Toxicology in Vitro 110 (2026) 106132

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Stronger prooxidative effects of chromium(VI) comparing to chromium(III) in endocrine and non-endocrine tissues with the thyroid being completely resistant to antioxidant protection

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ARTICLE INFO

Editor: Dr. I Maschmeyer

Keywords:
Chromium
Thyroid
Melatonin
Antioxidant
Oxidative damage
Carcinogen

ABSTRACT

Chromium (Cr) is a harmful heavy metal pollutant. Cr(VI) is a group 1 carcinogen (carcinogenic to humans), whereas Cr(III) is a group 3 carcinogen (not classifiable as to its carcinogenicity to humans). Cr is also documented to be an endocrine disrupting chemical. The study aimed to check whether Cr(VI) compound ($K_2Cr_2O_7$) or Cr(III) compound ($CrCl_3{\cdot}6H_2O$) induce oxidative damage to membrane lipids (lipid peroxidation, LPO) in homogenates of two endocrine (the thyroid and the ovary) and two non-endocrine (the kidney and the liver) tissues, and whether antioxidants, such as melatonin, indole-3-propionic acid (IPA) and 17β-estradiol, reveal protective effects. Of note, the healthy thyroid gland is characterized by relatively high oxidative stress. Porcine tissue homogenates were incubated in the presence of Cr(VI) (0.05–10.0 mM) or Cr(III) (5.0–200.0 mM) with/without melatonin (5 mM) or IPA (5 mM) or 17β-estradiol (1 mM). The malondialdehyde+4-hydroxyalkenals (MDA + 4-HDA) concentration (LPO index) was measured spectrophotometrically. Cr(VI) (≥0.1–1.25 mM) significantly increased LPO in all tissues but these damaging effects were not prevented by any antioxidant tested. In turn, Cr(III) (≥25 mM) also significantly increased LPO in all examined tissues. All antioxidants reduced Cr(III)-induced LPO in the ovary, kidney, and liver but had no protective effects in the thyroid. Our findings highlight chromium's strong prooxidative effects, especially in the thyroid. The inability of antioxidants to prevent Cr-induced damage, especially in the thyroid, underscores the need for further research to identify effective protective strategies.

1. Introduction

Environmental pollutants such as heavy metals, pesticides, and industrial chemicals are increasingly recognized as key contributors to carcinogenesis. Among them, chromium compounds—especially hexavalent chromium [Cr(VI)]—pose serious toxicological risks due to their ability to induce oxidative stress, DNA damage, and endocrine disruption (Silva et al., 2024; Jomova et al., 2025). Cr(VI) has been shown to cross biological barriers and cause damage in various tissues, including the brain and gastrointestinal tract, as demonstrated in aquatic models (Li et al., 2024; Zhao et al., 2025). Some of these pollutants act as endocrine-disrupting chemicals (EDCs), which interfere with hormonal signaling and may lead to developmental disorders, infertility, and hormone-related cancers (Damstra et al., 2002; Gore et al., 2015; Yang et al., 2023). Although these chemicals can impact the entire endocrine system, the thyroid gland—along with the broader hypothalamic-pituitary-thyroid axis—appears to be particularly susceptible [Calsolaro et al., 2017; Köhrle and Frädrich, 2021; Karbownik-Lewińska et al., 2022].

In general, EDCs include a huge number of heterogeneous chemicals, among others heavy metals. Among the heavy metals classified as endocrine-disrupting chemicals, chromium is of particular concern due to its widespread environmental presence and redox activity. Its main forms are trivalent chromium [Cr(III)] and hexavalent chromium [Cr

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https://doi.org/10.1016/j.tiv.2025.106132
Received 3 July 2025; Received in revised form 28 July 2025; Accepted 25 August 2025
Available online 26 August 2025

(VI)] (Zhao et al., 2024). Whereas Cr(III) was historically regarded as an essential element (Vincent, 2014; Vincent and Brown, 2019), more recent studies in humans and rodents indicate that it should instead be considered only as potentially pharmacologically active (Vincent, 2017; Henriksen and Bügel, 2023; Mattos Pereira and Nair, 2024). Cr(VI) is toxic and has carcinogenic properties [Yu et al., 2021]. Cr(VI) is considered as one of the most dangerous environmental pollutants, mainly due to its ability to cause DNA damage and induce oxidative stress in cells (Jadoon and Malik, 2017; DesMarais and Costa, 2019).

Chromium, depending on its chemical form, may interact with the hormonal system; therefore, it acts as an endocrine disruptor (IARC, 2012; Wuri et al., 2024). As it was mentioned above Cr is classified as a carcinogen, with Cr(VI) specifically placed in Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) Monographs (IARC, 2012). In contrast, Cr(III) is classified by IARC as a Group 3 agent (not classifiable as to its carcinogenicity to humans) (IARC, 1990). The carcinogenicity of Cr is believed to stem from macromolecular damage caused by reactive intermediates, such as reactive oxygen species (ROS), which are generated during its intracellular reduction to Cr(III). Consequently, Cr(VI) is more toxic, as it reacts with nearby molecules and is eventually reduced within the body to the more stable Cr(III).

Besides carcinogenic action in humans and animals and effecting hormonal system, toxic effects of Cr include allergic dermatitis, acute and chronic neurotoxicity, dermatotoxicity, genotoxicity, immunotoxicity (DesMarais and Costa, 2019; Long et al., 2021; Shehata et al., 2023; Sazakli, 2024). Chromium compounds are used in paints, metal finishes and stainless steel. An additional global problem is water contamination with this element (Dippong et al., 2024; Suljević et al., 2024).

Antioxidants, which capture and neutralize free radicals to reduce oxidative stress, are considered potentially protective against oxidative damage caused by Cr. Among the most effective antioxidants are indole substances. Melatonin (*N*-acetyl-5-methoxytryptamine), a primary metabolite of tryptophan, is a notable example of an indole substance. While melatonin's main physiological role is to regulate the circadian rhythm, it also exhibits numerous other activities (Reiter et al., 2013; Bonmati-Carrion et al., 2014). It is a powerful antioxidant and free radical scavenger (Karbownik et al., 2001), proven to be highly potent and effective in mitigating oxidative damage to macromolecules across various experimental conditions (Karbownik et al., 2001; Karbownik and Lewiński, 2003; Karbownik et al., 2005; Karbownik et al., 2006; Reiter et al., 2016; Iwan et al., 2021; Rynkowska et al., 2021; Stępniak et al., 2022a, 2022b; Esteban-Zubero et al., 2023; Gładysz et al., 2023). Some other protective effects of melatonin include regulation of DNA methylation, which may contribute to the prevention of cancer (Linowiecka et al., 2023), protection against inflammation caused by chemical stimulants and oxidative damage (Ramos et al., 2023), the beneficial effect on reproduction (Reiter et al., 2023), and action as coadjutant in the treatment of various diseases (González-Flores et al., 2023).

The other indole substance used in the present study is an indole-3-propionic acid (IPA). It has a chemical structure similar to melatonin, which also possesses effective antioxidant properties. Previous studies have shown that not only melatonin, but also IPA, provides effective protection against oxidative damage to biological macromolecules caused by potential carcinogens (Karbownik et al., 2001; Karbownik et al., 2005; Karbownik et al., 2006; Konopelski and Mogilnicka, 2022) or by selected EDCs (Gładysz et al., 2023). Another antioxidant used in the present study is 17β-estradiol, a hormone with antioxidant properties. The protective antioxidative effects of 17β-estradiol have been confirmed, among others, in previous experimental studies conducted in our department (Stepniak and Karbownik-Lewinska, 2016; Stepniak et al., 2018; Rynkowska et al., 2020).

The study aimed to assess whether, and to what extent, melatonin and other well-known antioxidants—such as indole-3-propionic acid (IPA) and 17β-estradiol—can prevent oxidative damage to membrane lipids (lipid peroxidation, LPO) induced by Cr(VI) in the form of potassium dichromate ($K_2Cr_2O_7$) or by Cr(III) in the form of chromium(III) chloride hexahydrate ($CrCl_3{\cdot}6H_2O$). This investigation was conducted using tissue homogenates from two endocrine organs, i.e. the thyroid and the ovary, as well as two non-endocrine organs, i.e. the kidney and the liver. Of note, the thyroid gland is characterized by relatively high oxidative stress (Karbownik-Lewińska and Kokoszko-Bilska, 2012; Kościuszko et al., 2023; Stępniak and Karbownik-Lewińska, 2024).

2. Materials and methods

2.1. Ethical considerations

According to the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes, dated January 15, 2015 (which implements Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010, on the protection of animals used for scientific purposes), using animals solely for organ or tissue collection does not require approval from the Local Ethics Committee. Moreover, in this study, no experimental animals were used; instead, porcine tissues were obtained from animals in a slaughterhouse as part of the routine slaughter process for consumption. Additionally, a separate statement from the Ethics Committee of the Medical University of Lodz, obtained on October 20, 2021, confirmed adherence to the above directive.

2.2. Chemicals

All chemicals used in the study were of analytical grade and were commercially sourced. Melatonin, indole-3-propionic acid (IPA), 17β-estradiol, potassium dichromate ($K_2Cr_2O_7$), and chromium(III) chloride hexahydrate ($CrCl_3{\cdot}6H_2O$) were purchased from Sigma (St. Louis, MO, USA). Ethanol (96 %) was obtained from Stanlab (Lublin, Poland), and the LPO-586 kit for lipid peroxidation (LPO) was acquired from Enzo Life Science (Farmingdale, NY, USA).

2.3. Animals

Porcine tissues were collected from twenty (20) pigs of both sexes slaughtered at the local slaughterhouse. Animals were treated according to the European Community Council Regulation (1099CE/2009) concerning protection of animals at the time of killing. All animals were sexually mature as determined by age (8–9 months) and body mass [118 ± 3.8 (SD) kg]. They were in good body condition and considered free of pathologies by the veterinary medical officer responsible for the health of animals and hygiene of the slaughterhouse. Immediately (in less than 5 min) after the slaughter, the tissues, i.e., thyroids, ovaries, kidneys (renal cortex) and livers (from left lateral lobe), were collected, frozen on solid CO_2, and stored at −80 °C till experimental procedure. Each experiment was repeated three times.

2.4. Experimental steps

Tissues from the thyroid, ovary, kidney, and liver were homogenized in a 50 mM Tris-HCl buffer (pH 7.4) at 10 % (*w/v*) concentration, kept ice-cold, and subsequently incubated for 30 min at 37 °C with the test substances. Melatonin, IPA, and 17β-estradiol were dissolved in absolute ethanol, with a final ethanol concentration of 1 % (*v*/v) in the incubation mixture. Chromium compounds were prepared using Tris-HCl buffer.

In initial tests, we evaluated whether the available chromium compounds could induce lipid peroxidation. In the main experiments, co-incubations were conducted with a single antioxidant and a chromium compound within homogenates of the thyroid, ovary, kidney, or liver, as detailed below:

Experiment 1: $CrCl_3{\cdot}6H_2O$ [Cr(III)] (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) with/without melatonin (5 mM) or IPA (5

mM), or 17β-estradiol (1 mM) in the thyroid.

Experiment 2: $K_2Cr_2O_7$ [Cr(VI)] (0.050, 0.075, 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) with/without melatonin (5 mM) or IPA (5 mM), or 17β-estradiol (1 mM) in the thyroid.

Experiment 3: $CrCl_3{\cdot}6H_2O$ [Cr(III)] (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) with/without melatonin (5 mM) or IPA (5 mM), or 17β-estradiol (1 mM) in the ovary.

Experiment 4: $K_2Cr_2O_7$ [Cr(VI)] (0.050, 0.075, 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) with/without melatonin (5 mM) or IPA (5 mM), or 17β-estradiol (1 mM) in the ovary.

Experiment 5: $CrCl_3{\cdot}6H_2O$ [Cr(III)] (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) with/without melatonin (5 mM) or IPA (5 mM), or 17β-estradiol (1 mM) in the kidney.

Experiment 6: $K_2Cr_2O_7$ [Cr(VI)] (0.050, 0.075, 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) with/without melatonin (5 mM) or IPA (5 mM), or 17β-estradiol (1 mM) in the kidney.

Experiment 7: $CrCl_3{\cdot}6H_2O$ [Cr(III)] (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) with/without melatonin (5 mM) or IPA (5 mM), or 17β-estradiol (1 mM) in the liver.

Experiment 8: $K_2Cr_2O_7$ [Cr(VI)] (0.050, 0.075, 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) with/without melatonin (5 mM) or IPA (5 mM), or 17β-estradiol (1 mM) in the liver.

The chromium concentrations used in the experiment represent a cross-section ranging from the highest concentrations achievable in vitro (limited by solubility) to lower concentrations that no longer produced measurable pro-oxidative effects. The concentrations of melatonin, indole-3-propionic acid, and 17β-estradiol were selected based on previous experiments conducted in our laboratory. These represent the highest concentrations achievable in vitro due to limited solubility and showed the most effective performance in our experimental model (Rynkowska et al., 2020; Rynkowska et al., 2021; Gładysz et al., 2023). All experiments were conducted in duplicate and repeated three times.

Each homogenate was prepared as a pooled biological sample consisting of tissues (the thyroid or the ovary or the kidney, or the liver) collected from 20 individual animals. As such, all experiments were performed on one composite biological sample per tissue, with each treatment condition tested in duplicate and repeated in three independent experimental runs to ensure technical reproducibility.

2.5. Measurement of lipid peroxidation (LPO) products

The concentration of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA) was measured as an indicator of lipid peroxidation (LPO) in tissue homogenates as described previously (Stepniak et al., 2021). In brief, homogenates were centrifuged at 3000 ×*g* for 10 min at 4 °C. After collecting the supernatant, each experiment was performed in duplicates. A 200 μL sample of the supernatant was combined with 650 μL of a methanol solution (1:3, *v*/v) containing the chromogenic agent *N*-methyl-2-phenylindole, then vortexed. Subsequently, 150 μL of methanesulfonic acid (15.4 M) was added, and the mixture was incubated at 45 °C for 40 min. The reaction between MDA + 4-HDA and *N*-methyl-2-phenylindole forms a chromophore, which was measured spectrophotometrically at 586 nm, using a 10 mM 4-hydroxynonenal (4-HNE) solution as a standard. Lipid peroxidation levels were expressed as the amount of MDA + 4-HDA (nmol) per mg of protein. Protein content was determined using the Bradford method (Bradford, 1976), with bovine serum albumin as the standard.

2.6. Statistical analyses

The data were analyzed statistically using a one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test, for cases involving more than two dependent variables (such as different concentrations of either Cr compound alone or Cr combined with an antioxidant). For comparisons between two independent variables (Cr compound versus Cr with an antioxidant), an unpaired Student *t*-test was applied. A level of $p < 0.05$ was considered statistically significant. Additionally, effect sizes (Cohen's *d*) were calculated to quantify the magnitude of differences between selected treatment groups. Results are expressed as the mean ± SE.

3. Results

In the Experiment 1, incubation of thyroid homogenates with Cr(III) compound, i.e. chromium (III) chloride hexahydrate ($CrCl_3{\cdot}6H_2O$), resulted in a concentration-dependent increase in LPO levels, which was statistically significant at Cr(III) concentrations of 25, 50, 75, 100, 125, 150, 175 and 200 mM. The effect sizes (Cohen's d) calculated for these comparisons ranged from 18.85 to 4.95, indicating very strong biological relevance of the observed oxidative damage. Neither melatonin (5 mM), nor indole-3-propionic acid (5 mM), nor 17β-estradiol (1 mM) demonstrated any protective effects (Fig. 1).

In the Experiment 2, incubation of thyroid homogenates with Cr(VI) compound, i.e. potassium dichromate ($K_2Cr_2O_7$), resulted in a concentration-dependent increase in LPO levels, which was statistically significant at Cr(VI) concentrations of 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM. The effect sizes (Cohen's d) calculated for these comparisons ranged from 16.29 to 3.28, indicating very strong biological relevance of the observed oxidative damage. Neither melatonin (5 mM), nor indole-3-propionic acid (5 mM), nor 17β-estradiol (1 mM) exhibited protective effects. Interestingly, adding an indole substance (melatonin or IPA) with Cr(VI) compound at seven highest concentrations (0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) with effect sizes ranged from 9.59 to 3.08, or adding 17β-estradiol with Cr(VI) at its two highest concentrations (7.5 and 10.0 mM) with effect sizes 4.49 and 5.28 respectively, resulted in an additional increase in LPO levels (Fig. 2).

Tissue homogenates were prepared from pooled samples consisting of thyroids collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

In the Experiment 3, incubation of ovary homogenates with Cr(III) compound, i.e. chloride hexahydrate ($CrCl_3{\cdot}6H_2O$), resulted in a concentration-dependent rise in LPO levels, which was statistically significant at Cr(III) concentrations of 25, 50, 75, 100, 125, 150, 175 and 200 mM with the effect sizes ranged from 13.63 to 4.45. The addition of melatonin (5 mM), or indole-3-propionic acid (5 mM), or 17β-estradiol (1 mM) partially reduced the prooxidative effects of Cr(III) added at the four highest concentrations (125, 150, 175 and 200 mM) with effect sizes ranged from 2.61 to 2.78. The statistically significant decrease in LPO levels, observed after the addition of IPA or 17β-estradiol together with Cr(III) at concentrations of 5.0 or 7.5 mM, lacks scientific relevance (Fig. 3).

Tissue homogenates were prepared from pooled samples consisting of ovaries collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

In the Experiment 4, incubation of ovary homogenates with Cr(VI) compound, i.e. potassium dichromate ($K_2Cr_2O_7$), resulted in a concentration-dependent increase in LPO levels, which was statistically significant at Cr(VI) concentrations of 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM with the effect sizes ranged from 29.91 to 4.46. Neither melatonin (5 mM), nor indole-3-propionic acid (5 mM), nor 17β-estradiol (1 mM) provided any protective effects. Notably, adding an indole substance (melatonin or IPA) with Cr(VI) at its eight highest concentrations (0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, and 10.0 mM) with effect sizes ranged from 5.45 to 2.85, or adding 17β-estradiol with Cr(VI) at its two highest concentrations (7.5 and 10.0 mM) with effect sizes 14.27 and 3.49 respectively, resulted in an additional increase in LPO levels (Fig. 4).

In the Experiment 5, incubation of kidney homogenates with Cr(III) compound, i.e. chloride hexahydrate ($CrCl_3{\cdot}6H_2O$), resulted in a concentration-dependent increase in LPO levels, which was statistically significant at Cr(III) concentrations of 25, 50, 75, 100, 125, 150, 175 and

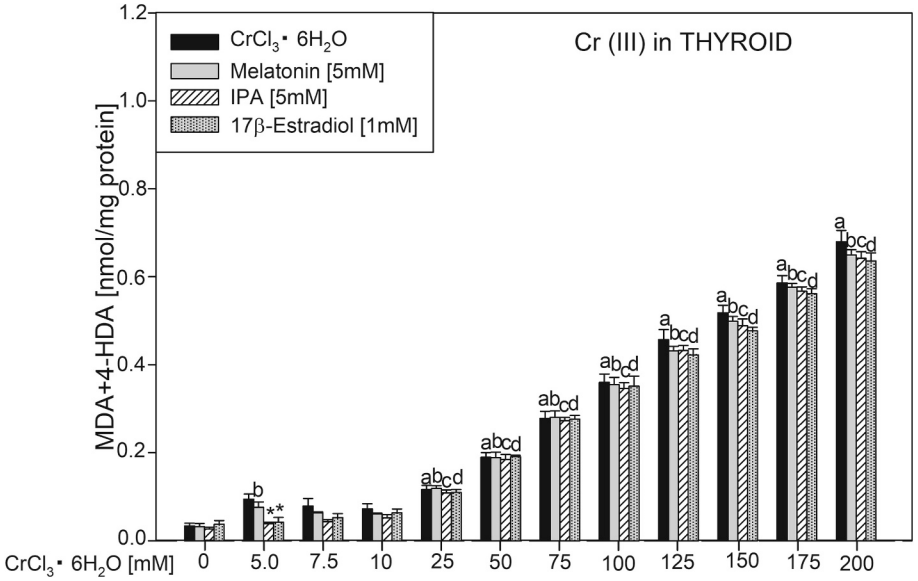

Fig. 1. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine thyroid homogenates, incubated in the presence of chromium(III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$) (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) alone (black bars), or $CrCl_3 \cdot 6H_2O$ with melatonin (5 mM) (grey bars) or $CrCl_3 \cdot 6H_2O$ with indole-3-propionic acid (IPA) (5 mM) (striped bars) or $CrCl_3 \cdot 6H_2O$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $CrCl_3 \cdot 6H_2O$ in the same concentration Tissue homogenates were prepared from pooled samples consisting of thyroids collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

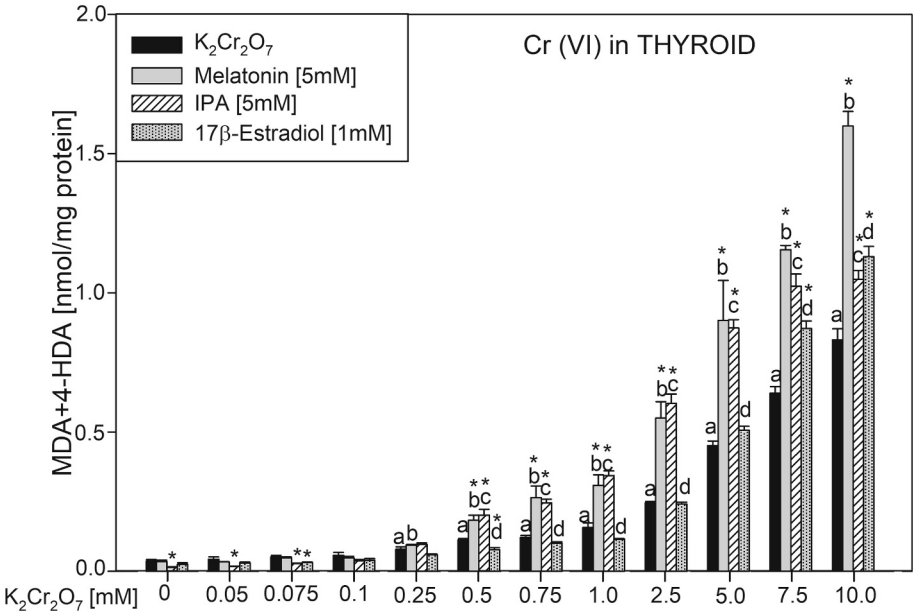

Fig. 2. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine thyroid homogenates, incubated in the presence of potassium dichromate ($K_2Cr_2O_7$) (0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) alone (black bars), or $K_2Cr_2O_7$ plus melatonin (5 mM) (grey bars) or $K_2Cr_2O_7$ plus indole-3-propionic acid (IPA) (5 mM) (striped bars) or $K_2Cr_2O_7$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $K_2Cr_2O_7$ in the same concentration.

200 mM with the effect sizes ranged from 13.63 to 4.45. The addition of melatonin (5 mM), or indole-3-propionic acid (5 mM), or 17β-estradiol (1 mM) partially reduced the prooxidative effects of Cr(III) added at its seven highest concentrations (50, 75, 100, 125, 150, 175 and 200 mM) with effect sizes ranged from 12.04 to 5.81. The statistically significant reduction in LPO levels observed with the addition of IPA at Cr(III) concentrations of 7.5 or 10 mM is considered to lack scientific relevance (Fig. 5).

Tissue homogenates were prepared from pooled samples consisting of kidneys (renal cortex) collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

In the Experiment 6, incubation of kidney homogenates with Cr(VI) compound, i.e. potassium dichromate ($K_2Cr_2O_7$), resulted in a concentration-dependent rise in LPO levels, which was statistically significant at Cr(VI) concentrations of 0.10, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM with the effect sizes ranged from 32.94 to 4.89. Neither

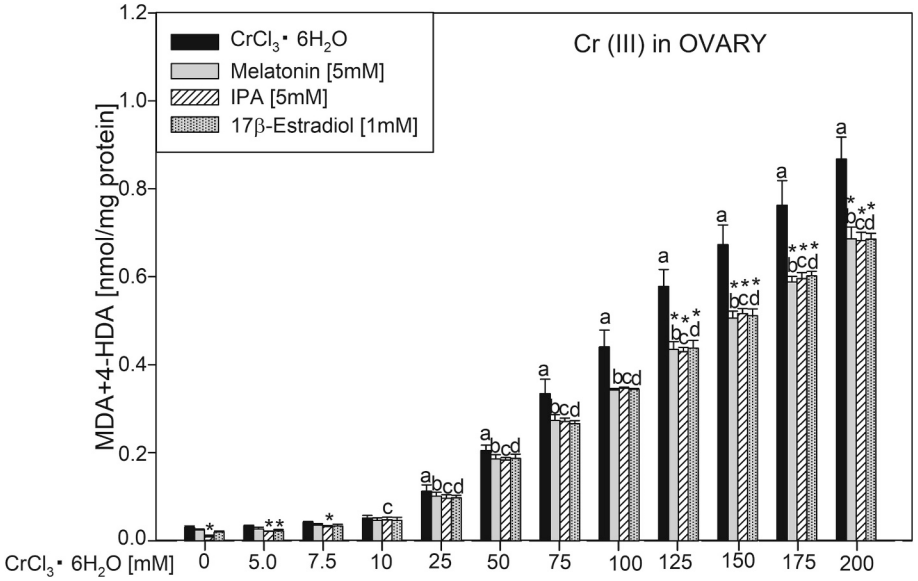

Fig. 3. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine ovary homogenates, incubated in the presence of chromium(III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$) (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) alone (black bars), or $CrCl_3 \cdot 6H_2O$ with melatonin (5 mM) (grey bars) or $CrCl_3 \cdot 6H_2O$ with indole-3-propionic acid (IPA) (5 mM) (striped bars) or $CrCl_3 \cdot 6H_2O$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $CrCl_3 \cdot 6H_2O$ in the same concentration.

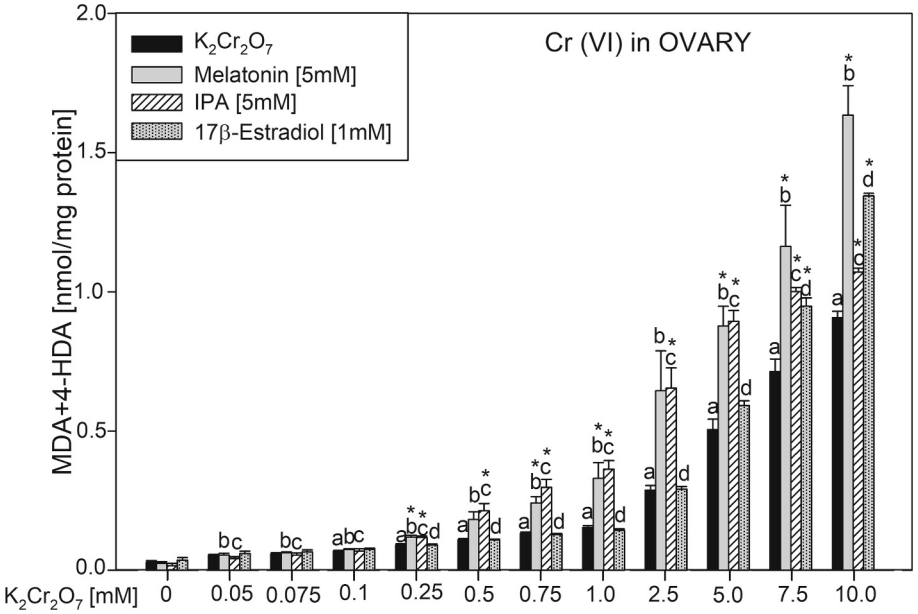

Fig. 4. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine ovary homogenates, incubated in the presence of potassium dichromate ($K_2Cr_2O_7$) (0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) alone (black bars), or $K_2Cr_2O_7$ plus melatonin (5 mM) (grey bars) or $K_2Cr_2O_7$ plus indole-3-propionic acid (IPA) (5 mM) (striped bars) or $K_2Cr_2O_7$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $K_2Cr_2O_7$ in the same concentration Tissue homogenates were prepared from pooled samples consisting of ovaries collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

melatonin (5 mM), nor indole-3-propionic acid (5 mM), nor 17β-estradiol (1 mM) demonstrated protective effects. Interestingly, adding melatonin together with $K_2Cr_2O_7$ at its eight highest concentrations (0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) with effect sizes ranged from 12.96 to 2.97, or 17β-estradiol together with Cr(VI) at its four highest concentrations (2.5, 5.0, 7.5 and 10.0 mM) with effect sizes ranged from 12.96 to 2.97, resulted in a further increase in LPO levels. The addition of indole-3-propionic acid did not alter the LPO levels induced by Cr(VI) treatment (Fig. 6).

In the Experiment 7, incubation of liver homogenates with Cr(III) compound, i.e. chromium (III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$), resulted in a concentration-dependent increase in LPO levels, which was statistically significant at Cr(III) concentrations of 50, 75, 100, 125, 150, 175 and 200 mM with the effect sizes ranged from 19.90 to 5.95. Melatonin (5 mM) or indole-3-propionic acid (5 mM) partially reduced the prooxidative effects of Cr(III) with effect sizes 2.86 and 3.43

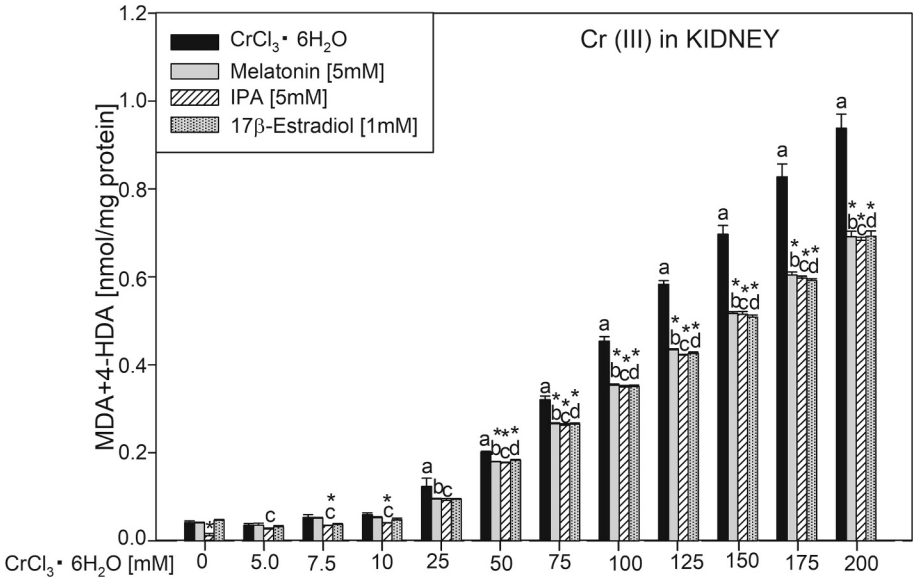

Fig. 5. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine kidney homogenates, incubated in the presence of chromium(III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$) (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) alone (black bars), or $CrCl_3 \cdot 6H_2O$ with melatonin (5 mM) (grey bars) or $CrCl_3 \cdot 6H_2O$ with indole-3-propionic acid (IPA) (5 mM) (striped bars) or $CrCl_3 \cdot 6H_2O$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $CrCl_3 \cdot 6H_2O$ in the same concentration.

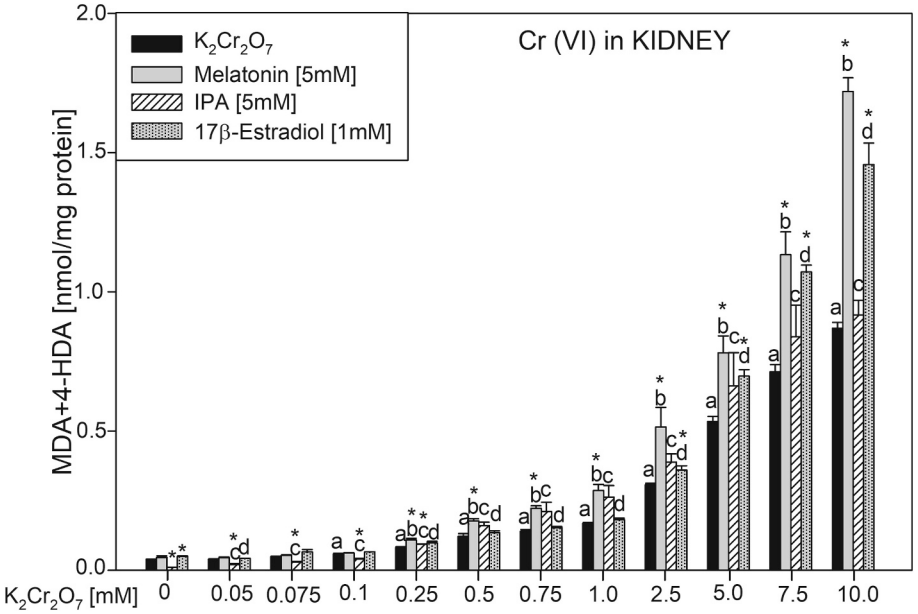

Fig. 6. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine kidney homogenates, incubated in the presence of potassium dichromate ($K_2Cr_2O_7$) (0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) alone (black bars), or $K_2Cr_2O_7$ plus melatonin (5 mM) (grey bars) or $K_2Cr_2O_7$ plus indole-3-propionic acid (IPA) (5 mM) (striped bars) or $K_2Cr_2O_7$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $K_2Cr_2O_7$ in the same concentration Tissue homogenates were prepared from pooled samples consisting of kidneys (renal cortex) collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

respectively at its highest concentration of 200 mM. A statistically significant reduction in LPO levels was observed after the addition of IPA together with Cr(III) at a concentration of 7.5 mM, though this finding lacks scientific significance. The addition of 17β-estradiol (1 mM) did not show any protective effects (Fig. 7).

In the Experiment 8, incubation of liver homogenates with Cr(VI) compound, i.e. potassium dichromate ($K_2Cr_2O_7$), resulted in a concentration-dependent increase in LPO levels, which was statistically significant at Cr(VI) concentrations of 0.10, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM with the effect sizes ranged from 36.23 to 4.36. Protective effects were observed with melatonin (5 mM), indole-3-propionic acid (5 mM), or 17β-estradiol (1 mM), and these effects were statistically significant at two concentrations of $K_2Cr_2O_7$ (0.10 and 0.25 mM) with effect sizes ranged from 4.00 to 3.31. Statistically significant decrease in LPO level after addition of melatonin/IPA/17β-estradiol to liver homogenates incubated with Cr(VI) in concentrations

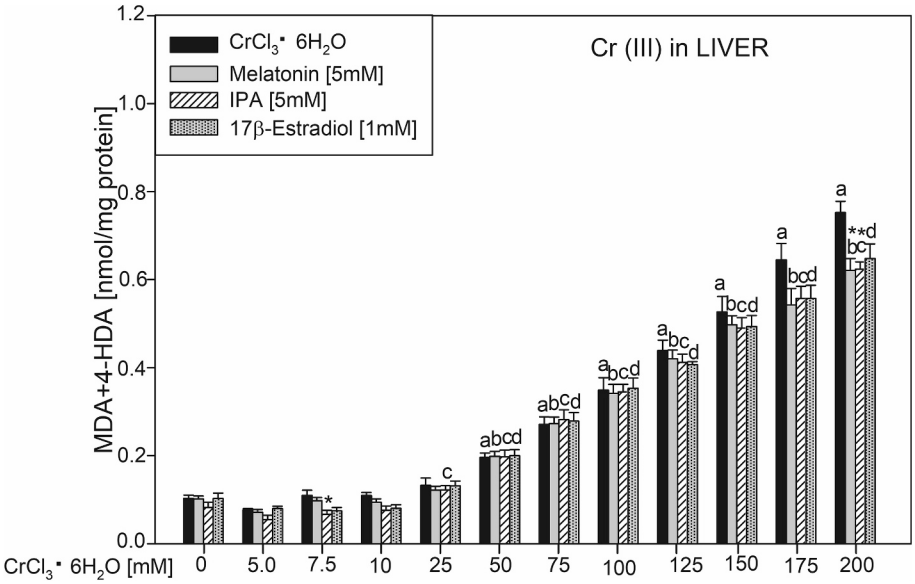

Fig. 7. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine liver homogenates, incubated in the presence of chromium(III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$) (5.0, 7.5, 10, 25, 50, 75, 100, 125, 150, 175 and 200 mM) alone (black bars), or $CrCl_3 \cdot 6H_2O$ with melatonin (5 mM) (grey bars) or $CrCl_3 \cdot 6H_2O$ with indole-3-propionic acid (IPA) (5 mM) (striped bars) or $CrCl_3 \cdot 6H_2O$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $CrCl_3 \cdot 6H_2O$ in the same concentration Tissue homogenates were prepared from pooled samples consisting of livers (left lateral lobe) collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

of 0.05 and 0.075 mM is without scientific significance. Unexpectedly, the addition of melatonin to liver homogenates together with Cr(VI) in its six highest concentrations (0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) or the addition of indole-3-propionic acid together with Cr(VI) in its five concentrations (0.75, 1.0, 2.5, 5.0 and 7.5 mM), or the addition of 17β-estradiol together with Cr(VI) in three highest concentrations (5.0, 7.5 and 10.0 mM), resulted in further increase in LPO levels (Fig. 8). The effect sizes for these interactions ranged from 2.05 to 10.05, indicating strong pro-oxidative responses.

Tissue homogenates were prepared from pooled samples consisting of livers (left lateral lobe) collected from 20 porcine animals. All conditions were tested in duplicate and repeated in three independent runs.

In Fig. 9, we present an overview of the key findings to provide a clear and concise summary of the main results.

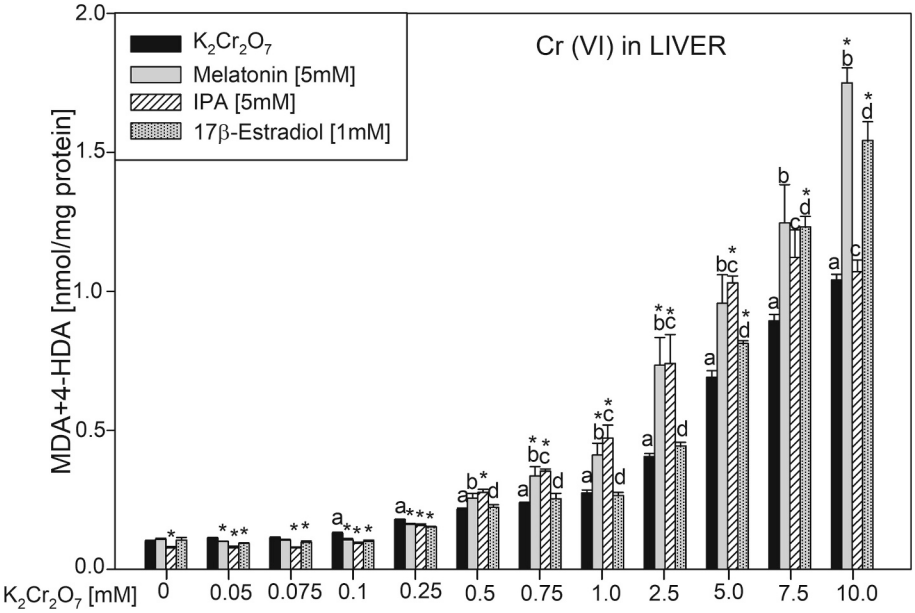

Fig. 8. Lipid peroxidation (LPO), measured as the level of malondialdehyde +4-hydroxyalkenals (MDA + 4-HDA), in porcine liver homogenates, incubated in the presence of potassium dichromate ($K_2Cr_2O_7$) (0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mM) alone (black bars), or $K_2Cr_2O_7$ plus melatonin (5 mM) (grey bars) or $K_2Cr_2O_7$ plus indole-3-propionic acid (IPA) (5 mM) (striped bars) or $K_2Cr_2O_7$ with 17β-estradiol (1 mM) (dot bars). LPO level is expressed in nmol/mg protein. Data are presented as mean ± SE (error bars). [a] $p < 0.05$ vs. control (without any substance); [b] $p < 0.05$ vs. melatonin (5 mM); [c] $p < 0.05$ vs. indole-3-propionic acid (5 mM); [d] $p < 0.05$ vs. 17β-estradiol (1 mM); * $p < 0.05$ vs. $K_2Cr_2O_7$ in the same concentration.

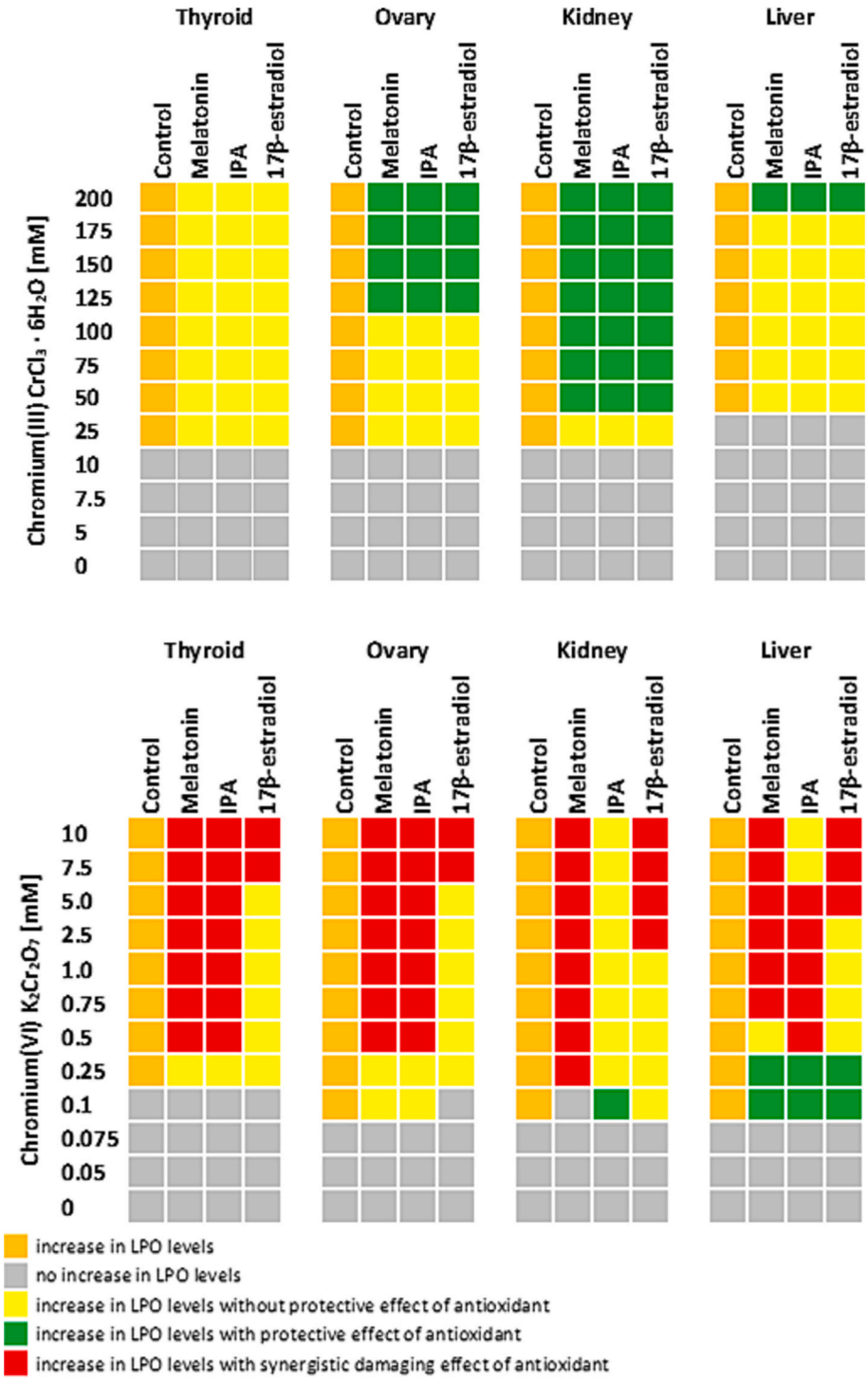

Fig. 9. Overview of the key findings.

4. Discussion

In the context of public health and environmental protection, it is important to understand the mechanisms of action of chromium and its interactions with potential protective factors, such as antioxidants. Chromium is a trace element essential for many organisms, excluding humans, in small amounts; however, its toxic forms pose significant health risks. Such knowledge can help to develop better risk management strategies and regulations to minimize exposure to this hazardous substance. The potential application of the current results may relate to health consequences of exposure of humans to large doses of chromium compounds, especially in the occupational environment, and to developing effective protective tools.

In this discussion, we analyze the current state of knowledge regarding the pro-oxidative effects of chromium, focusing on its two chemical forms, Cr(III) and Cr(VI), and the potential mechanisms through which these forms may affect human organs. Additionally, we have checked in the present study whether selected antioxidants protect against lipid peroxidation caused by Cr compounds in two endocrine glands and in two non-endocrine tissues.

It is important to consider that the differences observed in tissue response and antioxidant efficacy may stem from the fundamental

chemical and biological properties of the two chromium forms used—$CrCl_3 \cdot 6H_2O$ [Cr(III)] and $K_2Cr_2O_7$ [Cr(VI)]. Potassium dichromate [Cr(VI)] is highly soluble in water and dissociates into chromate and dichromate ions that can enter cells via anion channels due to their structural similarity to sulfate. Once inside the cell, Cr(VI) is reduced to Cr(III) through a series of redox reactions, during which multiple reactive oxygen species (ROS) are produced. This intracellular reduction is the primary mechanism of Cr(VI)-induced oxidative stress and cytotoxicity. In contrast, $CrCl_3 \cdot 6H_2O$ [Cr(III)] is less soluble and forms stable complexes in aqueous solutions, which limits its bioavailability and cellular uptake. Furthermore, Cr(III) does not readily participate in redox cycling under physiological conditions and does not directly produce ROS. These mechanistic differences are critical to understanding the more severe oxidative effects of Cr(VI) observed in our study, especially given that Cr(VI) was used at a 20-fold lower concentration than Cr(III), yet caused stronger oxidative damage. This highlights the markedly higher redox activity and toxicity of Cr(VI) compared to Cr (III).

Regarding the first issue, the results of the current study has confirmed that both Cr compounds are very strong prooxidative factor, at least for one biological macromolecule such as membrane lipids, in two endocrine and two non-endocrine tissues. A plausible mechanism underlying this effect involves Fenton-type reactions, in which Cr(III) directly or Cr(VI) indirectly catalyze the conversion of hydrogen peroxide (H_2O_2) into highly reactive hydroxyl radicals (•OH) (Shi et al., 1993). These radicals, characterized by a redox potential of approximately +2.8 V, possess an extremely strong oxidizing capacity and can readily accept electrons from a wide range of biological molecules (Luo et al., 1996). As a result, hydroxyl radicals aggressively attack cellular components by abstracting hydrogen atoms or adding to double bonds, triggering lipid peroxidation chain reactions, disrupting membrane integrity, and impairing mitochondrial function — changes that can ultimately lead to apoptosis or necrosis. The carcinogenic potential of Cr (III) may also be linked to this mechanism, particularly through oxidative DNA lesions such as 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a well-established marker of mutagenic damage (Shin et al., 2023). Similarly, another macromolecule, such as DNA (commercially available thymus DNA), was oxidatively damaged by Cr(III) (Burkhardt et al., 2001). Also Cr(VI) is able to generate •OH via Fenton reaction (Watwe et al., 2021).

Regarding the other issue, unfortunately, none of the antioxidants used were effective against Cr(VI)-induced oxidative damage in any of examined tissues. Additionally, melatonin and 17β-estradiol enhanced damaging effects of Cr(VI). In should be stressed that – as it was mentioned in the Introduction section – melatonin and also IPA usually effectively prevent oxidative damage to macromolecules caused by different prooxidative agents, carcinogen included (Rynkowska et al., 2021; Iwan et al., 2021; Gładysz et al., 2023; Stępniak and Karbownik-Lewińska, 2024). The lack of protective action of melatonin observed in the present study may result from mechanisms of Cr(VI) actions, which are different from oxidative mechanisms. It can also be taken into account that just prooxidative effects of Cr(VI) were so strong, that melatonin and other antioxidants were not able to prevent them. In turn, synergistic damaging effects of Cr(VI) plus antioxidant, such as melatonin and 17β-estradiol, is rather unexpected. This paradoxical response may be attributed to the dual role of these compounds as both antioxidants and, under certain conditions, prooxidants. Although melatonin primarily acts as a free radical scavenger, at high concentrations or in the presence of transition metals, its metabolites may participate in redox reactions that generate reactive oxygen species (Shin et al., 2023). Similarly, 17β-estradiol—through its catechol estrogen metabolites—can undergo redox cycling, promoting oxidative stress in specific redox environments. Both compounds may also alter the overall cellular redox state or interact directly with chromium ions, potentially modifying their redox behavior and enhancing toxicity. However, such an action of melatonin which makes oxidative damage even stronger when compared to the effect of a known prooxidant, was observed before. Namely, we observed in our earlier study that melatonin enhanced damaging effect of sodium chlorate ($NaClO_3$) in the thyroid, and hypothetical mechanisms, comprising prooxidative action of melatonin or bias issues associated with very high concentrations of both substances added together to the incubation medium, are described in our paper (Gładysz et al., 2023).

Concerning Cr(III), its damaging effects on membrane lipids were not prevented in the thyroid. Instead, in the ovary, in the kidney and – partially – in the liver, the antioxidants used in the present study did reveal protective action. This difference between the thyroid gland and other tissues may result from definitely high level of oxidative stress occurring in the thyroid, as oxidative reactions are required for normal thyroid hormone synthesis (Karbownik-Lewińska and Kokoszko-Bilska, 2012; Kościuszko et al., 2023; Stępniak and Karbownik-Lewińska, 2024). In the thyroid gland, where H_2O_2 is naturally generated as part of hormone biosynthesis, the presence of chromium compounds may intensify ROS production beyond the capacity of local antioxidant systems, thereby contributing to the oxidative damage even in thyroid tissue which thus not seem to be very susceptible to oxidative stress. In agreement with such an assumption, higher level of oxidative damage to macromolecules has been documented under basal conditions (corresponding to physiological conditions) in the thyroid gland comparing to other organs, such as the ovary (Rynkowska et al., 2020; Iwan et al., 2021), the kidney (Iwan et al., 2021; Stępniak et al., 2022a, 2022b), and the liver (Iwan et al., 2021; Stępniak et al., 2022a, 2022b; Gładysz et al., 2023). These observations can explain why the thyroid gland, when exposed to Cr(III), did not respond to protective effects of melatonin and other antioxidants, whereas the three other tissues did. Such conditions of oxidative damage caused in the thyroid by chromium being a very strong prooxidant may activate signaling pathways. Chromium-induced ROS can trigger mitogen-activated protein kinases (MAPKs), such as p38 and JNK, as well as the NF-κB pathway, all of which are involved in regulating inflammation, apoptosis, and cellular repair. Persistent activation of these pathways in a redox-disrupted environment may lead to chronic inflammation and abnormal cell proliferation (Brzezianska and Pastuszak-Lewandoska, 2011; Shen et al., 2023). In addition, chromium compounds, particularly Cr(VI), have been shown to affect PI3K/Akt and AMPK signaling, further contributing to mitochondrial imbalance and cell death mechanisms. Such processes are especially relevant to thyroid physiology and pathology, where redox regulation is tightly linked to hormone synthesis and proliferative control (Zhang et al., 2017; Haidar et al., 2023). It should be mentioned that in case of another macromolecule, such as DNA, when it was oxidatively damaged by Cr (III), melatonin revealed protective effects (Burkhardt et al., 2001).

Summarizing the above discussed issue, i.e. the difference between Cr(III) and Cr(VI) regarding protection against these compounds, it should be stated that Cr(VI) is much stronger prooxidant, at least with relation to membrane lipids. This statement is additionally supported by the fact that Cr(VI) was used in much lower concentrations than Cr(III) (maximal concentrations of 10 mM vs. 200 mM, respectively). Our observations are in agreement with well documented stronger carcinogenicity of Cr(VI) versus Cr(III) (IARC, 1990; IARC, 2012).

Whereas concentrations of Cr(III) and Cr(VI) were selected in the present study on the basis of their limited solubility, these concentrations should be discussed with relation to Cr concentrations found in living organisms, human organism included. In Chinese young adults blood concentration of chromium was found at the level of 1.83 ng/mL (equal to $3.519 \cdot 10^{-5}$ mM) (Feng et al., 2024), and in Polish healthy individuals it was 9.8 μg/L (equal to $1.8846 \cdot 10^{-4}$ mM), whereas in Polish population of oncological patients diagnosed with prostate cancer – 6.4 μg/L (equal to $1.231 \cdot 10^{-4}$ mM) (Drozdz-Afelt et al., 2024). The lowest concentrations applied in our in vitro studies were 5.0 mM for Cr (III), and 0.05 mM for Cr(VI). Therefore, concentrations of Cr used in the current study exceed several orders of magnitude those Cr concentrations which are normally found in human blood. Regarding Cr

concentrations in human organs (like the thyroid), they are not known. Thus, our in vitro model can mimic only the condition of extremely high exposure to Cr in the environment. These extremely high concentrations of Cr used in the present study can be the reason, why well known antioxidants did not reveal protective effects against Cr(VI) – induced damage and regarding Cr(III), the protective effects of the antioxidants were only partial in the ovary and the kidney, and very weak in the liver, and unfortunately in the thyroid no protective effect at all was observed. The latter observation regarding Cr(III) is of special importance because most structures in the organism, especially intracellular organelles, are usually exposed to Cr(III) (after reduction of Cr(VI)).

The practical significance of the current study should be discussed. Firstly, the observation that both Cr compounds, i.e. Cr(III) and Cr(VI), cause a significant increase of oxidative damage in tissue homogenate, strongly suggest that exposure to this heavy metal acting through various routes (inhalations, digestion, etc.) may directly affect human organs. Secondly, the protective effects of selected antioxidants against Cr(III)-induced damage observed in the ovary, kidney, and liver suggest that it is worth using appropriate supplementation with these antioxidants or, at least, keeping them at physiological levels (e.g., avoiding artificial light at night to maintain melatonin at physiological concentrations (Carlos et al., 2024; Reiter et al., 2024) or using postmenopausal replacement therapy with β-estradiol) (Tatarchuk et al., 2024). In turn, the lack of protective effects of these antioxidants in thyroid tissue against Cr(III)-induced damage suggests that chromium is a strong prooxidant and, possibly, a strong carcinogen for the thyroid, and that this field should be carefully explored in the future research.

The limitations of the present study are as follows. The most important is that the study was performed under in vitro conditions. It is important to note that we used porcine tissues, which are widely recognized as physiologically and anatomically similar to human tissues, particularly the thyroid gland, as it closely resembles the human thyroid in terms of size, weight, and structure. In addition, the porcine genome is more genetically similar to the human genome than that of commonly used rodent models, enhancing the relevance of our findings. However, our study was conducted using tissue homogenates rather than whole organs or intact cells. Therefore, our observations are limited to experimental conditions that differ from those in living organisms. Another limitation is that we have evaluated oxidative damage to only one macromolecule, i.e. membrane lipids, and that we have used only one method to measure oxidative damage to membrane lipids. Regarding protective effects of antioxidants, our study was limited to use only three selected antioxidative substances. Therefore, it is not excluded that other antioxidants would be more spectacular in revealing protective effects against Cr-inducted oxidative damage.

At present, the biological significance of our findings is uncertain, as they require validation in vivo. The use of tissue homogenates does not allow us to observe key cellular processes related to chromium metabolism, which are essential for understanding its full toxic potential. Nonetheless, our data demonstrate significant oxidative damage caused by both Cr(III) and Cr(VI), particularly in the thyroid, suggesting that chromium exposure poses a considerable risk. This is supported by existing literature, which indicates that the real risk of chromium-induced damage in humans is most likely to occur in occupational settings where exposure levels are high. Further studies are necessary to gain a clearer understanding of the biological impact and potential health risks associated with chromium exposure in humans, as well as the protective effects of melatonin.

5. Conclusions

Both Cr compounds, i.e. Cr(III) and Cr(VI), cause significant oxidative damage to membrane lipids in endocrine and non-endocrine tissues. These strong prooxidative effects can contribute, besides DNA damage, to carcinogenic action of chromium. While melatonin shows protective effects against Cr(III)-induced oxidative damage in the ovary, kidney, and liver, the thyroid does not respond to the antioxidative properties of this indoleamine. This may be attributed to the inherently oxidative nature of the thyroid, where relatively high levels of oxidative damage occur even under normal physiological conditions. Neither melatonin nor other antioxidants demonstrate protective effects against Cr(VI)-induced oxidative damage in the thyroid or in other tested tissues, further supporting the conclusion that Cr(VI) is more toxic. Our findings indicate stronger prooxidative effects of chromium(VI) comparing to chromium(III) to membrane lipids, especially in the thyroid, which confirms that exposure to chromium(VI) is more dangerous, also regarding the potential contribution to the process of carcinogenesis. Low effectiveness of antioxidants to prevent Cr-induced oxidative damage, especially in the thyroid, underscores the need for further research to identify more effective protective strategies.

CRediT authorship contribution statement

Aleksandra K. Gładysz: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Jan Stępniak:** Visualization, Software, Investigation, Formal analysis, Data curation. **Laura López-Pingarrón:** Validation, Conceptualization. **Joaquin J. Garcia:** Validation, Methodology, Conceptualization. **Małgorzata Karbownik-Lewińska:** Writing – review & editing, Validation, Supervision, Methodology, Data curation, Conceptualization.

Funding

This research was funded by statutory funds from the Medical University of Lodz, Poland (503/1–107-03/503–11-001).

Declaration of competing interest

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

Acknowledgments

This article is a revised and expanded version of a poster entitled “No responsiveness of the thyroid to protective effects of melatonin against chromium-induced oxidative damage – comparison to the ovary and non-endocrine tissues”, which was presented at the 46th Annual Meeting of the European Thyroid Association, Athens, Greece, 7th-10th September 2024 (Endocrine Abstracts (2024) 101 PS3-24-08 | DOI: https://doi.org/10.1530/endoabs.101.PS3-24-08).

Data availability

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author (malgorzata.karbownik-lewinska@umed.lodz.pl).

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