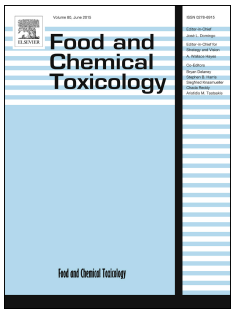


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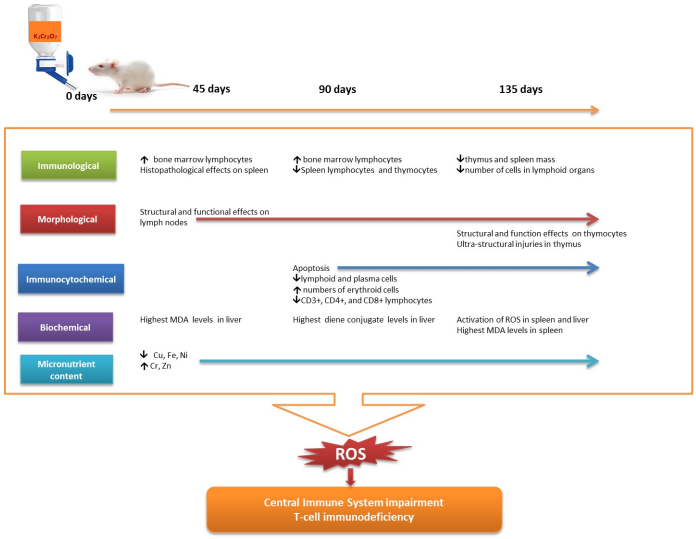

# Long-term effects of chromium on morphological and immunological parameters of Wistar rats

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[1] Abbreviations: Chromium (Cr), Hexavalent chromium [Cr (VI)] potassium dichromate ($K_2Cr_2O_{7)}$, No-Observed-Adverse-Effect-Level (NOAEL), Lowest-Observed-Adverse-Effect-Level (LOAEL), Agency for Toxic Substances and Disease Registry (ATSDR), minimal Risk Level (MRL), concanavalin A (ConA), enzyme-linked immunosorbent assay technique (ELISA), Mayer`s hematoxylin and eosin (H&E), superoxide dismutases (SOD) malondialdehyde (MDA), 2-thiobarbituric acid (TBA), diene conjugates (DC), iron (Fe), cooper (Cu), zinc (Zn), nickel (Ni), medians (Me), mean arithmetic values (M), ,standard error of the mean (SEM)

**Abstract**

Hexavalent chromium raises high concern because of its wide industrial applications and reported toxicity. Long-term (135 days) oral exposure of Wistar rats to chromium in the form of $K_2Cr_2O_7$ (exposed group~20mg/kg/day) led to a decrease in thymus mass and thymocytes' number and caused structural and functional changes in the lymph nodes and spleen, namely lymphoreticular hyperplasia and plasmocytic macrophage transformation. Programmed cell death was increased in both thymocytes and splenocytes and decreased in lymphocytes in the T-zones of spleen and lymph nodes. Moreover, Cr (VI) administration decreased myeloid cells' and neutrophils' number, while it increased lymphoid and erythroid cells' number in bone marrow. Cr (VI) immune system effects seem to be related to oxidative stress induction, as depicted by the increased levels of diene conjugates and malondialdehyde in the spleen and liver and by the decreased activity of catalase and superoxide dismutase in rats' erythrocytes. In addition, exposure to Cr (VI) decreased copper, nickel and iron concentrations in blood and liver, while Cr levels in blood, spleen and liver were increased, as expected. The observed changes in the series of immunological parameters studied contribute to the the development of new approaches for the prevention of low level Cr exposure toxicity.

**Keywords**
Chromium, immunotoxicity, rats, oxidative stress, microelements

## Introduction

It is known that the immune system can be suppressed leading to agent-induced secondary immunodeficiency after exposure to certain toxicants (Hartung and Corsini, 2013; Hartung 2016; Hultman, 2007; Tsiaoussis et al., 2019). Many different inorganic elements, chromium (Cr) being one of them, may act as toxicants. Among chromium compounds, hexavalent chromium [Cr (VI)] is of particular interest as it is widely used in different industries (in metal, leather, textile, chemical, paint, pharmaceutical, etc.). Furthermore, Cr (VI) markedly accumulates in the body and causes toxic effects at a wide range of concentrations (ECHA, 2015; EFSA, 2014; Holmes et al., 2008). It has been characterized as carcinogenic to humans (Group I) by the International Agency for Research on Cancer (IARC, 2012). Hence, applications of Cr (VI) in commercial products have been restricted under Regulation (EC) 1907/2006 in the European Union.

Apart from occupational exposure and exposure from commercial products, food and water are the important sources for the majority of toxic metals for humans, including Cr as well (González-Weller et al., 2013; Renieri et al., 2019; Taghizadeh et al., 2017). It is worth acknowledging however, that real life exposure scenarios concern exposures to multiple stressors, which can be challenging to study (Docea et al., 2018; Hernández & Tsatsakis, 2017; Kostoff, Goumenou, & Tsatsakis, 2018; A. Tsatsakis, Goumenou, Liesivuori, Dekant, & Hernández, 2019; A M Tsatsakis et al., 2017; Aristidis M. Tsatsakis, Docea, & Tsitsimpikou, 2016). The highest concentrations of chromium in food are found in mushrooms, oysters, liver, brewer's yeast, and black pepper while low contents are reported for meat, fruits, grain, and vegetables (González-Weller et al., 2013). Chromium in its normal oxidation state in biological tissues which is Cr (III) is an essential mineral believed to be a component

of the glucose tolerance factor with a role in the carbohydrate metabolism involved in cardiovascular risk and the metabolic syndrome (Hummel et al., 2007). Chromium toxicity is closely associated with its oxidation state, where hexavalent compounds are about 10–100 times more toxic than the trivalent ones, when administered orally (Soares et al., 2010). Trivalent forms of Cr (which are mainly present in food) have a low toxicity (Reilly, 2002). Concerning the risks that the presence of Cr (VI) in food and drinking water pose to public health, the European Food Safety Authority (EFSA) reported a No-Observed-Adverse-Effect-Level (NOAEL) of 7.8 mg Cr (VI)/kg b.w. per day and a Lowest-Observed-Adverse-Effect-Level (LOAEL) of 15.7 mg Cr (VI)/kg b.w. per day (EFSA, 2014). Furthermore, the Agency for Toxic Substances and Disease Registry (ATSDR) following animal studies, noted an oral Minimal Risk Level (MRL) of 0.005 mg/kg b.w. per day for intermediate exposure and nonneoplastic effects (ASTDR, 2012).

Cr (VI) introduced into the organism through the oral route, is reduced to Cr (III), thus giving rise to free radicals which are mainly implicated in Cr (VI) induced toxicity (ASTDR, 2012; EFSA, 2014; Shrivastava et al., 2002). Several animal studies, investigating various Cr (VI) oral toxicity endpoints, revealed that among major targets of Cr (VI) compounds are the immune system (Bucher, 2007), liver and kidneys, as well as the hematological system and the gastrointestinal tract (Vihol et al., 2012). Moreover, immunotoxicity of Cr (VI) has been reported through dermal exposure as well, following direct contact with the skin which causes a systemic reaction of the immune system (ASTDR, 2012; Shrivastava et al., 2002).

Cr (VI) effects on the immune system involve morphological and functional damage to its organs such as thymus, spleen lymph nodes, and bone marrow (Hultman, 2007). Immunologically active tissues can serve as toxicity biomarkers

because of the early reaction of the immune system following exposure to organic or inorganic chemicals (Chaturvedi et al., 2011). The most commonly investigated morphological endpoint of the immune system is the determination of the morpho-functional alterations in the lymphoid tissue after long-term exposure to different toxicants of organic and inorganic origin (Holmes et al., 2008).

Hence, the aim of the present work was to perform a comprehensive *in vivo* assessment of chronic effects of Cr (VI) on the morphological, immunological and oxidative stress parameters in Wistar rats' spleen and lymph nodes.

## 1. Materials and methods

### 2.1. Study design

Experimental studies were conducted in 280 healthy pubertal male Wistar rats (250-300 g) maintained on a standard diet. Animals were randomized into 2 groups, exposed, (n=210) and control (unexposed) group, (n=70). The exposed group received the following treatments for either 45, 90 or 135 consecutive days: potassium dichromate ($K_2Cr_2O_7$, Scientific-Production Enterprise "Polykhim", St. Petersburg, Russia) dissolved in drinking water, while the control group was receiving only drinking water and was kept under the same standard conditions (i.e. room temperature of 22°C, 50-60% humidity and 12 h night:12 h day photoperiod) as the exposed group during the entire experiment. Water and food was given ad libitum to both groups. The choice of dose, method of administration and the duration of the experiment were substantiated by previous studies (Utenin, 2002) and available literature (ASTDR, 2012). Based on rat daily average drinking water intake of 15-16 ml (Bucher, 2007) that contained 4-6 mg of the potassium dichromate dissolved, average Cr (VI) intake in exposed group approximately corresponded to

20mg/kg/day. On days 45 (n=72), 90 (n=68) or 135 (n=70) of the experiment, the animals from exposed group were sacrificed using an overdose of ketamine and the parameters described below were evaluated at all time points. All animals from control group were sacrificed at the end of the experiment (135$^{th}$ day). The work is approved by the expert examination of the Orenburg State Medical University Ethics Committee (protocol №9, 14.05.08).

2.2. Immunological parameters

On day 45, 90 and 135 of the experiment, rats' thymus mass and spleen mass along with number of thymus, spleen and bone marrow nuclear cells were determined; spleen and bone marrow cellular composition was assessed in accordance with the laboratory methods of experimental animal studies (Volchegorskij et al., 2000).

Blood was collected in test tubes ("Venosafe", Belgium) without EDTA, maintained 30 minutes and then centrifuged within 15 minutes at 1500 rpm. For preparation of the homogenate the tissue sample (1 g) was homogenized in 5 ml of the phosphatic buffer and protein content was assessed according to the method (Lowry, 1951).

Immunophenotyping of splenocytes was examined using monoclonal antibodies ("eBioscience", USA) against CD3, CD4, CD8 receptors. Percentage of CD3+, CD4+, CD8+ spleen lymphocytes was defined using the flow cytometer "FACS Canto II" with two lasers ("Becton Dickinson", USA). Cell cycle and apoptosis of splenocytes were assessed using the DNA fluorochrome staining method, followed by the cytofluorometry using the flow cytometer FACS Calibur (Sibirijak et al., 2008). The effect of Cr (VI) on the splenocytes' cytokine production (IL-4, IL-6, IL-10, and IFNγ) (pg/ml) was also studied in the supernatants of splenocytes' cultures with and

without stimulation with concanavalin A (ConA) using the enzyme-linked immunosorbent assay technique (ELISA) (Bender MedSystems, Austria), which was finally measured using a spectrophotometer at 450 nm (Multiskan, Labsystems, Finland).

2.3. Morphological parameters

Morphological investigations were conducted in 15 randomly selected rats of the Cr(VI) exposed group and in 9 rats of the control group on days 45, 90 and 135, respectively, using general morphometric and histological methods of investigation. Conducting morphological studies on 15 experimental and 9 control rats was justified by the fact that in each stage of the experiment (45, 90, 135 days) 5 animals were taken. This allowed us to produce 100 histological sections from each rat (500 sections for each stage, 1500 sections in total). Such a number of objects (histo-slices) ensured morphometry and statistical processing of quantitative data.

Tissue fixation was made using 10% neutral formalin solution. Paraffin sections were stained with Mayer`s hematoxylin and eosin (H&E). Morphometric studies were performed by ocular micrometer. In order to determine the pro-apoptotic protein p53 expression levels and the anti-apoptotic Bcl-2 protein levels, the avidin-biotin-peroxidase method was used as previously described (Geyer, 1973). Proteins' expression levels were expressed as the amount of positively stained cells per 1000 cells (permille) (Geyer, 1973).

Comparative analysis of the areas occupied by the germinal center and the peri-arterial sheath (in µm) in rats' spleen was performed in exposed and control animals. Area measurements were performed in the H&E stained paraffin-embedded slides of

spleen obtained from the three Cr (VI) exposed groups in three different time points post dichromate administration and from the control group.

## 2.4. Parameters of oxidative stress

Superoxide dismutases (SOD) activity was determined by adrenaline auto-oxidation in the alkaline environment. Oxidation speed of adrenaline was estimated based on kinetics of optical density change at 347 nm (Sirota, 1999).

Determination of catalase activity was carried out by a kinetic spectrophotometric method of direct registration of decomposition of a substrate of the enzyme - hydrogen peroxide. The amount of the enzyme was enough to cause falling of optical density from 0.45 to 0.4 in 17 seconds per unit of activity of a catalase (Zuck, 1963).

Concentration of malondialdehyde (MDA) homogenates of liver and spleen was determined via the 2-thiobarbituric acid (TBA) test (Himreaktivsnab, Russia) (Volchegorsky et al., 2000).

Concentration of the diene conjugates (DC) in liver and spleen homogenates was determined by a maximum of lipid solution absorption characteristic of DC, in system isopropanol-heptane (1:1) at 233 nm (Volchegorsky et al., 2000).

## 2.5. Microelements analysis

Microelements, i.e. iron (Fe), cooper (Cu), zinc (Zn), Cr and nickel (Ni)) content in blood, liver and spleen tissues was determined by atomic absorption spectroscopy using the spectrophotometer "KBAHT-2A" (company «LLC Korchek», Russia). To study the changes in the elemental composition caused by Cr intake: blood, liver, spleen of the experimental animals were isolated and frozen at –20 °C. Sample preparation of the selected samples was performed by dry ashing, followed by

dissolving the residue in a mixture of nitric and trichloroacetic acids. Selected blood samples, not less than 1 ml, and organs, not less than 5 g, were placed in a crucible and dried for 1.5 hours at a temperature of 110 ºC in a drying cabinet, then for 1.5 hours at a temperature of 250 ºC. After that, ammonium sulfate was added to the sample and at a temperature of 450-500 °C the sample was reduced to ashes in a muffle furnace. After cooling in a desiccator, 0.3-0.5 ml of concentrated nitric acid was added to the sample and evaporated to “wet salts”. Then, 5 ml of 1% nitric acid was added to the cooled residue and left for 30-40 minutes, filtered and transferred to a test tube with a ground up stopper (Onishchenko, 2011).

### 2.6. Statistical analysis

Results were analyzed using analysis of non-parametric Mann-Whitney test to compare groups since the data did not follow the normal distribution (according to Kolmogorov-Smirnov test). Study parameters were presented as medians (Me) and inter-quartile range ($25^{th}$ and $75^{th}$ percentile), as well as mean arithmetic values (M) ± the standard error of the mean (SEM). The statistical package STATISTICA 10 was used. Differences between treatment groups were considered significant when $p<0.05$.

## 3. Results

### 3.1. Body weight

The initial weight of the control and experimental rats was 180-200g. As they mature, the weight of the rats increased, especially in the control group. The average weight of rats was: for controls - 316 ± 8.66; on the 45th day - 252 ± 7.39 g; on the 90th - 306 ± 8.08 g; on the 135th - 270 ± 8.78 g.

3.2. Immunological analysis

In rats exposed to Cr (VI), no pathological changes in leukocytes' number and differential leukocyte count were observed after 3 months of exposure (animals sacrificed at 90$^{th}$ day). After 135 days of exposure, significant decreases in thymus (33.5%) and spleen (27.4%) weights were observed compared to controls; the populations of thymocytes (55%) and splenic karyocytes (42.9%) were also significantly reduced (Table 1).

**Table 1.** Mean (Me) mass of rats, thymus, spleen and number (No) of thymocytes and spleenocytes, marrow karyocytes populations in Wistar rats of the exposed and control group

| | **Exposed group** | | | | | | **Control group** | |
|---|---|---|---|---|---|---|---|---|
| | **45$^{th}$ day** | | **90$^{th}$ day** | | **135$^{th}$ day** | | | |
| | **Me** | **[25$^{th}$;75$^{th}$ percentile]** | **Me** | **[25$^{th}$;75$^{th}$ percentile]** | **Me** | **[25$^{th}$;27$^{th}$ percentile]** | **Me** | **[25$^{th}$;75th percentile]** |
| Rat mass,( g) | 250,0* n=29 | [239,4; 278,0] | 345,0* n=21 | [332,0; 360,0] | 260,0* n=13 | [250,0; 290,0] | 322,0 n=41 | [297,5; 360,0] |
| Thymus mass, (mg) | 257,0* n=30 | [160,0;283,0] | 208,0* n=22 | [180,0; 258,0] | 173,0 n=13 | [165,0; 175,0] | 243,0 n=54 | [196,0; 295,0] |
| No of thymocytes, x $10^6$ | 310,0* n=30 | [215,0; 400,0] | 350,0 n=22 | [330,0; 360,0] | 195,0* n=13 | [182,0; 205,0] | 464,0 n=54 | [328,0; 538,0] |
| Spleen mass, (mg) | 973,0* n=40 | [858,0; 1189,0] | 990,0 n=22 | [913,0; 1053,0] | 755,0* n=13 | [683,0; 766,0] | 1046,0 n=54 | [946,0; 1128,0] |
| Splenic karyocytes No x $10^6$ | 946,0* n=40 | [868,0; 1168,0] | 537,0* n=22 | [453,0; 74100] | 590,0* n=13 | [557,0; 621,0] | 1041,0 n=54 | [868,0; 165,0] |
| Bone marrow karyocyte No x $10^6$ | 82,0 n=21 | [75,0; 102,0] | 62,0 n=22 | [56,0; 74,0] | 85,0* n=13 | [75,0; 90,0] | 76,5 n=54 | [58,0; 95,0] |

Medians marked by * are significantly different from controls (Mann-Whitney test, $p<0.05$).

n represents the population number

At 45 days, no changes in the cellular composition of the spleen were found. Decreased numbers of lymphoid and plasma cells and increased numbers of erythroid cells were observed on the 90$^{th}$ day of the experiment. After 135 days, analogous alternative changes were observed, to the ones detected at 90 days (Figure 1).

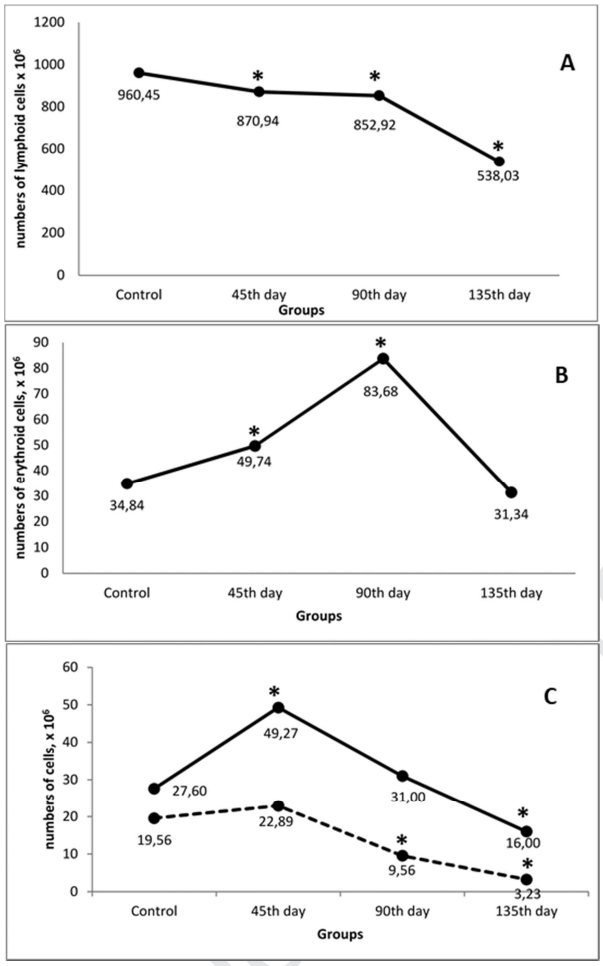


**Figure 1.** Cellular composition of the spleen (cells: lymphoid (A), erythroid (B); C – myeloid (—), plasma cells (-----) of the exposed and control group Wistar rats. * *Significant differences ($p<0.05$) with the control group*

In bone marrow, myeloid cells' and neutrophils' numbers were reduced, while the number of lymphoid and erythroid cells rose (Figure 2).

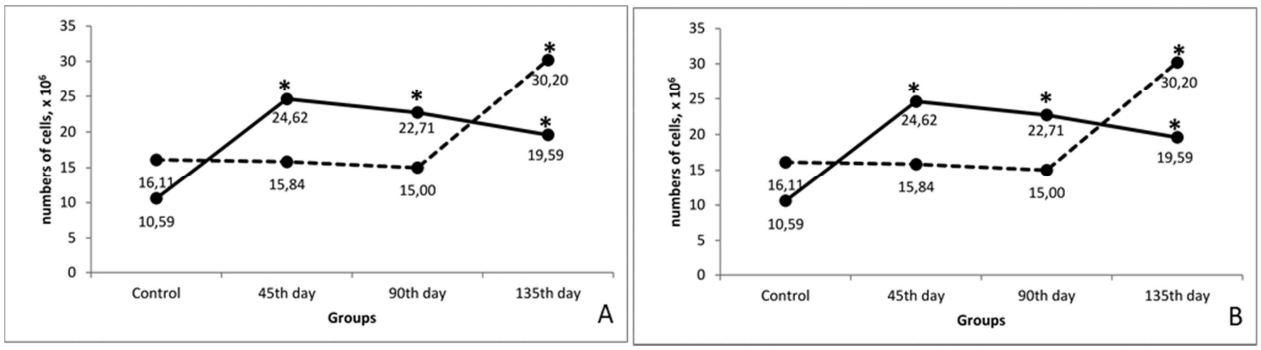


**Figure 2.** Cellular composition of the bone marrow (cells: A - lymphoid (—), erythroid (----); B - myeloid(—), neutrophils'(----) of the exposed and control group Wistar rats. * *Significant differences ($p<0.05$) with the control group*

Investigation of the pattern of spleen T-lymphocyte subpopulations of Cr (VI)-treated rats (Table 2) showed decreased counts of absolute $CD3^+$ and $CD4^+$ lymphocytes on the $90^{th}$ and the $135^{th}$ day of exposure, which indicates Cr (VI) immunosuppressive properties.

**Table 2.** Spleen lymphocyte subpopulations in Wistar rats of the exposed and control group

| | **Exposed group** | | | | | | **Control group** | |
|---|---|---|---|---|---|---|---|---|
| | **45th day(n= 20)** | | **90th day(n=11)** | | **135th day(n=5)** | | **(n=24)** | |
| | **Me** | **[25th;75th percentile]** | **Me** | **[25th;75th percentile]** | **Me** | **[25th;27th percentile]** | **Me** | **[25th;75th percentile]** |
| Splenocytes No | 940.00 | [869.00; 1022.00] | 671.00 | [492.00; 860.00] | 606.00* | [557.00; 713.00] | 883.50 | [727.00; 1110.00] |
| CD3+ (%) | 45.88 | [41.33; 51.30] | 46.30 | [44.60; 47.400] | 44.600 | [44.20; 52.400] | 49.20 | [44.80; 51.00] |
| CD3+ ( $x10^6$) | 419.87 | [372.19; 458.61] | 229.23* | [238.14; 376.36] | 270.28* | [245.76; 283.76] | 445.92 | [310.38; 544.32] |
| CD4+ ( %) | 32.75* | [27.50; 34.45] | 35.00 | [28.50; 36.10] | 37.60 | [28.50; 39.70] | 37.65 | [34.50; 40.25] |
| CD4+ ($x10^6$) | 307.62 | [230.85; 343.73] | 225.45* | [140.22; 257.36] | 221.84* | [138.23; 249.74] | 323.33 | [278.87; 411.20] |
| CD8+ (%) | 16.76 | [10.60; 19.70] | 7.00* | [6.40; 8.60] | 13.80 | [11.80; 18.20] | 13.50 | [9.60; 17.90] |
| CD8+ ($x10^6$) | 159.93 | [101.10; 182.62] | 54.08* | [42.94; 76.37] | 104.72 | [76.08; 117.64] | 99.77 | [73.39; 191.09] |

Medians marked by * are significantly different from controls (Mann-Whitney test, $p<0.05$).

n represents the population number

Figures 3 and 4 show the relative and absolute lymphocyte counts in the spleen and bone marrow at all time points of this experiment. The study revealed a

significant decrease in relative lymphocyte count (at days 45 and 90) and absolute lymphocyte count (at days 45, 90, 135). Both relative and absolute counts of bone marrow lymphocytes increased significantly even after 45 days of exposure and this increment persisted till the end of the experiment.

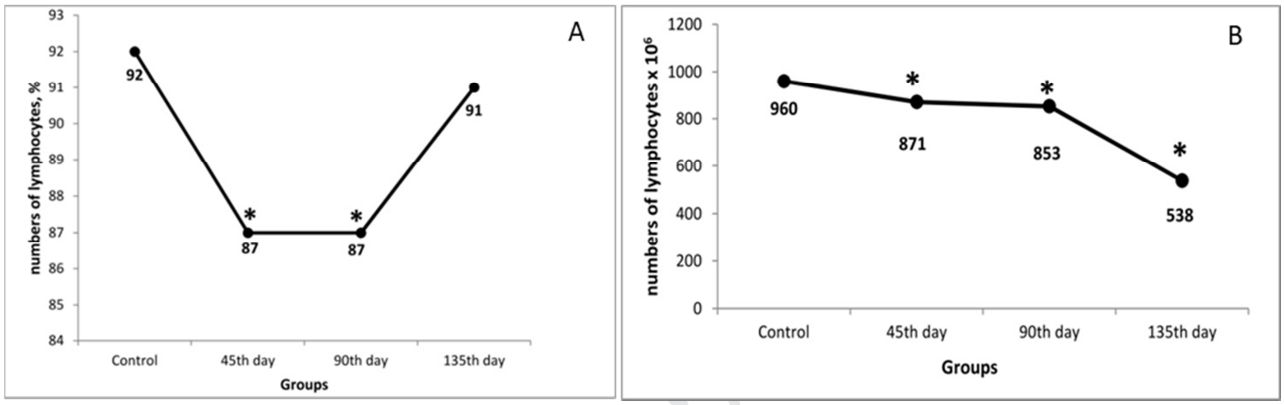


**Figure 3.** Number of spleen lymphocytes (A-relative number, B-absolute number) of the exposed and control group Wistar rats. * *Significant differences ($p<0.05$) with the control group*

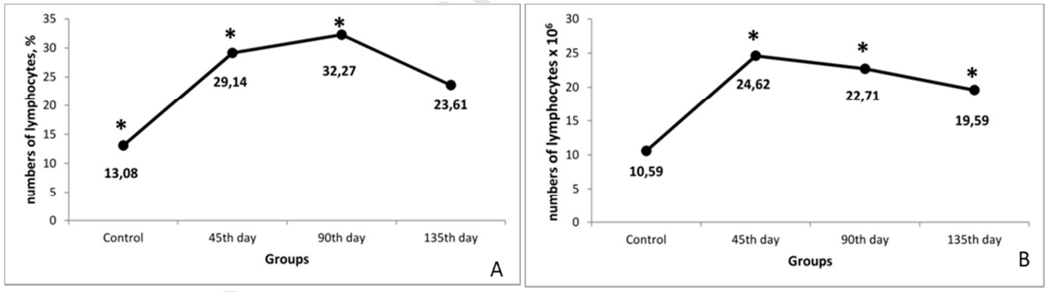


**Figure 4.** Number of bone marrow lymphocytes (A-relative number, B-absolute number) of the exposed and control group Wistar rats. * *Significant differences ($p<0.05$) with the control group*

## 3.3. Morphological analysis

Thymus' morphological evaluation of the Cr (VI)-treated rats indicated a number of structural-functional changes, namely parenchyma and stroma cellular re-organization, signs of ultra-structural injuries of the cytoplasmic components and increases in damaged forms of thymocytes and considerable decreases in thymocytes'

counts in thymus lobules' cortices were observed. Increased Hassall`s bodies and adipocytes' number, stromal fibrillogenesis, reactive changes in microcirculation vessels were found, while decreased function-specific reticular epitheliocytes were present with diminished association with T-lymphocytes (Figure 5). These changes can be attributed to a Cr (VI)-induced functional impairment of the central immune system.

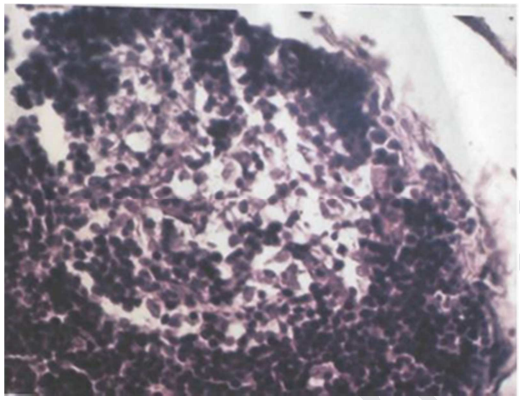

**Figure 5.** Morphological effects of Cr(VI) on Wistar rats. Lymphocyte decortication in the thymus of experimental animals. Stage: 90 days., Dye: hematoxylin-eosin. Magnification lens 40 eyepiece 10

Histopathological evaluation of Cr (VI)-exposed rats' spleens on days 45, 90 and 135 of the experiment, showed a plethora of the trabecular and pulpal vessels and an increase in lymphoid follicles' size. The hyperplastic reaction of the germinal zones was observed along with an accumulation of plasmocytes and macrophages. Morphometric evaluation showed an increase in the spleen's white pulp size (Table 3) accompanied by an increase of the B-zones. However significant increases in the T-dependent (periarterial) zones were not observed.

**Table 3.** Morphometric parameters of white pulp of spleen in Wistar rats of the exposed and control group. Comparative analysis of the areas occupied by germinal center and periarterial sheath (in μm) in the rats' spleens with and without dichromate administration in the drinking water

| | Exposed group | | | | | | Control group | |
|---|---|---|---|---|---|---|---|---|
| | 45th day(n= 15) | | 90th day(n=15) | | 135th day(n=14) | | (n=16) | |
| | *Me* | *[25th;75th percentile]* | *Me* | *[25th;75th percentile]* | *Me* | *[25th;27th percentile]* | *Me* | *[25th;75th percentile]* |
| B-zone | 887.00* | [883.00; 901.00] | 910.00* | [907.00; 911.00] | 1009.00* | [1007.00; 1014.00] | 812.00 | [810.00; 815.00] |
| T-zone | 45.88* | [41.33; 51.30] | 46.30 | [44.60; 47.400] | 44.60 | [44.20; 52.400] | 49.20 | [44.80; 51.00] |

Medians marked by * are significantly different from controls (Mann-Whitney test, $p<0.05$).

n represents the population number

Dimensional analysis of spleen lymphoid follicles' germinal centers and periarterial sheath was carried out for the evaluation of the T- and B-lymphocytes' state. It was found that Cr (VI) exposure led to a significant decrease (by 32.5±2.1%, $p<0.05$) of periarterial sheath (T-zone) (at day 45), while the germinal center (B-zone) increased at days 45, 90 and 135. B-zones increase was connected to the increased number of B-lymphocytes.

At each time point, the structural and functional evaluation of the lymph nodes of the exposed group found an increased size of the lymph nodes, attributed to a change of the cellular elements (Figure 5). The ultrastructural study of lymph nodes paracortex revealed insults to reticular cells and lymphocytes.

3.4. Immunocytochemical analysis

Cr (VI) induced lymphocytes' and thymocytes' programmed cell death in the spleen (periarterial lymphoid sheath) and lymph nodes' T-zones (paracortex) as presented in Table 4 and Figure 6a. Thymocytes' and lymphocytes' apoptosis in the spleen and lymph nodes T-dependent zones was identified at the ultra-structural level with the characteristic nucleus and cytoplasm changes (Figure 6b). At the 90$^{th}$ and the 135$^{th}$ day of the experiment, not only individual cells, but also cell clusters underwent apoptosis. Following 90 days of Cr (VI) treatment, increased speed of splenocytes' apoptosis were observed as depicted by the increased numbers of apoptotic cells

(Table 4) along with a decreasing tendency of mitotically active cells' counts, both of the pre-synthetic and in the resting cell cycle state cells.

**Table 4.** Content of pro-apoptotic protein p53 and anti-apoptotic protein bcl2 in the organs of the immune system of Wistar rats of the exposed and control group (permille)

| | **Exposed group** | | | | | | **Control group** | |
|---|---|---|---|---|---|---|---|---|
| | **45th day(n= 5)** | | **90th day(n=5)** | | **135th day(n=5)** | | **(n=5)** | |
| | **Me** | **[25th;75th percentile]** | **Me** | **[25th;75th percentile]** | **Me** | **[25th;27th percentile]** | **Me** | **[25th;75th percentile]** |
| P53 in thymus | 2.00* | [1.00; 2.00] | 3.00* | [1.00; 4.00] | 5.00* | [4.00; 6.00] | 0.00 | [0.00; 1.00] |
| Bcl2 in thymus | 3.00* | [3.00; 4.00] | 4.00* | [2.00; 4.00] | 5.00* | [4.00; 6.00] | 1.00 | [1.00; 2.00] |
| P53 in spleen | 3.00* | [1.00; 3.00] | 4.00* | [4.00; 5.00] | 5.00* | [4.00; 5.00] | 0.00 | [0.00; 1.00] |
| Bcl2 in spleen | 4.00* | [4.00; 4.00] | 5.00* | [4.00; 5.00] | 5.00* | [5.00; 5.00] | 1.00 | [1.00; 1.00] |
| P53 in lymph nodes | 3.00* | [3.00; 3.00] | 5.00* | [5.00; 6.00] | 6.00* | [6.00; 7.00] | 0.00 | [0.00; 0.00] |
| Bcl2 in lymph nodes | 4.00* | [3.00; 4.00] | 5.00* | [4.00; 5.00] | 6.00* | [5.00; 6.00] | 0.00 | [0.00; 0.00] |

Medians marked by * are significantly different from controls (Mann-Whitney test, $p<0.05$).

n represents the population number

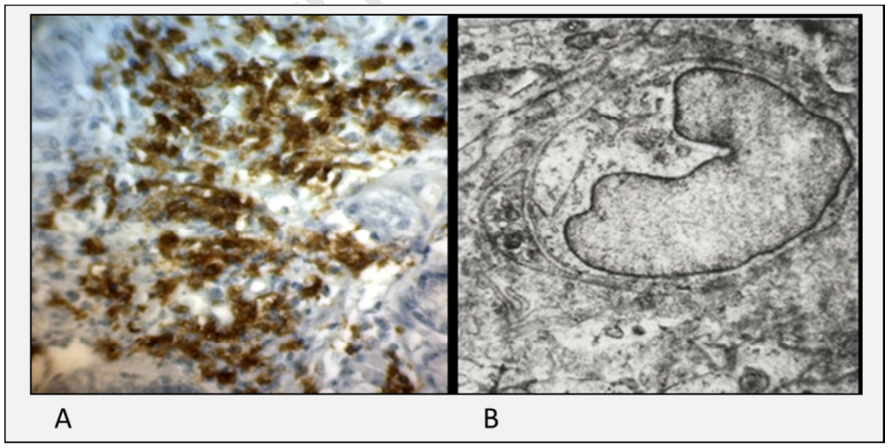


**Figure 6 (A),(B).** Immunocytochemical effects of Cr (VI) on lymphocytes and cytocytes of exposed Wistar rats. (a) Lymphocyte decortication in the thymus of experimental animals. Stage: 90 days., Color: hematoxylin-eosin. Magnification lens 40 eyepiece 10, (b) Thymocyte in contact with reticuloepithelium cells. Diffraction pattern. Increase x22500

Following exposure to potassium dichromate, the levels of IL-4, IL-6, IL-10, and IFN-γ secreted by the splenocytes were lower than the limits of detection of the ELISA kit. Con A induced splenocytes' cytokines production, as evidenced by increased IL-4 levels at each time point. IL-6 levels, on the contrary, reached their lowest values on the 135$^{th}$ day (Table 5). In terms of IL-10 and IFN-γ production, no significant differences were observed compared to control animals.

**Table 5.** Con A induced cytokine production (pg/ml) by splenocytes of Wistar rats of the exposed and control group.

| | **Exposed group** | | | | | | **Control group** | |
|---|---|---|---|---|---|---|---|---|
| | **45$^{th}$ day(n= 8)** | | **90$^{th}$ day(n=10)** | | **135$^{th}$ day(n=9)** | | **(n=18)** | |
| | **Me** | **[25$^{th}$;75$^{th}$ percentile]** | **Me** | **[25$^{th}$;75$^{th}$ percentile]** | **Me** | **[25$^{th}$;27$^{th}$ percentile]** | **Me** | **[25$^{th}$;75th percentile]** |
| **IL-4** | 10,3* | [5,5;16,9] | 8,8* | [5,4; 37,3] | 87,0* | [11,4; 94,7] | 3,1 | [1,4;7,1] |
| **IL-6** | 116,5 | [35,3; 165,5] | 113,6 | [35,3;126,0] | 98,4* | [47,9;110,7] | 112,2 | [99,4;145,8] |
| **IL-10** | 60,5 | [57,8;62,6] | 82,3 | [69,8;100,7] | 90,4 | [73,8; 105,4] | 57,4 | [47,7;109,0] |
| **IFN-γ** | 87,0 | [41,5;123,9] | 44,0 | [36,0;66,7] | 57,7 | [27,1;126,6] | 53,4 | [48,3;59,3] |

Medians marked by * are significantly different from controls (Mann-Whitney test, $p<0.05$).

n represents the population number

## 3.5. Analysis of oxidative stress parameters

In the current study, Cr (VI) effects on free radicals-induced lipid peroxidation, as well as the antioxidant status were evaluated. The liver showed the most pronounced increase (by 43%) in the levels of diene conjugates on the 90th day, while the maximum MDA content (222%) was observed on the 45th day (Table 6). In the spleen, the highest levels of diene conjugate were found on the 90$^{th}$ day (121%) while MDA rise was observed on the 135$^{th}$ day (308%).

**Table 6.** Effect of Cr (VI) on Wistar rats spleen and liver oxidative stress status (M ±m)

| Groups | Days | **Spleen** | | **Liver** | |
|---|---|---|---|---|---|
| | | Diene conjugates (unit wholesale. the square/mg of protein) | Malondialdehyde (protein nmol/mg) | Diene conjugates (unit wholesale. the square/mg protein) | Malondialdehyde (protein nmol/mg) |
| Control group | | 0,39±0,01 (28) | 1,33±0,09 (28) | 0,40±0,02 (6) | 3,73±0,53 (32) |

| | | | | | |
|---|---|---|---|---|---|
| | 45 | 0,34*±0,01 (10) | 2,26±0,40 (8) | 0,36*±0,01 (10) | 8,28*±1,71 (8) |
| Exposed group | 90 | 0,47*±0,01 (8) | 2,03±0,32 (12) | 0,57*±0,01 (8) | 3,86±0,60 (23) |
| | 135 | 0,33*±0,02 (8) | 4,10*±1,18 (9) | 0,36*±0,01 (8) | 5,96±2,19 (9) |

Means marked by * are significantly different from controls (Mann-Whitney test, $p<0.05$).

n represents the population number

In order to clarify whether oxidation activation via free radical formation is due to decreased activity of the scavenging enzymes, SOD and catalase activity were monitored. A significant decrease of scavenging enzymes' activity in the exposed rats' erythrocytes was found: SOD level by 46% (90th day) and catalase activity by 18% (45th day) and by 50% (135th day) (Table 7).

**Table 7.** Effect of Cr (VI) on the activity of antioxidant enzymes in erythrocytes of Wistar rats

| Groups | Days | SOD, RU/rHb | Catalase, RU/rHb |
|---|---|---|---|
| Control group | (n=20) | 227±25.6 | 257.4±8.49 |
| Exposed group | 45 (n=6) | 189±9.86 | 219*±3.75 |
| | 90 (n=6) | 123*±14.2 | 275±8.04 |
| | 135 (n=7) | 125±53.0 | 172*±20.8 |

Means marked by * are significantly different from controls (Student t test, $p<0.05$).

n represents the population number



## 3.6. Micronutrient analysis

Micronutrients' content in rats' blood and organs was assessed in an attempt to elucidate whether there is a connection between the effects on the immunological and oxidative stress parameters. At each time point, decreased levels of Cu, Fe, and Ni but increased levels of Cr and Zn in the Cr-treated rats' peripheral blood were observed. Noteworthy, these alterations were more pronounced on the 135th day (Table 8). Chromium concentration in spleen and liver of the exposed rats was increased and levels of Cu, Zn, and Fe were different at the various time points (Table 8).

**Table 8.** Micronutrient levels (µg/g) in blood, spleen, and liver of the exposed and control group Wistar rats (Me, the 25th and 75th percentile)

| | Micronutrient levels in blood | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Exposed group | | | | | | | | | Control group | |
| | 45th day(n= 20) | | | 90th day(n=11) | | | 135th day(n=5) | | | (n=24) | |
| | Me | [25th;75th percentile] | % | Me | [25th;75th percentile] | % | Me | [25th;27th percentile] | % | Me | [25th;75th percentile] |
| **Cu** | 0.83 | [0.74; 1.12] | 95 | 0.65* | [0.59; 0.71] | 75 | 0.52* | [0.36; 0.69] | 60 | 0.87 | [0,79; 1.02] |
| **Zn** | 5.32* | [4.48; 7.14] | 108 | 5.62* | [5.02; 6.7] | 114 | 3.88 | [2.68; 5.09] | 79 | 4.92 | [4.0; 5.47] |
| **Fe** | 319* | [276; 384] | 87 | 399 | [330; 473] | 108 | 202* | [125; 279] | 55 | 368 | [292; 477] |
| **Ni** | 0.03* | [0.02; 0.07] | 43 | 0.07 | [0.03; 0.08] | 100 | 0.02* | [0.02; 0.03] | 29 | 0.07 | [0.03; 0.20] |
| **Cr** | 0.33* | [0.22; 0.43] | | 0.23* | [0.09; 0.29] | | 0.52* | [0.36; 0.68] | | 0 | [0; 0.02] |
| | Micronutrient levels in spleen | | | | | | | | | | |
| | 45th day(n= 10) | | | 90th day(n=6) | | | 135th day(n=5) | | | (n=17) | |
| | Me | [25th;75th percentile] | % | Me | [25th;75th percentile] | % | Me | [25th;75th percentile] | % | Me | [25th;75th percentile] |
| **Cu** | 2.06 | [1.36; 3.72] | 113 | 3.83 | [2.35; 4.71] | 210 | 2.46 | [2.39; 2.53] | 135 | 1.82 | [1.58; 3.78] |
| **Zn** | 21.57 | [15.86; 25.52] | 116 | 12.6 | [8.20; 23.64] | 68 | 18.32 | [18.15; 18.48] | 99 | 18.53 | [15.7; 22.67] |
| **Fe** | 138 | [85; 231] | 49 | 141* | [87; 173] | 50 | 490* | [430; 549] | 175 | 280 | [127; 424] |
| **Ni** | 0.33 | [0.05; 0.99] | 69 | 1.24 | [0.72; 1.64] | 258 | 1.27 | [1.17; 1.37] | 265 | 0.48 | [0.12; 1.21] |
| **Cr** | 3.39* | [1.6; 7.65] | 1130 | 2.60 | [1.37; 5.71] | 867 | 18 | [13.85; 23.50] | 6000 | 0.3 | [0.02; 1.11] |
| | Micronutrient levels in liver | | | | | | | | | | |
| | 45th day(n= 15) | | | 90th day(n=8) | | | 135th day(n=5) | | | (n=19) | |
| | Me | [25th;75th percentile] | % | Me | [25th;75th percentile] | % | Me | [25th;75th percentile] | % | Me | [25th;75th percentile] |
| **Cu** | 2.28* | [2.07; 2.75] | 81 | 2.23 | [2.01; 2.65] | 79 | 3.23* | [2.81; 3.42] | 115 | 2.82 | [2.85; 3.11] |
| **Zn** | 17.04* | [13.78; 18.92] | 84 | 17.6* | [13.26; 20.36] | 87 | 32* | [23.66; 73.53] | 157 | 20.32 | [16.46; 25.24] |
| **Fe** | 42.44* | [27; 48] | 66 | 44.81 | [36; 49] | 70 | 103* | [98; 113] | 160 | 64.38 | [55; 88] |
| **Ni** | 0.08 | [0.03; 0.17] | 114 | 0.14* | [0.06; 0.19] | 200 | 0.05 | [0.03; 0.13] | 71 | 0.07 | [0.03; 0.08] |
| **Cr** | 3.32* | [2.61; 3.85] | 16600 | 3.03* | [1.94; 3.61] | 15150 | 10.72* | [8.65; 13.65] | 53600 | 0.02 | [0; 0.05] |

Medians marked by * are significantly different from controls (Mann-Whitney test, $p<0.05$).

n represents the population number
*% - the percentage of deviation of the values of the experimental groups from the control level*

## 4. Discussion

Reductions in thymus weight and thymocytes population observed throughout the experiment following exposure to Cr (VI) could be attributed to the redistribution of thymocytes due to the lymphocyte pool replenishment in peripheral blood and peripheral lymphoid organs (Yarilin, 2010). The immunotoxic property of Cr (VI) has been previously demonstrated (Dai et al., 2017) as it decreases T cells viability and

inhibits T cells activation. However, decreases in thymus cells' number in the current study can be attributed in part to apoptosis linked to p53 protein, a pro-apoptotic factor, whose thymus cortex levels was considerably increased on the 135$^{th}$ day post-exposure. Akbar et al. ( 2011) reported that apoptosis and subsequent inhibition of T-lymphocyte expansion is induced by Cr (VI) while Shrivastava et al. (2002) noted that modulation of apoptosis regulatory gene p53 is involved in Cr (VI)-induced toxicity. Appearance of such apoptotic cells (single and in groups), provides evidence of the intracellular induction of programmed cell death.

Furthermore, our data indicated a decreased T-lymphocyte activity that evidently reflects T-cell immunodeficiency. Probably this occurred because of immunocytes' dysfunction (in particular B-cell zones showed morphological signs of hyper-responsiveness). Thus, the developed morphological pattern in Cr (VI)-exposed animals shows the reciprocal responsiveness of central and peripheral organs of immunogenesis.

This responsiveness was reflected as lymphoreticular hyperplasia and plasmocytic macrophage transformation of the spleen and lymph nodes. Notably, the extent of the organ damage corresponds with the intensity of their infiltration by polymorhonuclear and mast cells, characterizing the non-specific cellular component of the inflammatory response.

Moreover, reduced numbers of the splenic cells population could be attributed to the suppression of lymphoproliferation (Kurlyandskiy et al., 2002). The increase in relative and absolute numbers of lymphoid cells in bone marrow, shown by the myelogram, can be explained by thymic and splenic lymphocytes ingress into bone marrow (O'Brien and Kortenkamp, 1994). At the same time, the observed increases in lymphoid cells count were probably due to the migration of extramedullary

lymphocytes into bone marrow, which is necessary for hematopoiesis stimulation. Reduction of myeloid cells and neutrophils could result from neutrophil mobilization from the bone marrow into the blood flow (Kurlyandskiy and Filov, 2002; Yarilin , 2010).

Also, it is possible that lymphoid organs' hypocellularity occurred following direct damaging effect of Cr which impairs the cells' energy exchange and leads to energy failure (Kurlyandskiy and Filov, 2002). Moreover, all Cr-induced changes are probably associated with the activation of the free radical oxidation process as one of the main mechanisms of cell damage in Cr exposure is thought to be the excessive activation of the free radical chain reactions, which subsequently lead to lipid peroxidation (Dlugosz et al., 2012; Shrivastava et al., 2002). Reduction/oxidation misbalance in cells is associated with an increase in lipid peroxidation and thus accumulation of oxygenates in rats' liver and spleen is observed. Thus, Cr exposure led to the activation of free radical oxidation and lipid peroxidation and to the reduction of scavenging enzymes activity (i.e. decreased activity of catalase and SOD in erythrocytes) and increased diene conjugates and MDA concentrations in rats' liver and spleen homogenates (Bagchi et al., 1995; Patlolla et al., 2009). The evidence of Cr-induced MDA concentration is also given in a study on mice in which mice were treated with $K_2Cr_2O_7$ for 30 days (Rao et al., 2009). Ability of toxic metals to produce oxidative stress in different organs and cell lines have been shown in many studies and is regarded as one of the most important mechanisms of their toxicity (Engin et al., 2017; Matović et al., 2015; Wallace et al., 2019).

In contrast to short term hexavalent chromium exposure studies following oral administration(Shipkowski et al., 2017)which showed rather few immunotoxic effects in female F344/N rats, SD rats, and B6C3F1 mice, long term exposure showed a variety of immunotoxic effects as the observed decrease in thymus mass and

thymocytes' number along with structural and functional changes in the lymph nodes and spleen, namely lymphoreticular hyperplasia and plasmocytic macrophage transformation.

Immune senescence is thought to be linked to cancer. The observed morphological changes in thymus and the reduction of thymocytes' number are associated with the immune senescence as thymus is the major site of T cell development and maturation (Hakim & Gress, 2007) Tissue Antigens. 2007; 70(3):179–189). Also it appears that an inverse relationship exists between immune function and the incidence of many forms of cancer (Foster, Sivarapatna, & Gress, 2011). Thymus microenvironment is essential for the adaptive immune system and altered thymus function can lead to increased risk for tumor relapse attributed to impaired immunological surveillance (Holländer, Krenger, & Blazar, 2010). At the same time the observed thymus changes can have implications for reproductive toxicity. The brain–pituitary–reproductive axis and the brain thymus–lymphoid axis are thought to communicate and several theories are postulated for a thymic control of reproductive physiology (Morale et al., 2003).

Spleen depletion can be mediated via suppression of lymphoproliferation (i.e. the loss of cell count may depend on decreased reproduction). T-lymphocytes spleen subpopulations were all suppressed in the current study. $CD4^+$ and $CD8^+$ T-lymphocytes are cells with distinct roles in immune system response, especially in cancer cases. In a previously conducted work on a similar model in Wistar rats treated with Cr (VI), it was found that a decrease in the relative and absolute number of CD3, CD4 splenocytes was accompanied by a decrease in the level of antibody-forming cells in the spleen (Smolyagin et al., 2013). Cr was found capable of inhibiting both $CD4^+$ and $CD8^+$ T cells proliferation upon activation (Dai et al., 2017). The observed changes in T-lymphocyte subpopulations in Cr-exposed rats' spleens could also be the result of high microsomal cytochrome P450 cells' content (Kurlyandskiy and Filov, 2002).

Cytokines are known to regulate the growth, differentiation, and activation of the immune cells (Dranoff, 2004). Recent review by Gangemi et al ( 2016) showed the impact of cytokine levels on health and the development of several chronic diseases. At the same time T-lymphocytes subsets have a specific pattern of cytokine

secretion (Mclaughlin et al., 2017; Simbirtsev, 2018). Similarly to our study, specific cytokines' expression was found decreased in an *in vitro* mouse spleen T cells' model of Cr exposure (Dai et al., 2017) while IL-2 production was reduced in a study of Cr exposure of primary human lymphocytes *in vitro* (Akbar et al., 2011). In our study, IL-2, IL-4 and IL-10 secretion by T-lymphocytes was found decreased after Cr exposure. Con A stimulation up-regulated IL-4 expression at splenocytes' cultures from Cr-exposed Wistar rats. Con A-induced changes in cytokines levels may be connected with different sensitivity of cells that secrete these cytokines to the effect of Cr, based on the peculiarities of folate receptors expression on the surface of these cells: Th1 (for IFNγ) and Th2 (for IL-4). Such hypothesis is supported by nonregular distribution (Valko et al., 2005) of one of the types of folate receptors (FR4) on the regulatory T-cells membranes, which allow identifying different subpopulations by their features.

The observed micronutrients imbalance can also be the cause of changes in immunological factors. Chronic intake of Cr (VI) leads to its accumulation in an organism, which simultaneously results in decreases in necessary micronutrients like Cu, Fe, and Zn. Such an effect was observed for Cr (VI) ions, which possess tetrahedral configuration and enter cells through isostructural phosphate and sulfate transport channels (Valco et al., 2005; Kudrin and Gromova 2007). Other studies report significant increases in total Cr concentrations in various tissues of rats and mice following 90 days of exposure to sodium dichromate dihydrate in drinking water (Thompson et al., 2011). Moreover, the increase of Cr concentration in tissues is apparently dose-dependent as was demonstrated by increased concentrations of Cr in the blood, kidney, and femur detected in rats, mice and guinea pigs administered a range of Cr (VI) levels in their drinking water for 21 days. Increased levels of Cr (VI)

with in a dose dependent manner were also observed in the liver and kidney of male and female mice (NTP, 2008). Rankov et al. (2010) reported a significant accumulation of Cr in genital organs and sexual accessory glands of white Wistar male rats exposed to drinking water containing 25, 50 or 75 mg Cr (VI)/ L. Furthermore, chronic intake of Cr dissolved in drinking water leads to intestinal malabsorption of other elements. It may be caused by Cr-induced oxidation of other ions, particularly oxidation of iron to the forms that cannot be absorbed. Cr (VI) oxidizing capacity is explained by its higher redox potential compared with the other elements.

Another cause may be the competition for metal transport proteins on the enterocyte membranes, which can contribute to displacement of other microelements following chronic intake of high doses of Cr (VI) (Deicher and Hörl, 2006). Besides, reduction of micronutrients content may relate to the disruption of ligand homeostasis mechanism, presented by the competition for metal binding sites in transport proteins, which consequently results in displacement of other elements by Cr. Iron and Cu deficiency found in blood plasma samples may negatively affect scavenging enzymes formation, as observed at the beginning of the experiment because these microelements act as cofactors. Similar results were presented by Suh et al. (2014) who reported responses of rats and mice to Cr (VI) exposure for 90 days, consistent with Fe deficiency, including significant induction of divalent metal transporter 1 (DMT1, Slc11a2) and transferrin receptor 1. Low scavenging enzymes activity, in turn, may be the cause of conspicuous activation of free radical oxidation processes and induction of oxidative stress (Dlugosz et al., 2012). Changes in micronutrient content may be also explained by their correlation with each other, presented as synergism or antagonism between toxic and essential elements (Buha et al., 2012;

Bulat et al., 2017, 2012). In this regard, antagonism between Zn and Cu, Fe and Cr, Zn and Cr, and synergism between Fe and Ni have been suggested (Tuormaa, 2000). Increased Cr (VI) in hematological parameters may be due to the antagonism between Cr and Fe in binding to transferrin (Bjørklund et al., 2017).

Similar results pointing to immunotoxicity were obtained in studies in which animals were treated with other toxic metals such as cadmium (Cd) and mercury (Hg). Cadmium administrated to rats daily by oral gavage for 2 weeks resulted in significant decreases in plasma levels of IgG and IgA, T-lymphocyte sub-types (CD4+, CD3+, CD56+, and CD8+), and in thymic and hepatic indices (relative weights) while it produced formation/release of pro-inflammatory cytokines (IL-1 and TNFα), and increase of the relative spleen weight (Salah-Abbès et al., 2015). In a study conducted in Wistar rats treated orally with the different doses of mixtures of Cd and decabrominated diphenyl ethers the significant increase in white blood cells count was observed suggesting inflammation (Curcic et al., 2017). Repeated exposure to Hg via subcutaneous injection during 14 days impaired several immune parameters such as the production of TNF α, IL-1, nitric oxide by macrophages and cytokines production. It can be postulated that immune system can be directly affected by toxic metals leading to decreased resistance to infections or tumors, as well as certain auto-immune disorders (Batista-Duharte et al., 2018).

## 5. Conclusion

Collectively, Cr (VI) exposure leads to the activation of free radical oxidation processes and to micronutrients' imbalance, which can contribute to the development of the observed changes in immunological and biochemical factors. Chromium capacity to activate free radical oxidation processes is essential in Cr-induced

cytotoxicity. It leads to the cell depletion in the organs of the immunogenesis and the development of immunological suppression. The observed changes in the series of immunological parameters studied provide new essential knowledge for the development of new approaches for the prevention of adverse effects due to Cr exposure.

**Conflict of Interest**

The authors declared no conflicts of interest.

Acknowledgments

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The work is approved by the expert examination of the Orenburg State Medical University Ethics Committee (protocol №9, 14.05.)



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**Highlights**

- Long-term Cr (VI) exposure leads to mass loss and decrease in cell number in rats spleen and thymus.
- Long-term Cr (VI) exposure causes structural and functional changes in spleen.
- Long-term Cr (VI) exposuredecreases the relative $CD3^+$-, $CD4^+$-, $CD8^+$- lymphocytes count in rats.
- Cr (VI) immune system effects could partly be attributed to oxidative stress induction.
- Cr (VI) immune system effects could partly be attributed to to changes in micronutrient status.

**Declaration of interests**

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

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

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On behalf of co-authors,
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