Iron is an essential microelement for the proper functioning of many organs, among others it is required for thyroid hormone synthesis. However, its overload contributes to the increased formation of reactive oxygen species via Fenton chemistry \((\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-)\), and it is potentially toxic. Individual organs/tissues are affected differently by excess iron. The excessive absorption of iron with subsequent deposition in various organs is associated with diseases such as hemochromatosis. Such an iron deposition also occurs in the thyroid gland where it can disturb thyroid hormone synthesis. In turn, melatonin is an effective antioxidant, which protects against oxidative damage. This study aims to check if lipid peroxidation resulting from oxidative damage to membrane lipids, is caused by Fenton reaction substrates, and if protective effects of melatonin differ between the thyroid and various non-endocrine porcine tissues (liver, kidney, brain cortex, spleen, and small intestine). To mimic the conditions of iron overload, \(\text{Fe}^{2+}\) was used in extremely high concentrations. Homogenates of individual tissues were incubated together with Fenton reaction substrates, i.e., \(\text{FeSO}_4\) (9.375, 18.75, 37.5, 75, 150, 300, 600, 1,200, 1,800, 2,100, 2,400, 3,000, 3,600, 4,200, and 4,800 \(\mu\text{M}) + \text{H}_2\text{O}_2\) (5 mM), either without or with melatonin (5 mM). The concentration of malondialdehyde+4-hydroxyalkenals (MDA+4-HDA), as the LPO index, was evaluated by a spectrophotometrical method. Fenton reaction substrates increased concentrations of LPO products in all chosen tissues. However, in the thyroid, compared to non-endocrine tissues, the damaging effect was generally weaker, it was not observed for the two lowest concentrations of iron, and the LPO peak occurred with higher concentrations of iron. Melatonin reduced experimentally induced LPO in all examined tissues (without differences between them), and these protective effects did not depend on iron concentration. In conclusion, membrane lipids in the thyroid compared to those in non-endocrine tissues are less sensitive to pro-oxidative effects of Fenton reaction substrates, without differences regarding protective effects of melatonin.

Keywords: Fenton reaction, lipid peroxidation, oxidative damage, thyroid, melatonin
INTRODUCTION

Iron is a critical micronutrient in mammalian organisms, and it is a cofactor for many biological reactions. As a redox-active transition metal, it acts as an electron donor and acceptor in a plethora of fundamental cellular processes, such as oxygen transport, DNA and RNA synthesis, cell proliferation, and energy metabolism. In mammals, all iron is obtained from digestion of food where it exists largely in two forms, i.e., non-heme iron in the relatively non-toxic ferric form (Fe\(^{3+}\)), derived mainly from plant-based foods and animal products, and heme iron in a more reactive and toxic form of ferrous ion (Fe\(^{2+}\)), derived from the breakdown of hemoglobin and myoglobin in animal tissues. Despite the fact that non-heme iron is usually much less well absorbed than heme iron, it constitutes most of the human dietary iron, even in meat-eating populations (Hurrell and Egli, 2010; Abbaspour et al., 2014).

The adult well-nourished human body contains approx. 3–5 g of iron. Under healthy conditions, most of it is bound to some form of a ligand. It is estimated that up to 80% of body iron is present in red blood cell hemoglobin, while another approximately 20% is stored in the form of ferritin and heme within hepatocytes and macrophages of the liver and spleen (Hentze et al., 2004). The remaining small amount (<1%) of the iron in the human body is found in various proteins such as cytochromes (Lane et al., 2015).

This iron compartmentalization is crucial for organism homeostasis since “free” (catalytically active) iron can react with hydrogen peroxide (H\(_2\)O\(_2\)) via the Fenton chemistry (Fe\(^{3+}\) + H\(_2\)O\(_2\) → Fe\(^{2+}\) + OH\(^-\) + O\(_2\)), the reaction directly related to the oxidative stress. The hydroxyl radical (‘OH) produced during this process is one of the most powerful oxidizing agents and can react—at a diffusion-controlled rate—with practically all subcellular components in the organism (Koppenol and Hider, 2019). The hydroxyl radical can react and consequently damage all biological macromolecules (lipids, proteins, nucleic acids, and carbohydrates) leading to cell dysfunction and death. It has been observed that the harmful effects of ‘OH may contribute to pathogenesis of cancer, atherosclerosis, or neurodegenerative diseases (Lipinski, 2011).

Improper iron compartmentalization and subsequent elevated levels of oxidative stress are often the results of accumulation and overload of this element. The human organism does not have any specific mechanism to remove excess iron. Iron elimination occurs only via non-regulated ways such as cell desquamation or bleeding; hence, its concentration is controlled only at the level of iron absorption. A number of diseases and pathological factors can lead to iron overload. The most common causes of iron overload are overconsumption of iron when it is in excess in the environment (Aranda et al., 2016), congenital disturbances of iron metabolism (hemochromatosis) (Girelli et al., 2021), or secondary hemochromatosis resulting from repeated blood transfusions in patients with beta-thalassemia (Ali et al., 2021) or with sickle cell anemia (Badawy et al., 2016). The growing literature demonstrates that also chronic hepatitis C may cause iron overload (Zou and Sun, 2017).

Iron overload and enhanced oxidative stress can lead to adverse effects in all tissues; however, it can be, especially severe in the thyroid when we take into consideration the “oxidative nature” of this gland. Hydrogen peroxide (one of the Fenton reaction substrates) is indispensable for biosynthesis of thyroid hormones, in which it serves as an electron acceptor at each stage of this process (Song et al., 2007). Therefore, H\(_2\)O\(_2\) is generated in the thyroid in high concentrations which, in turn, can create favorable conditions for the Fenton reaction to occur.

Until now, Fenton reaction substrates were used very commonly to experimentally induce oxidative damage to macromolecules in different tissues (e.g., ovary, thyroid, and skin) with very high Fe\(^{2+}\) concentrations (Rynkowska et al., 2020, 2021), equal to these used in the present study.

This study aims to check if lipid peroxidation (LPO) resulting from oxidative damage to membrane lipids caused by Fenton reaction substrates and protective effects of melatonin differs between the thyroid and various non-endocrine porcine tissues (liver, kidney, brain cortex, spleen, and small intestine). While it is currently known that certain hormones can be synthesized by and secreted from nontraditional endocrine organs, still specific organs classified as endocrine glands are separated. They are traditionally defined as ductless glandular structures that release their hormonal secretions into the extracellular space, from where they can eventually enter the bloodstream; examples of classic endocrine glands are the thyroid gland, the pituitary gland, the adrenal gland, and the ovary, etc. (Hsiao et al., 2017; Holt et al., 2021). Regarding tissues, such as liver, kidney, brain cortex, spleen, and small intestine, which are used in the present study, do not fulfill the criteria of an endocrine gland; therefore, they can be called non-endocrine tissues. In fact, experimental and clinical studies to describe various processes occurring in endocrine versus non-endocrine tissues have been published before (McCarthy et al., 2013; Xie et al., 2018). In the present study, we have chosen these non-endocrine tissues, which play important roles in iron absorption (intestine) and accumulation (liver and spleen) or iron overload. Iron overload plays a crucial role in the pathogenesis of diseases developing in the tissues, such as brain cortex (Belaidi and Bush, 2016) or kidney (Naknishii et al., 2019). To simulate conditions of iron overload, Fe\(^{2+}\) ion was used in the present study in extremely high concentrations.

MATERIALS AND METHODS

Ethical Considerations

In accordance with the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes from 15 January, 2015 (which implements Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes)—the use of animals to collect organs or tissues does not require the approval of the Local Ethics Committee. These animals are only subjected to registration by the center in which the organs or tissues were taken. Additionally, we did not use experimental animals; instead, porcine tissues were collected from animals at a slaughterhouse during the routine process of slaughter carried out for consumption.
Chemicals
All chemicals applied in the study are of analytical grade and come from the following commercial sources: melatonin, ferrous sulfate (FeSO₄), and hydrogen peroxide (H₂O₂)—Sigma (St. Louis, MO, United States); the ALDetect Lipid Peroxidation Assay Kit—Enzo Life Sciences, Inc. (Zandhoven, Belgium).

Tissue Collection
Porcine tissues were collected from pigs slaughtered at the local slaughterhouse. Animals were treated according to the European Community Council Regulation (CE1099/2009) 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., thyroid, spleen, liver (from left lateral lobe), brain cortex, small intestine (jejunum and ileum), and kidney (renal cortex), were collected, frozen on solid CO₂, and stored at −80°C till experimental procedure. Each experiment was repeated three times.

Incubation of Tissue Homogenates
We homogenized individual tissues (thyroid, spleen, liver, brain cortex, small intestine, and kidney) in ice-cold 20 mM Tris–HCl buffer (pH 7.4) (10%, w/v), and then tissues were incubated (37°C, 30 min) in the presence of FeSO₄ (9.375, 18.75, 37.5, 75, 150, 300, 600, 1,200, 1,800, 2,100, 2,400, 3,000, 3,600, 4,200, and 4,800 µM)+H₂O₂ (5 mM) without melatonin or with the addition of melatonin in its highest achievable in vitro concentration (due to limited solubility), i.e., 5 mM. After incubation, the samples were cooled on ice to stop the reaction.

Assay of Lipid Peroxidation
The ALDetect Lipid Peroxidation Assay Kit was used to measure concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA), being the index of lipid peroxidation. To obtain supernatants, samples of homogenates were centrifuged (5,000×g, 10 min, 4°C). Next, 200 µL of supernatant was mixed with methanol: acetonitrile (1:3, v/v) solution (650 µL), containing N-methyl-2-phenylindole as a chromogenic reagent, and after that the sample was vortexed. In the next step, methanesulfonic acid (150 µL, 15.4 M) was added, and then incubation was conducted again (45°C, 40 min). The product of the reaction between MDA+4-HDA and N-methyl-2-phenylindole was a chromophore, which was measured by the spectrophotometrical method (at an absorbance of 586 nm) with the use of a 4-hydroxynonenal solution (10 mM) as the standard. The amount of protein was measured with the use of Bradford’s method (the standard was bovine albumin) (Bradford, 1976). We expressed the level of lipid peroxidation as the concentration of MDA+4-HDA (nmol)/mg protein.

Statistical Analyses
We used the following statistical tests: one-way analysis of variance (ANOVA), followed by the Student–Neuman–Keuls test, or an unpaired t-test. p < 0.05 was accepted as the level of statistical significance. We presented the results as means ± SE.
RESULTS

LPO levels under basal conditions were higher in the brain cortex and spleen than in other examined tissues (Figure 1). Incubation with melatonin did not change basal levels of LPO significantly in any of the examined tissues (Figure 1).

Fenton reaction substrates increased LPO levels in all examined tissues except for the thyroid—except for the thyroid—in all used concentrations of Fe^{2+} (Figure 2). Namely, in the thyroid, the damaging effect was not observed for the two lowest concentrations of iron (9.375 and 18.75 µM) (Figure 2). Additionally, compared to that in non-endocrine tissues, Fenton reaction-induced damage in the thyroid was weak for the increasing Fe^{2+} concentrations up to 1,800 µM; although with the increasing Fe^{2+} concentrations above 1,800 µM, these differences gradually disappeared (Figure 3A). Importantly, the LPO peak occurred "later," i.e., with higher concentrations of iron, whereas the LPO peak was observed in the thyroid at an

FIGURE 2 | Concentrations of lipid peroxidation products (MDA + 4-HDA) in the homogenates of porcine tissues (thyroid, brain cortex, spleen, liver, kidney, and intestine), incubated without any substance (control; white bars) or with melatonin (5 mM) (gray bars) or with Fe^{2+} (9.375, 18.75, 37.5, 75, 150, 300, 600, 1,200, 1,800, 2,100, 2,400, 3,000, 3,600, 4,200, and 4,800 µM) + H_2O_2 (5 mM) (black bars) or with Fe^{2+} (9.375, 18.75, 37.5, 75, 150, 300, 600, 1,200, 1,800, 2,100, 2,400, 3,000, 3,600, 4,200, and 4,800 µM) + H_2O_2 (5 mM) with melatonin (5 mM) (striped bars). p < 0.05 vs. respective control (without any substance); *p < 0.05 vs. Fe^{2+} in the same concentration. Statistical differences between Fe^{2+} + H_2O_2 + melatonin (striped bars) vs. melatonin (gray bars) in particular tissues are not marked.
Fe²⁺ concentration of 2,400 μM. In the liver, the LPO peak was recorded at an Fe²⁺ concentration of 600 μM (four times lower than that in the thyroid), in the spleen—at an Fe²⁺ concentration of 1,200 μM (two times lower than that in the thyroid), and in the kidney, brain cortex, and intestine—at an Fe²⁺ concentration of 1,800 μM (lower by 25% than that in the thyroid) (Figures 3A,B).

Melatonin reduced LPO levels induced by Fenton reaction substrates in all tissues; these protective effects did not significantly differ between tissues, and the protective effects of melatonin did not depend on iron concentration (Figure 2).

**DISCUSSION**

In our earlier studies, we have observed that the thyroid gland is less sensitive to the damaging effects of iron than the ovary which is also an endocrine gland (Rynkowska et al., 2020). We have also evaluated the damaging effect of KIO₃ and have found that KIO₃-induced LPO is significantly lower in the thyroidal tissue than non-endocrine tissues and the ovary (Iwan et al., 2021a). Our present results confirm the results cited previously and clearly indicate that membrane lipids in the thyroid are less sensitive to pro-oxidative events occurring in this gland. Such findings suggest that the thyroid gland has developed more effective (than other tissues) protective mechanisms for maintaining redox homeostasis.

The thyroid gland constitutes a specific organ in such a sense that oxidative reactions are absolutely required for thyroid hormone synthesis. Hydrogen peroxide is generated for the needs of thyroid hormone biosynthesis by NADPH oxidases, especially by dual oxidase 2 (DUOX2) (Pachucki et al., 2004), and it is synthesized in a higher amount than this which is required for proper iodide incorporation into thyroid hormones. This apparent discrepancy may be associated with the relatively high Michaelis–Menten constant of thyroperoxidase (TPO, a clue enzyme in thyroid hormone synthesis) for H₂O₂, resulting in relatively higher concentrations of H₂O₂ (as a substrate) indispensable for proper activation of TPO (Song et al., 2007). Interestingly, in our experimental study on sexual dimorphism, we have found that thyroid follicular cells from female thyroids are exposed to higher H₂O₂ concentrations than male thyroids, which probably results from the higher activity of NOX/DUOX enzymes in the female thyroid (Stepniak et al., 2018), and it is in agreement with the assumption that higher prevalence of thyroid diseases in women is associated with stronger oxidative stress. H₂O₂ is primarily a toxic compound with a relatively long half-life which, as a non-polar molecule, is able to diffuse across biological membranes. Because it is an oxidizing agent, it is in power to induce damage to biological macromolecules such as DNA, lipids, and proteins, consequently leading to mutagenesis and apoptosis (Song et al., 2007). Therefore, to ensure the proper functioning of the thyroid, sources of H₂O₂...
and other potentially pro-oxidative compounds need to be under strict control. Therefore, some adaptive mechanisms have developed in the thyroid, such as specific localization of potentially dangerous processes (e.g., production of H$_2$O$_2$) and compartmentalization of potentially damaging elements (Szanto et al., 2019), as well as formation of antioxidants and ROS-scavenging systems. Regarding the last mentioned adaptive mechanism, responsible for this are mainly redox-controlling enzymes such as peroxiredoxins, glutathione peroxidases, and catalase. As catalase is rather weakly expressed in the thyroid, H$_2$O$_2$ degradation is carried out in this organ mainly by the enzymes from the glutathione peroxidases family (Song et al., 2007; Schweizer et al., 2008). This enzymatic antioxidant system is additionally supported by the contribution of molecules such as ascorbic acid, polyphenolic compounds, coenzyme Q10, β-carotene, retinol, and tocopherol (Kochman et al., 2021).

Under basal conditions, these thyroid systems effectively fulfill their antioxidant role. In our both studies (the present and the previous by Iwan et al., 2021a), we have observed that the basal level of LPO in the thyroid gland is not higher than that in other tissues. However, under any pathological condition associated either with endogenous abnormalities or exposure to exogenous pro-oxidants, this redox balance may be disrupted and, in consequence, the level of oxidative damage can be increased, resulting in various diseases (Valko et al., 2007), such as cancer (Ziech et al., 2011). A phenomenon that can definitely lead to disruption of redox homeostasis in the thyroid is iron overload. Consistently, we have shown earlier that Fe$^{2+}$, than H$_2$O$_2$, damages more strongly both nuclear DNA and membrane lipids (Stepniak et al., 2013). Hepatic iron concentration in patients with asymptomatic and symptomatic hemochromatosis is approximately 8 to 15 times higher than that in healthy subjects—36–550 μmol/g (36–550 mM) in dry weight tissue (Bacon et al., 2011). Whereas in healthy individuals blood iron concentration is below 150 μg/dl, it is 150–300 μg/dl (0.026–0.053 mM) in subjects with hemochromatosis (Bacon et al., 2011). To mimic conditions of iron overload, in the present study, we have used the range of iron concentrations from 0.009 to 4.8 mM, which corresponds to the aforementioned.

In case of disrupted redox homeostasis, different exogenous antioxidants can be considered to be used. One of the most famous antioxidants is melatonin (N-acetyl-5-methoxytryptamine), which is produced in the organism mostly by the pineal gland, but it is also available as an exogenous substance. This molecule is confirmed to be a very effective antioxidant and free radical scavenger which prevents oxidative damage not only in the thyroid (Lewinski and Karbownik, 2002; Karbownik et al., 2005; Kokoszko-Bilska et al., 2014; Zasada and Karbownik-Lewinska, 2015; Iwan et al., 2021a, 2021b; Stepniak et al., 2021) but also in many other tissues and organs (Karbownik et al., 2000, 2001a, 2001b, 2006; Gitto et al., 2001; Osuna et al., 2002; Mogulkoc et al., 2006; Reiter et al., 2017). In the current study, we have observed that melatonin reduced LPO induced by Fenton reaction substrates in all examined tissues, and this protective effect was independent of iron concentration. These results confirm a well-known fact that melatonin is very effective in protecting against even these effects of iron which are caused by its extremely high concentrations (corresponding to those iron concentrations found in patients with hemochromatosis). It should be underlined that in our study melatonin did not reduce LPO levels below the physiological threshold in control groups of any examined tissue. This additionally supports the statement that this indoleamine is a distinctive antioxidant, which does not affect physiological processes, whereas it is effective under conditions with additional oxidative abuse.

Our study is probably the first attempt to compare the damaging effects of high iron concentrations in the thyroid and in various non-endocrine tissues under in vitro conditions. It should be stressed that the lower sensitivity of thyroidal membrane lipids, compared to that of membrane lipids in non-endocrine tissues, in response to high iron concentrations, is similar to previously observed pro-oxidative effects of KIO$_3$ (Iwan et al., 2021a). Therefore, we suppose that membrane lipids (and possibly other biological macromolecules) in the thyroid reveal higher resistance to any external pro-oxidative agent than those in other tissues. These differences in sensitivities of given tissues to pro-oxidative factors result presumably from oxidative/antioxidative processes which normally (under physiological conditions) occur in the tissue. Because oxidative processes occur in the thyroid gland at a high level (Karbownik-Lewinska and Kokoszko-Bilska, 2012), this endocrine gland has developed an adaptive mechanism and, therefore, it is probably better prepared to protect against damaging effects of pro-oxidants.

This study has some limitations. First, our study was conducted using tissue homogenates, so our results may not be directly extrapolated under in vivo conditions, especially into the human organism. However, some directions of action of a given agent observed in vitro should be taken into account also under in vivo conditions. Second, we used only one experimental method to measure oxidative damage to membrane lipids, i.e., a spectrophotometric assay, evaluating lipid peroxidation by measuring MDA + 4-HDA. Although this experimental method has some disadvantages, it is very reliable and is commonly used in studies on oxidative stress.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

Conceptualization: MK-L; methodology: MK-L; software: JS; validation: MK-L; formal analysis: JS and AR; investigation: AR; writing—original draft preparation: JS; writing—review and editing: MK-L and JS; visualization: JS and AR; supervision: MK-L.

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Stepniak, J., Lewinski, A., and Karbownik-Lewinska, M. (2018). Sexual Dimorphism of NADPH Oxidase/H2O2 System in Rat Thyroid Cells; Effect of Exogenous 17β-Estradiol. *Ijms* 19, 4063. doi:10.3390/ijms19124063

Szanto, I., Pusztaféri, M., and Mavromati, M. (2019). H2O2 Metabolism in Normal Thyroid Cells and in Thyroid Tumorigenesis: Focus on NADPH Oxidases. *Antioxidants* 8, 126. doi:10.3390/antiox8050126

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., and Telser, J. (2007). Free Radicals and Antioxidants in Normal Physiological Functions and Human Disease. *Int. J. Biochem. Cell. Biol.* 39, 44–84. doi:10.1016/j.biocel.2006.07.001

Xie, J., Li, Z., and Tang, Y. (2018). Successful Management of Multiple-Systemic Langerhans Cell Histiocytosis Involving Endocrine Organs in an Adult. *Med. Baltim.* 97, e11215. doi:10.1097/md.0000000000011215

Zasada, K., and Karbownik-Lewinska, M. (2015). Comparison of Potential Protective Effects of Melatonin and Propylthiouracil against Lipid Peroxidation Caused by Nitrobenzene in the Thyroid Gland. *Toxicol. Ind. Health.* 31, 1195–1201. doi:10.1177/0748233713491799

Ziech, D., Franco, R., Pappa, A., and Panayiotidis, M. I. (2011). Reactive Oxygen Species (ROS)--induced Genetic and Epigenetic Alterations in Human Carcinogenesis. *Mutat. Research/Fundamental Mol. Mech. Mutagen.* 711, 167–173. doi:10.1016/j.mrfmmm.2011.02.015

Zou, D.-M., and Sun, W.-L. (2017). Relationship between Hepatitis C Virus Infection and Iron Overload. *Chin. Med. J. Engl.* 130, 866–871. doi:10.4103/0366-6999.202737

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