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CONTENTS

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Heavy Metals Effect on the Rat Uterus and Effectiveness of Vitamin E Treatment

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest: The authors report no conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Research involving Human Participants and/or Animals: All applicable international, national, and/or institutional ethical guidelines for the care and use of animals were followed.

Data availability: All data generated and analyzed during this study are included in this published article and its supplementary information files.

Abstract

Environmental pollution by heavy metals (HMs) is an increasingly critical problem that is posing a growing threat to reproductive health. Consequently, the aim of the current research was to study changes in rat uterus under 90 days of HMs exposure and estimate the efficacy and benefits of vitamin E treatment.

Female rats were randomly divided into three groups: untreated animals (control group); animals orally treated with the HMs mixture (HM group); and animals treated simultaneously with HMs and vitamin E (HM+E group). The toxic effects of the HMs (comprising Zn, Cu, Mn, Fe, Pb, and Cr) on the uterus of rats were investigated by histological, morphometrical, spectrophotometrical, and statistical methods.

Long-term HMs exposure triggered pathological (degenerative, inflammation, and atrophic) changes in the rat uterus together with a significant reduction of the uterine-wall thickness (37.99%, $p<0.0001$) compared to the control. In contrast, there was a lower intensity of morphological lesions and wall thickness decrease (26.03%, $p<0.0001$) in the uterus, in rats that underwent treatment with vitamin E. A substantial bioaccumulation of zinc, copper, manganese, iron, lead, and chromium general levels in the rat uterus was demonstrated in both the HM group (74.46%, $p<0.0001$) and the HM+E group (49.81%, $p<0.0001$), as compared to the control group. The lowest accumulative potential belonged to Zn and the highest to Pb. The results obtained showed a significant decline in the weight of animals treated by HMs in both HM (18,21%, $p<0.01$) and HM+E (13,09%; $p<0.05$) groups compared to the control. Our findings have demonstrated that treatment with vitamin E in HM-induced intoxication has a significant restrain of HMs accumulation (up to 16.46%, $p<0.0001$) together with morphometric variations (less on 16.17%, $p<0.01$).

In summary, long-term exposure to the HMs mixture had a pernicious toxic effect on the morphology and chemical content of the uterus of rats (strong negative correlations). Treatment with vitamin E significantly reversed the HMs impact on the uterus but did not demonstrate absolute protection.

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Keywords: uterus; heavy metals; reproductive health; vitamin E; antioxidant; detox treatment

1. Introduction

In recent years, significant insights have been gained in understanding the roles of the uterus in menstruation, fertility, and pregnancy together with orchestrating the development, differentiation, and maturation of the reproductive system. However, in the context of the deterioration of reproductive health and infertility increase (up to 21.9%), the study of uterus pathology became relevant (Cedars et al., 2017; Ho et al., 2017; Nik Hazlina et al., 2022). Indeed, certain conditions and diseases of the uterus can cause reproductive-health disorders and dysfunction of the synergistic action of the procreative system, disrupting the reproduction and survival of species (Peters et al., 2016; Cedars et al., 2017; Hanson et al., 2017). Any disturbance of estrogenic/anti-androgenic endocrine activity and enzyme mechanisms that can act by mimicking or inhibiting the actions of endogenous hormones may be accompanied by uterus lesions (e.g., benign and malignant tumors, congenital uterine malformation, infertility, ectopic gestation, pregnancies abort and poor pregnancy outcomes). Moreover, such damage of the uterus can be manifested in subsequent generations (Newbold *et al.*, 2006; Gore *et al.*, 2015; Rosenfeld, 2015; Katz *et al.*, 2016; Hanson *et al.*, 2017; Ho *et al.*, 2017). In addition, uterus macroscopic and microscopic lesions (atrophic and hyperplastic) can be caused by the development of pathological processes in this organ (e.g., endometritis, endometriosis, reproductive tract infection (about 33% in females), microflora imbalance, and benign and malignant tumors) or in other organs (e.g., diabetes, obesity, cardiovascular disease, oxidative stress, chronic stress, reproductive tract obstruction, and neurological disorders) (Gore *et al.*, 2015; Peters *et al.*, 2016; Katz *et al.*, 2016; Cedars *et al.*, 2017; Chen *et al.*, 2017; Hanson *et al.*, 2017; Lin *et al.*, 2018; Vannuccini and Petraglia, 2019). It is important to note that uterine lesions can also be provoked by factors with exogenous origins, such as viruses, bacteria, parasites, ionizing/non-ionizing radiation, and pollutants (Newbold *et al.*, 2006; Gore *et al.*, 2015; Cedars *et al.*, 2017; Chen *et al.*, 2017; Hanson *et al.*, 2017; Ho *et al.*, 2017; Lytvynenko *et al.*, 2017; Lin *et al.*, 2018; Nwosu *et al.*, 2018). It is known that long-term exposure of the organism to exogenous pollutants can contribute to epigenetic modifications that are reflected in subsequent generations (i.e., transgenerational epigenetic inheritance). The effects of various chemicals (e.g.., heavy metals (HMs), bisphenol A, genistein, phytoestrogens, diethylstilbestrol, phthalates, and polyaromatic hydrocarbons) can simulate the impact of sex hormones and morphogens (Romaniuk *et al.*, 2015; Katz *et al.*, 2016; Ho *et al.*, 2017; Mohammad Hosseini *et al.*, 2019).

The increased morbidity risk due to xenobiotic contamination of the environment has encouraged the study of their effects. Among the most common pollutants that have a detrimental effect on organisms are HMs (Romaniuk *et al.*, 2015; Hamid *et al.*, 2016; Romaniuk *et al.*, 2017; Mohammad Hosseini *et al.*, 2019). However, HMs are not always toxic — most of them are essential trace elements. They are involved in numerous enzymatic, hormonal, redox, and other processes at all developmental stages. However, exceeding the threshold level in the body results in their accumulation in tissues, and they can acquire toxic properties (Singh *et al.*, 2011; Hamid *et al.*, 2016; Nwosu *et al.*, 2018). In addition, some metals are always toxic (Pb, Cd, Cr, Ti, Si, Rb, Sr, Al, As, and Sn). The HMs effect depends on their properties, concentration, type, density, duration of exposure, molecular stability, partition coefficient, polarity, the interaction between metals, distribution, and transport into the ecosystem (Singh *et al.*, 2011; Jaishankar *et al.*, 2014; Nakade *et al.*, 2015; Hamid *et al.*, 2016; Nwosu *et al.*, 2018; Mohammad Hosseini *et al.*, 2019).

It is important to note that the long-term effects of various HMs are reflected in the abnormal variability of biochemical, functional (inhibition of menstruation, decrease in the frequency of implanted ova and of pregnancies, intrauterine growth restriction, preterm delivery, and spontaneous abortions), morphological (histopathological changes in the endometrium, myometrium and perimetrium; inflammation; reduction in the uterine gland, and decrease in the height of columnar cells, etc.), and molecular genetic (degeneration of hormones receptors and decrease of their sensitivity, oxidative stress, altering enzymes, growth factors, proliferation activities, tumor suppressor genes, cytokines, lymphokines, transport proteins and proteases, etc.) parameters of the uterus (Jaishankar *et al.*, 2014; Nakade *et al.*, 2015; Hamid *et al.*, 2016; Katz *et al.*, 2016; Hanson *et al.*, 2017; Ho *et al.*, 2017; Mohammad Hosseini *et al.*, 2019). However, some recent data revealed discrepancies regarding these changes due to the effect of the most common HMs and their accumulation (Nakade *et al.*, 2015; Mohammad Hosseini *et al.*, 2019; Lee et al., 2021). On the one hand, this might have been due to the one or several effects of HMs. On the other hand, these changes might have depended on the variability of the xenobiotic concentrations (Singh *et al.*, 2011; Jaishankar *et al.*, 2014; Hamid *et al.*, 2016; Su *et al.*, 2017; Mohammad Hosseini *et al.*, 2019; Lee *et al.*, 2021). In addition, most previous research has considered pollutants and their concentrations in specific geographic locations. HMs accumulation in the organism differs globally depending on the pollution source and the ways of environmental spread (Singh *et al.*, 2011; Jaishankar *et al.*, 2014; Romaniuk *et al.*, 2015; Nakade *et al.*, 2015; Hamid *et al.*, 2016; Romaniuk *et al.*, 2017; Su *et al.*, 2017; Lee *et al.*, 2021). Consequently, the HMs effects on the body are extremely unpredictable and may negatively affect reproductive health (such as breast, endometrial, fallopian tubes or ovarian cancers, endometriosis, endometritis, menstrual disorders, infertility and spontaneous abortions, as well as pre-term deliveries, stillbirths) (Jaishankar et al., 2014; Peters et al., 2016; Hamid et al., 2016; Cedars et al., 2017; Doncova et al., 2019; Dutta S et al., 2022).

Nevertheless, there have been increasing numbers of reports of the successful use of various (natural or artificial compounds) supplementation, which can withstand the adverse impact of HMs. These agents have detoxifying and antioxidant properties and can reduce the intensity of xenobiotics' effects as prophylactics and for the treatment of HMs-related disorders. Most of these protective substances have a direct antagonistic relationship with

HMs and have high efficiency (Al-Attar, 2011; Jaishankar *et al.*, 2014; Romaniuk *et al.*, 2018; Sahiti *et al.*, 2020). However, multiple mechanisms of action of each trace element (especially in combination) can complicate the search for universal natural compounds that will neutralize the accumulation of HMs in body tissues and/or completely block their effect.

One of the most discovered naturally occurring supplementation is vitamin E (α-tocopherol) which consists of tocopherols and tocotrienols. This effective lipid-soluble non-enzymatic antioxidant can reduce radical-induced peroxidation in biological membranes and blood, stimulate the activation of antioxidant enzymes, suppress inflammation, accelerate structural recovery, protect cellular membranes and reduce the intensity of oxidative stress caused by HMs-induced toxicity. This enables free radicals to acquire a hydrogen atom from antioxidant molecules, effectively countering lipid peroxidation and safeguarding unsaturated membrane lipids due to its oxygen-scavenging capability. (Al-Attar, 2011; Mohd Mutalip *et al.*, 2018; Sahiti *et al.*, 2020). Moreover, various studies have shown that adequate intake of vitamin E solves reproductive health problems (an essential dietary factor required to maintain normal reproduction), such as enhancing term delivery, sustaining the endometrial membrane, preventing breast cancer growth, decreasing the level of fetal death and spontaneous abortion, etc. (Al-Attar, 2011; Mohd Mutalip *et al.*, 2018; Sahiti *et al.*, 2020). Based on these, vitamin E is often used in researches that describe its effectiveness and protective effects from oxidative stress specifically caused by the impact of various HMs (Al-Attar, 2011; Romaniuk *et al.*, 2018; Sahiti *et al.*, 2020). However, till today, there is no clear information regarding the beneficial effect of vitamin E on the uterus induced by HMs exposure.

Summarizing all the above, the *aim* of our current research was to study the changes in rats' uterus under 90 days of HMs exposure and estimate the efficacy and benefits of vitamin E treatment.

2. Materials and Methods

2.1. Animals

For this study, we used 12-week-old healthy Wistar female rats with an average weight of 221.7±17.1 g, which were purchased from the Animal Experimental Unit of the Medical Institute, Sumy, Ukraine. The rats were selected after physical and behavioral examinations (body weight and health state, posture, and response to handling). The animals were acclimated for 7 days before any experimental procedures. All animals were housed in same-sex sub-groups (4 animals in 1 cage) in polypropylene cages with individual ventilation and were maintained under environmentally controlled laboratory conditions of temperature 22°C±1°C, relative humidity 55±5%, and 12 hours light/dark cycle. During the experiment, the animals had *ad libitum* access to standard pellets and water. Cage cleaning was performed daily. Individual animal bodyweights were recorded at weekly intervals. All necessary procedures were adopted to keep the rodents free from stress. Nulliparous and non-pregnant female rats were used in the study. The estrous-cycle monitoring was performed by daily vaginal smears. These were collected every morning at 9.00 and were analyzed by light microscopy (Sikora *et al.*, 2021). The results before and during experiments were presented according to four phases (proestrus, estrus, metestrus, and diestrus). However, we used data only from the estrus phase to avoid the cyclic hormonal changes in female rats that could be associated with the estrous cycle and confound the results.

2.2. Experimental design

The female rodents were randomly assigned to three groups (eight rats per group). Group I (Control) comprised normal (untreated) rats that received ordinary food and drinking water. Group II (HM) comprised rodents that were orally treated with HMs substances for 90 days. Group III (HM+E) comprised animals that received water with HMs and vitamin E within 90 days. The experimental animals were euthanized by CO_2 inhalation followed by cervical dislocation and their uteruses were immediately exposed by low abdominal midline incision. The uteruses were then collected and trimmed of fascia and fat. From each rat, the uterine wall of 1.0 cm in length was excised from each uterine horn (proximal part) in the direction from the partial caudal fusion to the ovaries. One random uterine horn was assigned to atomic absorption spectrometry and the other horn was fixed in formaldehyde for later use. A total of 48 uterine horns from 24 rats were assigned to the investigation.

2.3. Experimental substances

The experimental model comprised six of the most common (dangerous and potentially dangerous) HMs (Jaishankar *et al.*, 2014; Nakade *et al.*, 2015; Romaniuk *et al.*, 2017; Su *et al.*, 2017; Romaniuk *et al.*, 2018; Lee *et al.*, 2021) at the following concentrations: zinc ($ZnSO_4 \times 7H_2O$) – 5 mg/l, copper ($CuSO_4 \times 5H_2O$) – 1 mg/l, iron ($FeSO_4$) – 10 mg/l, manganese ($MnSO_4 \times 5H_2O$) – 0.1 mg/l, lead ($Pb(NO_3)_2$) – 0.1 mg/l, and chromium ($K_2Cr_2O_7$) – 0.1 mg/l. The concentrations of mentioned above HMs were comparable to those found in the environment according to the results of the epidemiological examination of the environment of the Northern regions of Ukraine and in accordance with preliminary reports (Romaniuk *et al.*, 2015; Romaniuk *et al.*, 2017). The list of chemical elements and their concentrations were confirmed and approved by the Bioethics Committee of the Medical Institute of Sumy State University (No. 2/10 from 10.10.2019). The HMs mixture was dissolved in ordinary water and prepared each three days. Contaminated water was supplied in a drinking bottle in *ad libitum* access for oral administration annually within 90 days.

As antagonist supplementation, we used alpha-tocopherol (vitamin E) due to its antioxidant properties at an average daily prophylactic dose (9.1 mg/kg to rats' bodyweight considering species' characteristics). Conversion of human doses to rat doses was as following: Animal equivalent dose (mg/kg) = Human dose (mg/kg) × Km ratio (Nair and Jacob, 2016). Based on this, considered the coefficient of species characteristics of rats (6.0) and humans (37.0) with average human body weight (70 kg), the dose for rats was followed: 37,0/6,0 = 6,2; 1,47 mg/kg x 6,2= 9,1 mg/kg. The average weight of animals was 221.7 ± 17.1 g. Therefore, animals received vitamin E at an average dose of 2.02 mg per rat bodyweight. Animals

were administered vitamin E via the oral gavage technique (daily at 10.00 am) for 90 days. Selected antioxidant was estimated based on the literature and manufacturer's recommendations (Al-Attar, 2011; Nair and Jacob, 2016; Romaniuk *et al.*, 2018; Sahiti *et al.*, 2020).

2.4. Tissue processing, histology, and morphometric scoring of the uterus

The fresh rat uterine horns were fixed in 10% neutral buffered formaldehyde for 24 hours, dehydrated in ethanol (70–96%), and embedded in paraffin wax blocks. Formalin-fixed paraffin-embedded tissue blocks were sectioned using a rotational microtome Shandon Finnesse 325 (Thermo Scientific, USA). Transverse sections of uterine horns with a thickness of 5 μm were placed on SuperFrost Plus™ Adhesion slides (Thermo Scientific, USA) and dried overnight. The next day, the samples were submerged in xylene (dewaxing – 2 times per 5 minutes each), descending grades of ethanol (rehydration – 100% (1 time per 5 minutes), 95% (1 time per 5 minutes), 70% (1 time per 5 minutes)) and washed in running tap water (2 times per 5 minutes each). Immediately after, samples were immersed in hematoxylin solution for 4 min and followed by immersion in eosin solution for 2 min. The sample were washed in running tap water (2 times per 10 minutes each) after incubation of both hematoxylin and eosin solutions. Finally, sections were dipped in 96% (2 times per 5 minutes) and 100% (1 time per 5 minutes) ethanol, cleared up with xylene (1 time per 5 minutes), and mounted on Histomount Mounting Solution (Thermo Scientific, USA). Two independent pathologists additionally evaluated the histopathological examination. In case, two pathologists did not reach a consensus regarding the results, we sought the help of a third pathologist. The microscopy and morphometric scoring of the rats' uteruses were performed with the Zeiss Axio Primo Star microscope, Zeiss AxioCam ERc 5s digital camera, and ZEN 2 (blue edition) software package (Germany).

2.5. Atomic absorption spectrometry of uterine tissues

The atomic absorption spectrometry of uterine tissues was performed according to the following protocol. Tissue samples were scaled, weighed on an analytical balance, dried (at 105°C), and burnt in porcelain crucibles at 450°C (48 h). Ash was dissolved into hydrochloric and nitric acids at 50°C overnight. After dilution with distilled water, the samples were measured. The sample solution was evaporated with the flame atomizer. Thereafter, the electrothermal atomic absorption spectrophotometer C-115M1 (Ukraine) with the analytic software package AAS SPEKTR (Ukraine) was used to determine the number of chemical elements according to their wavelength as follows: zinc (213.9 nm), copper (324.7 nm), iron (248.3 nm), manganese (279.4 nm), lead (283.3 nm), and chromium (357.9 nm).

2.6. Statistical analysis

All results are expressed as the mean ± standard deviation (M ± SD). Distribution type was estimated with the Shapiro–Wilk test. The differences between groups for normally distributed datasets were determined by the independent student's t-test. The one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc comparisons test was performed to compare variables among groups. Analysis of the strength and direction of the relationships between two variables was performed using the Pearson's (r) correlation coefficient. Differences in values were considered significant at $p<0.05$. Data analysis and graphs were prepared with GraphPad Prism® 6.0.

2.7. Ethics approval

All animals handling and experimental procedures fully adhere to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0 (Percie *et al.*, 2020). The experiment has been conducted in the European Community Guide for the Care and Use of Laboratory Animals guidelines, ethical and responsible manner, and is in full compliance with all relevant codes of experimentation (institutional and national) and legislation. This study was approved by the Bioethics Committee of the Medical Institute of Sumy State University (No. 2/10 from 10.10.2019).

3. Results

3.1. Liveweight, histopathologic and morphometric changes in rat uterus caused by HMs

HMs administration induced body weight loss in both experimental groups. Indeed, the liveweight proportion of female rats was less in HM group (HMs exposure only) (decrease of 18,21%; $p < 0.01$) and in HM+E group (HMs exposure with vitamin E treatment) (decrease of 13,09%; $p < 0.05$) than in the Control group. There was no significant difference between HM group and HM+E group. A difference in body weight was first noticed in the third week of the experiment and it increased in the following weeks. Other visual changes were not detected.

Long-term HMs exposure (HM group) contributed to pathological changes in the initial part of the uterine horn and reduction of the organ's wall thickness (see Figure 1 and Figure 2). However, these changes were found in both the endometrium and the myometrium. Detailed morphological analysis indicated a nonspecific versatility of these changes: dystrophy of the prismatic (vacuolar degeneration) and exocrine cells (cystic transformation of goblet cells) of the mucous membrane and myometrial myocytes. Atrophic changes in the epithelium were also observed — that are, reduced and uneven height of the superficial (cylindrical epithelium changes to cubic) and glandular epithelium due to a reduction of cytoplasm volume; reduction of endometrial gland number, size, and lumen; and cystic enlargement of single glands. The uterine mucosa had a decreased number of folds and a significantly increased intrauterine lumen. Focal inflammatory infiltration was found mainly in the endometrium. However, the myometrium was also locally involved in the inflammatory process. This was accompanied by connective tissue disorganization, microcirculatory disorders, and slight edema along the entire wall of the uterine horn.

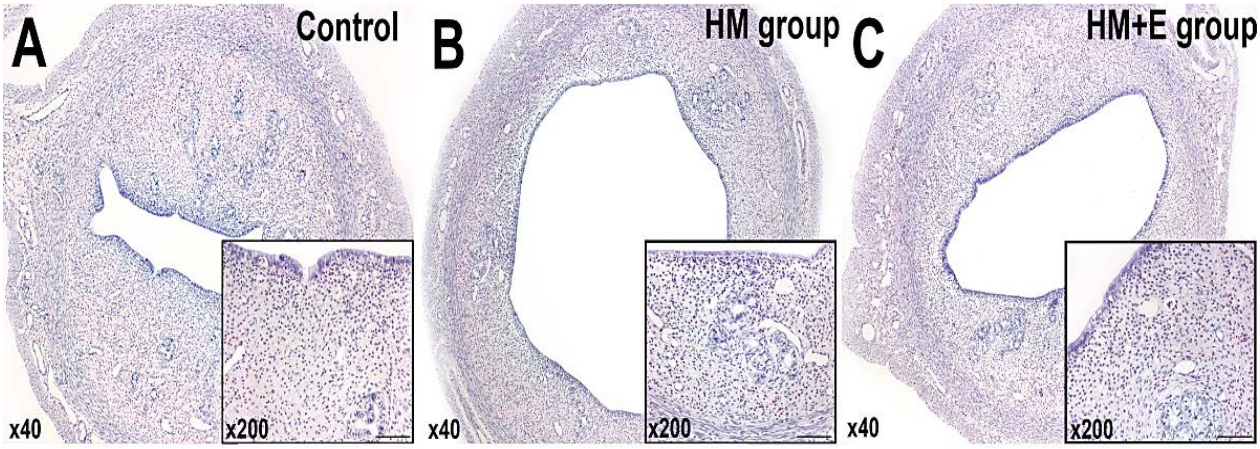

Figure 1. The HMs (Zn, Cu, Mn, Fe, Pb, and Cr) effect on the histopathologic changes in rats uterine horns: control group (A), HM group (B), and HM+E group (C). Staining with hematoxylin and eosin. Magnification: ×40 and ×200. Scale bar – 50 µm.

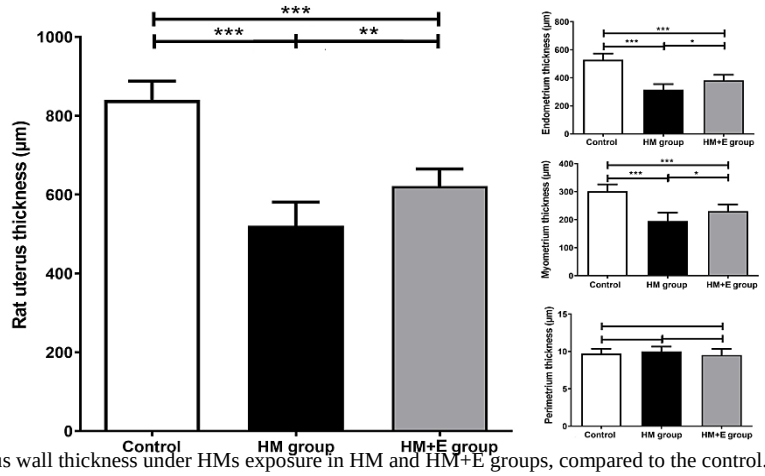

Figure 2. Variability of rat uterus wall thickness under HMs exposure in HM and HM+E groups, compared to the control. Data are expressed as Mean ± SD (Bars – T style with above direction). Values were analyzed by One-way ANOVA followed by Bonferroni's post-hoc comparisons test (n=24): *p < 0.05; **p < 0.01; ***p < 0.001.

Additionally, compared to the control group, the morphometric analysis revealed a significant reduction of the uterine-wall thickness (37.99%, p<0.0001) under HMs long-term exposure. Among all of the uterine layers, the endometrium had the highest reduction of the thickness (40.28%, p<0.0001), followed by the myometrium (35.28%, p<0.0001), and the perimetrium (2.88%, p>0.05).

In contrast, we detected moderate degenerative and atrophic changes in the rat uteruses of the HM+E group. On the one hand, the intensity of pathological transformations and morphometric variations (less on 16.17%, p<0.01) of the uterus wall were lower than those in the HM group. On the other hand, the morphometric scoring showed a reduction of rat uterine thickness (26.03%, p<0.0001) in the HM+E group due to a decrease of the endometrium and myometrium sizes by 27.88% and 23.56% (p<0.0001), respectively, compared to the control group. The difference in perimetrium thickness was not statistically significant (1.95%, p>0.05).

3.2. Imbalance of chemical contents in rat uterus tissues caused by HMs

The detection limits, distribution, and variability of the HMs concentration levels in rat uteruses are shown in Figure 3 and Table 1. The HM and HM+E groups had a wide range of variations of HMs accumulation in uterus tissues. According to atomic absorption spectrometry, the mean concentration of each element (Zn, Cu, Fe, Mn, Pb, and Cr) differed significantly (74.46%, p<0.0001) from the control values, even in the group with treatment by vitamin E (49.81%, p<0.0001). The highest concentration was estimated for Fe and the lowest for Pb. However, we detected a tendency for a general increase of HMs in rat uterus tissues as follows (listed in descending order): Pb (88.11%, p<0.0001) > Fe (86.26%, p<0.0001) > Cr (73.09%, p<0.0001) > Mn (63.6%, p<0.0001) > Cu (61.8%, p<0.0001) > Zn (49.34%, p<0.0001) for HM group vs Pb (62.24%, p<0.0001) > Fe (58.81%, p<0.0001) > Cr (55.58%, p<0.0001) > Cu (46.17%, p<0.0001) > Mn (44.77%, p<0.0001) > Zn (29.4%, p<0.0001) for the HM+E group relative to the control group. Therefore, the HMs bioaccumulation in the HM+E group was lower than that in the HM group (16.46%, p<0.0001).

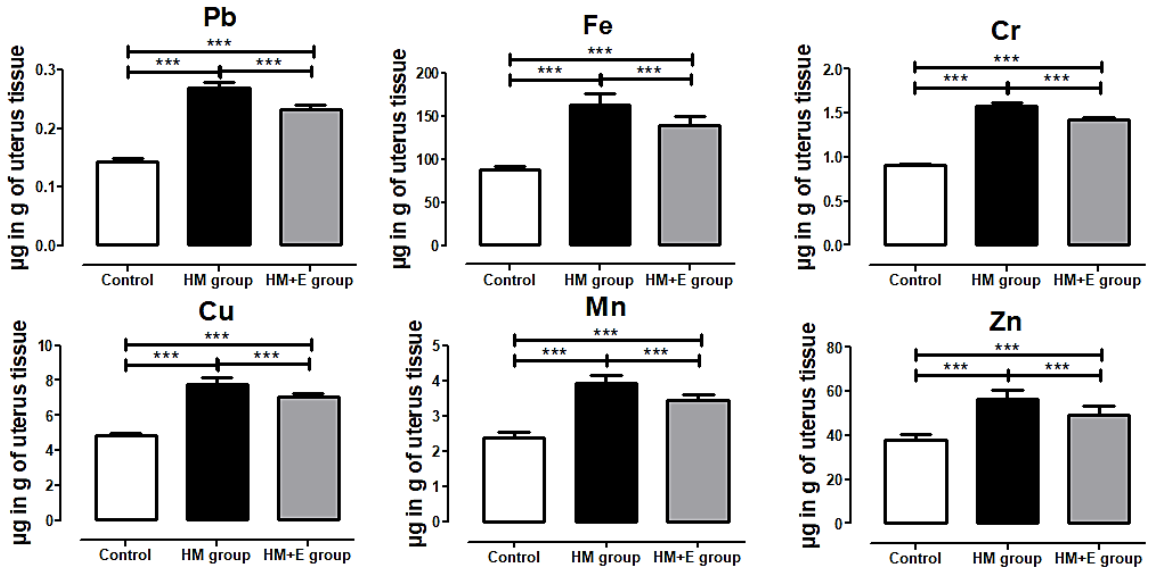

Figure 3. The imbalance of HMs concentration in rat uterine tissues in HM and HM+E groups. The Zn, Cu, Mn, Fe, Pb, and Cr concentrations were significantly higher in the HM group than in the control and HM+E group. The HMs concentration in the HM+E group was significantly higher than in the control group. Data are expressed as Mean ± SD (Bars – T style with above direction). Values were analyzed by One-way ANOVA followed by Bonferroni's post-hoc comparisons test (n=24): *p < 0.05; **p < 0.01; ***p < 0.001.

Table 1. Variability of HMs concentration (µg/g) in rats uterine tissues.

	HM group	HM+E group	Control group
Pb	0.269 ± 0.009***[1]/***[2]	0.232 ± 0.009***[1]	0.143 ± 0.006
Fe	163.18 ± 12.650***[1]/***[2]	139.13 ± 10.37***[1]	87.61 ± 4.38
Cr	1.582 ± 0.037***[1]/***[2]	1.422 ± 0.034***[1]	0.914 ± 0.01
Mn	3.91 ± 0.23***[1]/***[2]	3.46 ± 0.15***[1]	2.39 ± 0.15
Cu	7.78 ± 0.35***[1]/***[2]	7.06 ± 0.21***[1]	4.83 ± 0.15
Zn	56.69 ± 3.8***[1]/***[2]	49.74 ± 4.01***[1]	37.96 ± 2.45
Total	233.41 ± 8.84***[1]/***[2]	200.42 ± 9.86***[1]	133.83 ± 5.5

Note: [1] – compared to control group. [2] – compared to HM+E group. *p < 0.05; **p < 0.01;***p < 0.001.

3.3. Correlation analysis

There were strong negative correlations between rats' uterine thickness and HMs accumulation in both experimental groups — HM and HM+E (see Table 2). Thus, each individual metal had a different strength of influence as follows: Zn (r=−0.89), Cu (r=−0.93), Mn (r=−0.91,), Fe (r=−0.95), Pb (r=−0.93), Cr (r=−0.95) vs Zn (r=−0.77), Cu (r=−0.95), Mn (r=−0.87), Fe (r=−0.97), Pb (r=−0.91), Cr (r=−0.93) (p<0.0001), respectively. It is important to note that the strength of these relationships was different in the endometrium, myometrium, and perimetrium. The correlation between the uterus membranes thickness and the HMs concentration was slightly lower in the HM+E group (r=−0.91, p<0.0001), compared to the HM group (r=−0.96, p<0.0001).

Table 2. The strength of Pearson's correlations (r) between HMs accumulation and uterus thickness.

	Endometrium		Myometrium		Perimetrium	
	HM group	HM+E group	HM group	HM+E group	HM group	HM+E group
Pb	-0.92***	-0.85***	-0.87***	-0.84***	0.16	-0.21
Fe	-0.94***	-0.85***	-0.9***	-0.83***	0.38	-0.19
Cr	-0.93***	-0.87***	-0.9***	-0.84***	0.24	-0.14
Mn	-0.9***	-0.8**	-0.85***	-0.82***	0.14	-0.18
Cu	-0.91***	-0.9***	-0.87***	-0.84***	0.25	-0.05
Zn	-0.88***	-0.74**	-0.81***	-0.65***	0.13	-0.05
Total	-0.95***	-0.86***	-0.9***	-0.82***	0.33	-0.17

Note: *p < 0.05; **p < 0.01; ***p < 0.001 – compared to the control group.

4. Discussion

Industrialization and urbanization have affected many organisms' natural lifestyles, violating the evolutionarily programmed organism existence and corresponding complex (genetic) diseases (Saeb and Al-Naqeb, 2016). The progressive accumulation of pollutants in the environment poses a great threat. Therefore, humanity should focus on mitigating (degassing and deactivation) existing and preventing future pollution (Jaishankar *et al.*, 2014; Hamid *et al.*, 2016; Ho *et al.*, 2017; Zhang *et al.*, 2019; Sahiti *et al.*, 2020). Many previous studies have shown the effects of chemical toxins on organisms. Such 'coexistence' may depend on the origin, ways of pollutants influence, individual characteristics of each species, the effectiveness of individual protection or prevention, social behavior, health outcomes, social and demographic features (Saeb and Al-Naqeb, 2016). In general, four factors can contribute to the violation of physiological homeostasis in the body: genetic, hormonal, ontogenetic, and life/health factors (Jaishankar *et al.*, 2014; Saeb and Al-Naqeb, 2016; Ho *et al.*, 2017; Su *et al.*, 2017; Zhang *et al.*, 2019; Lee *et al.*, 2021).

HMs are among the top exogenous pollutants worldwide that can spread and bioaccumulate in terrestrial, aquatic, and airborne environments. In such natural conditions, essential and toxic trace elements have a long half-life, and they can accumulate and change their nature (Singh *et al.*, 2011; Jaishankar *et al.*, 2014; Romaniuk *et al.*, 2015; Nakade *et al.*, 2015; Hamid *et al.*, 2016; Romaniuk *et al.*, 2017; Nwosu *et al.*, 2018; Mohammad Hosseini *et al.*, 2019; Zhang *et al.*, 2019; Sahiti *et al.*, 2020). It should be noted that the geochemical cycling of HMs on the planet has both artificial and natural compounds (weathering of metal-bearing rocks and volcanic eruptions, etc.). This increases their spread even in regions with low urbanization and technological progress levels (Singh *et al.*, 2011; Jaishankar *et al.*, 2014; Nakade *et al.*, 2015; Hamid *et al.*, 2016; Ali *et al.*, 2019; Zhang *et al.*, 2019; Lee *et al.*, 2021). Moreover, polyelemental additive metal contamination can contribute to the suppression and/or stimulation of each compound or even change its properties (Lodovici and Bigagli, 2011; Singh *et al.*, 2011; Jaishankar *et al.*, 2014; Nwosu *et al.*, 2018; Ali *et al.*, 2019).

The main effects of HMs on the body are the development of oxidative stress (hyperproduction of free radicals, reactive oxygen and nitrogen forms and lipid peroxidation, and inhibition of antioxidant mechanisms), inhibition of enzymatic activity, hormonal disorders, disruption of cell integrity, imbalance of cell division and apoptosis, impaired gene expression (blocking of signal pathways), chromosomal aberrations, pathological methylation and accumulation of damaged DNA, etc. (Singh *et al.*, 2011; Jaishankar *et al.*, 2014; Romaniuk *et al.*, 2015; Hamid *et al.*, 2016; Romaniuk *et al.*, 2017; Chen *et al.*, 2019). However, the mechanisms of HMs effect on the reproductive system are not fully understood. On the one hand, the toxicity at low exposure concentrations of metals such as cadmium, lead, aluminum, metalloid arsenic is more or less clear (cytotoxic, carcinogenic, and genotoxic effects). On the other hand, the excessive concentrations of essential elements (such as copper, zinc, manganese, nickel, iron, etc.) can act through complex direct and indirect pathways (Fenton-type reaction, or depletion of antioxidant systems). It is related to their mandatory physiological participation in antioxidant protection (Bielen *et al.*, 2013; Jaishankar *et al.*, 2014; Hamid *et al.*, 2016; Romaniuk *et al.*, 2017; Chen *et al.*, 2019).

Antioxidant deficiency, chronic diseases, or toxicants exposure are accompanied by an increased free radical concentration. It contributes to the violation of redox regulation and the development of oxidative stress, damage to the integrity of lipids, proteins, and DNA. On the one hand, the imbalance of redox systems occurs by direct inhibition of enzymatic protective (antioxidant) mechanisms (superoxide dismutase, ascorbate peroxidase, catalase, and glutathione peroxidase), non-enzymatic metabolic antioxidants (lipoic acid, glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc.) and nutrient non-enzymatic antioxidants (vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc.). On the other hand, it is achieved by stimulation of enzymes that produce free radicals (hydroxyl (OH•), superoxide ($O^{2}\bullet^{-}$), nitric oxide (NO•), nitrogen dioxide ($NO_2\bullet$), peroxyl (ROO•) and lipid peroxyl (LOO•)) and other non-radical reactive derivatives (hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH)) (Shao *et al.*, 2007; Pham-Huy *et al.*, 2008; Bielen *et al.*, 2013; Phaniendra *et al.*, 2015).

Vitamins and trace metals co-factors have an essential role in the non-enzymatic antioxidant mechanisms (Singh *et al.*, 2011; Bielen *et al.*, 2013; Phaniendra *et al.*, 2015; Su *et al.*, 2017). Artificial induction of non-enzymatic antioxidants leads to the counteraction of free radicals by direct and/or indirect ways (Bielen *et al.*, 2013; Hamid *et al.*, 2016; Mohammad Hosseini *et al.*, 2019; Sahiti *et al.*, 2020). Antioxidants can block the action of free radicals on the cell surface and in the blood. Tocopherol locates on the biological membrane of cells reduces the risk of free radicals entering the cell. Also, when combined with other antioxidants, vitamin E promotes faster cell "cleansing" and protection. They are also able to neutralize free radicals by transferring them positively charged atoms. Based on this, in our study, we used vitamin E for treatment because it is considered as the most powerful exogenous antioxidant and free-radical scavenger (Pham-Huy *et al.*, 2008; Al-Attar, 2011; Sahiti *et al.*, 2020). It is also known that stabilization of one antioxidant can lead to exhibiting cooperative behavior and enhance other's antioxidant mechanisms (Al-Attar, 2011; Bielen *et al.*, 2013; Sahiti *et al.*, 2020). For example, vitamin C has a regenerative effect on vitamin E from α-tocopherol radicals damage to membranes, zeaxanthin synthesis in the xanthophyll cycle, and inhibits activation of the caspase cascade and DNA damage, etc. (Shao *et al.*, 2007; Al-Attar, 2011; Bielen *et al.*, 2013;Sahiti *et al.*, 2020).

The results of our study indicated a pernicious toxic effect of HMs on the rat uterus. Histopathological studies on uteri of different exposure groups in the present study revealed its dose-dependent deleterious effects in all structural elements. Thus, the heterogeneity of uterine transformation was represented mainly by degenerative

and atrophic changes (degeneration and decrease in the height of luminal and glandular epithelium, decrease in the number of glands, their size and lumen), interstitial edema, inflammatory cells infiltration, microcirculatory disorder, connective tissue disorganization, and uterus wall thinning. Simultaneously, there was a decrease of the uterus thickness on the 37.99 % ($p < 0,0001$) vs 26.03 % ($p < 0,0001$), HM vs HM+E groups compared to the control. The reduction of the uterine wall (37.99%, $p<0.0001$) was caused by thinning of mucous and muscular membranes, respectively. It resulted in an increased intrauterine lumen and a decreased number of endometrial folds. This can complicate the movement of sperm and oocyte fixation (Höfer *et al.*, 2009; Lukacinova *et al.*, 2012; Nakade *et al.*, 2015; Hamid *et al.*, 2016; Cedars *et al.*, 2017; Hanson *et al.*, 2017; Chen *et al.*, 2019; Doncova *et al.*, 2019; Mohammad Hosseini *et al.*, 2019). It seems that this reaction of the uterus was caused by an increased concentration of Zn, Cu, Fe, Mn, Pb, and Cr in the organ tissue. It was also confirmed by the correlation between spectrophotometric and morphometric values imbalance.

Similar morphological and morphometric changes in the uterus have been described in other studies (Höfer *et al.*, 2009; Lukacinova *et al.*, 2012; Nakade *et al.*, 2015; Nasiadek *et al.*, 2018; Doncova *et al.*, 2019). However, different HMs combinations (mono- and/or polyelemental), their concentrations, and exposure time were used. In contrast, it was reported about the opposite effect of pollutants on the uterus, which was manifested by hyperplasia and dystrophy of the uterine mucosa (Höfer *et al.*, 2009; Nasiadek *et al.*, 2018). Authors indicate that HMs accumulation in the body can both stimulate and inhibit the activity of sex hormones and their effect on receptors in the uterus (Höfer *et al.*, 2009; Chatterjee and Chatterji, 2010; Katz *et al.*, 2016; Hanson *et al.*, 2017). Moreover, we have previously found an HMs effect on developing dystrophic/atrophic and/or oncological changes in other organs (the bladder, bone marrow, breast, and others) (Romaniuk *et al.*, 2015; Romaniuk *et al.*, 2017; Romaniuk *et al.*, 2018).

Our results point out that in contrast to HMs exposure of rats in HM group, the less significant histopathological lesions were identified in the rats' uterus after vitamin E treatment (HM+E group). Thus, after vitamin E treatment, moderate atrophy (decrease in height of columnar cells and fibrosis) and inflammation in the uterus were observed. Moreover, treatment in HM+E group caused the less pronounced reduction of rat uterine thickness (less on 16.17%, $p<0.01$) against the background of suppression of the accumulation of the metal in the uterus tissue, compared to HM group. Based on this, a vitamin E supplement may be beneficial in slowing progressive uterus damage. Such morphological results coincide with the data on the effectiveness and importance of the natural or artificial compounds with detoxifying and antioxidant properties (Pham-Huy *et al.*, 2008; Bielen *et al.*, 2013; Yadav *et al.*, 2016; Romaniuk *et al.*, 2019; Romaniuk *et al.*, 2018; Sahiti *et al.*, 2020). Unfortunately, a definitive defense mechanism against the impact of pollutants on the organism has not been identified or reported.

HMs exposure (both short- and long-term) leads to their accumulation in the organs (Hamid *et al.*, 2016; Romaniuk *et al.*, 2017; Su *et al.*, 2017; Nwosu *et al.*, 2018; Ali *et al.*, 2019; Mohammad Hosseini *et al.*, 2019). However, the imbalance of their concentrations in the uterus and other organs differed among reports (Höfer *et al.*, 2009; Lukacinova *et al.*, 2012; Rzymski *et al.*, 2016; Nasiadek *et al.*, 2018). Our study showed an increase of HMs accumulation ($p<0.001$) in rats' uterus. Moreover, the concentration and intensity of their accumulation differed in each case. Thus, the lowest relative bioaccumulation in both groups (HM and HM+E groups) belonged to Zn and the maximum to Pb (Pb>Fe>Cr>Mn>Cu>Zn for HM group and Pb>Fe>Cr>Cu>Mn>Zn for the HM+E group in descending order). It should be noted that corrector treatment caused the change in Mn and Cu accumulation order. In the HM+E group, the HMs levels were higher than control levels but were significantly lower (for Zn, Cu, Fe, Mn, Pb, and Cr; $p<0.0001$) compared to the HM group. This difference may be due to several factors, such as the properties of each individual metal, competition bonds (synergistic and antagonistic) both between metals and between metals with vitamin E, accumulative characteristics of HMs and bioaccumulative characteristics of the uterus, and others (Jaishankar *et al.*, 2014; Nakade *et al.*, 2015; Hamid *et al.*, 2016; Rzymski *et al.*, 2016; Romaniuk *et al.*, 2018; Mohammad Hosseini *et al.*, 2019; Sahiti *et al.*, 2020; Wang *et al.*, 2020). In addition, we demonstrated the relationship between the excess concentration of HMs in uterine tissue and the variability of morphometric values of the uterine wall. Strong negative correlations were found between organ thickness and HMs accumulation in the uterine tissues in both groups (HM group ($r=-0.96$, $p<0.0001$) and HM+E group ($r=-0.91$, $p<0.0001$)). In this case, the greatest influence on uterine wall thickness had Pb and Cr. On the other hand, the lowest influence had Zn and Cu. The essential HMs are prone to lower accumulation in the uterus on the background of detoxification by vitamin E. At the same moment, toxic or potentially dangerous metals have bigger accumulative properties. The lower HMs accumulation and reduced morphological changes in uterine tissue (HM+E group compared to HM group) validated the feasibility of the use of natural supplementation with antioxidant and detoxifying properties.

Based on our results and analysis of the literature, as a general concept, it has become clear that rats' uterus changes depending on the HMs influence. The long-term intake of low HMs doses and their accumulation in the uterus (as in other organs) led to the gradual imbalance of intracellular homeostasis, atrophy, inflammation, suppression of cellular transduction mechanisms, disruption of compensatory defense mechanisms, redox imbalance, and oxidative stress (decrease of the cellular antioxidants and increased oxidative DNA damage, lipid peroxidation, and reactive oxygen species) (Lodovici and Bigagli, 2011; Jaishankar *et al.*, 2014; Hamid *et al.*, 2016; Diantin *et al.*, 2018). The inflammation, provoked by the HMs action on the background of already existing pathological changes, also increased the free radical generation. Under the influence of chronic stress, adaptive mechanisms were exhausted and led to the development of atrophic changes of uterus cells. The aggravated cellular hypoxia leads to the progression of periglandular and perivascular fibrosis. Most likely, oxidative stress appeared to be one of the main mechanisms of the HMs effect on the body, which led to morphological transformations in the uterus. It was confirmed by reports

on the long-term adverse effects of free radicals under oxidative stress on the background of antioxidant capacity depletion (Shao *et al.*, 2007; Pham-Huy *et al.*, 2008; Phaniendra *et al.*, 2015; Yadav *et al.*, 2016; Romaniuk *et al.*, 2017; Romaniuk *et al.*, 2019). Vitamin E allows free radicals to abstract a hydrogen atom from the antioxidant molecule rather than from polyunsaturated fatty acids, thus breaking the chain of free radical reactions, the resulting antioxidant radicals being a relatively unreactive species. At the same time, the prolongation of the experimental conditions caused activation of adaptive mechanisms and the subsequent start of the recovery processes (Al-Attar, 2011; Yadav *et al.*, 2016; Romaniuk *et al.*, 2018; Mohammad Hosseini *et al.*, 2019; Albishtue *et al.*, 2020; Sahiti *et al.*, 2020; Tahtamouni *et al.*, 2020).

Such histopathological transformations of the uterine wall can disrupt the estrous cycle, cause hormonal imbalance, and reduce fertility (Höfer *et al.*, 2009; Lukacinova *et al.*, 2011; Doncova *et al.*, 2019). Moreover, it has been shown that low doses of various metals (lead, mercury, and cadmium) are associated with metal-specific reproductive system lesions (Doncova *et al.*, 2019). However, long-term HMs exposure activates epigenetic and adaptive mechanisms as evidenced by an increase in the total number of litters and neonates (i.e., vulnerable groups showed increased reproductive activity). HMs can impair reproductive function by affecting other organs in both female and male rats. Moreover, the HMs accumulation in different organs is much higher than that in the uterus (Höfer *et al.*, 2009; Lukacinova *et al.*, 2012; Sahiti *et al.*, 2020; Wang *et al.*, 2020; Shraideh *et al.*, 2021). In addition, the proven reprotoxic properties of HMs have been manifested as deteriorations of physical and reproductive health parameters in subsequent generations (Lukacinova *et al.*, 2011; Doncova *et al.*, 2019; Mohammad Hosseini *et al.*, 2019;). Also, from the results of this study, there was group-dependent body weight loss. The most pronounced weight loss was observed in the HM group. Such results can be explained by chronic HM intoxication, which is accompanied by endocrine disorders of the thyroid gland, atrophic changes in internal organs, alteration of electrolyte balance and lipid metabolism, injury of hepatic function and the induction of neurobehavioral function (Su *et al.*, 2017; Fiati Kenston *et al.*, 2018).

Increased environmental pollution is reflected in increased risks of deterioration of plants, animals, and humans. This has been confirmed by links between HMs excesses in the organs (including the uterus) of wild animals (from potentially contaminated areas) and human population density, age, season, and extent of territory contamination (Wirth *et al.*, 2010; Jaishankar *et al.*, 2014; Hamid *et al.*, 2016; Ljungvall *et al.*, 2017; Romaniuk *et al.*, 2017; Avilova *et al.*, 2018; Shah *et al.*, 2020; Lytvynenko *et al.*, 2021). Based on the variability of the consequences of pollutants, the prediction of the development of pathological changes is a complex process. This requires a consideration of HMs combinations and concentrations, their exposure time, the intake way, the features of local environmental pollution, the presence of concomitant pathologies and so on.

5. Conclusions

Long-term exposure (within 90 days) to the HMs combination had a pernicious toxic effect on the rats' uterus. A strong negative correlation between the accumulation of HMs (zinc, copper, iron, manganese, lead, and chromium) in uterus tissue with morphological (degenerative and atrophic) and morphometric (reduced uterine-wall thickness) changes was detected. Uncontrolled exposure to HMs was found to lead to serious complications and adverse reproductive health risks. The HMs exposure combined with vitamin E treatment was accompanied by significantly lower accumulation of chemical elements in the uterine wall and restraint of morphological lesions in the rats' uterus. This could be an important step towards the development of preventive and protective approaches to addressing toxic pollutants.

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