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A dissertation for Ph.D. degree

Title: **Potential protective effects of indole substances against oxidative damage to membrane lipids caused by KIO_3 in porcine thyroid – *in vitro* studies**.

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Dissertation content:

1. **Iwan P, Stepniak J, Karbownik-Lewinska M. Melatonin reduces high levels of lipid peroxidation induced by potassium iodate in porcine thyroid. Int J Vitam Nutr Res. 2021 Jun;91(3-4):271-277. doi: 10.1024/0300-9831/a000628. Epub 2019 Dec 17. PMID: 31842692.**
   IF: 2.560 , ministerial points: 100

2. **Iwan P, Stepniak J, Karbownik-Lewinska M. Cumulative Protective Effect of Melatonin and Indole-3-Propionic Acid against KIO_3-Induced Lipid Peroxidation in Porcine Thyroid. Toxics. 2021 Apr 21;9(5):89. doi: 10.3390/toxics9050089. PMID: 33919052; PMCID: PMC8143077.**
   IF: 4.472, ministerial points: 70

3. **Iwan P, Stepniak J, Karbownik-Lewinska M. Pro-Oxidative Effect of KIO_3 and Protective Effect of Melatonin in the Thyroid-Comparison to Other Tissues. Life (Basel). 2021 Jun 21;11(6):592. doi: 10.3390/life11060592. Erratum in: Life (Basel). 2022 Jul 07;12(7): PMID: 34205777; PMCID: PMC8234753**.
   IF: 3.253, ministerial points: 70

Total IF: 10.285
Total ministerial points: 240

ABSTRACT

Introduction

Reactive oxygen species (ROS) and free radicals participate in metabolic processes. Under physiological conditions, there is a balance between production and detoxification of ROS. Any imbalance between these processes may result in different pathological conditions.

The thyroid gland is an organ of "oxidative nature", in which oxidative processes are necessary for example for thyroid hormone biosynthesis. For this reason, the thyroid gland is characterized by high level of oxidative stress, which – in response to additional oxidative abuse caused by exogenous or endogenous pro-oxidants – may lead to different thyroid diseases, including thyroid cancer.

Iodine is a micronutrient playing an essential role in thyroid hormone synthesis. Under normal iodine supply, calculated physiological iodine concentration in the thyroid is approx. 9 mM. Its deficiency may lead to goiter formation and – in case of severe iodine deficiency – to hypothyroidism, and in pregnant patients – to impaired infant neurobehavioral development. Correction of iodine deficiency may ensure adequate thyroid hormone synthesis, decrease the prevalence of goiter and shift thyroid cancer subtypes towards a less malignant form.

To eliminate iodine deficiency, iodized salt is used in most countries in iodine prophylaxis. Programs of salt iodization are based on the use of either potassium iodide (KI) or potassium iodate (KIO_3). These two main iodine compounds have different pro- and antioxidative properties. KI is less reactive whereas KIO_3 reveals stronger oxidizing properties. Despite this, KIO_3 has GRAS ("generally recognized as safe") status given by Food and Drug Administration (FDA). However, KIO_3 was found to reveal oxidative damage to macromolecules under certain experimental in vitro conditions.

Indole substances, with their main representative melatonin (5-methoxy-N-acetyltryptamine), are very effective antioxidants and free radical scavengers. Indole-3-propionic acid (IPA) is another indole substance, similar in structure and biochemical properties to melatonin. Both are safe and it is generally accepted that they do not reveal side effects.

Melatonin has been shown to prevent experimentally-induced oxidative damage to macromolecules in different tissues, among others in the thyroid gland. This substance also inhibits thyroid growth processes. For this reason it should be considered as a potential protective agent against thyroid diseases, thyroid cancer included.

Aims of the study

The first aim of the study was to evaluate potential protective effects of melatonin against oxidative damage to membrane lipids (lipid peroxidation) induced by either KIO_3 or KI in porcine thyroid homogenates (original paper 1: **Iwan P, Stepniak J, Karbownik-Lewinska M. Melatonin reduces high levels of lipid peroxidation induced by potassium iodate in porcine thyroid. Int J Vitam Nutr Res. 2021 Jun;91(3-4):271-277**).

The subsequent aim was to analyze the protective effect of indole-3-propionic acid (IPA) and the cumulative effect of melatonin+IPA (in their highest achievable *in vitro* concentrations resulting from their limited solubility) against lipid peroxidation caused by KIO_3 in porcine thyroid homogenates (original paper 2: **Iwan P, Stepniak J, Karbownik-Lewinska M. Cumulative Protective Effect of Melatonin and Indole-3-Propionic Acid against KIO_3-Induced Lipid Peroxidation in Porcine Thyroid. Toxics. 2021 Apr 21;9(5):89**).

At the last step protective effects of melatonin against KIO_3-induced oxidative damage to membrane lipids in the thyroid were compared to those ones found in various other porcine tissues, such as the ovary, the spleen, the liver, the brain, the small intestine, and the kidney (original paper 3: **Iwan P, Stepniak J, Karbownik-Lewinska M. Pro-Oxidative Effect of KIO_3 and Protective Effect of Melatonin in the Thyroid-Comparison to Other Tissues. Life (Basel). 2021 Jun 21;11(6):592. Erratum in: Life (Basel). 2022 Jul 07;12(7)**).

Materials and methods

The studies were performed in *in vitro* conditions using homogenates of porcine tissues (the thyroid gland (in all original papers: 1, 2 and 3), and additionally the ovary, the spleen, the liver, the brain, the small intestine, and the kidney (original paper 3)).

The concentrations of KI (500; 250; 100; 50 mM), KIO_3 (200; 100; 50; 25; 20; 18.75; 17.5; 16.25; 15; 13.75; 12.5; 11.25; 10; 8.75; 7.5; 5.0; 2.5; 1.25 mM), melatonin (5.0; 2.5; 1.25; 1.0; 0.625 mM), 17ß-estradiol (1.0 mM) and IPA (10; 7.5; 5.0; 2.5; 1.25; 0.625 mM) were chosen on the basis of the results of previous studies (Karbownik et al., J Cell Biochem 2003, 90, 806–811; Karbownik et al., J Cell Biochem 2005, 95, 131–138; Milczarek et al., Thyroid Res 2013, 6, 10; Karbownik-Lewinska et al., Eur J Nutr 2015, 54, 319–323; Stepniak et al., Syst Biol Reprod Med 2016, 62, 17–21).

The concentrations of malondialdehyde+4-hydroxyalkenals (MDA+4-HDA), as an index of lipid peroxidation, were measured in homogenates spectrophotometrically with the use of ALDetect Lipid Peroxidation Assay Kit.

The data were statistically analyzed, using a one-way analysis of variance (ANOVA), followed by the Student-Neuman-Keuls' test, or using an unpaired t-test. Statistical significance was determined at the level of $p < 0.05$. Results are presented as means ± SE.

Results

Original paper 1

Potassium iodide (KI), in all used concentrations (i.e. 500; 250; 100; 50 mM), did increase lipid peroxidation in concentration-dependent manner. Potassium iodate (KIO_3) did increase lipid peroxidation in all used concentrations (i.e. 200; 100; 50; 25; 10; 5.0; 2.5 mM) with the strongest damaging effect to membrane lipids at concentrations of 10 mM and 25 mM. When thyroid homogenates were incubated in the presence of either KI or KIO_3 plus melatonin (5.0 mM), significant reduction of lipid peroxidation was observed only when KIO_3 was used at the concentration of 10 mM.

As in the above experiment melatonin did not protect against KI-induced lipid peroxidation, in next steps we used only KIO_3.

In the subsequent experiment we decided to use additional concentrations of KIO_3 (i.e. 20; 15; 7.5; 1.25 mM) to clarify unexpected results obtained in the first step of experiments. After using additional concentrations of KIO_3, the strongest damaging effect to membrane lipids was observed for KIO_3 concentration of around 15 mM with the highest LPO level confirmed for concentrations of 15 mM and of 20 mM.

Melatonin reduced, in concentration-dependent manner, KIO_3-induced lipid peroxidation, but only when this pro-oxidant was used at concentrations of 10 mM (melatonin was protective in concentrations of 5.0 mM and 2.5 mM) or of 7.5 mM (melatonin was protective in concentrations of 5.0; 2.5; 1.25; 1.0 mM); it should be recalled that KIO_3 concentrations of 10 mM and of 7.5 mM correspond to physiological iodine concentrations in the thyroid (calculated as approx. 9 mM).

The incubation of porcine thyroid homogenates in the presence of melatonin only (in concentrations of 5.0; 2.5; 1.25; 1.0; 0.625 mM) did not change the basal lipid peroxidation.

In the present study we decided to compare protective effects of melatonin with a well-known endogenous antioxidant – 17ß-estradiol. 17ß-estradiol, used at the concentration of 1.0 mM, being the highest possible concentration to be used in our model (due to its limited

solubility), did not cause any protective effects against KIO_3-induced lipid peroxidation, whereas melatonin, used in the same concentration of 1.0 mM, reduced lipid peroxidation induced by KIO_3 (7.5 mM).

Original paper 2

In the Experiment I, IPA (10 mM) and melatonin (5.0 mM), applied separately, reduced KIO_3-induced lipid peroxidation when this pro-oxidant was used at concentrations of 10 mM, 7.5 mM or 5.0 mM. However, in Experiment II with the use of additional concentrations of KIO_3, IPA revealed protective effects against higher concentration of KIO_3 (16.25 mM) than melatonin did (KIO_3 in the concentration of 15 mM).

Additionally, protective effects of IPA were stronger than those of melatonin against oxidative damage caused by KIO_3 at concentrations of 13.75 mM or lower.

The most important observation is that melatonin used together with IPA revealed stronger protective effects than each of these antioxidants used separately, but only when lipid peroxidation was induced by KIO_3 in concentrations of 15 mM and 10 mM (Experiment I) or in the range of concentrations from 18.75 mM to 8.75 mM (Experiment II). These cumulative protective effects of melatonin+IPA are especially evident at higher KIO_3 concentrations, i.e., 18.75 mM and 17.5 mM, against which no protection was seen when either melatonin or IPA were used separately.

It has also been observed that melatonin did not change the basal lipid peroxidation, whereas IPA or IPA+melatonin decreased the basal lipid peroxidation.

Original paper 3

The basal level of LPO was lower in the ovary than in all other tissues, which was statistically confirmed for the thyroid, spleen, liver, and kidney. In turn, the basal level was higher in the spleen than in other tissues, which was statistically confirmed for the thyroid, ovary, and kidney. The incubation with melatonin decreased the basal level of lipid peroxidation only in ovary tissue.

KIO_3 increased lipid peroxidation in all examined tissues (i.e., the thyroid, the ovary, the spleen, the liver, the brain, the small intestine, and the kidney) with the strongest damaging effect observed at concentrations of 20 mM, of 15 mM, and of 10 mM. It should be stressed, however, that in thyroid tissue the damaging effect of KIO_3 was not observed at its lowest concentration of 5.0 mM. Additionally, lipid peroxidation induced by KIO_3 at

concentrations of 10 mM and 7.5 mM was significantly lower in the thyroid than in other examined tissues (except the kidney).

Melatonin (5.0 mM) reduced KIO_3-induced lipid peroxidation in all examined tissues when this pro-oxidant was used at concentrations of 10 mM, 7.5 mM and 5.0 mM. An important observation is that in the thyroid gland, melatonin revealed a protective effect also against a higher concentration of KIO_3, i.e., 15 mM. The lipid peroxidation level resulting from KIO_3+melatonin treatment was lower in the thyroid than in other tissues. The latter two observations suggest that the protective effect of melatonin was the strongest in the thyroid.

CONCLUSIONS

1. **Melatonin and IPA are able to reduce very strong oxidative damage to membrane lipids caused by KIO_3 when this compound is used in concentrations close to physiological iodine concentrations in the thyroid.**

2. **Melatonin and IPA exert cumulative protective effects against oxidative damage in the thyroid caused by KIO_3, when this pro-oxidant is used in concentrations close to physiological iodine concentrations in the thyroid; this suggests that these two indoles should be administered simultaneously for more effective protection.**

3. **Comparing to other tissues the thyroid gland is less sensitive to pro-oxidative effects of KIO_3; on the other hand, the strongest protective effects of melatonin against KIO_3-induced oxidative damage was observed in the thyroid, which suggests that this endocrine gland responds more effectively to antioxidative action of melatonin.**

GENERAL CONCLUSION

Melatonin and IPA, especially when applied simultaneously, should be considered to be used to avoid the potential damaging effects in the thyroid (but also in other tissues) caused by iodine compounds applied in iodine prophylaxis.