A Response of the Pineal Gland in Sexually Mature Rats under Long-term Exposure to Heavy Metal Salts

Nataliia Hryntsova[1], Ingrid Hodorová[2], Josef Mikhaylik[2], Anatoly Romanyuk[3]

[1]Department of Morphology, Medical Institute, Sumy State University, Sumy, Ukraine;
[2]Department of Anatomy, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Košice, Slovak Republic;
[3]Department of Pathological Anatomy, Medical Institute, Sumy State University, Sumy, Ukraine

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Abstract: Pollution with heavy metal salts is an important environmental problem today, having an adverse effect on public health. The endocrine system maintains homeostasis in the body. The antioxidant protection (GPX-1) of the pineal gland in mature rats was studied. Animals of the experimental group represented a model of microelementosis, achieved by adding a mixture of heavy metal salts for 90 days to drinking water: 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. Morphological, statistical and immunohistochemical (GPX-1) research methods were used. Long-term (90-days) intake of heavy metal salts mixture in the body of experimental animals brought about development of the general adaptation syndrome, the stage of chronic stress "subcompensation" in the pineal gland. Morphological rearrangements were of nonspecific polymorphic nature as severe hemodynamics disorder in the

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Mailing Address: Assoc. Prof. Nataliia Hryntsova, PhD., Department of Morphology, Medical Institute, Sumy State University, Sanatorium 33, 40000 Sumy, Ukraine; Phone: +38(095)39 28 837; e-mail: natalia.gryntsova@gmail.com

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organ, impairment of vascular wall morphology, development of tissue hypoxia and oxidative stress, accompanied by processes of accelerated apoptosis in part of pinealocytes, by a significant decrease in glutathione peroxidase level in the organ and reactive astrogliosis as a response to the damaging agent's action. Along with the negative changes in the pineal gland, a compensatory-adaptive processes with signs of functional stress also occurred. A sufficiently high degree of glutathione peroxidase activity in 39% of pinealocytes located perivascularly, active adaptive glial reaction and activation of synthetic processes in some pinealocytes were also observed.

Introduction

Pollution with heavy metal salts is an important environmental problem today, having an adverse effect on public health. Such a negative effect determines development and course of oncological pathology, disorders of the body homeostasis and morphological transformations in various tissues (Romanjuk et al., 2018a, 2019), as each trace element with excessive exposure can be potentially toxic (Bharti et al., 2014). It is believed that the formation of free radicals, lipid peroxidation and changes in the antioxidant defence system play an important role in the toxic effects of heavy metals. Thus, heavy metals are able to penetrate freely into any cell due to their good fat solubility, to cause various modifications in the structure of DNA and RNA with changes in the function of many transcription factors, to raise lipid peroxidation of membrane formations, bind sulfhydryl groups of enzymes and proteins, and to change the electrolyte balance with accumulation of intracellular calcium ions. Under the influence of metals, the functional balance between oxidative and antioxidant mechanisms generally shifts in favour of the former with a decrease in the activity of superoxide dismutase, catalase, glutathione peroxidase and a decrease in the level of natural antioxidants such as glutathione. The set of serious lesions also includes damage to mitochondria and the microsomal apparatus of cells (Hemdan et al., 2005; Valko et al., 2005). Various human diseases and toxicity are often associated with oxidative stress. This pathophysiological stress has multiple effects, but is particularly characterized by decreased enzymatic activity, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX-1) and glutathione reductase (GR) (Rzeuski et al., 1998; Bharti et al., 2014).

The human pineal gland and oxidative stress

At present, the human pineal gland is the least studied endocrine gland, which occupies one of the central places in the endocrine regulation of all organs and systems' vital activity, carries out adaptive reactions of the body to changing environmental conditions. It is known that the melatonin hormone (indole metabolite of the tryptophan amino acid, which is mainly produced by the pineal gland) is the strongest natural inhibitor of free radical processes in the body (Russel, 2000; Bharti et al., 2014). In experiments *in vitro* melatonin reduces the formation

of hydroxyl radical by 5 to 14 times more efficiently than other known inhibitors such as glutathione (Arushanyan, 2004). In both *in vitro* and *in vivo* experiments, it has been shown that epiphyseal indoles, and in particular melatonin, are able to neutralize the manifestations of oxidative stress that occurs when intoxicated by various metal compounds. It is known that melatonin protects endocrine tissue from peroxidation initiated by ferrous sulphate (Karbownik and Lewinski, 2003), copper (Daniels et al., 1998), chromium (Susa et al., 1997), lead acetate (El-Missiry, 2000; El-Sokkary et al., 2003). At the same time melatonin acted as a "trap" for free radicals and simultaneously improved the functional state of the natural antioxidant system by activating the enzymes that were part of it: superoxide dismutase, catalase, glutathione peroxidase (Arushanyan and Elbekyan, 2006). In this case, according to the literature, the origin of melatonin antitoxic effect may be due to the direct chemical interaction of the hormone and individual metals. Thus, using electrochemical methods it has been shown that various metals (aluminium, copper, iron, zinc) can form complexes with melatonin, which acts as their chelator (Limson et al., 1998; Arushanyan and Elbekyan, 2006).

Heavy metal salts, particularly lead, have a multiple toxic effect on the blood and the cardiovascular systems. Thus, lead inhibits enzymes involved in the synthesis of haem and globin and as a result reduces the amount of hemoglobin, which affects the formation of erythrocytes. Impairment of porphyrin synthesis and due to iron and aminolevulinic acid accumulation may be one of the causes for activation of oxidative stress and lipid peroxidation (Trachtenberg et al., 2015). The hemocoagulation system is also sensitive to the action of lead in low doses. Increased hemostasis activity, development of hypercoagulation syndrome and disseminated intravascular blood coagulation, inhibition of fibrinolytic activity, which indicates the activation of thrombosis, have been experimentally established (Trachtenberg et al., 2010, 2015).

As a result of the hemotoxic effect of lead, hemic and circulatory hypoxia occurs, which results in the development of tissue hypoxia in the body, activation of free radical oxidation and oxidative stress, which causes implementation of lead's vasotoxic effect (Apikhtina et al., 2012; Trachtenberg et al., 2015). A characteristic feature of oxidative stress is that when the damage increases above a certain critical level, the cell's self-elimination program – apoptosis – is activated (Trachtenberg et al., 2001).

The activity of oxidative stress reactions depends on the absolute or relative content of endogenous antioxidants in the tissues (tocopherols, ascorbic acid, thio- and selenium-containing compounds). Their increased or decreased concentration may affect the intensity of oxidative stress (Belenichev, 2014).

Family of glutathione peroxidases (GPX)

Among selenoproteins, highly reported on, there is the family of glutathione peroxidases (GPX), which are involved in the regulation of the redox state and in

protection against oxidative damage. The glutathione peroxidase activity in the pineal gland is higher compared to other brain structures (Razygraev, 2004). Relatively high concentrations of selenium were found in the pineal gland, which is due to its antioxidant properties, in particular to its ability to neutralize reactive oxygen intermediates formed during the synthesis of melatonin (Reiter, 1996; Kravtsiv and Yanovich, 2008). The GPX-1 enzyme is a homotetramer and contains selenocysteine. This means that GPX-1 expression is affected by the level of selenium in the studied tissue or organ.

Astroglia of the pineal gland

In the pineal gland, the degree of oxidative stress and pinealocytes apoptosis development can be impliedly judged by the quantitative indices of pinealocytes and glial elements present in the gland, as well as by the morphological features of the pinealocytes' nuclear apparatus (Gubina-Vakulik, 2006). After all, according to modern views on the functions and morphology of astroglia, astrocytes are able to synthesize gas transmitters, including carbon monoxide (CO), which is involved in the mechanisms of inflammation and apoptosis. In addition, the increase in the number of glial elements in the pineal gland certainly has a compensatory-adaptive value, particularly in the processes of RNA, amino acids, growth factors transfer to pinealocytes and by controlling water-ion homeostasis in the gland (Goryainov et al., 2013). It is impossible to overestimate the contribution of astrocytes in the protection of the gland's parenchyma from oxidative stress through the synthesis of hydrogen sulphide (H_2S). This gas gliotransmitter has synaptic modulator and neuroprotective properties, protecting against oxidative stress (Goryainov et al., 2013). Therefore, activity assessment of antioxidant enzymes and markers of free radical damage in the pineal gland is important for assessing the toxicity of heavy metal salts mixture for the antioxidant system of the pineal gland. According to the authors, the effect of heavy metal salts mixture on the antioxidant system of the pineal gland is an urgent problem and requires a detailed study.

The purpose of the work is to study the system of the pineal gland's antioxidant protection in sexually matured male rats under the conditions of long-term exposure to a mixture of heavy metal salts.

Material and Methods

Animals

The experiment was performed on 24 white sexually mature male rats weighing 200–250 g, aged 7–8 months, which were divided into 2 groups (the control and the experimental ones). Animals of both groups were kept in the normal vivarium conditions, where equal keeping conditions, nutrition, proper care and natural light (day/night) were maintained, with a constant ambient temperature (20–22 °C). The animals had free access to drinking water. The study was carried out in the autumn-winter period.

Experimental microelementosis model

The experimental group included rats, which for 90 days received drinking water with a mixture of heavy metal salts: 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 selected concentration of salts in the mixture was due to the presence of such concentrations of these salts in the soil and drinking water of some regions in Ukraine according to literature sources (Romanjuk et al., 2018a, b, 2019).

Termination of microelementosis induction

After the 90th day of the experimental procedure, animals of both groups were anesthetised by thiopental injection (at the dose of 30–40 mg/10 g body weight) before the subsequent surgical and histological procedures. All the animal studies were conducted in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals for Experimental and Scientific Purposes (Strasbourg, 1986) and the "General Ethical Rules for Animal Experiments" approved by the First National Congress of Bioethics (Kyiv, 2001, Ukraine), Protocol No. 4 of 06/03/2020 Commission of Bioethics of Sumy State University. The subject of the study is the pineal gland of experimental and control animals.

Pineal gland extraction technique and histological studies

To study the morphological changes in the structural components of the pineal gland conventional procedures of microanatomical (histological) study method were used. In order to carry out morphological, morphometric and immunohistochemical studies of the pineal gland, the organ was extirpated and histological tissue specimens were made according to the original method developed by the authors (Hryntsova and Romanyuk, 2020; Hryntsova et al., 2020). In this case, for the purpose of atraumatic extirpation, the pineal gland was not completely dissected

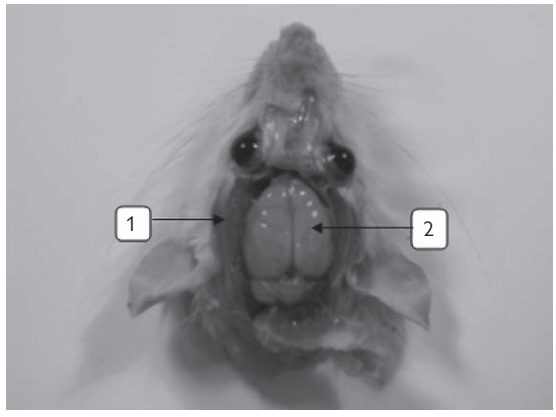

Figure 1 – Circumferential dissection of the skull on the parietotemporal bones (1), exposure of the brain (2) (digital photo).

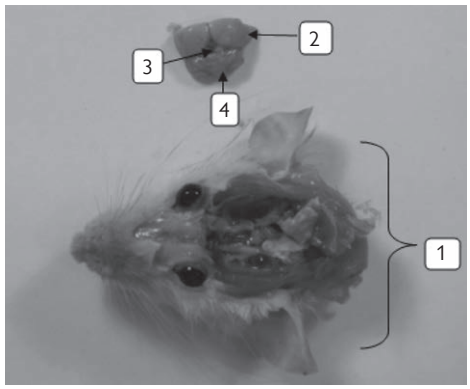

Figure 2 – Extirpation of the pineal gland with the organo-complex (together with fragments of the brain and cerebellum adjacent to the pineal gland) (digital photo).

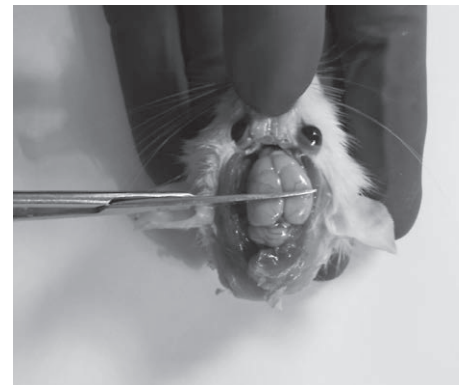

Figure 3 – Head of a decapitated rat with extirpated brain (1). Organo-complex: a fragment of the brain (2), the pineal gland (3), a fragment of the cerebellum (4) before fixation (digital photo).

from the epithalamus of diencephalon, instead, it was extirpated together with fragments of the brain and cerebellum adjacent to it (Figures 1–3).

The pineal gland, together with the adjacent tissues, was immersed in the fixing fluid (5% formalin solution) for the period of 12–24 hours (Figure 3).

The dehydration process was carried out in a number of ethyl alcohol portions with ascending concentrations of 70°, 80°, 90°, 96°, after which the objects were embedded in paraffin. Sections from 4 to 5 μm thick were made of histological blocks with a rotary microtome, followed by making permanent histological preparations and staining with hematoxylin-eosin according to standard procedure. The developed method permitted to rationally prepare the pineal gland, which prevented its injury and permitted to prepare high-quality histological specimens for light-optical, morphometric and immunohistochemical study methods.

Assessment of the pineal gland's morphological state was performed by a number of microscopic indices: state of stromal and parenchymal components, state of the vascular bed, changes in blood rheology, state of pinealocytes and astrocytic glia, including determination of neurocytoglial index and GPX-1 immunohistochemical marker, which is one of the most important antioxidative enzymes in mammals. To determine the gliocyto-neuronal index the ratio of the neuroglia location density to the density of pinealocytes location was found. To determine the density of pinealocytes and neuroglia in the pineal gland their absolute number was counted in the microscope field of view (ob. 40, oc. 10), studying at least 30 fields of view (Salkov et al., 2015). In this case viable pinealocytes only were taken into account.

General morphological and morphometric analysis was performed using the "Leica DM 500" light-optical microscope, with ×4, ×10, ×40 lenses, binoculars 7, 10. Photo documentation of the results obtained was performed with a digital video camera

"Leica DM IC C50 HD Camera". "Leica Application Suite LAS EZ version 20.0 (Build: 292) Copyright @ 2010" software was used.

Marker expression research GPX-1 method

To study the changes of glutathione peroxidase activity in the cytoplasm of pinealocytes the immunohistochemical method was used.

Pineal glands were embedded in paraffin blocks using standard procedures.

1) Sections of 5 μm were cut, deparaffinised (dehydration in xylene and rehydration in alcohols in decreasing concentrations) and rinsed in EnVision Flex Wash Buffer (#K800721-2, Agilent Dako, Santa Clara, CA, USA, later in the text referred to as wash buffer). Endogenous peroxidase activity was blocked by incubation of slides in a mixture of methanol and hydrogen peroxide. After another rinsing in wash buffer, antigens were revitalized in the microwave.
2) Blocking
   To block staining of non-specific structures slides were rinsed in wash buffer, and 2% milk blocking solution in Tris buffer was added.
3) Antigen-antibody reaction
   Rabbit polyclonal GPX-1 antibodies produced by Bioss Antibodies Inc. (USA) were used in the reaction in a dilution of 1:250, serial number bs-3882R, reactivity: human, mouse and rat, isotype: IgG. Immunohistochemical reactions were performed in accordance with the Bioss Antibodies Inc. protocol (USA). The primary anti-GPX-1 rabbit polyclonal antibody was applied overnight at 4 °C, followed by rinsing in wash buffer. Subsequently, Biotinylated Link (#K0675, Agilent Dako, Santa Clara, CA, USA) was used, then slides were rinsed with wash buffer, and Streptavidin-HRP (#K0675, Agilent Dako, Santa Clara, CA, USA) was applied.
4) Final rendering
   The tissue sections were again washed in wash buffer, and 3,3-diaminobenzidine (DAB) (#K5207, Agilent Dako, Santa Clara, CA, USA) was applied. The slides were rinsed in tap water, counterstained by hematoxylin, and embedded into Pertex® Mounting Medium 00811-EX (manufactured by Histolab Products AB).

The negative controls were created by omitting the primary antibody. Two observers independently evaluated the results of the immunostaining under a light microscope.

The level of GPX-1 marker of antioxidant activity expression was revealed immunohistochemically in the cytoplasm of pinealocytes. The expression of the GPX-1 marker was assessed by the number of the gland's cells whose cytoplasm had the colour characteristic for GPX-1.

Statistical analysis

Assessment of GPX-1 expression was performed semi-quantitatively by counting the number of stained cells per 100 cells in three fields of view, the result was expressed

as a percentage and assessed on the accepted scale: 1) no expression of GPX-1 (–), 2) 0–20% – low expression of GPX-1 (+), 3) 21–50% – moderate expression of GPX-1 (++), 4) 51–100% – significant expression of GPX-1 (+++) (Lutsik and Yashchenko, 2018). Processing of digital results was performed by applied statistical methods using the Microsoft Word Excel 2010 text editor with AtteStat 12.0.5 application. Reliability of the difference between the experimental and control data of morphometric and immunohistochemical parameters was assessed using the Student's *t*-test, the probability of error less than 5% ($p \leq 0.05$) was considered sufficient.

Results

After 90 days of the experiment, the pineal gland of the experimental animals had an oval shape, maintained its anatomical integrity and connection with the vascular plexus. Heavy metal salts caused noticeable negative changes in all structural components of the gland: stromal, vascular and parenchyma. The capsule of the gland was slightly thickened, the intertrabecular spaces were expanded, in some areas – significantly. Quite pronounced morphological changes were observed in the vascular bed of dystonic nature compared to the control animals. In some areas, the lumen of the vessels was expanded, and in others, the vessels were spasmodic. Large vessels of the subcapsular zone and deep areas of parenchyma were full-blooded, with signs of impaired blood rheological properties. Erythrocytes completely filled the lumen of blood vessels, in some places they were very close to each other, their contours were not clearly delineated, blood stasis was formed, erythrocyte aggregation, sludge phenomenon were observed. The vascular wall

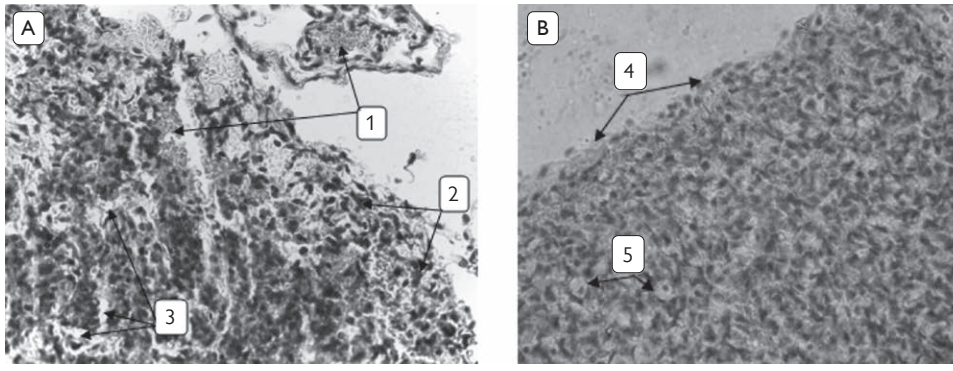

Figure 4 – Morphological rearrangements of the structural components in the pineal gland of experimental (A) and control (B) animals under the conditions of 90-day exposure to of heavy metal salts. A: 1 – plethora of the subcapsular zone vessels with signs of the blood rheological properties impairment; 2 – reactive astrogliosis; 3 – expansion of intertrabecular spaces; B: 4 –connective tissue capsule; 5 – light pinealocyte (hematoxylin-eosin staining, ×400).

was also affected, especially in the large afferent vessels of the epiphysis in the subcapsular zone. There was the vascular wall's thickening and its permeability increase, which was manifested in the release of erythrocytes into the extravascular space with the formation of diapedetic hemorrhages of different areas, expansion of the perivascular space. Around the vessels of the subcapsular zone, especially with impaired permeability of the vascular wall, and hemorrhages, a clearly pronounced active glial reaction was observed in the form of reactive astrogliosis. In addition to local, there was also a general diffuse glial reaction observed (Figure 4).

The results of light optical studies were confirmed by the previous immunohistochemical studies of Ki-67 proliferation marker (Romanjuk et al., 2018b), according to which the assessment of the Ki-67 protein's expression level revealed its moderate proliferative activity in peripheral astrocytes (35–40%). The intensity of cell nuclei staining was assessed as moderate (++).

Vascular plethora of the gland was accompanied by edema. Intertrabecular capillaries in some areas were spasmodic and were not visualized, possibly due to edema and disintegration of the fibrous component in the connective tissue trabeculae. In other fields of view, the lumen of the capillaries was significantly expanded with erythrocytes in their lumen, forming "rouleau" (Figure 5).

The pineal gland's parenchyma of experimental animals was distinct in some sponginess and accumulation of edematous fluid in the intertrabecular spaces. Discomplexation of cellular trabeculae and some disorders of cytoarchitectonics were observed. In some fields of view around the pinealocytes pericellular edema was formed. The structure of the pineal gland's parenchyma was characterized by a mosaic nature of morphological changes. The tissue specimens were dominated by

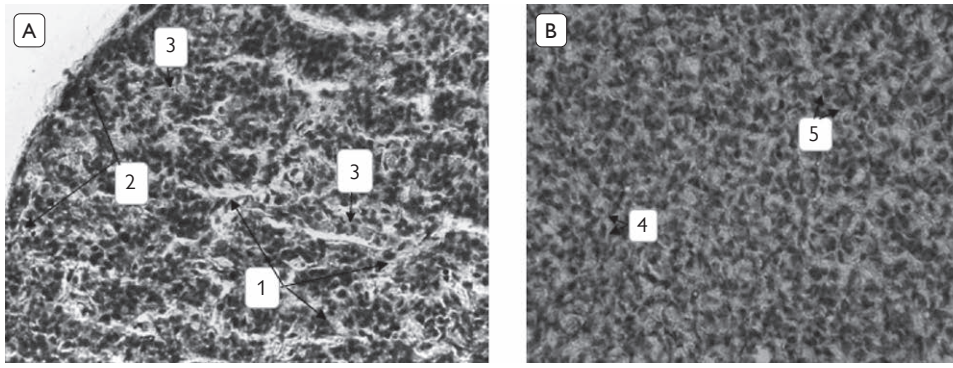

Figure 5 – Morphological rearrangements of the structural components in the pineal gland of experimental (A) and control (B) animals under the conditions of 90-day exposure to heavy metal salts. A: 1 – edema and disintegration of the fibrous component in the connective tissue trabeculae of the gland; 2 – reactive astrogliosis; 3 – "rouleau" in the lumen of the capillaries; B: 4 – light pinealocyte; 5 – dark pinealocyte (hematoxylin-eosin staining, ×400).

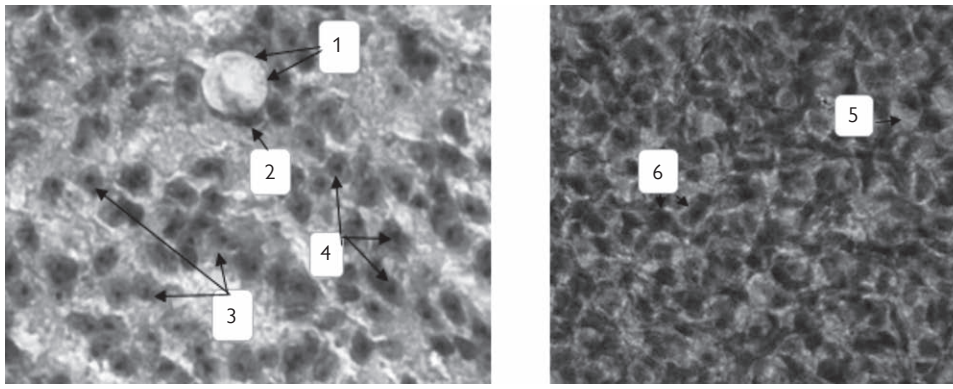

Figure 6 – Morphological rearrangements of the structural components in the pineal gland of experimental (A) and control (B) animals under the conditions of 90-day exposure to heavy metal salts. A: 1 – light pinealocyte with vacuolated cytoplasm; 2 – the nucleus with an eccentrically position; 3 – hypertrophied pinealocyte nuclei; 4 – hyperchromic, hypertrophied nucleolus; B: 5 – light pinealocyte; 6 – dark pinealocyte (hematoxylin-eosin staining, ×800).

light pinealocytes with cleared, often vacuolated cytoplasm and nuclei, both of oval and slightly deformed, angular shape. In vacuolated cells, the nuclei were shifted to the periphery, located eccentrically. On the periphery there were dark pinealocytes, frequently surrounded by astrocytic glia. In some cells, the size of the nuclei was increased compared to that of the control, their chromatin network was cleared, with a hyperchromic, hypertrophied nucleolus located in the center of the nucleus. Such pinealocytes were frequently found near the hemocapillary wall. In some nuclei, the nucleoli were slightly shifted to the karyomembrane. A relatively small

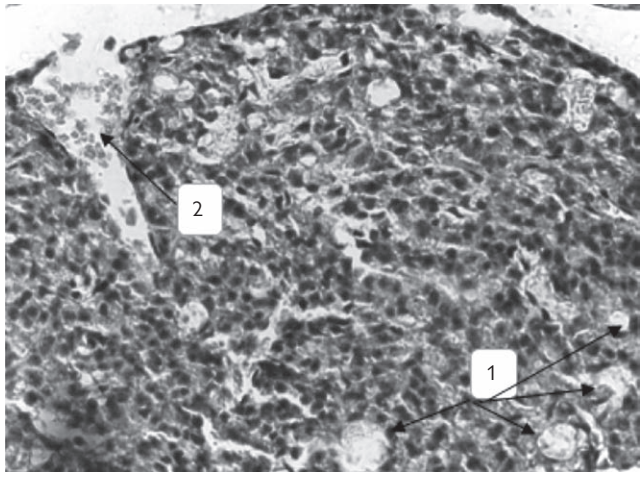

Figure 7 – Morphological rearrangements of structural components in the pineal gland of experimental animals under the conditions of 90-day exposure to heavy metal salts. 1 – multiple polymorphic cysts in the gland's parenchyma; 2 – blood rheological properties (hematoxylin-eosin staining, ×400).

proportion of cells had deformed, hyperchromic nuclei, frequently with signs of pyknotic rearrangements. Their chromatin network was homogeneous; nucleoli were not visualized (Figure 6).

Small oxyphilic secretory granules were visualized in the cytoplasm of a moderate part of pinealocytes, as well as in the lumen of blood vessels. The gland's parenchyma had a moderate number of small, medium, and in some areas of single large sized cysts, stained by oxyphilic method (Figure 7).

In some nuclei chromatin margination and an increase in the number of nuclei with nucleoli were observed. The presence of pinealocytes with angular nuclei and highly vacuolated cytoplasm in the parenchyma of the gland, according to a number of authors indicates the synthesis and accumulation of indolamines in these cells (Bondarenko et al., 2013). However, most of the pinealocytes showed signs of polypeptide synthesis in them.

In order to study one of the links of the antioxidant system of the pineal gland protection, a comparative immunohistochemical study of the pineal gland tissue micropreparations obtained from experimental and control animals on the level of GPX-1 expression was performed. In the control animals' specimens, a significant number of pinealocytes was found with the presence of the GPX-1 marker in their cytoplasm, which correlates with the literature data (Razygraev, 2004). When assessing the results of the reaction, the presence of the studied marker in the cytoplasm of almost all pinealocytes (88% of GPX-1 – positive cells with diffuse location in the cytoplasm of the GPX-1 marker was determined (Figure 8).

However, the degree of expression had some signs of mosaicism. Thus, a particularly significant number of such glandulocytes was found in the peripheral areas of the pineal gland, taking into account the greater synthetic activity of pinealocytes in these areas of the body. Here, cells with a significant level of GPX-1

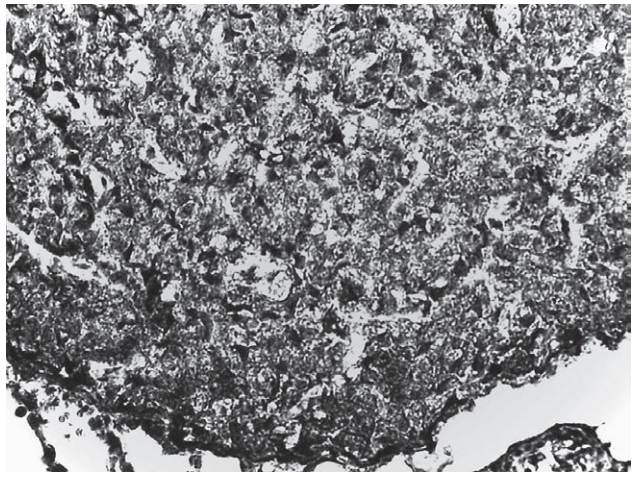

Figure 8 – Immunohistochemical study of GPX-1 expression in the cytoplasm of pinealocytes in the control animals (magnification ×400).

Table 1 – Expression level of GPX-1 in pinealocytes of the pineal gland in experimental and control animals, n=12

GPX-1 expression	Control group total number 12 animals	Experimental group total number 12 animals
Low positive 1	17.22 ± 0.84	–
Moderately positive 2	31.55 ± 0.27	13.88 ± 1.14
Strong positive 3	39.23 ± 0.15	25.12 ± 1.09

(+++) expression in the cytoplasm were identified, while in the parenchyma and its central regions, the expression level was defined as + and ++ (Table 1).

In the pineal parenchyma of the experimental animals, the expression of GPX-1 was lower in the experimental group than in the control group, as evident from Table 1.

Against the background of the general negative reaction of pinealocytes (–), 39% of GPX-1-positive secretory-active cells with a diffuse location of the GPX-1 marker in the cytoplasm with a moderate (++) and high (+++) level of GPX-1 expression were visualized, especially in the peripheral areas of the gland (Table 1). A correlation was found between the presence of the GPX-1 marker in the pinealocytes cytoplasm and the presence of secretory granules in their cytoplasm, as well as the location of such secretory-active cells in the perivascular spaces (Figure 9).

In order to quantify cellular structures of the pineal gland, the density of pinealocytes and neuroglia, as well as the neurocytoglial index of control and experimental animals were determined by comparing the results of the study. In this

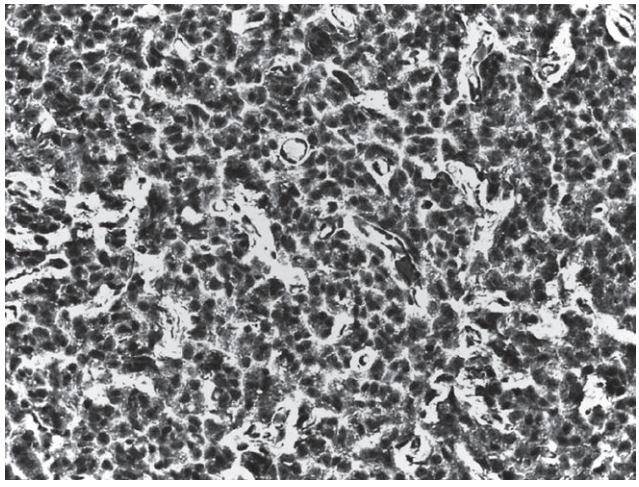

Figure 9 – Immunohistochemical study of GPX-1 expression in the cytoplasm of experimental animals pinealocytes after 90 days of consuming heavy metal salts (magnification ×400).

Table 2 – Morphometric parameters of pinealocytes and astrocytic glia of the pineal gland in experimental and control animals, n=12

Index	Study groups of animals	
	control animals	experimental animals
Absolute number of pinealocytes	115.25 ± 11.8418	85.00 ± 6.0139*
Absolute number of astrocytic glia cells	38.75 ± 5.2341	84.75 ± 10.9725**
Glyocyto-neuronal index	0.336 ± 0.4420	0.997 ± 1.8245

Reliable in comparison with the control: *p≤0.05; **p≤0.01

case, the morphometric indices of the density of pinealocytes in the experimental animals were significantly reduced by 26% (p≤0.05, t = 2.277626) compared to the control animals. At the same time, the density of glial elements in the pineal gland of experimental animals was significantly increased in comparison with the control by 2.2 times (p≤0.01, t = 3.783842). Glyocyto-neuronal index in the pineal gland of experimental animals increased by 2.96 times (p≥0.05) compared to that of the control animals (Table 2).

Discussion

Thus, the obtained data of the immunohistochemical study indicate a high level of antioxidant protection of GPX-1 in the pineal gland of control animals. Prolonged exposure of experimental animals to the mixture of heavy metal salts (including lead salts) caused negative changes in all structural components of the gland: stromal, vascular, parenchymal. These changes have signs of a homeostasis shift in the form of impaired rheological properties of blood, morphology of the vascular wall, its increased permeability, full blood vessels, which leads according to (Shafran et al., 2004) to the development of oxidative stress (Hryntsova et al., 2022) and to manifestations of tissue hypoxia in the gland's parenchyma.

Ischemic damage to the pineal gland certainly had negative consequences for the secretory activity of pinealocytes and mechanisms of hormone diffusion into the blood as a result of disorders of the plasmalemma membrane in pinealocytes and morphological changes in the vascular wall. This is evidenced by the increased number of pinealocytes with vacuolated cytoplasm and the presence of cristae with different sizes and shapes in the pineal gland's parenchyma of the experimental animals. Such morphological properties of the gland's cells and parenchyma, in our opinion, may indicate a delay in the evacuation of hormones into the vascular bed and impairment of the antioxidant defence system of the body as a whole. However, considering the pathogenesis of dystrophic changes in the parenchyma of the pineal gland, including pinealocytes, it is impossible not to mention the possibility of direct toxic effects of heavy metals directly on pinealocytes, given the anatomical absence of blood-brain barrier in this organ. The study of morphological

and morphometric features of pinealocytes and astrocytic glia in the pineal gland of experimental animals permits to indirectly assess the degree of oxidative stress and apoptosis processes in cells (Gubina-Vakulik, 2006). In some nuclei a chromatin margination and an increase in the number of nuclei with nucleoli were observed. which according to the authors is a sign of some activation in synthetic cell activity (Shkorbatov, 2005), but other authors still consider chromatin margination as one of the manifestations of pinealocytes' accelerated apoptosis (Gubina-Vakulik, 2006).

The authors suggest that the cause of the death of some pinealocytes by apoptosis may be long-term exposure of heavy metals on the pineal gland, including such well-known mechanisms of action of heavy metals on the living organism as activation of free radical oxidation, initiation of peroxidation of proteins, lipids and development of oxidative stress (Shahid et al., 2014).

These processes are indicated by a significant decrease in the density of pinealocytes of experimental animals compared to that of control animals, and at the same time a significant increase in the density of glial elements (as confirmed by immunohistochemical studies of the Ki-67 marker – Romanjuk et al., 2018b) and gliocyto-neuronal index.

The formed perivascular astroglial proliferates represent an adaptive response of glia to the action of the damaging agent (Drozdova et al., 2017) and may indirectly indicate more intensive processes of pineal cell apoptosis in these animals (Gubina-Vakulik, 2006). In addition, the increased number of glial elements in the pineal gland certainly has a definite compensatory-adaptive value, especially in the processes transferring RNA, amino acids and growth factors (Goryainov et al., 2013).

All these signs indicate, directly or indirectly, activation of apoptotic processes in the pineal gland in response to the damaging agent. However, it is impossible to neglect other properties of astrocytic neuroglia in glial proliferates, which, in our opinion, are aimed at achieving water-ion homeostasis in the gland by improving the trophism of pinealocytes, barrier function, preventing the penetration of heavy metals into the parenchyma of the gland.

The study of GPX-1 enzymatic activity in the cytoplasm of experimental animals' pinealocytes after 90 days of heavy metal salts consuming led to a decrease in the number of pinealocytes positive for this immunohistochemical marker in comparison with the indicators of control animals. In pineal preparations of experimental animals, a relationship between the presence of GPX-1 expression in the cytoplasm of pinealocytes and the presence of secretory granules in their cytoplasm was revealed. This location of secretory-active pinealocytes with moderate (++) and high (+++) levels of GPX-1 expression in the cytoplasm may be explained by their better supply of oxygen and nutrients compared to cells in deeper areas. The obtained results indicate the development of complex compensatory and adaptive processes in the pineal gland, which are parallel to the indicated negative changes.

It seems likely that long-term exposure to heavy metal salts caused increased oxidative stress in most cells of the pineal gland.

With regard to pathogenic mechanisms of imbalance development in the antioxidant system of the pineal gland, it is possible to assume that such a decrease in glutathione peroxidase activity of pinealocytes also developed due to antagonism between heavy metals and selenium. After all, it is well known that selenium plays an important role in endocrine functions. GPX-1 contains selenocysteine, selenium-dependent glutathione peroxidase. Relatively high concentrations of selenium were found in the pineal gland, which was associated with its antioxidant properties, in particular with its ability to neutralize reactive oxygen intermediates formed during the synthesis of melatonin (Reiter, 1996). This means that the expression of GPX-1 was affected by the level of selenium in the pineal gland, as the action of melatonin was modulated depending on the content of selenium in the gland. The most informative index of selenium supply of the body is the concentration of SePP (selenoprotein P) in the blood and the activity of glutathione peroxidase (Köhrle et al., 2005; Kravtsiv and Yanovich, 2008).

According to the literature, metals are able to form strong sulphide bonds, which lead to blocking of functional SH-groups (sulfhydryl groups) in a number of antioxidant enzymes (Trachtenberg et al., 2010, 2015), including glutathione peroxidase, which contains selenocysteine. The mechanism of GPX-1 inhibition is based on the binding of heavy metals to reduced glutathione (GSH) in the active site of these enzymes (Rusetskaya and Borodulin, 2015).

The high degree of GPX-1-positive pinealocytes' cytoplasm colour intensity and the presence of secretory granules in their cytoplasm may indicate, in our opinion, the maximum stress degree of adaptive secretory processes in these pinealocytes.

In addition, in our opinion, there is a very interesting correlation between the presence of the GPX-1 marker in the cytoplasm of pinealocytes and the presence of secretory granules in their cytoplasm, as well as the location of such secretory active cells in the perivascular spaces. In our opinion, this can be explained by their better supply of oxygen and nutrients due to active glial proliferates, which increased number was found in the peripheral areas of the gland compared to deeper areas. Adaptive processes in the gland included an increase in the number of cells with nuclei in which the nucleoli were visualized, which is also a sign of increased synthetic activity of cells.

Conclusion

Thus, the long-term (90-days) intake of heavy metal salts mixture leads in the experimental animals to the development of the general adaptation syndrome, that represents a stage of chronic stress "subcompensation" in the pineal gland.

Morphological rearrangements are of nonspecific polymorphic nature as severe hemodynamics disorder in the organ, impairment of vascular wall morphology, development of tissue hypoxia and oxidative stress, processes of accelerated apoptosis in part of pinealocytes, a significant decrease in glutathione peroxidase level in the organ and reactive astrogliosis as a response to the damaging agent's action.

Along with the negative changes in the pineal gland, there also occur compensatory-adaptive processes with signs of functional stress. A sufficiently high degree of glutathione peroxidase activity in 39% of pinealocytes located perivascularly, active adaptive glial reaction and activation of synthetic processes in some pinealocytes were observed.

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