%	↓23.3%
NEU # (10^9/L)	↑3.2%	↑19.6%	↑6.0%	↑2.7%	↓11.3%
EOS #(10^9/L)	↓20.3%	↓8.1%	↓11.7%	↑10.8%	↓3.9%
BAS #(10^9/L)	↓37.2%	↑9.1%	↓8.3%	↑46.1%	↓15.9%
LYM # (10^9/L)	↑4.4%	↓1.4%	↑4.6%	↑0.2%	↑6.0%
MON # (10^9/L)	↓2.8%	↓5.0%	↓9.9%	↓7.3%	↓5.1%
PLT (10^9/L)	↓6.4%	↓11.9%	↑1.7%	↑8.6%	↑15.5%
MPV (fL)	↑1.6%	↑3.3%	↓6.1%	↓7.6%	↓9.1%
PCT (mL/L)	↓6.8%	↓9.1%	↓6.8%	0.0%	↑2.5%

Note: [Ŧ] Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests – $P < 0.05$. RBC: red blood cells; HGB: haemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cells; NEU: neutrophil; EOS: eosinophil granulocytes; BAS: basophil granulocytes; LYM: lymphocytes; MON: monocytes; PLT: platelets; MPV: mean platelet volume and PCT: plateletcrit.

the presence of vitamin deficiency, and the effect of the vitamin deficiency was compensated (remaining only the increasing effect for the pesticide mixture). The decrease of the maximum weight after exposure to the pesticide mixture can be explained by the additive effects of the low doses of imazamox that may reduce food consumption (Authority, 2016) and imazethapyr that may decrease the body weight gain (Authority, 2017).

Vitamin deficiency combined with exposure to a low dose of pesticide mixture for 6 months determined a significant increase (~65%) in ALKP levels relative to control rats with adequate vitamin intake. Increased ALKP alone in the absence of other findings is not sufficient to suggest hepatotoxicity, but might be related with hepatic enzyme induction of xenobiotic metabolising enzymes (see below). Although this effect (hepatic enzyme induction) is often observed in rodent studies on pesticide (Yokoyama et al., 2019), the histopathological assessment performed in this study failed to show hepatocellular hypertrophy, which is consistent with a non-significant 3.7% increase in liver microsomal protein found. In addition, while the T-100 group did not show significant differences in ALKP relative to C-100 (only 2.4% decreased levels), the C-25 group showed statistically significant greater

Table 9
Oxidative stress parameters, xenobiotic-metabolising enzymes and lysosomal enzymes in the 4 groups of rats expressed as mean ± SD.

Parameter	GROUPS			
	Control 100	Control 25	Test 100	Test 25
Total antioxidant activity (FRAP) in liver	8.136 ± 0.6494	8.448 ± 1.0075	8.22 ± 0.3453	8.328 ± 0.4008
Erythrocyte Catalase (μmol/min × mg protein)	0.5869 ± 0.0773	0.6018 ± 0.0959	0.5979 ± 0.0863	0.5942 ± 0.054
Erythrocyte Superoxide dismutase (U/min × mg protein)	1.4361 ± 0.1147	1.3992 ± 0.2277	1.5018 ± 0.2298	1.5273 ± 0.2942
Erythrocyte Glutathione peroxidase (mmol/min × mg protein)	389.0088 ± 48.8217	392.6981 ± 36.884	375.5921 ± 37.0662	356.6077 ± 39.1465
Liver Ethoxyresorufin (CYP1A1) (pmol/min × mg protein)	16.4447 ± 5.2472	15.8968 ± 6.7144	16.1421 ± 4.6691	26.7638 ± 11.7638$^{\#}$
Liver Methoxyresorufin (CYP1A2) (pmol/min × mg protein)	39.721 ± 18.171	38.3711 ± 10.4736	44.422 ± 16.4201	108.982 ± 63.9213$^{Ŧ,\#}$
Liver Pentoxyresorufin (CYP2B1) (pmol/min × mg protein)	10.284 ± 3.8705	11.604 ± 6.4978	11.736 ± 7.5075	17.9 ± 7.3484$^{\#}$
Liver Glutathione-S transferase (GST) (μmol/min × mg protein)	0.9162 ± 0.0743	0.899 ± 0.116	0.9328 ± 0.1006	1.0646 ± 0.1135$^{Ŧ,\#}$
Total activity of Arylsulfatase A and B (μmol/min × g of liver tissue)	3.739 ± 0.4089	3.641 ± 0.7519	3.524 ± 0.2349	3.785 ± 0.6679
Unsedimentable activity of liver Arylsulfatase A and B (%)	5.007 ± 0.777	5.323 ± 1.1671	5.036 ± 0.7136	5.28 ± 0.5673
Total activity of liver β-Glucuronidase (μmol/min × g of tissue)	2.495 ± 0.3786	2.311 ± 0.2491	2.305 ± 0.2064	2.6 ± 0.4597
Unsedimentable activity of liver β-Glucuronidase (%)	4.581 ± 0.5394	5.137 ± 0.7624	4.83 ± 1.1986	4.744 ± 0.9818
Total activity of liver β-Galactosidase (μmol/min × g of tissue)	2.401 ± 0.3576	2.375 ± 0.3115	2.434 ± 0.3094	2.326 ± 0.2348
Unsedimentable activity of liver β-Galactosidase (%)	7.29 ± 0.9348	7.404 ± 0.8947	7.406 ± 1.3662	7.901 ± 1.174
Liver Microsomal protein (mg/g of tissue)	17.4 ± 2.1427	17.88 ± 1.7492	16.37 ± 1.5225	18.54 ± 1.2158
Cytosolic protein (mg/g of liver tissue)	78.15 ± 2.4052	79.24 ± 5.0213	77.07 ± 3.9839	75.91 ± 3.5225
Malondialdehyde (MDA) in erythrocytes (mmol/mL)	7.351 ± 1.8882	7.1086 ± 1.3369	7.3625 ± 1.7674	5.9258 ± 1.1264
Malonedialdehyde (MDA) in serum (mmol/mL)	4.6614 ± 1.1944	4.8229 ± 1.0752	5.2267 ± 1.4676	5.1229 ± 0.9518
Malonedialdehyde (MDA) in liver (mmol/g)	379.4868 ± 24.0458	352.1791 ± 48.7743	363.2048 ± 43.0588	397.3073 ± 30.8963

Note: Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests: ^{Ŧ} – $P < 0.05$ when the group is compared with C25; $^{\#}$ - $P < 0.05$ when the group is compared with C100.

levels (26%) than the C-100. Hence, the 30.7% increased ALKP activity in the T-25 group related to C-25 is largely due to the hypovitaminosis conditions.

The increased levels of serum lipase may indicate the presence of a condition affecting the pancreas, usually acute inflammation. Serum lipase may also rise as a result of a reduced clearance from the circulation because of an altered hepatic function or a renal impairment. Other non-pancreatic conditions can also be responsible for increased lipase levels, such as gall bladder inflammation, kidney diseases, diabetes, drugs and infection (Hameed et al., 2015). As amylase levels were not raised in pesticide-treated rats, the increase in lipase should be related to mechanisms other than pancreatic inflammation. Taking into account the low levels of the individual pesticide in the mixture, the reduced hepatic or renal clearance may play a relevant role for the raised serum lipase levels. The decrease in total serum proteins observed in vitamin-deficient rats exposed to the pesticide mixture largely affected globulins rather than albumin (see Table 3). Because only a minor and non-significant effect was observed in the T-100 group relative to C-100 and in the C-25 group compared to C-100, it may result from the combined effect of the pesticide mixture and vitamin-deficiency. This decrease in total proteins can be explained either by a greater protein catabolism or by a reduced liver synthesis function supported by the decrease of total cholesterol levels, as the majority of cholesterol synthesis also occurs in the liver. Imazethapyr has been shown to produce disturbances in oxidative stress parameters that lead to increase protein catabolism in *C.carpio* after 90 days of exposure (Moraes et al., 2011). Apryatin et al. showed that the lack of vitamin B1, B6 and B2 from rat diet for 35 days caused a decrease in the triglycerides and total albumin levels (Apryatin et al., 2018). In our study, the vitamin-deficiency diet combined with exposure to pesticide mixture had an impact on serum lipid profile with the effect being higher than additive for cholesterol and less than additive for triglycerides, with both showing decreased levels after pesticides exposure. Exposure to pesticides may impair lipid absorption in the gut and lipid storage in the liver and adipose tissue, thus changing the serum lipid profile (He et al., 2020). Panthothenic acid (B5) is the substrate of coenzyme A (CoA) and its deficiency determines a decrease in CoA synthesis. CoA is required for cholesterol synthesis and intracellular reactive oxygen species (ROS) regulation (Slyshenkov et al., 2004). The increase of ROS due to B5 deficiency is associated with osteoclast differentiation and bone resorption (Bax et al., 1992), which is supported by the increased urinary calcium levels found in this study. However, serum levels were normal likely because of regulatory mechanisms for calcium homeostasis. Vitamin B6 deficiency is associated with an increased lipid peroxidation in the kidney that leads to enhanced calcium levels (Ravichandran and Selvam, 1990). Vitamin K has been involved in prevention of osteopenia, by stimulating bone formation and attenuating bone resorption, and also stimulates tubular reabsorption of calcium (Iwamoto et al., 2006). Thereby, the increased urinary calcium levels observed in our study can be associated with deficiency of vitamin K.

At the haematological level, neither vitamin-deficiency alone nor exposure to the pesticide mixture alone had any significant effect on complete blood count parameters. However, the combination of the two stressors decreased the number of RBC along with HCT and HBG levels.

Table 10
Percent changes in oxidative stress parameters, xenobiotic-metabolising enzymes and lysosomal enzymes in the 4 groups of rats.

Parameter	C25 vs. C100	T100 vs. C100	T25 vs. C100	T25 vs. C25	T25 vs. T100
Total antioxidant activity (FRAP)	↑3.8%	↑1.0%	↑2.4%	↓1.4%	↑1.3%
Erythrocyte Catalase	↑2.5%	↑1.9%	↑1.2%	↓1.3%	↓0.6%
Erythrocyte Superoxide dismutase	↓2.6%	↑4.6%	↑6.4%	↑9.2%	↑1.7%
Erythrocyte Glutathione peroxidase	↑1.0%	↓3.5%	↓8.3%	↓9.2%	↓5.1%
Liver Ethoxyresorufin (CYP1A1)	↓3.3%	↓1.8%	↑62.7% [Ŧ]	↑68.4%	↑65.8%
Liver Methoxyresorufin (CYP1A2)	↓3.4%	↑11.8%	↑174.4%[Ŧ]	↑184.0%[Ŧ]	↑145.3% [Ŧ]
Liver Pentoxyresorufin (CYP2B1)	↑12.8%	↑14.1%	↑74.1%[Ŧ]	↑54.3%	↑52.5%
Liver GlutathioneS-transferase (GST)	↓1.9%	↑1.8%	↑16.2%[Ŧ]	↑18.4%[Ŧ]	↑14.1%
Total activity of liver Arylsulfatase A and B	↓2.6%	↓5.8%	↑1.2%	↑4.0%	↑7.4%
Unsedimentable activity of liver Arylsulfatase A and B	↑6.3%	↑0.6%	↑5.5%	↓0.8%	↑4.9%
Total activity of liver β-Glucuronidase	↓7.4%	↓7.6%	↑4.2%	↑12.5%	↑12.8%
Unsedimentable activity of liver β-Glucuronidase	↑12.1%	↑5.4%	↑3.6%	↓7.7%	↓1.8%
Total activity of liver β-Galactosidase	↓1.1%	↑1.4%	↓3.1%	↓2.1%	↓4.4%
Unsedimentable activity of liver β-Galactosidase	↑1.6%	↑1.6%	↑8.4%	↑6.7%	↑6.7%
Liver Microsomal protein	↑2.8%	↓5.9%	↑6.6%	↑3.7%	↑13.3%
Cytosolic protein	↑1.4%	↓1.4%	↓2.9%	↓4.2%	↓1.5%
Malondialdehyde (MDA) in erythrocytes	↓3.3%	↑0.2%	↓19.4%	↓16.6%	↓19.5%
Malondialdehyde (MDA) in serum	↑3.5%	↑12.1%	↑9.9%	↑6.2%	↓2.0%
Malondialdehyde (MDA) in liver	↓7.2%	↓4.3%	↑4.7%	↑12.8%	↑9.4%

Note: [Ŧ] Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests – P < 0.05.

Table 11
Organ weight in the 4 groups of rats expressed as mean ± SD.

Parameter	GROUPS			
	Control 100	Control 25	Test 100	Test 25
Kidneys (relative to 100 g bw)	0.4536 ± 0.062	0.435 ± 0.0537	0.4359 ± 0.0422	0.527 ± 0.0718[Ŧ,#]
Adrenal (relative to 100g bw)	0.0046 ± 0.0009	0.0048 ± 0.0013	0.0051 ± 0.0011	0.006 ± 0.0011[#]
Testes (relative to 100g bw)	0.621 ± 0.0976	0.669 ± 0.134	0.6313 ± 0.1089	0.7953 ± 0.0922[#]
Lungs (relative to 100g bw)	0.3491 ± 0.0391	0.3485 ± 0.0342	0.3671 ± 0.0557	0.3729 ± 0.0416
Liver (relative to 100g bw)	2.5988 ± 0.2617	2.6789 ± 0.3324	2.6526 ± 0.4912	2.7916 ± 0.3452
Thymus (relative to 100g bw)	0.1152 ± 0.0289	0.0994 ± 0.0365	0.108 ± 0.009	0.077 ± 0.0158[#]
Spleen (relative to 100g bw)	0.2763 ± 0.0584	0.249 ± 0.0393	0.2831 ± 0.0397	0.3172 ± 0.0848
Heart (relative to 100g bw)	0.2276 ± 0.0309	0.2211 ± 0.0174	0.2247 ± 0.0211	0.2431 ± 0.0237
Prostate (relative to 100g bw)	0.0963 ± 0.0165	0.101 ± 0.0385	0.0798 ± 0.0233	0.106 ± 0.0132
Brain (relative to 100g bw)	0.3637 ± 0.0639	0.3895 ± 0.0476	0.3881 ± 0.0674	0.4653 ± 0.0744
Pituitary (relative to 100g bw)	0.002 ± 0.0003	0.0021 ± 0.0004	0.0019 ± 0.0004	0.0023 ± 0.0004

Note: Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests: [Ŧ] – P < 0.05 when the group is compared with C25; [#] - P < 0.05 when the group is compared with C100.

Table 12
Percent changes in organ weight in the 4 groups of rats.

Parameter	C25 vs. C100	T100 vs. C100	T25 vs. C100	T25 vs. C25	T25 vs. T100
Kidneys (relative to 100g bw)	↓4.1%	↓3.9%	↑16.2%[Ŧ]	↑21.1%[Ŧ]	↑20.9% [Ŧ]
Adrenal (relative to 100g bw)	4.4%	10.9%	↑30.4%[Ŧ]	↑25.0%	↑17.7%
Testes (relative to 100g bw)	↑7.7%	↑1.7%	↑28.1%[Ŧ]	↑18.9%	↑26.0%
Lungs (relative to 100g bw)	↓0.2%	↑5.2%	↑6.8%	↑7.0%	↑1.6%
Liver (relative to 100g bw)	↑3.1%	↑2.1%	↑7.4%	↑4.2%	↑5.2%
Thymus (relative to 100g bw)	↓13.7%	↓6.3%	↓33.2%[Ŧ]	↓22.5%	↓28.7% [Ŧ]
Spleen (relative to 100g bw)	↓9.9%	↑2.5%	↑14.8%	↑27.4%	↑12.1%
Heart (relative to 100g bw)	↓2.9%	↓1.3%	↑6.8%	↑10.0%	↑8.2%
Prostate (relative to 100g bw)	↑4.9%	↓17.1%	↑10.1%	↑5.0%	↑32.8/% [Ŧ]
Brain (relative to 100g bw)	↑7.1%	↑6.7%	↑27.9%[Ŧ]	↑19.5%	↑19.9%
Pituitary (relative to 100g bw)	↑5.0%	↓5.0%	↑15.0%	↑9.5%	↑21.1%

Note: [Ŧ] Posthoc pairwise comparisons after significant ANOVA/Kruskal-Wallis tests – P < 0.05.

These findings can be explained by the supra-additive effects of the pesticide mixture and vitamin deficiency on the haematopoetic function of the bone marrow, which impacted only peripheral erythrocytes but spared leukocytes and platelets. A potential involvement of pesticide-induced oxidative stress on peripheral erytrhocytes can be ruled out because both erythrocyte MDA and antioxidant enzymes were unaffected (see Tables 9 and 10). Vitamin B12 and folate deficiencies are associated with anemia (Soofi et al., 2017). Thiamine deficiency has been reported to decrease RBC, HTC and HGB levels in rats (Hobara and Yasuhara, 1981). Diquat induced lipid peroxidation (Magalhães et al., 2018b) can be associated with the adverse effects on haematopoetic function. *In vitro* studies showed that bentazone can increase lipid peroxidation of erythrocytes (Abudayyak et al., 2014), so exposure to low doses of diquat in combination with vitamin deficiency might explain

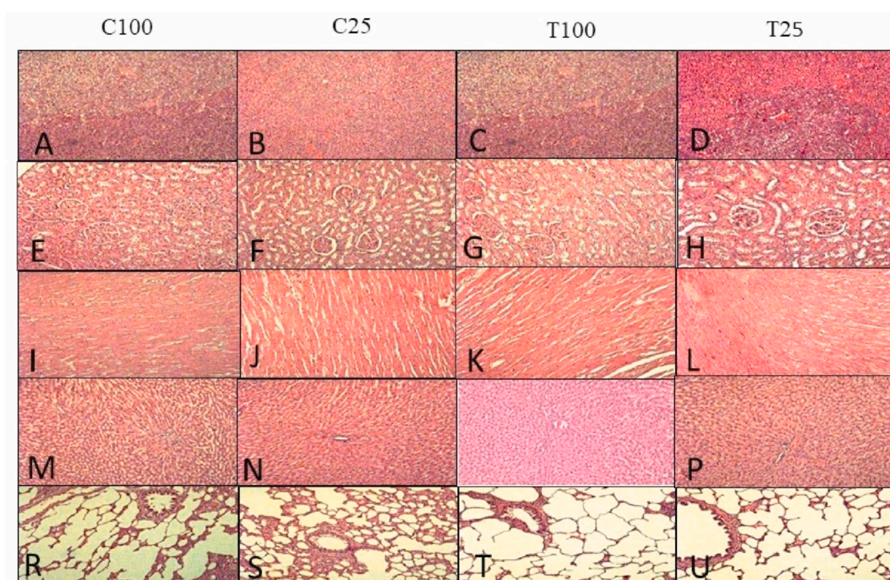

Fig. 2. A, B, C, D - adrenals in C100, C25, T100 and T25 respectively (× 100) Cross section of rat adrenal showing cortex and medulla. The cortex contains large cells with voluminous granular cytoplasm. Nuclei are vesicular with a single nucleolus. Vascularity is prominent. Clusters of small medullary cells with deeply staining nuclei regularly distributed in vascular cortex. E, F, G, H - kidney in C100, C25, T100 and T25 respectively (× 100) Micrograph of well-developed renal cortex corticis. Micrograph of a well-developed cortical layer of the kidneys. The nephrons (renal corpuscles, transverse sections of the proximal and distal tubules) and collecting ducts are visible. The structure of the renal glomeruli is preserved, the condition of the capillaries is normal. Bowman-Shumlyansky's capsule of normal thickness, without signs of sclerosis. There are no focal changes. I, J, K, L - heart in C100, C25, T100 and T25 respectively (× 100) Striation is visible on the sections. The heart muscle fibers branch and form anastomoses between themselves, which are joined into a common network. The spaces between the fibers of the heart muscle are filled with vascularized connective tissue. Capillaries are clearly visible. Cardiac muscle cells (cardiomyocytes) have only one nucleus. M,N,O, P - liver in C100, C25, T100 and T25 respectively (× 100) Normal rats liver showing some portal tracts and terminal hepatic venule. Terminal hepatic venule and portal tracts surrounded by converging hepatocyte plates and sinusoids. Portal tract contain bile duct, hepatic arteriole and portal venule. Hepatic lobules are clearly distinguishable. R, S, T, U- lung in C100, C25, T100 and T25 respectively (× 100) Most part of the section is represented by the pulmonary alveoli and alveolar ducts. The blood vessels, bronchi and bronchioles are visible. Alveoli and alveolar saccules are located along the alveolar ducts and are their continuation.

the observed effects on haematopoetic function; however, as mentioned above, the oxidative stress parameters were unaffected in both erythrocytes and serum (Tables 9 and 10).

Interestingly, exposure to the pesticide mixture in vitamin-deficient rats determined a raised activity of CYP450 enzymes, likely as a result of liver enzyme induction. The affected activities corresponded to CYP1A1, CYP1A2, and CYP2B1, which play a pivotal role in phase I xenobiotic metabolism. An apparent upregulation of GST, a relevant phase II detoxification enzyme, was also observed. Studies have shown that pyridoxine deficiency is associated with increased CYP1A1 hepatic levels (Tangjarukij et al., 2009) and Biotin deficiency with an increased expression of CYP1A2 activity (Báez-Saldaña et al., 2009). The upregulation of CYP450 enzymes has several implications in xenobiotic metabolism, including the increase in toxicity of chemicals biotransformed into active metabolites through CYP450 enzymes. One example is diquat, which is metabolized by CYP450 reductase to unstable diquat monocation free radical that, in turn, increases the generation of superoxide radicals. If the antioxidant protective mechanisms are overwhelmed, oxidative stress ensues (Magalhães et al., 2018b). However, this study found induction of GST activity in liver that can, to some extent, counteract the potentially hazardous metabolites generated by the induced CYP450 isoforms.

Neither the vitamin-deficiency nor the pesticide mixture groups showed significant histopathological changes when administered alone or in combination. A slight significant increase in organ weight was observed for kidneys, adrenals, testes, prostate and brain in vitamin-deficient rats exposed to the pesticide mixture relative to control rats with adequate vitamin intake. Although this effect was not correlated with organic changes, it will be further evaluated in the follow-up of this study at 12 and 24 months of exposure.

The pesticides used in this study were commercial formulations (i.e., plant protection products) instead of active substances; thereby further studies should address the effects of the mixture of active substances which were actually used to calculate the ADI and may not necessarily elicit the same toxicological properties (Mesnage et al., 2019). Furthermore, future studies should use high-throughput 'omics' technologies to get a better understanding of the toxicity pathways involved as did a recent study comparing the effects of a glyphosate-based herbicide to glyphosate alone (Bayen et al., 2004).

5. Conclusions

Six-month exposure of rats to a mixture of diquat, imazamox, imazethapyr, tepraloxydin, bentazone and acifluorfen, at doses 100 times below their corresponding NOAELs, together with a vitamin-deficient diet (B vitamins, folates and vitamin K) resulted in increased ALKP levels, abnormal serum lipid profile (decreased triglycerides and cholesterol and increased lipase), decreased total blood proteins levels, and increased urinary calcium levels. The combination the two stressors also decreased the number of RBC, HCT and HBG levels. Regarding xenobiotic metabolising enzymes, the two stressors in combination increased CYP1A1, CYP1A2, CYP2B1 and GST levels, likely as a result of liver enzyme induction. No significant effects on the clinical chemistry parameters studied were observed after exposure to a low dose pesticide mixture alone (with the exception of increased serum lipase) or following a continuous vitamin deficiency intake.

The results of this study, along with other similar studies using different chemical mixtures, clearly show that exposure to a pesticide mixture at doses considered safe for consumers in combination with other stressors (i.e., a vitamin-deficient diet) can produce detrimental effects at several organ levels. The classical risk assessment approach, aimed to investigate the single-chemical effects in order to set safety limits, does not take into account all the risks that individuals are exposed to in real-life, which can modify the response of the organism to chemicals. Clearly there is a need to move forward from single chemical testing to a more complex approach that should take into consideration the multitude of stressors that can influence, and challenge, the setting of real safety levels.

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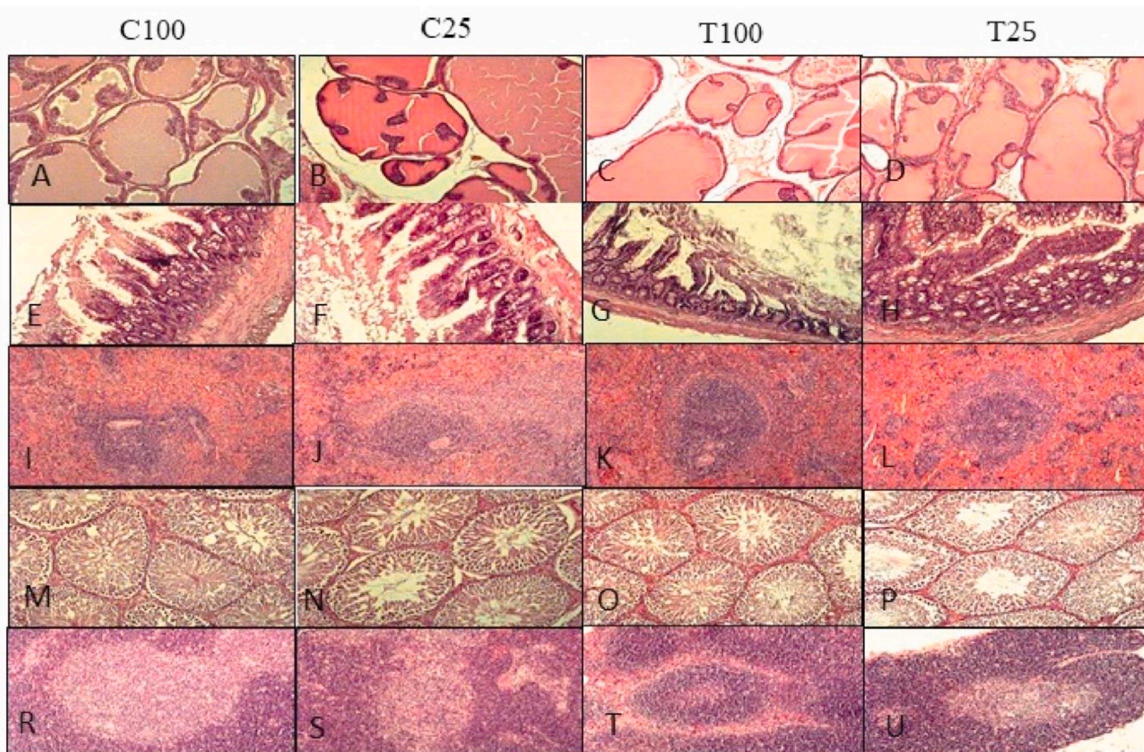

Fig. 3. A, B, C, D - prostate in C100, C25, T100 and T25 respectively (× 100). Low-power view o prostatic central zone architecture; large glands with complex luminal in oldings and distinct intraluminal bridges. Prostatic acini with basal cells demonstrated normal form. E, F, G, H - small intestine in C100, C25, T100 and T25 respectively (× 100). The surface of the ileum villus is covered by a single layer of tall columnar epithelial cells. The underlying lamina propria contains lymphoid and plasma cells and a connective tissue framework, including a lymphatic vessel and a subepithelial capilary network. I, J, K, L-spleen in C100, C25, T100 and T25 respectively (× 100). The red and white pulps are clearly distinguished. The red pulp is full-blooded. Lymphoid nodules and periarteriolar lymphocyte sheath are clearly visible in the white pulp. Lymphoid nodules of normal size, have a normal structure. Periarterial zone, germinal center, mantle and marginal zones are clearly distinguishable. M, N, O, P- testicles in C100, C25, T100 and T25 respectively (× 100). A cross-sectional view of seminiferous tubule and interstitium. Germ cell maturation is variable around the tubule, a normal finding. R, S, T, U- thymus in C100, C25, T100 and T25 respectively (× 100). Normal lobular architecture of the thymus demonstrating clear separation between cortex and medulla. There are numerous cortical thymocytes, most of which have small nuclei with densely packed chromatin. The medulla of the thymus contains less lymphocytes than the cortex. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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CRediT authorship contribution statement

Aristidis Tsatsakis: Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **Nadezhda V. Tyshko:** Methodology, Investigation, Writing – review & editing, Supervision. **Marina Goumenou:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **Svetlana I. Shestakova:** Investigation, Data curation, Writing – original draft. **El'vira O. Sadykova:** Investigation, Data curation, Writing – original draft. **Valentin M. Zhminchenko:** Investigation, Data curation, Writing – original draft. **Ovidiu Zlatian:** Investigation, Data curation, Writing – original draft. **Daniela Calina:** Investigation, Writing – review & editing, Supervision. **Valentina A. Pashorina:** Investigation, Data curation, Writing – original draft. **Nikolaj S. Nikitin:** Investigation, Data curation, Writing – original draft. **Marina D. Trebukh:** Investigation, Data curation, Writing – original draft. **Maria S. Loginova:** Investigation, Data curation, Writing – original draft. **Eleanora N. Trushina:** Investigation, Data curation, Writing – original draft. **Oksana K. Mustafina:** Investigation, Data curation, Writing – original draft. **Ludmila I. Avrenyeva:** Investigation, Data curation, Writing – original draft. **Galina V. Guseva:** Investigation, Data curation, Writing – original draft. **Nikita V. Trusov:** Investigation, Data curation, Writing – original draft. **Lidiya V. Kravchenko:** Investigation, Data curation, Writing – original draft. **Antonio F. Hernández:** Methodology, Investigation, Writing – review & editing, Supervision. **Anca Oana Docea:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

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

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