INSTYTUT CENTRUM ZDROWIA MATKI POLKI W ŁODZI

Rozprawa na stopień doktora nauk medycznych

„Potencjalne ochronne efekty substancji indolowych przed oksydacyjnymi uszkodzeniami lipidów błon komórkowych wywołanymi przez KIO_3 w gruczole tarczowym – badania *in vitro*"

Lek. Paulina Iwan
Uniwersytet Medyczny w Łodzi
Zakład Endokrynologii Onkologicznej

Miejsce pracy: Wojewódzki Zespół Zakładów Opieki Zdrowotnej – Centrum Leczenia Chorób Płuc i Rehabilitacji, Łódź

Promotor pracy:
Prof. dr hab. n. med. Małgorzata Karbownik-Lewińska

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Pani Profesor dr hab. n. med. Małgorzacie Karbownik-Lewińskiej

za zaufanie, wyrozumiałość, nieocenioną pomoc i opiekę naukową,

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Rodzicom i Najbliższym

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1. Wykaz publikacji stanowiących rozprawę doktorską:

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2. Streszczenie

Wstęp

Reaktywne formy tlenu (RFT) i wolne rodniki uczestniczą w wielu procesach metabolicznych. W warunkach fizjologicznych utrzymuje się równowaga pomiędzy wytwarzaniem a neutralizowaniem RFT. Jednakże zaburzenie tej równowagi może powodować niepożądane dla organizmu skutki.

Gruczoł tarczowy jest narządem, w którym procesy oksydacyjne odgrywają ważną rolę i są niezbędne m.in. do syntezy hormonów tarczycy. Z tego względu gruczoł tarczowy charakteryzuje się stałym wysokim poziomem stresu oksydacyjnego, który może być dodatkowo zwiększany w odpowiedzi na różne egzo- i endogenne substancje (prooksydanty) i przyczyniać się wówczas do różnych stanów chorobowych, na przykład raka tarczycy.

Jod jest pierwiastkiem niezbędnym do prawidłowego funkcjonowania organizmu. Jego kluczową rolą jest udział w syntezie hormonów tarczycy. Oszacowano fizjologiczne stężenie jodu w gruczole tarczowym, które w warunkach odpowiedniej podaży wynosi ok. 9 mM. Niedobór jodu może powodować poważne skutki zdrowotne, m.in. powstanie wola lub niedoczynność tarczycy, a jeśli jest stwierdzany u ciężarnych – także zaburzenia rozwoju płodu. Dlatego tak ważna jest odpowiednia suplementacja jodu, która zapewnia odpowiednią syntezę hormonów tarczycy, zmniejsza częstość występowania wola i zmienia dystrybucję poszczególnych postaci raka tarczycy z obniżeniem odsetka postaci o gorszym rokowaniu.

Jodowanie soli kuchennej jest w wielu krajach najpopularniejszą metodą profilaktyki niedoboru jodu. Światowe programy suplementacji jodu polegają na dodawaniu do soli kuchennej jodku potasu (KI) albo jodanu potasu (KIO_3). Związki te charakteryzują się różnymi właściwościami oksydacyjnymi – KI jest mniej reaktywny, podczas gdy KIO_3 wykazuje silniejsze właściwości prooksydacyjne. Mimo to KIO_3 uzyskał status „GRAS” (*generally recognized as safe* – generalnie uznany za bezpieczny), nadawany przez FDA (*Food and Drug Administration*). Jednakże w pewnych eksperymentalnych warunkach *in vitro* KIO_3 wykazywał zdolność do oksydacyjnych uszkodzeń makrocząsteczek biologicznych.

Związki indolowe, z ich głównym reprezentantem melatoniną (5-metoksy-N-acetyltryptaminą), są efektywnymi antyoksydantami i zmiataczami wolnych rodników. Kwas indolo-3-propionowy (IPA) jest substancją indolową, podobną do melatoniny pod względem

struktury chemicznej i właściwości biochemicznych. Oba te związki są uznawane za bezpieczne i nie wykazują istotnych działań ubocznych.

W licznych badaniach udowodniono, że melatonina wykazuje działanie ochronne wobec eksperymentalnie wyindukowanych oksydacyjnych uszkodzeń lipidów błon komórkowych w różnych tkankach, ze szczególnym uwzględnieniem gruczołu tarczowego. Melatonina wpływa również hamująco na wzrost i czynność tarczycy. Z tego powodu może być uznawana jako potencjalny czynnik ochronny przed różnymi chorobami tarczycy, włącznie z nowotworami tego gruczołu.

Cel pracy

Pierwszym celem pracy była ocena potencjalnego działania ochronnego melatoniny przed oksydacyjnymi uszkodzeniami lipidów błon komórkowych (czyli peroksydacją lipidów – LPO) indukowanymi przez KI oraz KIO_3 w homogenatach tarczycy wieprzowej (praca oryginalna 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;91:271-277**).

Następnym celem pracy było zbadanie ochronnego efektu kwasu indolo-3-propionowego (IPA) oraz efektów łącznego zastosowania melatoniny i IPA (w najwyższych, możliwych do uzyskania w warunkach *in vitro*, stężeniach, wynikających z ich ograniczonej rozpuszczalności) przed peroksydacją lipidów wyindukowaną przez KIO_3 w homogenatach tarczycy wieprzowej (praca oryginalna 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;9:89**).

W ostatniej części pracy porównywano ochronne działanie melatoniny przed wyindukowanymi przez KIO_3 oksydacyjnymi uszkodzeniami lipidów błon komórkowych w tkance tarczycy i w innych tkankach zwierzęcych (tj. jajnik, śledziona, wątroba, mózg, jelito cienkie i nerka) (praca oryginalna 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;11:592. Erratum in: Life (Basel). 2022 Jul 07;12(7)**).

Materiały i metody

Badania zostały przeprowadzone w warunkach *in vitro*, z użyciem homogenatów tkanek wieprzowych (tarczyca (we wszystkich pracach oryginalnych: 1, 2, 3) oraz dodatkowo: jajnik, śledziona, wątroba, mózg, jelito cienkie i nerka (praca oryginalna 3)).

Użyte stężenia 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), melatoniny (5.0; 2.5; 1.25; 1.0; 0.625 mM), 17β-estradiolu (1.0 mM) oraz IPA (10; 7.5; 5.0; 2.5; 1.25; 0.625 mM) zostały wybrane na podstawie wyników wcześniej opublikowanych badań naszego zakładu (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 Repred Med 2016, 62, 17–21).

Stężenie dialdehydu malonowego+4-hydroksyalkenali (MDA+4-HDA), jako wskaźnika peroksydacji lipidów, zmierzono spektrofotometrycznie z użyciem *ALDetect Lipid Peroxidation Assay Kit*.

Wyniki poddano analizie statystycznej, używając metody jednoczynnikowej analizy wariancji (ANOVA), a następnie testu Neuman-Keulsa, lub używając t-testu dla dwóch prób niezależnych. Istotność statystyczną określano na poziomie $p<0.05$. Wyniki przedstawiono jako średnie ± SE.

Wyniki

Praca oryginalna 1

Jodek potasu (KI), we wszystkich użytych stężeniach (tj. 500; 250; 100; 50 mM) i w stopniu zależnym od stężenia, spowodował wzrost poziomu peroksydacji lipidów. Także jodan potasu (KIO_3) podwyższył poziom peroksydacji lipidów we wszystkich zastosowanych stężeniach (tj. 200; 100; 50; 25; 10; 5.0; 2.5 mM), przy czym najsilniejszy efekt uszkadzający zaobserwowano przy stężeniach 10 mM i 25 mM. Po inkubacji homogenatów tarczycy z KIO_3 lub KI łącznie z melatoniną (5.0 mM), istotne obniżenie poziomu peroksydacji lipidów było zauważalne jedynie w przypadku KIO_3 użytego w stężeniu 10 mM.

Ponieważ w powyższym modelu nie odnotowano ochronnego działania melatoniny przed peroksydacją lipidów wyindukowaną przez KI, w kolejnych etapach doświadczenia wykorzystywano jedynie KIO_3.

W dalszej części doświadczenia zastosowano dodatkowe stężenia KIO_3 (tj. 20; 15; 7.5; 1.25 mM) aby wyjaśnić niespodziewane wyniki uzyskane w pierwszym etapie badania. Po użyciu dodatkowych stężeń KIO_3, najsilniejszy efekt uszkadzający lipidy błon komórkowych obserwowano przy stężeniach KIO_3 zbliżonych do 15 mM, z najwyższym poziomem LPO potwierdzonym dla stężeń 15 mM i 20 mM.

Melatonina, w stopniu zależnym od stężenia, zredukowała wyindukowaną przez KIO_3 peroksydację lipidów, ale tylko wówczas, gdy ten prooksydant był zastosowany w stężeniach

10 mM (melatonina użyta w stężeniach: 5.0 mM i 2.5 mM działała ochronnie) i 7.5 mM (melatonina użyta w stężeniach: 5.0; 2.5; 1.25 i 1.0 mM działała ochronnie). Należy podkreślić, że powyższe stężenia KIO_3 (tj. 10 mM i 7.5 mM) odpowiadają fizjologicznemu stężeniu jodu w tarczycy (wyliczonemu na ok. 9 mM).

Inkubacja homogenatów tarczycy wieprzowej jedynie z melatoniną zastosowaną w stężeniach 5.0; 2.5; 1.25; 1.0; 0.625 mM nie zmieniła podstawowej peroksydacji lipidów.

W dalszej części badania zdecydowaliśmy się porównać efekt ochronny melatoniny z potencjalnym działaniem ochronnym innej znanej substancji antyoksydacyjnej – 17β-estradiolu. 17β-estradiol, użyty w stężeniu 1.0 mM będącym najwyższym stężeniem możliwym do uzyskania w warunkach *in vitro*, nie wykazywał korzystnych efektów wobec indukowanej przez KIO_3 peroksydacji lipidów, podczas gdy melatonina, zastosowana w tym samym stężeniu (tj. 1.0 mM), istotnie obniżyła poziom peroksydacji lipidów wyindukowanej przez KIO_3 (7.5 mM).

Praca oryginalna 2

W Eksperymencie I, IPA (10 mM) i melatonina (5.0 mM) użyte osobno, obniżyły poziom peroksydacji lipidów wyindukowanej przez KIO_3 w stężeniach 10 mM, 7.5 mM i 5.0 mM. Jednakże w Eksperymencie II, po zastosowaniu dodatkowych stężeń KIO_3 wykazano, że IPA wywołuje efekt ochronny przy wyższych stężeniach jodanu potasu (16.25 mM) w porównaniu z efektem ochronnym melatoniny (istotne obniżenie LPO przy stężeniu KIO_3 15 mM).

Dodatkowo, efekt ochronny wywołany przez IPA był silniejszy w porównaniu z działaniem wywołanym przez melatoninę przy stężeniach KIO_3 13.75 mM i niższych.

Jednak najważniejszą obserwacją było, że melatonina użyta łącznie z IPA wykazywała silniejsze działanie niż każdy z antyoksydantów zastosowany osobno. Efekt ten był widoczny przy stężeniach KIO_3 15 mM i 10 mM (w Eksperymencie I), a po użyciu dodatkowych stężeń w Eksperymencie II w zakresie stężeń od 18.75 mM do 8.75 mM. Ten kumulacyjny efekt ochronny melatoniny+IPA był szczególnie zauważalny przy wyższych stężeniach KIO_3, tj. przy 18.75 mM i 17.5 mM, przy których ani melatonina, ani IPA użyte osobno nie wykazywały działania protekcyjnego.

Podobnie jak wykazano w pracy oryginalnej 1, także w omawianym badaniu potwierdzono, że melatonina nie zmienia podstawowej peroksydacji lipidów, podczas gdy zarówno IPA, jak i melatonina+IPA obniżyły podstawową peroksydację lipidów.

Praca oryginalna 3

Poziom podstawowej peroksydacji lipidów był niższy w tkance jajnika niż w pozostałych badanych tkankach, co potwierdzono statystycznie w odniesieniu do tkanki tarczycy, śledziony, wątroby i nerki. Z kolei poziom podstawowej peroksydacji lipidów był wyższy w śledzionie niż w innych tkankach (istotność statystyczna w porównaniu z tkanką tarczycy, jajnika i nerki). Inkubacja w obecności melatoniny obniżyła poziom podstawowej peroksydacji lipidów jedynie w tkance jajnika.

Porównując efekt działania KIO_3 na homogenaty tkanek wieprzowych zaobserwowano, że KIO_3 zwiększa poziom peroksydacji lipidów we wszystkich badanych tkankach (tj. tarczycy, jajniku, śledzionie, wątrobie, mózgu, jelicie cienkim, nerce), z najsilniejszym efektem uszkadzającym stwierdzanym przy stężeniach KIO_3 20 mM, 15 mM i 10 mM. Należy jednak podkreślić, że w tkance tarczycy nie stwierdzono efektu uszkadzającego przy najniższym stężeniu KIO_3 – 5.0 mM. Ponadto poziom LPO indukowany przez KIO_3 w stężeniach 10 mM i 7.5 mM był istotnie niższy w tarczycy niż w innych badanych tkankach (wyłączając tkankę nerki).

Melatonina (w stężeniu 5.0 mM) obniżyła indukowaną przez KIO_3 (10 mM, 7.5 mM i 5.0 mM) peroksydację lipidów we wszystkich badanych tkankach. Ważną obserwacją jest to, że w gruczole tarczowym melatonina wykazywała działanie ochronne także przy wyższym stężeniu KIO_3, tj. 15 mM. Poziom LPO po inkubacji w obecności KIO_3+melatonina był istotnie niższy w gruczole tarczowym niż w innych badanych tkankach. Dwie ostatnie obserwacje sugerują, że ochronny efekt melatoniny był najsilniejszy w tkance tarczycy.

WNIOSKI

1. **Melatonina i kwas indolo-3-propionowy bardzo wyraźnie obniżają poziom oksydacyjnych uszkodzeń lipidów błon komórkowych spowodowanych działaniem jodanu potasu (KIO_3) użytego w stężeniach odpowiadających fizjologicznym stężeniom jodu w tarczycy.**

2. **Melatonina i kwas indolo-3-propionowy wywierają kumulacyjny efekt ochronny przed oksydacyjnymi uszkodzeniami lipidów błon komórkowych tkanki tarczycy wywołanymi przez KIO_3 użyty w stężeniach odpowiadających fizjologicznym stężeniom jodu w tarczycy; sugeruje to, że te dwie substancje indolowe powinny być stosowane jednocześnie w celu uzyskania lepszego efektu ochronnego przed stresem oksydacyjnym.**

3. **W porównaniu z innymi tkankami, gruczoł tarczowy jest mniej wrażliwy na prooksydacyjne działanie KIO_3. Z drugiej strony, najsilniejsze działanie ochronne melatoniny wykazano właśnie w tkance tarczycy, co sugeruje, że gruczoł ten skuteczniej odpowiada na antyoksydacyjne działanie melatoniny.**

WNIOSEK OGÓLNY

Melatonina i kwas indolo-3-propionowy, w szczególności przyjmowane jednocześnie, powinny być rozważane w celu zapobiegania możliwym uszkodzeniom oksydacyjnym w gruczole tarczowym (a także w innych tkankach) wywołanym przez związki jodu stosowane w profilaktyce jodowej.

4. Streszczenie w języku angielskim – Summary

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.

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Original Communication

Melatonin reduces high levels of lipid peroxidation induced by potassium iodate in porcine thyroid

Paulina Iwan[1], Jan Stepniak[1], and Malgorzata Karbownik-Lewinska[1,2]

[1] Department of Oncological Endocrinology, Medical University of Lodz, Lodz, Poland
[2] Polish Mother's Memorial Hospital – Research Institute, Lodz, Poland

Abstract: Iodine is essential for thyroid hormone synthesis. Under normal iodine supply, calculated physiological iodine concentration in the thyroid is approx. 9 mM. Either potassium iodide (KI) or potassium iodate (KIO_3) are used in iodine prophylaxis. KI is confirmed as absolutely safe. KIO_3 possesses chemical properties suggesting its potential toxicity. Melatonin (N-acetyl-5-methoxytryptamine) is an effective antioxidant and free radical scavenger. Study aims: to evaluate potential protective effects of melatonin against oxidative damage to membrane lipids (lipid peroxidation, LPO) induced by KI or KIO_3 in porcine thyroid. Homogenates of twenty four (24) thyroids were incubated in presence of either KI or KIO_3 without/with melatonin (5 mM). As melatonin was not effective against KI-induced LPO, in the next step only KIO_3 was used. Homogenates were incubated in presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25 mM) without/with melatonin or 17ß-estradiol. Five experiments were performed with different concentrations of melatonin (5.0; 2.5; 1.25; 1.0; 0.625 mM) and one with 17ß-estradiol (1.0 mM). Malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) concentration (LPO index) was measured spectrophotometrically. KIO_3 increased LPO with the strongest damaging effect (MDA + 4-HDA level: ≈1.28 nmol/mg protein, $p < 0.05$) revealed at concentrations of around 15 mM, thus corresponding to physiological iodine concentrations in the thyroid. Melatonin reduced LPO (MDA + 4-HDA levels: from ≈0.97 to ≈0,76 and from ≈0,64 to ≈0,49 nmol/mg protein, $p < 0.05$) induced by KIO_3 at concentrations of 10 mM or 7.5 mM. Conclusion: Melatonin can reduce very strong oxidative damage to membrane lipids caused by KIO_3 used in doses resulting in physiological iodine concentrations in the thyroid.

Keywords: Melatonin, potassium iodate, lipid peroxidation, thyroid, antioxidant

Introduction

Reactive oxygen species (ROS) and free radicals participate in metabolic processes [1]. Under physiological conditions, there is a balance between the production and detoxification of ROS. Thyroid gland is an organ of "oxidative nature" [2], in which oxidative processes are necessary, e.g. for thyroid hormone biosynthesis. In turn, an enhanced oxidative stress, defined as an imbalance between oxidants and antioxidants, may result in different thyroid diseases [2–5].

Iodine is a micronutrient that is essential for the synthesis of thyroid hormones [6]. The only natural source of iodine is the diet. Salt iodization is one of the safest and most effective methods of achieving iodine sufficiency across a population [6, 7]. Either potassium iodide (KI) or potassium iodate (KIO_3) are used for salt iodization [8].

It has been documented that two main iodine compounds, i.e. KI and KIO_3, have different chemical properties, resulting in different pro-/anti-oxidative properties [9]. KI is less reactive whereas KIO_3 reveals stronger oxidizing properties. This difference may be associated with main chemical properties of these two compounds, namely KI is the reductant whereas KIO_3 is the oxidant and, as one of halogenate salts, it may react very easily with oxidisable substances. Before IO_3^- can be effectively used in human body it should be reduced to I^- [10]. Potassium iodide may prevent oxidative damage to membrane lipids in the thyroid gland when used in doses recommended in iodine prophylaxis, although in higher concentrations (>50 mM) it increased lipid peroxidation [11]. Additionally, in the process of inducing oxidative stress, KI stimulates simultaneously a well-known antioxidative enzyme, i.e. it increases peroxiredoxin 3 protein expression, in Fischer rat thyroid cell line [12]. In contrast, KIO_3 increased lipid peroxidation in concentrations > 2.5 mM with the strongest damaging effect at the concentration of 10 mM [11], which corresponds to the physiological concentration of iodine in the thyroid.

As previously mentioned, thyroid gland has the "oxidative nature", therefore the antioxidative defence system

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should prevent the build-up of excessive ROS [2, 4]. Different exogenous antioxidants were founded to prevent experimentally-induced oxidative damage to macromolecules in the thyroid [2], with melatonin being the first antioxidant tested [13]; it is worth mentioning others, e.g. indole-3-propionic acid (IPA) [14, 15], propylthiouracil [14, 16], estrogens [17].

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine produced mainly, but not exclusively, in the pineal gland and possesses excellent antioxidative properties [18–26]. Its protective effects against oxidative damage to macromolecules have been confirmed in numerous studies [13–15, 18–20, 27–29]. However, melatonin effects are concentration dependent. As it was documented recently, this indoleamine may also reveal at certain concentrations some prooxidative effects; melatonin at concentrations lower than 100 μM induced lipid peroxidation, Band 3 protein expression, and cell shape alterations in human erythrocytes [29]. However, according to the point of view of most experts working on melatonin, this indoleamine reveals almost exclusively antioxidative effects, and single observations, such as mentioned above [29], should not confirm its prooxidative nature.

It is worth mentioning that melatonin has been documented to reveal inhibitory effects on thyroid growth and function [30–32].

The 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.

Materials and methods

Chemicals

Potassium iodide (KI), potassium iodate (KIO_3), melatonin and 17ß-estradiol were purchased from Sigma (St. Louis, MO, USA). The ALDetect Lipid Peroxidation Assay Kit was obtained from Enzo Life Sciences, Inc. (Zandhoven, Belgium). All the used chemicals were of analytical grade and came from commercial sources.

Animals

Porcine thyroids were collected from twenty four (24) animals at a slaughter-house, frozen on solid CO_2 and stored at -80° until assay. Each experiment was repeated three to four times. Therefore, three to four tissue pools were prepared, with six (6) thyroid glands used for each homogenate pool.

Assay of lipid peroxidation

Thyroid tissue was homogenized in ice cold 20 mM Tris-HCl buffer (pH 7.4) (10%, w/v) and then incubated for 30 min at 37° in the presence of either KI (500; 250; 100; 50 mM) or KIO_3 (200; 100; 50; 25; 10; 5.0; 2.5 mM) without or with addition of melatonin (5 mM).

As melatonin did not reveal any protective effect against KI-induced lipid peroxidation, in the next step only KIO_3 was used.

In the next step thyroid homogenates were incubated in the presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25 mM) without/with addition of melatonin or 17ß-estradiol. Five experiments were performed with different concentrations of melatonin, i.e. 5.0; 2.5; 1.25; 1.0; 0.625 mM and one experiment with 17ß-estradiol in concentration of 1.0 mM.

The concentrations of KI and KIO_3 [11], 17ß-estradiol [17] and melatonin [14] were chosen on the basis of the results of our previous studies.

The reactions were stopped by cooling the samples on ice. Each experiment was run in duplicate.

Measurement of lipid peroxidation products

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA), as an index of lipid peroxidation, were measured in thyroid homogenates, with the ALDetect Lipid Peroxidation Assay Kit. The homogenates were centrifuged at 5,000 x g for 10 min at 4°. After obtaining supernatant, each experiment was carried out in duplicate. The supernatant (200 μl) was mixed with 650 μl of a methanol: acetonitrile (1:3, v/v) solution, containing a chromogenic reagent, N-methyl-2-phenylindole, and vortexed. Following the addition of 150 μl of methanesulfonic acid (15.4 M), the incubation was carried out at 45° for 40 min. The reaction between MDA + 4-HDA and N-methyl-2-phenylindole yields a chromophore, which is spectrophotometrically measured at the absorbance of 586 nm, using a solution of 10 mM 4-hydroxynonenal as the standard. The level of lipid peroxidation is expressed as the amount of MDA + 4-HDA (nmol) per mg protein. Protein was measured using Bradford's method [33], with bovine albumin as the standard.

Statistical analyses

Results are expressed as means ± SE. The data were statistically analyzed, using a one-way analysis of variance (ANOVA) followed by the Tukey's test or unpaired t-test. Normality of distribution was confirmed by the use of

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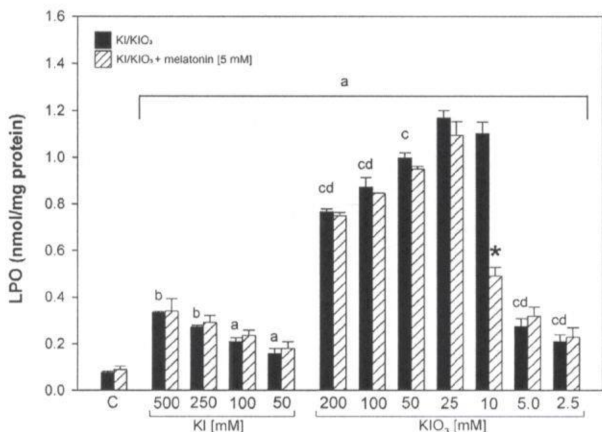

Figure 1. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates prepared from twenty four (24) porcine thyroids, incubated for 30 min in the presence of either KI (500; 250; 100; 50 mM) or KIO_3 (200; 100; 50; 25; 10; 5.0; 2.5 mM) with (striped bars) or without (black bars) melatonin (5.0 mM). The experiment was repeated three to four times. Therefore, three to four tissue pools were prepared, with six (6) thyroid glands used for each homogenate pool. Data are expressed as nmol/mg protein. Values are expressed as mean ± SE (error bars). *$p < 0.05$ vs. KIO_3 in the same concentration without melatonin. [a]$p < 0.05$ vs. respective control. [b]$p < 0.05$ vs. any other KI concentration. [c]$p < 0.05$ vs. KIO_3 at the concentration of 25 mM. [d]$p < 0.05$ vs. KIO_3 at the concentration of 10 mM. C – control.

Shapiro-Wilk test, whereas the equality of variance was confirmed by the use of Levene's test. The level of $p < 0.05$ was accepted as statistically significant.

Results

In the present study we have chosen those concentrations of KI (500; 250; 100; 50 mM) or KIO_3 (200; 100; 50; 25; 10; 5.0; 2.5 mM) which revealed stimulatory effects on lipid peroxidation in thyroid homogenates in our previous study [11]. Similarly to results of this previous study [11], potassium iodide, in all used concentrations, did increase lipid peroxidation in concentration-dependent manner (Figure 1). In turn, potassium iodate did increase lipid peroxidation in all used concentrations with the strongest damaging effect to membrane lipids at concentrations of 10 mM and 25 mM (Figure 1).

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 (Figure 1).

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 and in our previous study [11].

After using additional concentrations of KIO_3, the strongest damaging effect to membrane lipids was observed for KIO_3 concentration of around 15 mM (Figures 2–4) with the highest LPO level confirmed for concentrations of 15 mM and 20 mM (Figures 3 and 4).

Melatonin reduced, in concentration-dependent manner, KIO_3-induced lipid peroxidation, but only when this prooxidant was used at concentrations of 10 mM or of 7.5 mM. Namely, melatonin, at concentrations of 5.0 mM or 2.5 mM, decreased lipid peroxidation induced by KIO_3 in concentrations either of 10 mM or of 7.5 mM (Figure 2).

Lower concentrations of melatonin, i.e. 1.25 mM and 1.0 mM, decreased KIO_3-induced lipid peroxidation but only when this prooxidant was used at the concentration of 7.5 mM (Figure 3). The lowest concentration of melatonin used in our study, i.e. 0.625 mM, was not protective in our model (Figure 3).

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 (Figures 1–3).

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 (Figure 4), whereas melatonin, used in the same concentration of 1.0 mM, reduced lipid peroxidation induced by KIO_3 (7.5 mM) (Figure 3).

Discussion

On the basis of experimental findings the concentration of inorganic iodine in human or rat thyroid was calculated to be approx. 9 mM [34–36]. Due to similarity between human and porcine thyroid (volume, hormone synthesis, etc.) [37], it may be estimated that iodine concentration in porcine thyroid is at similar level. Such relatively high level of iodine in the thyroid is expected, especially that much lower iodine levels have recently been found in human placenta, i.e. 1.38 μg/g equal to approx. 10 μM [38] or in human blood serum, i.e. 99.1 μg/L equal to almost 1 μM [39]. It is worth emphasizing that the highest lipid peroxidation caused by KIO_3 was observed in the present study for the concentration of around 15 mM, which is of the same order of magnitude as physiological concentration of iodine in the thyroid.

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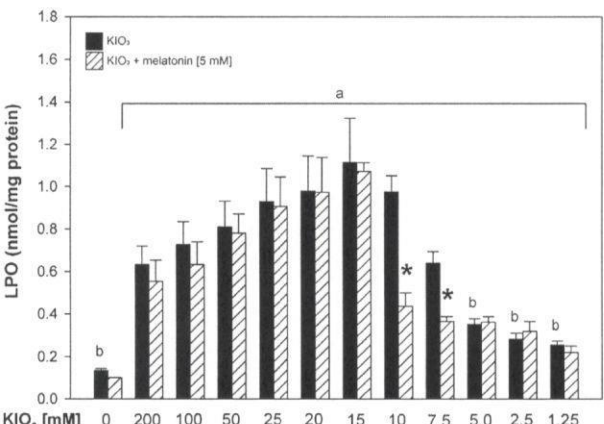


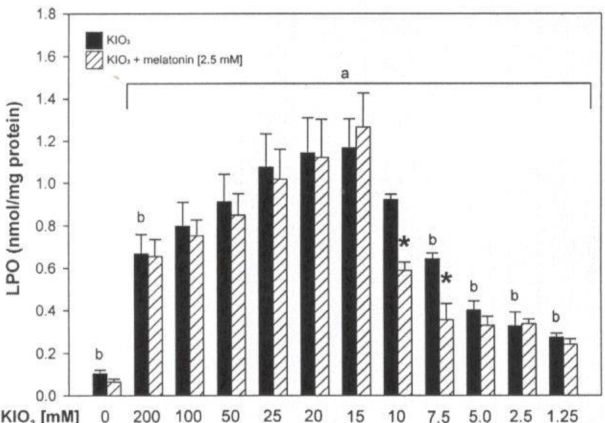
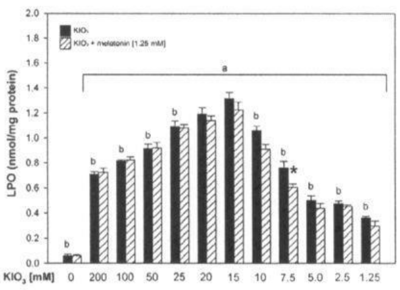
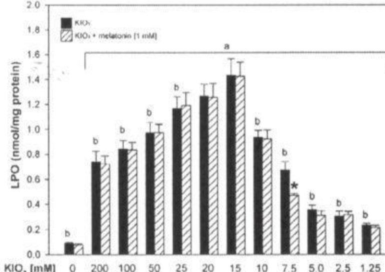


Figure 2. Lipid peroxidation, measured as MDA + 4-HDA level, in porcine thyroid homogenates, incubated in the presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25 mM) with (striped bars) or without (black bars) melatonin (5.0 mM or 2.5 mM). The experiment was repeated three to four times. Therefore, three to four tissue pools were prepared, with six (6) thyroid glands used for each homogenate pool. Data are expressed as nmol/mg protein. Values are expressed as mean ± SE (error bars). *$p < 0.05$ vs. KIO_3 in the same concentration without melatonin. [a]$p < 0.05$ vs. respective control. [b]$p < 0.05$ vs. KIO_3 at the concentration of 15 mM.





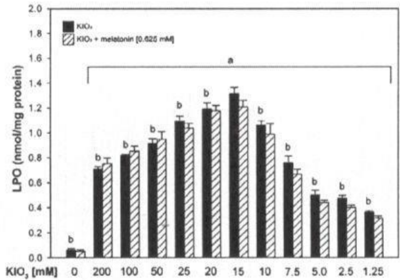


Figure 3. Lipid peroxidation, measured as MDA + 4-HDA level, in porcine thyroid homogenates, incubated in the presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25 mM) with (striped bars) or without (black bars) melatonin (1.25 mM, 1.0 mM or 0.625 mM). The experiment was repeated three to four times. Therefore, three to four tissue pools were prepared, with six (6) thyroid glands used for each homogenate pool. Data are expressed as nmol/mg protein. Values are expressed as mean ± SE (error bars). *$p < 0.05$ vs. KIO_3 in the same concentration without melatonin. [a]$p < 0.05$ vs. respective control. [b]$p < 0.05$ vs. KIO_3 at the concentration of 15 mM.

It should be stressed that at this concentration of iodine, KI did not increase the level of lipid peroxidation in porcine thyroid homogenates [11].

Because iodate is more stable than iodide (iodide is easily oxidized to I_2 and then lost by evaporation), some health authorities preferentially recommend iodate as an additive to salt for correcting iodine deficiency [9]. On the other hand, the superiority of KI over KIO_3 may rely on its stronger protective effects against oxidative damage to mtDNA [40]. Although iodate has been conferred GRAS ("generally recognized as safe") status by the FDA [9], available publications show "dual nature" of KIO_3.

Considerations concerning potential toxicity of KIO_3 are as follows

Iodic acid (HIO_3), together with chloric acid and bromic acid, belongs to the class of oxohalogen acids. Halogenate salts are stable under most conditions, but due to their oxidative properties they may react rapidly with easily oxidisable substances. As previously mentioned, KIO_3 belongs to the group of GRAS, but due to its similarity to $KBrO_3$ (known potential carcinogen belonging to the group 2B according to IARC [41] it is justified to check their mutagenic and cancerogenic potential. On the other hand, iodate has a lower oxidative potential than bromate has, and it did not induce toxic effects under conditions in which bromate did [9, 15].

It is probable that KIO_3-caused lipid peroxidation in porcine thyroid results from direct oxidative effects of this compound on cellular membranes. However, it should be stressed that probably also other macromolecules in thyroid cells can be directly affected by KIO_3, as it has been documented for nDNA and mtDNA in our earlier studies [40]. Additionally, similar direct effects can be exerted by KIO_3 in other tissues, but that should be experimentally proven.

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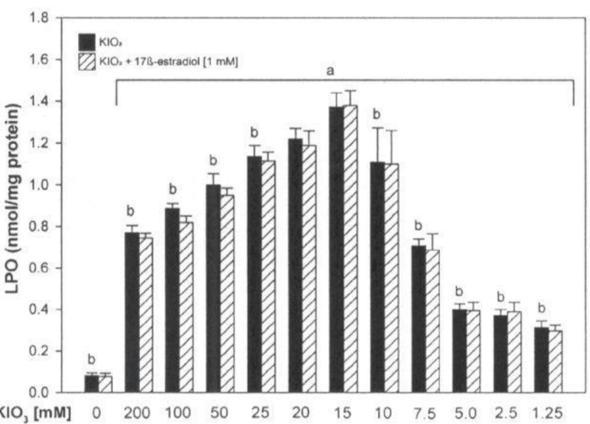

Figure 4. Lipid peroxidation, measured as MDA + 4-HDA level, in porcine thyroid homogenates, incubated in the presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25 mM) with (striped bars) or without (black bars) 17ß-estradiol (1.0 mM). The experiment was repeated three to four times. Therefore, three to four tissue pools were prepared, with six (6) thyroid glands used for each homogenate pool. Data are expressed as nmol/mg protein. Values are expressed as mean ± SE (error bars). $^{a}p < 0.05$ vs. respective control. $^{b}p < 0.05$ vs. KIO_3 at the concentration of 15 mM.

Iodate was tested for its potential toxicity, but that was not confirmed till now in humans.

However, taking into account chemical properties of iodate and its prooxidative effects documented in our previous [11] and present studies, it cannot be excluded that this compound is potentially dangerous.

For this reason it is advisable to search for new potential protective tools against prooxidative nature of KIO_3.

In the present study we have shown that melatonin, in concentrations usually used in in vitro studies (1.0 mM–5.0 mM), significantly reduced lipid peroxidation induced by KIO_3, when this compound was used at doses corresponding to physiological concentrations of iodine in the thyroid. The concentrations of melatonin used in the current study should be treated as corresponding to pharmacological doses, as they exceed even by three orders of magnitude the highest physiological concentrations of the indoleamine [42]. However, on the basis of the present results it is still advisable to maintain high concentrations of melatonin to prevent oxidative damage in the thyroid gland. It is suggested to avoid factors, which decrease melatonin concentrations in organisms, such as strong light at night [43, 44]. Furthermore, it may be beneficial to use exogenous melatonin by elderly, because physiological concentration of melatonin decreases with age [45].

We chose melatonin for our research, because protective effects of this compound against oxidative stress have been known for a long time [18, 20–24, 46]. These effects were observed in both in vivo and in vitro experiments. In the thyroid gland for example, melatonin reduced lipid peroxidation caused by Fenton reaction substrates (Fe^{2+} + H_2O_2) [13] and potassium bromate [14].

The mechanisms by which melatonin protects against lipid peroxidation involve direct or indirect antioxidative effects and free radical scavenging activities of this indoleamine [18, 21, 47, 48]. Melatonin, which is highly lipid soluble, is believed to be widely distributed in cellular membranes, where it may intercalate between the polar heads of fatty acids – this property simplifies melatonin to diminish prooxidative damage to lipids [21].

One of the most important issues is that melatonin, when used in very high pharmacological doses to either humans [49] or animals [19], has never revealed any undesirable effects.

In the present study melatonin did not reduce KI-induced lipid peroxidation. Although melatonin is usually highly protective against a prooxidant-induced damage, that indoleamine rarely does not prevent oxidative damage, especially under in vitro conditions. At the same time the lack of in vitro antioxidative effects caused by melatonin does not exclude such protective effects in in vivo conditions. However, taking into account that KI is absolutely safe in living organisms, it is not necessary to look for any protective action against this compound. It should be stressed again, that at concentrations close to physiological concentrations of iodine, KI did not induce lipid peroxidation in thyroid homogenates [11].

The observation from the present study, which should be discussed, is that melatonin was effective only when KIO_3 was used at concentrations of 10 mM and 7.5 mM; these concentrations correspond to physiological iodine concentration in the thyroid. Although the iodine concentration in the thyroid differs depending on the age, such differences are presumably not huge; therefore it can be stated that these two effective concentrations of KIO_3 correspond to physiological iodine concentration in the thyroid at any age. Prooxidative effects of KIO_3 were not reduced by melatonin when the prooxidant was used either in higher or in lower concentrations than 10 mM and 7.5 mM. We are not able to present clear explanation of these rather unexpected results. However, it can be hypothesized that during phylogenetical development in mammals, protective mechanisms have been developed to protect against well recognized toxic agents, to which organisms are potentially exposed for a long period of time. That can be the reason why melatonin reduced lipid peroxidation induced by KIO_3 in concentrations corresponding to physiological concentration of iodine in the thyroid. Although the thyroid and the whole organism can be exposed to much higher concentrations of iodine (e.g. resulting from pharmacological treatment), that is not a common epidemiological or any other individual situation. Thus it can be hypothesized that

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protective mechanisms have not been developed against these rare conditions.

In the present study we compared potential protective effects of 17ß-estradiol used in the concentration of 1.0 mM (the highest achievable concentration) with protective effects of melatonin used in the same concentration. We have found that 17ß-estradiol, well known endogenous antioxidant, is not protective at all in our model. Thus, melatonin appeared to be better potential protective agent.

As previously mentioned, salt iodization is one of the most effective methods to achieve iodine sufficiency across a population. However, KIO_3 frequently used for salt iodization, reveals oxidizing properties.

Major strengths of our study are as follows. We documented that KIO_3 causes oxidative damage to membrane lipids, with strongest effects observed at KIO_3 concentrations, resulting in physiological concentration of iodine in the thyroid. This finding is of great importance as KIO_3, frequently used for salt iodization, should be absolutely safe. Additionally, we observed protective effects of melatonin against prooxidative nature of KIO_3. This finding is again of great importance, as exogenous melatonin is confirmed to be absolutely safe even in very high pharmacological doses, either in humans or in animals. The weakness of our study is that we used only in vitro model; however, this is always the first step of any study.

Conclusions

Melatonin is able to reduce very strong oxidative damage to membrane lipids caused by potassium iodate when this compound is used in doses resulting in physiological concentrations of iodine in the thyroid. Our study is the first one to show protective effects of melatonin against prooxidative effects of iodates.

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Conflict of interest

The authors confirm that this article content has no conflict of interest.

Publication ethics

The procedures, used in the study, were approved by the Ethics Committee of the Medical University of Lodz, Poland.

ORCID

Karbownik-Lewinska Malgorzata
https://orcid.org/0000-0002-3045-5669

Prof. Malgorzata Karbownik-Lewinska
Medical University of Lodz
7/9 Zeligowski St.
90-752 Lodz
Poland
mkarbownik@hotmail.com

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Article

Cumulative Protective Effect of Melatonin and Indole-3-Propionic Acid against KIO_3—Induced Lipid Peroxidation in Porcine Thyroid

Paulina Iwan [1], Jan Stepniak [1] and Malgorzata Karbownik-Lewinska [1,2,*]

[1] Department of Oncological Endocrinology, Medical University of Lodz, 7/9 Zeligowski St., 90-752 Lodz, Poland; paulina.iwan@op.pl (P.I.); jan.stepniak@umed.lodz.pl (J.S.)
[2] Polish Mother's Memorial Hospital—Research Institute, 281/289 Rzgowska St., 93-338 Lodz, Poland
* Correspondence: mkarbownik@hotmail.com

Abstract: Iodine deficiency is the main environmental factor leading to thyroid cancer. At the same time iodine excess may also contribute to thyroid cancer. Potassium iodate (KIO_3), which is broadly used in salt iodization program, may increase oxidative damage to membrane lipids (lipid peroxidation, LPO) under experimental conditions, with the strongest damaging effect at KIO_3 concentration of ~10 mM (corresponding to physiological iodine concentration in the thyroid). Melatonin and indole-3-propionic acid (IPA) are effective antioxidative indoles, each of which protects against KIO_3-induced LPO in the thyroid. The study aims to check if melatonin used together with IPA (in their highest achievable in vitro concentrations) reveals stronger protective effects against KIO_3-induced LPO in porcine thyroid homogenates than each of these antioxidants used separately. Homogenates were incubated in the presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25; 0.0 mM) without/with melatonin (5 mM) or without/with IPA (10 mM) or without/with melatonin + IPA, and then, to further clarify the narrow range of KIO_3 concentrations, against which melatonin + IPA reveal cumulative protective effects, the following KIO_3 concentrations were used: 20; 18.75; 17.5; 16.25; 15; 13.75; 12.5; 11.25; 10; 8.75; 7.5; 0.0 mM. Malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) concentration (LPO index) was measured spectrophotometrically. Protective effects of melatonin + IPA were stronger than those revealed by each antioxidant used separately, but only when LPO was induced by KIO_3 in concentrations from 18.75 mM to 8.75 mM, corresponding to physiological iodine concentration in the thyroid. In conclusion, melatonin and indole-3-propionic acid exert cumulative protective effects against oxidative damage caused by KIO_3, when this prooxidant is used in concentrations close to physiological iodine concentrations in the thyroid. Therefore, the simultaneous administration of these two indoles should be considered to prevent more effectively oxidative damage (and thereby thyroid cancer formation) caused by iodine compounds applied in iodine prophylaxis.

Keywords: melatonin; indole-3-propionic acid; potassium iodate; KIO_3; lipid peroxidation; thyroid cancer; antioxidant; salt iodization

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1. Introduction

Free radicals are highly reactive transient molecules, which have an odd number of electrons and are generated in vivo as byproducts of normal metabolism [1,2]. Reactive oxygen species (ROS) include both oxygen radicals (e.g., superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical (•OH), and hydroperoxyl radical (•OOH)) and certain nonradical oxidizing agents (i.e., hydrogen peroxide (H_2O_2), peroxynitrite anion ($ONOO^-$), hypochlorous acid (HOCl) and ozone (O_3)) easily converted into radicals [1,2]. Under physiological conditions, there is a balance between beneficial and harmful effects of free radicals, which is essential for the survival of organisms and their health [1–3]. Any imbalance between

these processes may result in different pathological conditions. However, modulation of oxidative stress can serve as a strategy against diseases, cancer included [3].

Oxidative reactions occur practically in all tissues and organs, including thyroid gland, in which ROS play a particular role. This is due to the fact that different factors, such as H_2O_2, iron or iodine, are indispensable for thyroid hormone synthesis [4]. For this reason, thyroid gland is characterized by high level of oxidative stress, which—in response to additional oxidative abuse caused by exogenous or endogenous prooxidants—may lead to different thyroid diseases, including cancer [5].

Numerous evidence suggest that environmental factors, including endocrine disruptors, can contribute to thyroid cancer [6]. One of the major risk factors for goiter and, consequently, for thyroid cancer, is iodine deficiency [7]. Moreover, correction of iodine deficiency decreases the prevalence of goiter [8] and might shift thyroid cancer subtypes toward less malignant forms [7]. On the other hand, iodine excess may lead to thyroiditis, thyroid dysfunction, and also to papillary thyroid cancer [9].

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) [10]. It is known that these two main iodine compounds have different pro- and antioxidative properties. KIO_3, in contrast to KI, is the oxidant and thereby may react easily with oxidizable substances [11]. It has been documented recently that KIO_3 and KI reveal different in vitro effects on oxidative damage to macromolecules in the thyroid [12–15]. In these studies, KIO_3 did not reveal any protective effects; instead, it damaged by itself membrane lipids with the strongest damaging effect observed at concentrations of 10 mM [12] or of 15 mM [14,15], which both correspond to physiological iodine concentration in the thyroid [16–18]. However, KIO_3 has still GRAS ("generally recognized as safe") status given by FDA [19].

The increased oxidative stress can be diminished by antioxidants. Indole substances belong to very effective antioxidants. The most important representative of indole substances is melatonin (5-methoxy-*N*-acetyltryptamine). Melatonin is mainly produced by the pineal gland; it is a tryptophan metabolite which is repeatedly documented to reduce oxidative stress [20]. Melatonin effectively scavenges different free radicals and ROS; it is one of the strongest scavengers of •OH [21]. Additionally, its metabolites (*N*1-acetyl-*N*2-formyl-5-methoxykynuramine (AFMK), *N*-acetyl-5-methoxyknuramine (AMK), and cyclic-3-hydroxymelatonin (c3OHM)) have been also found to protect cells from ROS [22–24]. It has been demonstrated that one melatonin molecule has the capacity to scavenge up to 10 molecules of ROS [22]. Numerous studies revealed protective effects of melatonin against oxidative damage to macromolecules caused by potential carcinogens, as it has been summarized by us previously [25,26].

The main physiological function of melatonin is to regulate circadian rhythm [27]. Melatonin also causes positive effects on other physiological processes, such as for example bone formation, body mass regulation, reproduction, regulation of immune system and cardiovascular system, as well as it serves as a pharmacological agent [28]. Doses between 1 mg and 6 mg appear to be effective for improving sleep in older adults [29]. In clinical trials investigating anxiety, melatonin in doses varied from 3 to 10 mg probably reduced preoperative anxiety in adults, which is potentially clinically relevant [30]. Available studies show, that short-time use of melatonin, even in very high doses, is safe. Some randomized clinical studies revealed only mild side effects during long-time administration of this drug, comparable to placebo treatment, i.e., sleepiness, dizziness, headache or nausea [31].

Indole-3-propionic acid (IPA), an indole substance possessing a chemical structure similar to that of melatonin, is another effective antioxidant. Similar to melatonin, it scavenges effectively •OH [32]. Indole-3-propionic acid has been documented to protect against oxidative damage to membranes caused by such potential carcinogens as potassium bromate, iron or chromium [33–36]. Its potential favorable properties in humans include, among others, therapeutic strategy for Alzheimer disease [37].

Melatonin has been shown to prevent experimentally-induced oxidative damage to macromolecules in the thyroid gland [33,38,39]. This indole substance also inhibits thyroid growth and thyroid function [40]. As melatonin is confirmed to prevent the increased oxidative damage in the thyroid and to inhibit growth processes in this gland, it should be considered as a potential protective agent against thyroid cancer.

In our previous studies we have observed that not only melatonin [14], but also IPA [15] are able—in concentration-dependent manner—to reduce oxidative damage to membrane lipids caused by KIO_3, when this compound was used in doses close to physiological iodine concentrations in the thyroid. In the present study we decided to check if melatonin used together with IPA (in their highest achievable in vitro concentrations resulting from their limited solubility) reveals stronger protective effects against KIO_3-induced oxidative damage to membrane lipids in porcine thyroid homogenates comparing to protective effects of each antioxidant used separately.

2. Materials and Methods

2.1. Chemicals

Potassium iodate (KIO_3), melatonin and indole-3-propionic acid (IPA) were purchased from Sigma (St. Louis, MO, USA). The ALDetect Lipid Peroxidation Assay Kit was obtained from Enzo Life Sciences, Inc. (Zandhoven, Belgium). All the used chemicals were of analytical grade and came from commercial sources.

2.2. Animals

Porcine thyroids were collected from eighteen (18) animals at a slaughter-house, frozen on solid CO_2 and stored at $-80°$ until assay. Each experiment was repeated three times. Therefore, three tissue pools were prepared, with six (6) thyroid glands used for each homogenate pool.

2.3. Assay of Lipid Peroxidation

Thyroid tissue was homogenized in ice cold 20 mM Tris-HCl buffer (pH 7.4) (10%, w/v) and then incubated for 30 min at 37° in the presence of examined substances.

In Experiment I thyroid homogenates were incubated in the presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25; 0.0 mM) without any antioxidant or with addition of either melatonin (5 mM) or IPA (10 mM) or both (melatonin 5 mM + IPA 10 mM).

In Experiment II, to further clarify the range of KIO_3 concentrations, against which melatonin + IPA reveal cumulative effects, the following KIO_3 concentrations were used: 20; 18.75; 17.5; 16.25; 15; 13.75; 12.5; 11.25; 10; 8.75; 7.5; 0.0 mM. Therefore, thyroid homogenates were incubated in the presence of KIO_3 (in above concentrations) without any antioxidant or with addition of either melatonin (5 mM) or IPA (10 mM) or both (melatonin 5 mM + IPA 10 mM).

The concentrations of KIO_3 [12,14,15], of melatonin and of IPA [14,15,33,38] were chosen on the basis of the results of our previous studies; the highest achievable concentrations of melatonin and IPA resulting from their limited solubility were used.

The reactions were stopped by cooling the samples on ice.

2.4. Measurement of Lipid Peroxidation Products

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA), as an index of lipid peroxidation, were measured in thyroid homogenates, with the ALDetect Lipid Peroxidation Assay Kit. The homogenates were centrifuged at 5000× g for 10 min at 4°. After obtaining supernatant, each experiment was carried out in duplicate. The supernatant (200 μL) was mixed with 650 μL of a methanol: acetonitrile (1:3, v/v) solution, containing a chromogenic reagent, N-methyl-2-phenylindole, and vortexed. Following the addition of 150 μL of methanesulfonic acid (15.4 M), the incubation was carried out at 45° for 40 min. The reaction between MDA + 4-HDA and N-methyl-2-phenylindole yields a chromophore, which is spectrophotometrically measured at the absorbance of

586 nm, using a solution of 10 mM 4-hydroxynonenal as the standard. The level of lipid peroxidation is expressed as the amount of MDA + 4-HDA (nmol) per mg protein. Protein was measured using Bradford's method, with bovine albumin as the standard [41].

2.5. Statistical Analyses

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.

3. Results

In the Experiment I, IPA (10 mM) and melatonin (5 mM), applied separately, reduced KIO_3-induced lipid peroxidation when this prooxidant was used at concentrations of 10 mM, 7.5 mM or 5.0 mM (Figure 1), which is in line with the results of our previous studies [14,15].

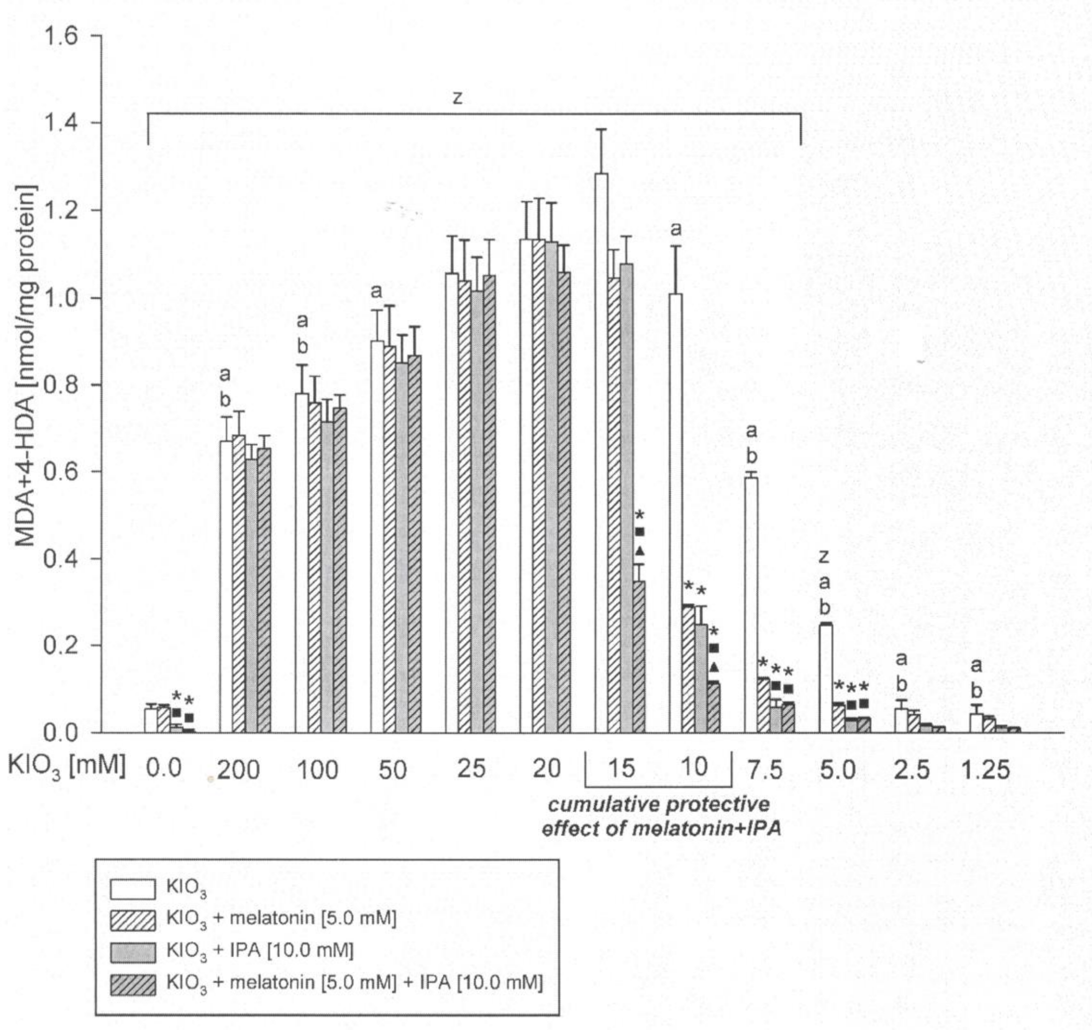

Figure 1. Lipid peroxidation, measured as MDA + 4-HDA level, in porcine thyroid homogenates, incubated in the presence of KIO_3 (200; 100; 50; 25; 20; 15; 10; 7.5; 5.0; 2.5; 1.25; 0.0 mM) (white bars), or KIO_3 + melatonin [5 mM] (striped bars), or KIO_3 + IPA [10 mM] (grey bars), or KIO_3 + melatonin [5 mM] + IPA [10 mM] (striped grey bars). *—$p < 0.05$ vs. KIO_3. a—$p < 0.05$ vs. KIO_3 [15 mM]. b—$p < 0.05$ vs. KIO_3 [10 mM, 20 mM, and 25 mM]. z—$p < 0.05$ vs. respective control. **■—$p < 0.05$ vs. KIO_3 in the same concentration + melatonin. ▲—$p < 0.05$ vs. KIO_3 in the same concentration + IPA.**

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) (Figure 2). 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 (Figure 2).

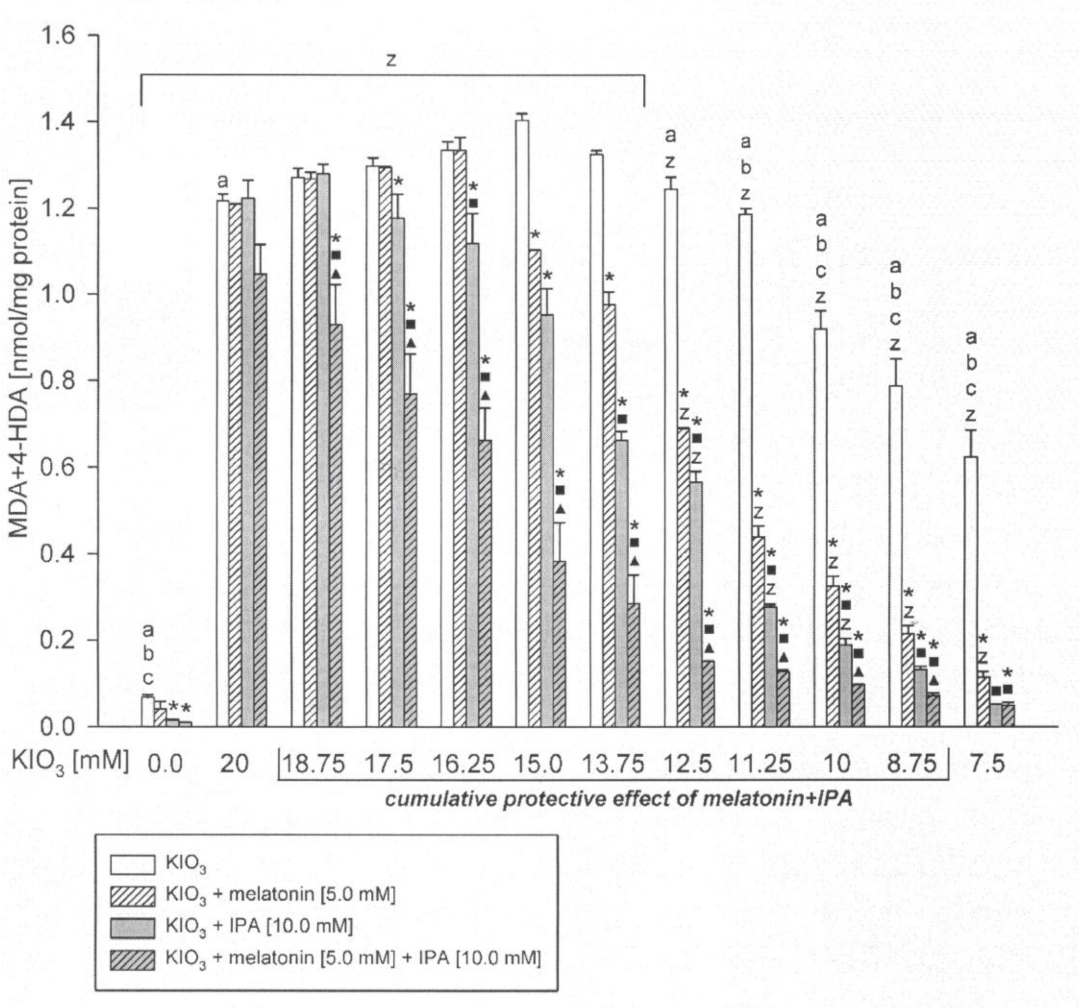

Figure 2. Lipid peroxidation, measured as MDA + 4-HDA level, in porcine thyroid homogenates, incubated in the presence of KIO_3 (20; 18.75; 17.5; 16.25; 15; 13.75; 12.5; 11.25; 10; 8.75; 7.5; 0.0 mM) (white bars), or KIO_3 + melatonin [5 mM] (striped bars), or KIO_3 + IPA [10 mM] (grey bars), or KIO_3 + melatonin [5 mM] + IPA [10 mM] (striped grey bars). *—$p < 0.05$ vs. KIO_3. a—$p < 0.05$ vs. KIO_3 [15 mM]. b—$p < 0.05$ vs. KIO_3 [16.25 mM]. c—$p < 0.05$ vs. KIO_3 [13.75 mM]. z—$p < 0.05$ vs. respective control. **■—$p < 0.05$ vs. KIO_3 in the same concentration + melatonin [5 mM]. ▲—$p < 0.05$ vs. KIO_3 in the same concentration + IPA [10 mM].**

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 LPO was induced by KIO_3 in concentrations of 15 mM and 10 mM (Experiment I, Figure 1) or in the range of concentrations from 18.75 mM to 8.75 mM (Experiment II, Figure 2). 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 been also observed that melatonin did not change the basal LPO level, whereas IPA or IPA + melatonin decreased the basal LPO level (Figures 1 and 2).

4. Discussion

This study is a continuation of our research on the antioxidative properties of melatonin and other indole substances. Taking into account properties of these substances we decided to use concomitantly two effective antioxidants—melatonin and IPA—in their highest achievable in vitro concentrations, i.e., 5 mM for melatonin and 10 mM for IPA, to evaluate their cumulative effect against oxidative damage caused by KIO_3.

Because iodate has been conferred GRAS status by FDA [19,42] and due to its greater chemical stability comparing to iodide, most health authorities recommend using preferentially the former iodine compound as an additive to salt for correcting iodine deficiency [43]. Iodate was tested for its potential toxicity, but it has not been confirmed till now in humans. However, taking into account that iodic acid (HIO_3) belongs to the class of oxohalogen acids, has similar chemical structure to that one of $KBrO_3$ (known potential carcinogen belonging to the group 2B according to IARC [44]) and reveals prooxidative effects documented in our previous studies [12,14,15], it cannot be excluded that this compound may be potentially dangerous.

Currently, despite the worldwide strategies for the prevention and control of iodine deficiency, it is still a widespread public health issue, especially in pregnant women. Severe iodine deficiency may be associated with many adverse effects, such as the increased risk of pregnancy loss and infant mortality, neonatal hypothyroidism, cretinism and neuropsychomotor retardation [45,46]. Moreover, as it was mentioned above, iodine deficiency may lead to goiter—a risk factor for thyroid cancer [7]. As KIO_3 is broadly used for salt iodization and as potential toxicity of KIO_3 has been observed in experimental studies, it is justified to look for safe factors, which can prevent any damage potentially caused by KIO_3. For this reason, we continue our research on the antioxidative properties of melatonin and other indole substances with relation to protection against oxidative damage to membrane lipids caused by KIO_3.

In the present study either melatonin or IPA decreased lipid peroxidation induced by KIO_3, what is in agreement with our previous observations [14,15]. The most important observation is, however, that melatonin used together with IPA revealed even stronger protective effects than each of these antioxidants used separately. It should be stressed that the protective effects of either melatonin or IPA [14,15] as well as of both indole substances used simultaneously (the present study) were observed only when KIO_3 was applied in concentrations (from 10 mM to 7.5 mM in [14,15]; from 18.75 mM to 8.75 mM in the present study) corresponding to physiological iodine concentration in the thyroid, which obviously result from recommended iodine supply. The physiological iodine concentration in rat and human thyroid was calculated to be approx. 9.0 mM [16–18] (being in the range of KIO_3 concentrations from 18.75 mM to 8.75 mM). Taking into account similarity between porcine and human thyroid, it can be assumed that concentration of iodine in porcine thyroid is similar.

Antioxidative effects of melatonin have been known for a long time [21,24,28]. These effects were observed not only in the thyroid gland [38], but also in other tissues, both in vivo and in vitro experiments [47,48]. Mechanisms by which melatonin protects against LPO are as follows: melatonin stimulates antioxidative enzymes, i.e., glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase, upregulates synthesis of glutathione (another intracellular antioxidant) and cooperates with free radical scavengers [20,24]. Moreover, melatonin is able to detoxify practically all free radicals and reactive species, such as •OH [21], nitric oxide and $ONOO^-$, and to suppress nitric oxide synthase [20,24]. Furthermore, metabolites of melatonin (AMK, AFMK and c3OHM) can protect against oxidative damage, as all three are highly effective scavengers of the devastatingly reactive •OH, and c3OHM is highly effective in scavenging the •OOH [22–24].

Melatonin is regarded as the strongest known antioxidant, but there are available studies, which showed superiority of IPA over melatonin [49]. IPA, similar to melatonin, is an endogenous electron donor that detoxifies the •OH, quenches the $O_2^{\bullet-}$ and acts synergistically with glutathione [32]. Its side chain cannot be decarboxylated, and thus, unlike other indoles, it cannot be converted to a reactive prooxidant intermediate [50].

Both substances, melatonin and IPA, are recognized as safe and do not reveal any adverse effects [31,37].

We proved, that IPA and melatonin, used together in very high doses, intensified antioxidative effect, at least under in vitro conditions. Therefore, they can be used together, when stronger protective action is expected but none of them can be used separately in higher dose due to their limited solubility.

As it was mentioned in the Introduction, exogenous melatonin is applied therapeutically in doses between 2 and 10 mg. In available studies the highest dose of melatonin used in clinical trials was 25 mg [51]. The intravenous administration of melatonin in a dose of 25 mg resulted in blood concentration of ~7.52×10^5 pg/mL [51]. In another study melatonin used in a dose of 10 mg intravenously resulted in blood concentration of ~3.9×10^5 pg/mL and when used orally, in concentration of ~3.5×10^3 pg/mL [52]. Relating these concentrations to those used by us (5 mM of melatonin is equivalent to ~1.16×10^9 pg/mL) it can be concluded that the concentrations used in the present experiment exceed the standard doses by several orders of magnitude. Unfortunately, similar studies with IPA have not been performed. It should be stressed, that our results concerning protective in vitro effects of melatonin used together with IPA cannot be directly extrapolated into in vivo conditions.

In the context of our results, it is worth recalling that both melatonin and IPA are regarded as interesting chemical compounds with potential properties for use in many fields of medicine. Oncostatic effects of melatonin have been reported in breast cancer, ovarian and endometrial carcinoma, prostate cancer, intestinal tumors or melanoma; melatonin may be used in psychiatric and neurodegenerative disorders (i.e., Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis), diabetes and metabolic syndrome or sepsis [20,24,28,53,54]. The research currently under way evaluates potential protective effects of melatonin against COVID-19 [55–58]. Concerning IPA, this indole substance—as it was mentioned above—may be regarded as a potential treatment option for Alzheimer's disease [37].

The current study is the next one in which we observed antioxidative effects of melatonin [14] and IPA [15] against oxidative damage caused by KIO_3 used at concentrations close to physiological iodine concentration in the thyroid. However, the current study is the first to document that protective effect of one indole substance can be enhanced by the simultaneous use of another indole substance. The importance of the present finding relies on the fact that due to limited solubility of indole substances it is possible to increase the effectiveness of a chosen substance only by the use of another indole substance. The future studies should focus on to check if the simultaneous use of more than two indole substances can still increase protective effects against experimentally-induced oxidative damage caused by different prooxidants.

The important limitation of our study is that the obtained results cannot be directly extrapolated into in vivo conditions. It is worth mentioning, that in in vivo conditions IO_3^- should be reduced to I^- by nonenzymatic reactions before it can become available to the body as iodide [43]. Recent studies showed that in rats even high doses of IO_3^- were completely reduced to I^- in vivo within 30 min [59]. The results suggest that IO_3^- may be reduced in the digestive tract before I^- enters the blood, but this mechanism is still unexplained [59]. Similar effects were observed in rat homogenates—IO_3^- was reduced to I^-— in vitro [11]. However, whereas KIO_3 decreased total antioxidative activity and NADPH concentration in tissues in vitro [11], this effect of KIO_3 has not been confirmed in vivo, i.e., KIO_3 did not affect the total antioxidative activity in blood serum and in other tissues [59]. These differences between results obtained in vivo and in vitro require further

research to better understand KIO_3 effects in various conditions. At this moment we can state that presumably, except for the gastrointestinal mucosa, exposure of other tissues (including the thyroid gland) to iodate (after its systemic administration) might be minimal. At the same time, however, it is not excluded that even minimal exposure of prooxidative agent can produce toxic effects.

In our previous studies [14,15] we tried to answer the question, why melatonin and IPA were effective against these concentrations of KIO_3 which correspond to physiological iodine concentration in the thyroid. We proposed a hypothesis, that during phylogenetical development in mammals, some protective mechanisms have been developed to protect against well recognized toxic agents, to which organisms are potentially endangered for years. However, much higher concentrations of iodine, resulting e.g., from pharmacological treatment, are not a common and physiological state; that is why protective mechanisms have not been developed against these rare conditions. The results of our current study also seem to confirm this hypothesis.

5. Conclusions

Melatonin and indole-3-propionic acid exert cumulative protective effect against oxidative damage caused by KIO_3, when this prooxidant is used in concentrations close to physiological iodine concentrations in the thyroid. Therefore, the simultaneous administration of these two indoles should be considered to prevent more effectively oxidative damage (and thereby thyroid cancer formation) caused by iodine compounds applied in iodine prophylaxis.

Author Contributions: P.I. designed the study, conducted the experiments, analyzed statistically the data, prepared graphical presentation of the results, and wrote the first version of the manuscript. J.S. participated in statistical analyses and in manuscript editing. M.K.-L. supervised all steps of the study, especially the conceptualization of the study, revised the manuscript critically for important intellectual content and gave final approval for the version to be published. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the fact that—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 subject to registration by the center in which the organs or tissues were taken. Additionally, we have not used experimental animals; instead, porcine thyroids were collected from animals at a slaughter-house during the routine process of slaughter carried out for consumption.

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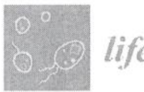
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Pro-Oxidative Effect of KIO_3 and Protective Effect of Melatonin in the Thyroid—Comparison to Other Tissues

Paulina Iwan [1], Jan Stepniak [1] and Malgorzata Karbownik-Lewinska [1,2,*]

[1] Department of Oncological Endocrinology, Medical University of Lodz, 7/9 Zeligowski St., 90-752 Lodz, Poland; paulina.iwan@op.pl (P.I.); jan.stepniak@umed.lodz.pl (J.S.)
[2] Polish Mother's Memorial Hospital—Research Institute, 281/289 Rzgowska St., 93-338 Lodz, Poland
* Correspondence: mkarbownik@hotmail.com

Abstract: Not only iodine deficiency, but also its excess may contribute to thyroid cancer. Potassium iodate (KIO_3), which is broadly used in the salt iodization program, can increase oxidative damage to membrane lipids (lipid peroxidation, LPO) under experimental conditions, with the strongest damaging effect at KIO_3 concentration of ~10 mM (corresponding to physiological iodine concentration in the thyroid). Melatonin is an effective antioxidant, which protects against KIO_3-induced LPO in the thyroid. This study aimed to compare the protective effects of melatonin, used in the highest achievable in vitro concentration, against KIO_3-induced oxidative damage to membrane lipids in various porcine tissues (thyroid, ovary, liver, kidney, brain, spleen, and small intestine). Homogenates were incubated in the presence of KIO_3 (20; 15; 10; 7.5; 5.0; 0.0 mM) without/with melatonin (5 mM). The malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) concentration (LPO index) was measured spectrophotometrically. KIO_3 increased the LPO in all examined tissues; in the thyroid, the damaging effect of KIO_3 (10; and 7.5 mM) was lower than in other tissues and was not observed for the lowest concentration of 5 mM. Melatonin reduced LPO induced by KIO_3 (10, 7.5, and 5 mM) in all tissues, and in the thyroid it was also protective against as high a concentration of KIO_3 as 15 mM; the LPO level resulting from KIO_3 + melatonin treatment was lower in the thyroid than in other tissues. In conclusion, the thyroid is less sensitive tothe pro-oxidative effects of KIO_3 compared to other tissues. The strongest protective effect of melatonin was observed in the thyroid, but beneficial effects were significant also in other tissues. Melatonin should be considered to avoid the potential damaging effects of iodine compounds applied in iodine prophylaxis.

Keywords: melatonin; potassium iodate; KIO_3; lipid peroxidation; antioxidant; salt iodization; thyroid

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1. Introduction

Free radicals and reactive oxygen species (ROS) are highly reactive transient molecules produced by almost all aerobic cells [1]. ROS include both oxygen radicals (e.g., superoxide anion radical—$O_2^{\bullet-}$, hydroxyl radical—$^{\bullet}OH$, and hydroperoxyl radical—$^{\bullet}OOH$) and certain nonradical oxidizing agents easily converted into radicals (e.g., ozone—O_3, hydrogen peroxide—H_2O_2, hypochlorous acid—HOCl) [2,3]. There is a balance between production and detoxification of ROS under physiological conditions in living organisms [1]. Any imbalance between these processes may result in oxidative stress which, in turn, may cause oxidative damage to membrane lipids, DNA and proteins [3,4]. The importance of oxidative stress is commonly emphasized in the pathogenesis of various degenerative diseases, such as cardiovascular and neurodegenerative diseases, kidney diseases, diabetes or cancer [5].

Due to the high level of polyunsaturated fatty acids (PUFAs) in cellular and organelle membranes, they are especially susceptible to lipid peroxidation (LPO), a process in which free radicals remove electrons from lipids and subsequently produce reactive intermediates.

LPO damages phospholipids directly—it can act as a cell death signal—and it is implicated in various degenerative processes, including cancer [4,6].

Although oxidative reactions occur in almost all tissues and organs, the thyroid gland is the organ of "oxidative nature" [7]. ROS are essential for thyroxine (T_4) synthesis since, H_2O_2 produced in thyroid follicular cells is indispensable in attaching iodine atoms to thyroglobulin [8]. Therefore, the thyroid gland is characterized by a high level of oxidative stress, which—in response to additional oxidative abuse caused by various prooxidants—may lead to different thyroid diseases, such as thyroid cancer [7]. Additionally, excess of iodine, as an exogenous pro-oxidant, may induce apoptosis in thyroid follicular cells [9].

An important role in iodine homeostasis is played by the sodium/iodide symporter (NIS). This protein is responsible for active transport of iodide (I^-) into the thyroid gland at the level of the basolateral membrane [10]. NIS was documented to mediate I^- transport not only in the thyroid gland, but also in other tissues, which are able to concentrate radioiodine, such as lactating breast, salivary glands, stomach, and small intestine [11,12]. Thyrotropin (TSH) is the primary regulator of I^- uptake and NIS expression, but only in thyroid follicular cells [11]. It is worth mentioning that NIS mRNA has also been found in other tissues, such as colon, ovaries, uterus or spleen, but the role and significance of NIS in these tissues is still unclear [10].

Iodine is a micronutrient playing an essential role in metabolism. Its deficiency may lead to goiter and hypothyroidism and in pregnant patients to impaired infant neurobehavioral development [13–15]. Correction of iodine deficiency may decrease the prevalence of goiter and shift thyroid cancer subtypes towards a less malignant form and ensure adequate thyroid hormone synthesis [15]. However, not only iodine deficiency, but also its excess may cause pathological phenomena such as thyroiditis, hypo- or hyperthyroidism, and papillary thyroid cancer [13].

Universal salt iodization is widely recognized as the most cost-effective method to reduce iodine deficiency [16]. Programs of salt iodization are based on the use of either potassium iodide (KI) or potassium iodate (KIO_3), with the latter—due to its higher stability—being the most commonly used iodine compound for this process [16]. Both KI and KIO_3 have different pro- and antioxidative properties; KI is the reductant, whereas KIO_3 is the oxidant and may react with oxidizable substances [17]. The differences between the oxidative properties of KI and KIO_3 and their effects on oxidative damage to macromolecules in the thyroid gland were documented recently [18–20]. KI, used in the doses recommended in iodine prophylaxis, may prevent oxidative damage to membrane lipids in the thyroid [18]. In turn, KIO_3 damages membrane lipids in the thyroid with the strongest damaging effect observed at concentrations of around 10 mM [18] and 15 mM [20–22], which correspond to the physiological iodine concentration in the thyroid [23–25].

The total body iodine content in humans was estimated to be 12–25 mg, of which 5–15 mg is stored in the thyroid [26], although data concerning this issue do vary. In another study using pigs, the distribution of iodine in the organism was similar, i.e., the thyroid contained about 80% of the total body iodine, internal organs and blood (14%), muscle and fat (5%), and bones (1%) [27]. Compared to the thyroid gland, the extrathyroidal tissues contain only traces of iodine. The ratio of the iodine concentration in kidney, liver, muscle and skin to that in the thyroid gland was calculated as 1 to 100,000 [28]. However, even in tissues with a low level of iodine concentrations such as the gastrointestinal tract, kidneys or liver, high doses of KIO_3 have shown potential toxicity [29].

Melatonin, N-acetyl-5-methoxytryptamine, being a tryptophan metabolite, mainly produced by the pineal gland, is very strong and effective in reducing oxidative stress [30]. It is considered that melatonin exists possibly in all animal and plant species. Probably melatonin appeared 3.0–2.5 billion years ago in photosynthetic cyanobacteria as an antioxidant [31]. It is documented that melatonin reveals protective effects against oxidative stress not only in the thyroid gland [20,22], but also, as was even earlier found, in many other tissues and organs, among others in kidney [32], spleen [33], ovary [34], liver [35] or erythrocytes [36].

Although the antioxidant capacity of melatonin has been proven both in vitro and in vivo conditions, there are few studies in which melatonin revealed pro-oxidative properties. It has been found, for example, that melatonin promotes the generation of ROS when used in a certain range of concentrations (mainly from μM to mM) and, additionally, depending on duration of the treatment under in vitro conditions [37]. What is of great importance is that this pro-oxidative action of melatonin was observed mostly in cancer cells and promoted inflammatory responses and apoptosis [37]. This observation has not been confirmed until now under in vivo conditions, but the ability of melatonin to induce apoptosis in tumor cells might have important therapeutic implications [37].

In our previous studies [20,22] we observed, that melatonin was able to reduce oxidative damage to membrane lipids caused by KIO_3, when this prooxidant was used in doses close to physiological concentrations of iodine in the thyroid. In the present study we decided to compare the protective effects of melatonin against KIO_3-induced oxidative damage to membrane lipids in various porcine tissues, i.e., in the thyroid, the ovary, the liver, the kidney, the brain, the spleen, and the small intestine. KIO_3 was used in the range of concentrations comprising those corresponding to physiological iodine concentration in the thyroid, whereas melatonin was used in the highest achievable in vitro concentration (i.e., 5 mM).

2. Materials and Methods

2.1. Chemicals

Potassium iodate (KIO_3) and melatonin were purchased from Sigma (St. Louis, MO, USA). The ALDetect Lipid Peroxidation Assay Kit was obtained from Enzo Life Sciences, Inc. (Zandhoven, Belgium). All used chemicals were of analytical grade and came from commercial sources.

2.2. Animals

Porcine tissues (i.e., thyroid, ovary, spleen, liver, brain, small intestine, and kidney) were collected from fifteen (15) female animals at a slaughter-house, frozen on solid CO_2 and stored at −80 °C until assayed. Each experiment was repeated three times.

2.3. Incubation of Tissue Homogenates

Porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, and kidney) were homogenized in ice cold 20 mM Tris-HCl buffer (pH 7.4) (10%, w/v) and then incubated for 30 min at 37 °C in the presence of KIO_3 (20; 15; 10; 7.5; 5.0, 0.0 mM) without or with addition of melatonin in a concentration of 5 mM (the highest achievable concentration resulting from its limited solubility).

The concentrations of KIO_3 and melatonin were chosen on the basis of the results of our previous studies [18–20,22].

The reactions were stopped by cooling the samples on ice.

2.4. Measurement of Lipid Peroxidation Products

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA), as an index of lipid peroxidation, were measured in homogenates with the ALDetect Lipid Peroxidation Assay Kit. The homogenates were centrifuged at 5000× g for 10 min at 4 °C. After obtaining supernatant, each experiment was carried out in duplicate. The supernatant (200 μL) was mixed with 650 μL of a methanol:acetonitrile (1:3, v/v) solution, containing a chromogenic reagent, N-methyl-2-phenylindole, and vortexed. Following the addition of 150 μL of methanesulfonic acid (15.4 M), the incubation was carried out at 45 °C for 40 min. The reaction between MDA + 4-HDA and N-methyl-2-phenylindole yields a chromophore, which is spectrophotometrically measurable at an absorbance of 586 nm, using a solution of 10 mM 4-hydroxynonenal as the standard. The level of lipid peroxidation is expressed as the amount of MDA + 4-HDA (nmol) per mg protein. Protein was measured using Bradford's method, with bovine albumin as the standard [38].

2.5. Statistical Analyses

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.

3. Results

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 significant and confirmed for thyroid, ovary, and kidney. The incubation with melatonin decreased the basal level of LPO only in ovary tissue (Figure 1).

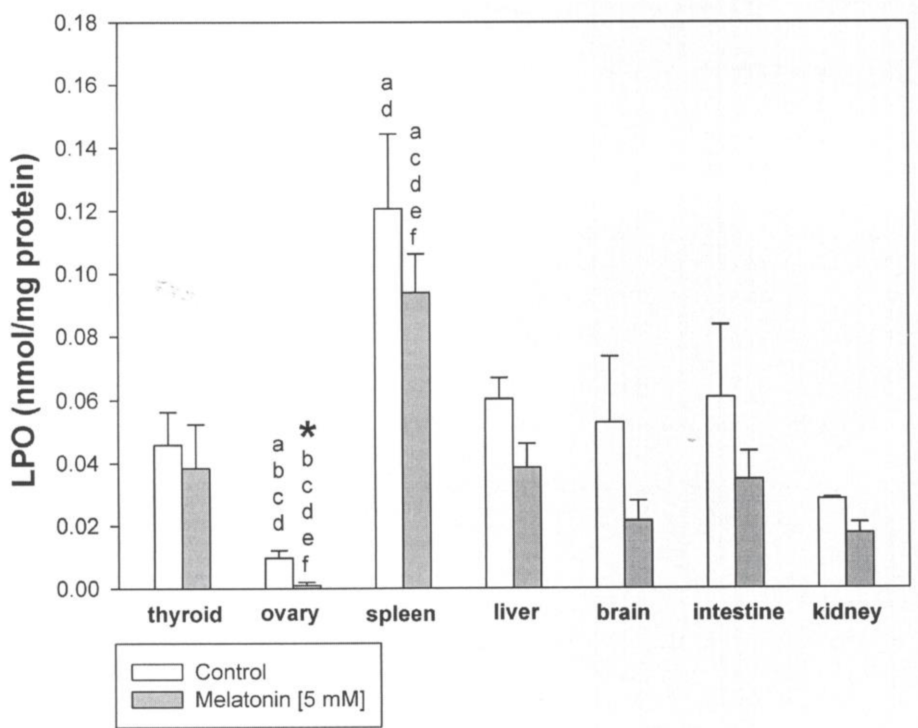

Figure 1. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates of porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, kidney), incubated without any substance (control; white bars), or with melatonin [5 mM] (grey bars). *—$p < 0.05$ vs. control (without melatonin) in the same tissue; **a**—$p < 0.05$ vs. respective bar (control or melatonin) in the thyroid; **b**—$p < 0.05$ vs. respective bar (control or melatonin) in the spleen; **c**—$p < 0.05$ vs. respective bar (control or melatonin) in the liver; **d**—$p < 0.05$ vs. respective bar (control or melatonin) in the kidney; **e**—$p < 0.05$ vs. respective bar (control or melatonin) in the brain; **f**—$p < 0.05$ vs. respective bar (control or melatonin) in the intestine.

KIO_3 increased the lipid peroxidation in all examined tissues (i.e., thyroid, ovary, spleen, liver, brain, small intestine, and kidney) with the strongest damaging effect observed at concentrations of of 20 mM (Figures 2 and 3), of 15 mM (Figures 2 and 4), and of 10 mM (Figures 2 and 5) vs. 7.5 mM and 5.0 mM in all tissues, and at concentrations of 20 mM (Figures 2 and 3), of 15 mM (Figures 2 and 4) vs. 10 mM in the thyroid and the liver. It should be stressed, however, that in thyroid tissue the damaging effect of KIO_3 was not observed at its lowest concentration of 5 mM (Figure 2). Additionally, LPO 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 (Figures 5–7).

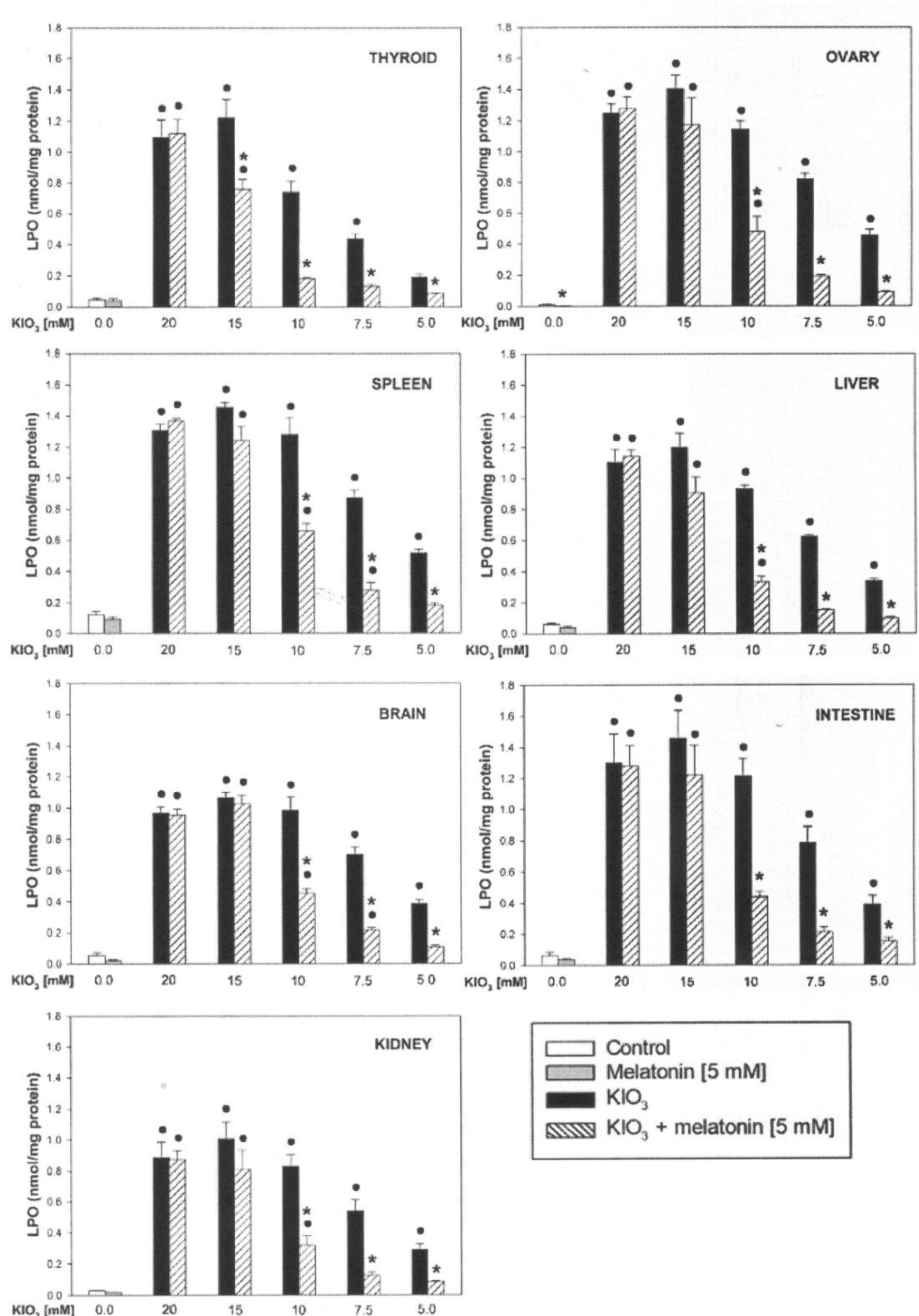

Figure 2. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates of porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, kidney), incubated without any substance (control; white bars), or with melatonin (5 mM) (grey bars), or with KIO_3 (20; 15; 10; 7.5; 5.0 mM) (black bars), or with KIO_3 (20; 15; 10; 7.5; 5.0 mM) + melatonin (5 mM) (striped bars). •—$p < 0.05$ vs. respective control (either without any substance or with melatonin); *—$p < 0.05$ vs. KIO_3 in the same concentration.

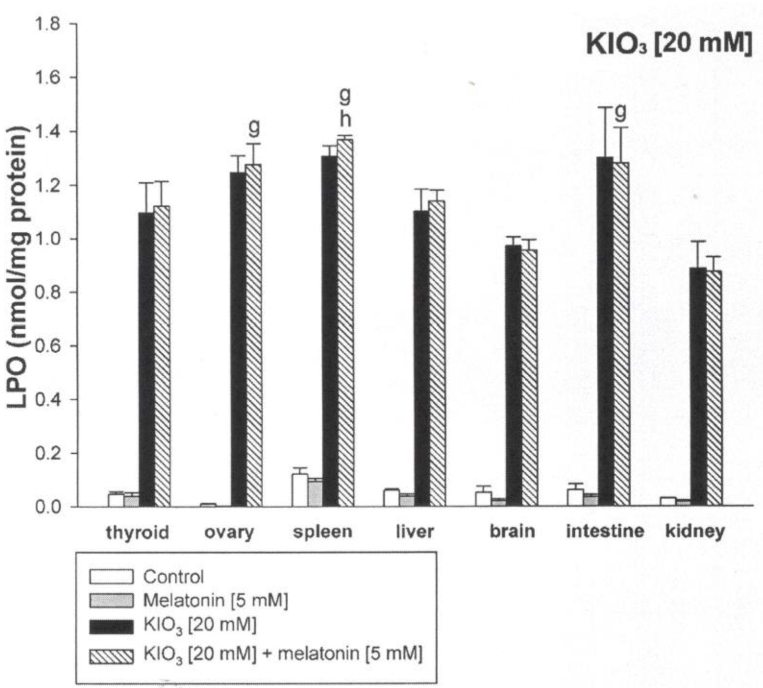

Figure 3. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates of porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, kidney), incubated without any substance (control; white bars), or with melatonin (5 mM) (grey bars), or with KIO_3 (20 mM) (black bars), or with KIO_3 (20 mM) + melatonin (5 mM) (striped bars). **g**—$p < 0.05$ vs. KIO_3 (20 mM) + melatonin (5 mM) in kidney; **h**—$p < 0.05$ vs. KIO_3 (20 mM) + melatonin (5 mM) in brain.

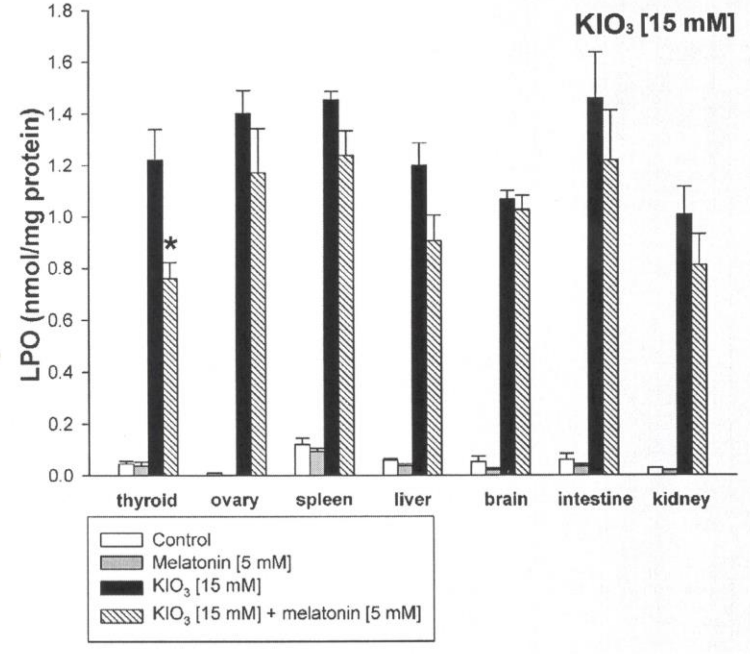

Figure 4. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates of porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, kidney), incubated without any substance (control; white bars), or with melatonin (5 mM) (grey bars), or with KIO_3 [15 mM] (black bars), or with KIO_3 (15 mM) + melatonin (5 mM) (striped bars). *—$p < 0.05$ vs. KIO_3 in the same tissue.

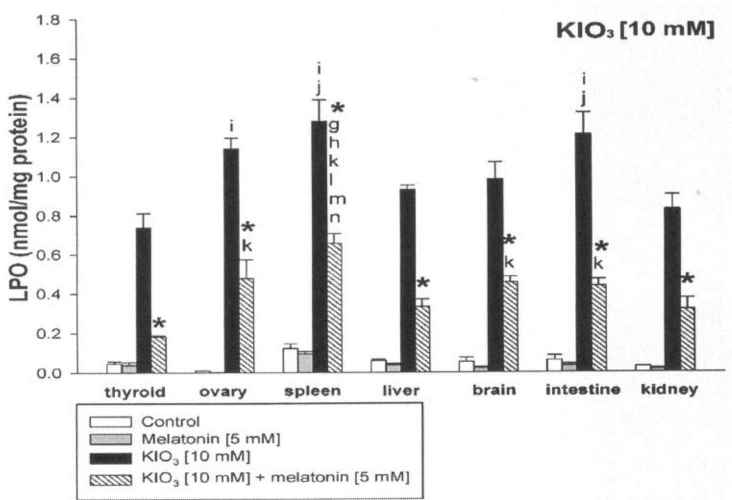

Figure 5. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates of porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, kidney), incubated without any substance (control; white bars), or with melatonin [5 mM] (grey bars), or with KIO_3 [10 mM] (black bars), or with KIO_3 [10 mM] + melatonin [5 mM] (striped bars). *—$p < 0.05$ vs. KIO_3 in the same tissue; **i**—$p < 0.05$ vs. KIO_3 [10 mM] in thyroid; **j**—$p < 0.05$ vs. KIO_3 [10 mM] in kidney; **g**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in kidney; **h**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in brain; **k**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in thyroid; **l**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in ovary; **m**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in liver; **n**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in intestine.

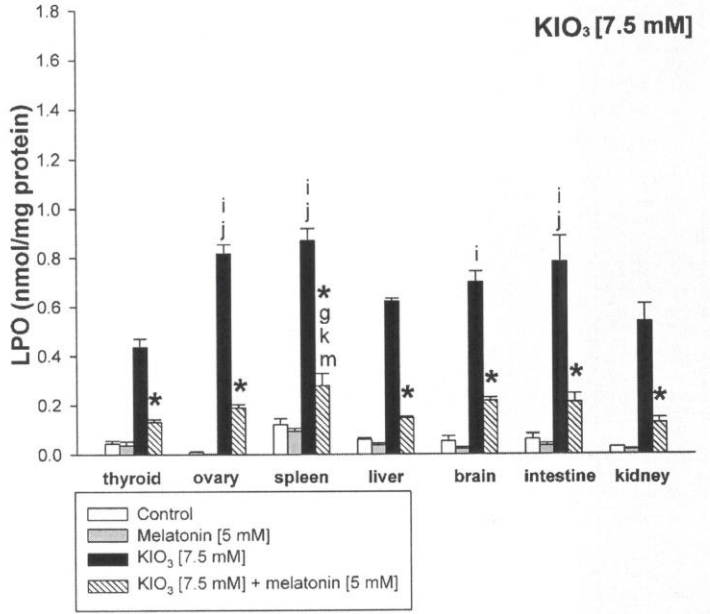

Figure 6. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates of porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, kidney), incubated without any substance (control; white bars), or with melatonin [5 mM] (grey bars), or with KIO_3 [7.5 mM] (black bars), or with KIO_3 [7.5 mM] + melatonin [5 mM] (striped bars). *—$p < 0.05$ vs. KIO_3 in the same tissue; **i**—$p < 0.05$ vs. KIO_3 [10 mM] in thyroid; **j**—$p < 0.05$ vs. KIO_3 [10 mM] in kidney; g—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in kidney; **k**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in thyroid; **m**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in liver.

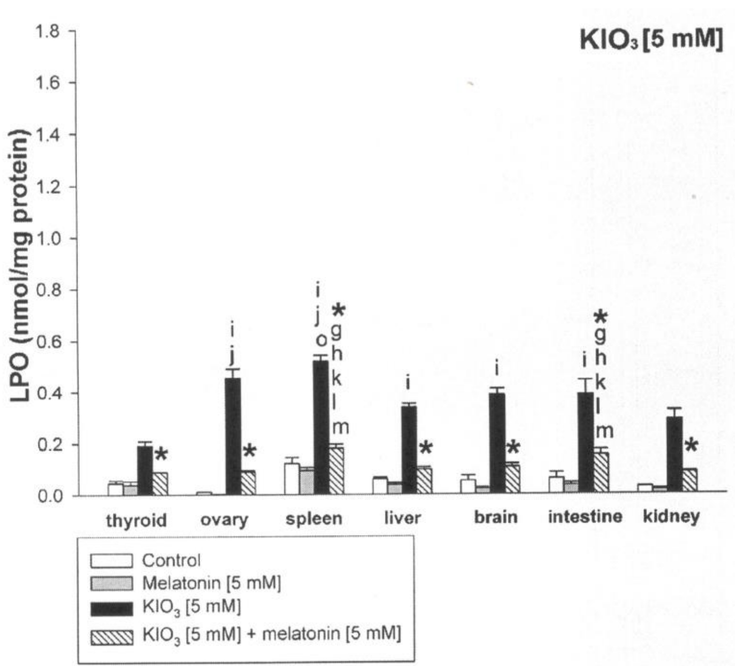

Figure 7. Lipid peroxidation, measured as MDA + 4-HDA level, in homogenates of porcine tissues (thyroid, ovary, spleen, liver, brain, small intestine, kidney), incubated without any substance (control; white bars), or with melatonin [5 mM] (grey bars), or with KIO_3 [5 mM] (black bars), or with KIO_3 [5 mM] + melatonin [5 mM] (striped bars). *—$p < 0.05$ vs. KIO_3 in the same tissue; i—$p < 0.05$ vs. KIO_3 [10 mM] in thyroid; **j**—$p < 0.05$ vs. KIO_3 [10 mM] in kidney; o—$p < 0.05$ vs. KIO_3 [10 mM] in liver; **g**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in kidney; **h**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in brain; **k**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in thyroid; **l**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in ovary; **m**—$p < 0.05$ vs. KIO_3 [10 mM] + melatonin [5 mM] in liver.

Melatonin (5 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 mM (Figure 2). 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 (Figures 2 and 4). The LPO level resulting from KIO_3 + melatonin treatment was lower in the thyroid than in other tissues (Figures 5–7). The latter two observations suggest that the protective effect of melatonin was the strongest in the thyroid.

4. Discussion

This study is the next in line, in which we evaluated antioxidative properties of melatonin against oxidative damage caused by KIO_3, and presumably the first attempt to compare the protective effects of melatonin in various porcine tissues. For the present study we chose the concentrations of KIO_3 (i.e., 20; 15; 10; 7.5; 5.0 mM) which had revealed the strongest damaging effect to membrane lipids in thyroid homogenates in our previous studies [18–20,22]. Due to the similarity between human and porcine thyroid (hormone synthesis, volume) [39] we decided to continue our experimental model also using other porcine tissues.

Although concentrations of iodine in all other tissues are much lower than in the thyroid gland [28], damaging effects of KIO_3 were observed in all tissues examined in our study.

When we compared the damaging effect of KIO_3 we observed that LPO induced by this compound was significantly lower in the thyroid gland than in any other examined porcine tissues (except kidney). This observation illustrates the fact that the thyroid gland has adapted to maintain large concentrations of iodine. As the thyroid constitutes an organ,

in which oxidative processes are indispensable for proper functioning and thyroid hormone synthesis, some protective mechanisms have been developed to protect this gland against the huge amount of iodine. One of the thyroidal adaptations to iodine excess is the Wolff–Chaikoff effect. This effect, still not completely explained, was observed in rats exposed to high amounts of iodide, which resulted in transient reduction in the thyroid hormone synthesis; the block lasted approx. 24 h [40]. This adaptation is associated with a decrease in expression of the sodium-iodide symporter (NIS), resulting in reduced intrathyroidal iodine concentration; thus, this is the next mechanism contributing in maintaining proper thyroid function. NIS is an intrinsic membrane protein, found mainly in the basolateral membrane of thyroid follicular cells; its regulator is not only TSH, but also I^- itself [40–43].

The basal level of LPO was lower in the ovary than in the thyroid homogenates, which was confirmed also in our previous studies [44]; and observations from two different studies [45,46]. On the other hand, LPO induced by KIO_3, similar to LPO induced by Fenton reaction substrates [44], was higher in the ovary than in the thyroid homogenates. This observation also confirms the hypothesis, that in physiological conditions oxidative stress in the thyroid (resulting mostly from oxidative reactions indispensable for thyroid hormone synthesis) is at a substantially higher level than in other tissues. At the same time this physiologically high level of oxidative stress in the thyroid makes this organ less vulnerable to pro-oxidative agents, such as iodate or iron (used in the Fenton reaction).

We also observed a significantly lower LPO level induced by KIO_3 in the kidney compared to other tissues (Figures 5–7). This observation may be justified by the following reason. Potassium bromate ($KBrO_3$)—halogenate salt, belonging to the same class (oxohalogen acids) together with chloric ($HClO_3$) and iodic (HIO_3) acids, have been known to be potential carcinogens, experimentally inducing renal tumors [47]. This compound has been classified as possibly carcinogenic to humans (group 2B according to IARC) [48]. Although KIO_3 has been conferred GRAS status by the FDA [29], it was not listed as a carcinogen with IARC, ACGIH, NTP, or OSHA [49] and did not induce toxic effects under conditions in which the bromate did [29], although similar to $KBrO_3$—kidney tissue is presumably more resistant to iodate than other tissues. However, separate studies should be performed to clarify the mechanism of lower kidney sensitivity to iodate.

In the present study we showed that melatonin significantly reduced LPO induced by KIO_3, when this compound was used at doses corresponding to physiological concentrations of iodine in the thyroid (approx. 9.0 mM) [23–25], which is in line with our previous publications [20–22]. It should be stressed that the protective effects of melatonin were observed in all examined tissues (when KIO_3 was applied in concentrations of 10 mM, 7.5 mM and 5 mM), but the most important observation is that melatonin revealed the strongest protective effect in the thyroid gland—it was the only tissue, in which beneficial results of melatonin were observed against as high a KIO_3 concentration as 15 mM. Additionally, LPO levels resulting from KIO_3 + melatonin exposure were lower in the thyroid compared to other tissues, but these differences may be due to a weaker damaging effect of KIO_3 in the thyroid. Further studies are required to clarify the mechanisms responsible for stronger effectiveness of melatonin against KIO_3-induced damage observed in the thyroid compared to other tissues.

The relationship between melatonin (or its main source, i.e., the pineal gland) and the thyroid gland has been studied for many years. Large experimental evidence suggests the inhibitory action of melatonin on thyroid growth and function [50,51]. These effects were observed when using different experimental models, such as chronic and short-term melatonin administration in vivo, light restriction, pinealectomy or exposure to melatonin under in vitro conditions [50,51]. The inhibitory action of melatonin on the hypothalamic–pituitary–thyroid axis occurs at all three levels, i.e., at the hypothalamic level (inhibition of synthesis and release of thyrotropin releasing hormone (TRH)), at the pituitary level (inhibition of thyrotropin (TSH) release), and directly at the thyroid level, resulting, among other effects, in decreased blood concentrations of thyroid hormones [50,51].

Melatonin is considered as one of the most effective known antioxidants. Mechanisms by which melatonin protects against lipid peroxidation are as follows. Melatonin acts directly by detoxification of reactive oxygen and nitrogen species, like $^{\bullet}OH$, $O_2^{\bullet-}$, H_2O_2, singlet oxygen ($^1O_2$), HOCl, nitric oxide ($NO^{\bullet}$) or peroxynitrite ($ONOO^-$). As an indirect antioxidant, melatonin can stimulate antioxidative enzymes (glutathione peroxidases, glutathione reductase, superoxide dismutase, and catalase) while suppressing the activity of prooxidant enzymes [30]. Furthermore, its metabolites (i.e., AMK—N1-acetyl-5-methoxykynuramine, AFMK—N1-acetyl-N2-formyl-5-methoxykynuramine, and c3OHM—cyclic-3-hydroxymelatonin) can protect against oxidative damage, as similar to melatonin, they are scavengers of hydroxyl- (AMK, AFMK, c3OHM) and hydroperoxyl- (c3OHM) radicals [52,53].

Except for antioxidative properties, melatonin is a regulator of the circadian rhythm and immune system and is also involved in blood pressure and autonomic cardiovascular regulation. Its therapeutic effects have been reported in certain tumors (e.g., breast cancer, ovarian and endometrial carcinoma, prostate cancer, hepatoma and intestinal tumors), cardiovascular diseases or psychiatric disorders [54].

It is worth emphasizing, that short-term use of melatonin, both in animals and humans, is safe, even in extreme doses. Only mild adverse effects (i.e., sleepiness, headache, dizziness or nausea) have been reported [55].

In the current study melatonin was used at a concentration of 5 mM, which, due to its limited solubility, is the highest achievable in vitro concentration and, after all, it is commonly used in experimental studies. This concentration (i.e., 5 mM) is equivalent to ~1.16×10^9 pg/mL. The physiological blood concentration of melatonin in humans is 0–20 pg/mL in the daytime and at night it reaches even 40–200 pg/mL and decreases with age (e.g., [56]). Exogenous melatonin is applied therapeutically in doses between 2 and 10 mg, and the highest dose of melatonin used in clinical trials was 25 mg [57]. The intravenous administration of melatonin at a dose of 25 mg resulted in a blood concentration of ~7.52×10^5 pg/mL [57]. Relating the abovementioned melatonin concentrations to those used by us it should be concluded that the concentrations used in the current experiment exceed by several orders of magnitude the physiological melatonin concentrations and even those resulting from standard doses of exogenous melatonin application; therefore they should be treated as pharmacological.

The melatonin level declines gradually over the life-span, which may cause disorders related to an altered circadian rhythm, such as sleeping disorders, delirium or disorders of cognitive functioning, especially characteristic for the elderly [58]. Moreover, available studies show that disruption of the circadian rhythm or clock gene expression may lead to liver diseases, such as liver steatosis, inflammation or cancer development. These facts may suggest, that supplementation of melatonin not only prevents oxidative stress-induced liver damage (induced e.g., by alcohol drinking or excess fatty acid diet), but also through restoring the circadian rhythm may be a promising therapeutic strategy for liver diseases [59].

In addition, other tissues, examined in the present work, are susceptible to oxidative stress. Especially the brain, with its high oxygen consumption and lipid-rich content can be very prone to this kind of damage [60]. In the ovary, oxygen radicals play important physiological roles, but its cyclic production over years may lead to an increased cumulative risk of ovarian pathology [61]. The small intestine is the main organ involved in the digestion and absorption of nutrients and is directly exposed to drugs and toxic food contaminants [62].

In physiological conditions there is a balance between production of ROS and RNS and their elimination by protective mechanisms, but with aging or under certain conditions, defense mechanisms are not sufficient, which may result in numerous pathologies. For this reason, it is advisable to look for new potential pharmacological agents against known prooxidants. In our opinion melatonin—a safe and strong antioxidant—should be considered

as a potential protective agent against oxidative damage to membrane lipids caused by KIO_3 not only in the thyroid gland, but also in other tissues, especially in older people.

Concerning clinical conditions associated with the exposure to KIO_3 excess, the following should be taken into consideration. Uncontrolled supplementation of tablets or drops containing microgram doses of KIO_3 seems to be a very probable situation, especially in the older population, while a variety of mineral waters, both with standardized and unstandardized iodine concentration, when drunk in huge amounts may contribute to iodine excess. Iodine contrast agents used in diagnostics and different medications, such as eye drops or antiseptics, commonly used in the general population, contain a very high amount of iodine compounds. Tablets with milligram doses of KIO_3 used at the time of nuclear emergency, although generally safe, may potentially cause some pro-oxidative effects. Overconsumption of iodized salt does not seem to constitute a strong risk factor of excessive exposure of an individual to iodine, however it should be also taken into account at least at the population level. In such situations of increased exposure to iodine compounds and other external factors with pro-oxidative properties, the potential beneficial effects of antioxidants such as melatonin could be very important. However, it should not be forgotten that our experiment was performed in in vitro conditions; therefore, it may not be directly extrapolated into in vivo situations and, consequently, it may not have a direct impact in clinical practice, at least at the current stage of research.

To our knowledge, our study is the first attempt to compare the protective effects of melatonin against experimentally-induced oxidative damage in various porcine tissues. The differences observed in this work should be confirmed by using other methods and other markers of oxidative damage (not only to membrane lipids but also to DNA and proteins) and, whenever possible, by using additional tissues. We intend to expand our research in this area in the future.

5. Conclusions

The thyroid gland is less sensitive to pro-oxidative effects of KIO_3 when compared to other tissues. Melatonin reveals a strong protective effect against oxidative damage caused by KIO_3, when this pro-oxidant is used in doses resulting in physiological concentrations of iodine in the thyroid. The strongest protective effect was observed in the thyroid gland which suggests that this organ responds stronger to antioxidative effects of melatonin. However, beneficial effects were significant also in other tissues. Melatonin, as a very safe agent, should be considered to avoid the potential damaging effects of iodine compounds applied in iodine prophylaxis.

Author Contributions: P.I. designed the study, conducted the experiments, analyzed the data statistically, prepared the graphical presentation of the results, and wrote the first version of the manuscript. J.S. participated in statistical analyses and in manuscript editing. M.K.-L. supervised all steps of the study, especially the conceptualization of the study, revised the manuscript critically for important intellectual content, and gave final approval for the version to be published. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the fact that—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 subject to registration by the center in which the organs or tissues were taken. Additionally, we did not use experimental animals; instead, porcine thyroids were collected from animals at a slaughter-house during the routine process of slaughter carried out for consumption.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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5. Komentarz do cyklu prac w języku polskim

Wstęp

Reaktywne formy tlenu (RFT) i wolne rodniki uczestniczą w wielu procesach metabolicznych. W warunkach fizjologicznych utrzymuje się równowaga pomiędzy wytwarzaniem a neutralizowaniem RFT. Jednakże zaburzenie tej równowagi może powodować niepożądane dla organizmu skutki [1].

Gruczoł tarczowy jest narządem, w którym procesy oksydacyjne odgrywają ważną rolę i są niezbędne m.in. do syntezy hormonów tarczycy [2]. Z tego względu gruczoł tarczowy charakteryzuje się stałym wysokim poziomem stresu oksydacyjnego, który może być dodatkowo zwiększany w odpowiedzi na różne egzo- i endogenne substancje (prooksydanty) i przyczyniać się wówczas do różnych stanów chorobowych, na przykład raka tarczycy.

Jod jest pierwiastkiem niezbędnym do prawidłowego funkcjonowania organizmu. Jego kluczową rolą jest udział w syntezie hormonów tarczycy. Oszacowano fizjologiczne stężenie jodu w gruczole tarczowym, które w warunkach odpowiedniej podaży wynosi ok. 9 mM [3-5]. Niedobór jodu może powodować poważne skutki zdrowotne, m.in. powstanie wola lub niedoczynność tarczycy, a jeśli jest stwierdzany u ciężarnych – także zaburzenia rozwoju płodu [6]. Dlatego tak ważna jest odpowiednia suplementacja jodu, która zapewnia odpowiednią syntezę hormonów tarczycy, zmniejsza częstość występowania wola i zmienia dystrybucję poszczególnych postaci raka tarczycy z obniżeniem odsetka postaci o gorszym rokowaniu.

Jodowanie soli kuchennej jest w wielu krajach najpopularniejszą metodą profilaktyki niedoboru jodu [7]. Światowe programy suplementacji jodu polegają na dodawaniu do soli kuchennej jodku potasu (KI) albo jodanu potasu (KIO_3) [7]. Związki te charakteryzują się różnymi właściwościami oksydacyjnymi – KI jest mniej reaktywny, podczas gdy KIO_3 wykazuje silniejsze właściwości prooksydacyjne. Mimo to KIO_3 uzyskał status „GRAS" (*generally recognized as safe* – generalnie uznany za bezpieczny), nadawany przez FDA (*Food and Drug Administration*) [8]. Jednakże w pewnych eksperymentalnych warunkach *in vitro* KIO_3 wykazywał zdolność do oksydacyjnych uszkodzeń makrocząsteczek biologicznych.

Związki indolowe, z ich głównym reprezentantem melatoniną (5-metoksy-N-acetyltryptaminą), są efektywnymi antyoksydantami i zmiataczami wolnych rodników. Kwas

indolo-3-propionowy (IPA) jest substancją indolową, podobną do melatoniny pod względem struktury chemicznej i właściwości biochemicznych [9-13]. Oba te związki są uznawane za bezpieczne i nie wykazują istotnych działań ubocznych [12,13].

W licznych badaniach udowodniono, że melatonina wykazuje działanie ochronne wobec eksperymentalnie wyindukowanych oksydacyjnych uszkodzeń lipidów błon komórkowych w różnych tkankach, ze szczególnym uwzględnieniem gruczołu tarczowego [9]. Melatonina wpływa również hamująco na wzrost i czynność tarczycy. Z tego powodu może być uznawana jako potencjalny czynnik ochronny przed różnymi chorobami tarczycy, włącznie z nowotworami tego gruczołu.

Cel pracy

Pierwszym celem pracy była ocena potencjalnego działania ochronnego melatoniny przed oksydacyjnymi uszkodzeniami lipidów błon komórkowych (czyli peroksydacją lipidów – LPO) indukowanymi przez KI oraz KIO_3 w homogenatach tarczycy wieprzowej (praca oryginalna 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;91:271-277**).

Następnym celem pracy było zbadanie ochronnego efektu kwasu indolo-3-propionowego (IPA) oraz efektów łącznego zastosowania melatoniny i IPA (w najwyższych, możliwych do uzyskania w warunkach *in vitro*, stężeniach, wynikających z ich ograniczonej rozpuszczalności) przed peroksydacją lipidów wyindukowaną przez KIO_3 w homogenatach tarczycy wieprzowej (praca oryginalna 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;9:89**).

W ostatniej części pracy porównywano ochronne działanie melatoniny przed wyindukowanymi przez KIO_3 oksydacyjnymi uszkodzeniami lipidów błon komórkowych w tkance tarczycy i w innych tkankach zwierzęcych (tj. jajnik, śledziona, wątroba, mózg, jelito cienkie i nerka) (praca oryginalna 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;11:592. Erratum in: Life (Basel). 2022 Jul 07;12(7)**).

Materiały i metody

Badania zostały przeprowadzone w warunkach *in vitro*, z użyciem homogenatów tkanek wieprzowych (tarczyca (we wszystkich pracach oryginalnych: 1, 2, 3) oraz dodatkowo: jajnik, śledziona, wątroba, mózg, jelito cienkie i nerka (praca oryginalna 3)).

Użyte stężenia 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), melatoniny (5.0; 2.5; 1.25; 1.0; 0.625 mM), 17β-estradiolu (1.0 mM) oraz IPA (10; 7.5; 5.0; 2.5; 1.25; 0.625 mM) zostały wybrane na podstawie wyników wcześniej opublikowanych badań naszego zakładu (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 Repred Med 2016, 62, 17–21).

Stężenie dialdehydu malonowego+4-hydroksyalkenali (MDA+4-HDA), jako wskaźnika peroksydacji lipidów, zmierzono spektrofotometrycznie z użyciem *ALDetect Lipid Peroxidation Assay Kit*.

Wyniki poddano analizie statystycznej, używając metody jednoczynnikowej analizy wariancji (ANOVA), a następnie testu Neuman-Keulsa, lub używając t-testu dla dwóch prób niezależnych. Istotność statystyczną określano na poziomie $p<0.05$. Wyniki przedstawiono jako średnie ± SE.

Wyniki

Praca oryginalna 1

Jodek potasu (KI), we wszystkich użytych stężeniach (tj. 500; 250; 100; 50 mM) i w stopniu zależnym od stężenia, spowodował wzrost poziomu peroksydacji lipidów. Także jodan potasu (KIO_3) podwyższył poziom peroksydacji lipidów we wszystkich zastosowanych stężeniach (tj. 200; 100; 50; 25; 10; 5.0; 2.5 mM), przy czym najsilniejszy efekt uszkadzający zaobserwowano przy stężeniach 10 mM i 25 mM. Po inkubacji homogenatów tarczycy z KIO_3 lub KI łącznie z melatoniną (5.0 mM), istotne obniżenie poziomu peroksydacji lipidów było zauważalne jedynie w przypadku KIO_3 użytego w stężeniu 10 mM.

Ponieważ w powyższym modelu nie odnotowano ochronnego działania melatoniny przed peroksydacją lipidów wyindukowaną przez KI, w kolejnych etapach doświadczenia wykorzystywano jedynie KIO_3.

W dalszej części doświadczenia zastosowano dodatkowe stężenia KIO_3 (tj. 20; 15; 7.5; 1.25 mM) aby wyjaśnić niespodziewane wyniki uzyskane w pierwszym etapie badania. Po użyciu dodatkowych stężeń KIO_3, najsilniejszy efekt uszkadzający lipidy błon komórkowych

obserwowano przy stężeniach KIO_3 zbliżonych do 15 mM, z najwyższym poziomem LPO potwierdzonym dla stężeń 15 mM i 20 mM.

Melatonina, w stopniu zależnym od stężenia, zredukowała wyindukowaną przez KIO_3 peroksydację lipidów, ale tylko wówczas, gdy ten prooksydant był zastosowany w stężeniach 10 mM (melatonina użyta w stężeniach: 5.0 mM i 2.5 mM działała ochronnie) i 7.5 mM (melatonina użyta w stężeniach: 5.0; 2.5; 1.25 i 1.0 mM działała ochronnie). Należy podkreślić, że powyższe stężenia KIO_3 (tj. 10 mM i 7.5 mM) odpowiadają fizjologicznemu stężeniu jodu w tarczycy (wyliczonemu na ok. 9 mM).

Inkubacja homogenatów tarczycy wieprzowej jedynie z melatoniną zastosowaną w stężeniach 5.0; 2.5; 1.25; 1.0; 0.625 mM nie zmieniła podstawowej peroksydacji lipidów.

W dalszej części badania zdecydowaliśmy się porównać efekt ochronny melatoniny z potencjalnym działaniem ochronnym innej znanej substancji antyoksydacyjnej – 17β-estradiolu. 17β-estradiol, użyty w stężeniu 1.0 mM będącym najwyższym stężeniem możliwym do uzyskania w warunkach *in vitro*, nie wykazywał korzystnych efektów wobec indukowanej przez KIO_3 peroksydacji lipidów, podczas gdy melatonina, zastosowana w tym samym stężeniu (tj. 1.0 mM), istotnie obniżyła poziom peroksydacji lipidów wyindukowanej przez KIO_3 (7.5 mM).

Praca oryginalna 2

W Eksperymencie I, IPA (10 mM) i melatonina (5.0 mM) użyte osobno, obniżyły poziom peroksydacji lipidów wyindukowanej przez KIO_3 w stężeniach 10 mM, 7.5 mM i 5.0 mM. Jednakże w Eksperymencie II, po zastosowaniu dodatkowych stężeń KIO_3 wykazano, że IPA wywołuje efekt ochronny przy wyższych stężeniach jodanu potasu (16.25 mM) w porównaniu z efektem ochronnym melatoniny (istotne obniżenie LPO przy stężeniu KIO_3 15 mM).

Dodatkowo, efekt ochronny wywołany przez IPA był silniejszy w porównaniu z działaniem wywołanym przez melatoninę przy stężeniach KIO_3 13.75 mM i niższych.

Jednak najważniejszą obserwacją było, że melatonina użyta łącznie z IPA wykazywała silniejsze działanie niż każdy z antyoksydantów zastosowany osobno. Efekt ten był widoczny przy stężeniach KIO_3 15 mM i 10 mM (w Eksperymencie I), a po użyciu dodatkowych stężeń w Eksperymencie II w zakresie stężeń od 18.75 mM do 8.75 mM. Ten kumulacyjny efekt ochronny melatoniny+IPA był szczególnie zauważalny przy wyższych stężeniach KIO_3, tj. przy 18.75 mM i 17.5 mM, przy których ani melatonina, ani IPA użyte osobno nie wykazywały działania protekcyjnego.

Podobnie jak wykazano w pracy oryginalnej 1, także w omawianym badaniu potwierdzono, że melatonina nie zmienia podstawowej peroksydacji lipidów, podczas gdy zarówno IPA, jak i melatonina+IPA obniżyły podstawową peroksydację lipidów.

Praca oryginalna 3

Poziom podstawowej peroksydacji lipidów był niższy w tkance jajnika niż w pozostałych badanych tkankach, co potwierdzono statystycznie w odniesieniu do tkanki tarczycy, śledziony, wątroby i nerki. Z kolei poziom podstawowej peroksydacji lipidów był wyższy w śledzionie niż w innych tkankach (istotność statystyczna w porównaniu z tkanką tarczycy, jajnika i nerki). Inkubacja w obecności melatoniny obniżyła poziom podstawowej peroksydacji lipidów jedynie w tkance jajnika.

Porównując efekt działania KIO_3 na homogenaty tkanek wieprzowych zaobserwowano, że KIO_3 zwiększa poziom peroksydacji lipidów we wszystkich badanych tkankach (tj. tarczycy, jajniku, śledzionie, wątrobie, mózgu, jelicie cienkim, nerce), z najsilniejszym efektem uszkadzającym stwierdzanym przy stężeniach KIO_3 20 mM, 15 mM i 10 mM. Należy jednak podkreślić, że w tkance tarczycy nie stwierdzono efektu uszkadzającego przy najniższym stężeniu KIO_3 – 5.0 mM. Ponadto poziom LPO indukowany przez KIO_3 w stężeniach 10 mM i 7.5 mM był istotnie niższy w tarczycy niż w innych badanych tkankach (wyłączając tkankę nerki).

Melatonina (w stężeniu 5.0 mM) obniżyła indukowaną przez KIO_3 (10 mM, 7.5 mM i 5.0 mM) peroksydację lipidów we wszystkich badanych tkankach. Ważną obserwacją jest to, że w gruczole tarczowym melatonina wykazywała działanie ochronne także przy wyższym stężeniu KIO_3, tj. 15 mM. Poziom LPO po inkubacji w obecności KIO_3+melatonina był istotnie niższy w gruczole tarczowym niż w innych badanych tkankach. Dwie ostatnie obserwacje sugerują, że ochronny efekt melatoniny był najsilniejszy w tkance tarczycy.

Dyskusja

Całkowita zawartość jodu w organizmie ludzkim została oszacowana na ok. 12-25 mg, z czego 5-15 mg jest magazynowane w gruczole tarczowym [14]. Podczas gdy tarczyca zawiera ok. 80% całkowitego jodu obecnego w organizmie, to narządy wewnętrzne i krew zawierają 14%, mięśnie i tkanka tłuszczowa – 5%, a kości jedynie 1% [15]. W porównaniu z tarczycą, w innych tkankach znajdują się jedynie śladowe ilości jodu. Dla przykładu, stosunek stężenia jodu w nerkach, wątrobie, mięśniach bądź skórze do stężenia jodu w tarczycy

wyliczono na 1:100,000. Jednak nawet w tkankach o małym stężeniu jodu, takich jak przewód pokarmowy, nerki czy wątroba, wysokie dawki KIO_3 wykazywały potencjalną toksyczność.

Obliczono, że stężenie jodu nieorganicznego w tarczycy ludzkiej i szczurzej wynosi ok. 9 mM [3-5]. Ze względu na podobieństwo między tarczycą ludzką i wieprzową (podobna objętość gruczołu i synteza hormonów tarczycy) można oszacować, że stężenie jodu w tarczycy wieprzowej jest na zbliżonym poziomie.

Obecnie, pomimo ogólnoświatowych strategii zapobiegania i kontroli niedoboru jodu, jest to nadal powszechny problem w zakresie zdrowia publicznego, szczególnie u ciężarnych. Ciężki niedobór jodu może wiązać się z wieloma poważnymi skutkami zdrowotnymi, takimi jak zwiększone ryzyko utraty ciąży i śmiertelności niemowląt, niedoczynność tarczycy u noworodków, kretynizm bądź opóźnienie neuropsychoruchowe. Ponadto niedobór jodu może prowadzić do powstania wola – czynnika ryzyka raka tarczycy [6].

Programy jodowania soli kuchennej oparte są o dodawanie do soli jodku potasu (KI) lub jodanu potasu (KIO_3) [7]. Ponieważ jodan jest cząsteczką bardziej stabilną niż jodek (jodek łatwo utlenia się do I_2, a następnie jest tracony przez odparowanie), część ekspertów preferuje KIO_3 jako nośnik jodu dodawany do soli w celu uzupełnienia niedoboru tego pierwiastka. Z drugiej strony przewaga KI nad KIO_3 może polegać na jego silniejszym działaniu ochronnym przed oksydacyjnymi uszkodzeniami mtDNA. Chociaż FDA (*Food and Drug Administration)* nadało KIO_3 status GRAS *(„generalnie uznany za bezpieczny”),* dostępne publikacje wskazują na „podwójną naturę” KIO_3.

Rozważania dotyczące potencjalnej toksyczności KIO_3 są następujące: kwas jodowy (HIO_3) wraz z kwasem chlorowym ($HClO_3$) i kwasem bromowym ($HBrO_3$) należą do klasy kwasów oksohalogenowych. Sole halogenowe w większości warunków są związkami stabilnymi, ale ze względu na swoje właściwości utleniające mogą gwałtownie reagować z substancjami łatwo utleniającymi się. Jak wcześniej wspomniano, KIO_3 należy do grupy GRAS, jednak ze względu na swoje podobieństwo strukturalne do bromianu potasu ($KBrO_3$) – znanego potencjalnego kancerogenu, należącego do grupy 2B według IARC (I*nternational Agency for Research on Cancer* – Międzynarodowa Agencja Badań nad Rakiem), uzasadniona jest ocena jego potencjału mutagennego i kancerogennego. Z drugiej strony, jodan ma niższy potencjał utleniający niż bromian i w dotychczas przeprowadzonych badaniach nie wywoływał efektów toksycznych w warunkach, w których bromian był szkodliwy [8].

Warto podkreślić, że najsilniejszy efekt uszkadzający lipidy błon komórkowych obserwowano w obecnych badaniach przy stężeniach KIO_3 zbliżonych do stężenia 15 mM, które jest podobne do fizjologicznego stężenia jodu w tarczycy (wyliczonemu na ok. 9 mM). Należy podkreślić, że KI w stężeniu 15 mM nie powodował wzrostu poziomu peroksydacji lipidów w homogenatach tarczycy wieprzowej [16].

Chociaż stężenia jodu w innych tkankach są znacznie niższe niż w gruczole tarczowym, w obecnych badaniach zaobserwowano uszkadzający efekt KIO_3 we wszystkich analizowanych tkankach (tj. w tarczycy, jajniku, śledzionie, wątrobie, mózgu, jelicie cienkim i nerce).

Porównując efekt działania KIO_3 na homogenaty różnych tkanek wieprzowych stwierdzono, że peroksydacja lipidów indukowana przez KIO_3 była istotnie niższa w tkance tarczycy niż w innych badanych tkankach (poza nerką). Obserwacja ta może ilustrować fakt, że gruczoł tarczowy jest zaadaptowany do utrzymywania wysokich stężeń jodu. Ponieważ tarczyca jest narządem, w którym procesy oksydacyjne są niezbędne do właściwego funkcjonowania i syntezy hormonów tarczycy, w toku ewolucji rozwinęły się mechanizmy chroniące ten gruczoł przed nadmiarem jodu. Jednym z nich jest efekt Wolffa-Chaikoffa. Efekt ten, wciąż nie do końca wyjaśniony, obserwowano u szczurów narażonych na bardzo duże ilości jodu, co powodowało przejściowe zmniejszenie syntezy hormonów tarczycy; taki blok trwał około 24 godziny. Ta adaptacja jest związana ze zmniejszeniem ekspresji symportera sodowo-jodowego (NIS), czego skutkiem jest zmniejszenie wewnątrztarczycowego stężenia jodu; jest to więc kolejny mechanizm przyczyniający się do utrzymania prawidłowej funkcji tarczycy. NIS jest białkiem transbłonowym, występującym głównie w błonie podstawnej komórek pęcherzykowych tarczycy; jego regulatorem jest nie tylko TSH, ale także sam jod [17].

Zaobserwowano również istotnie niższy poziom LPO indukowany przez KIO_3 w nerce w porównaniu z pozostałymi badanymi tkankami. Ta obserwacja może mieć następujące uzasadnienie: jak wspomniano, KIO_3 należy do soli oksohalogenowych, podobnie jak $KBrO_3$ – związek chemiczny, który w warunkach doświadczalnych indukował powstawanie guzów nerki i został sklasyfikowany jako potencjalnie kancerogenny dla ludzi (grupa 2B według IARC). Chociaż KIO_3 ma status GRAS nadany przez FDA i nie został wymieniony jako czynnik rakotwórczy na listach IARC, a także nie wywoływał efektów toksycznych w warunkach, w których odnotowano uszkadzające efekty bromianu, to tkanka nerki – w

porównaniu z innymi tkankami – jest prawdopodobnie bardziej odporna na oksydacyjne uszkodzenia wywołane przez jodan potasu.

Poziom podstawowej peroksydacji lipidów był niższy w homogenatach jajnika niż tarczycy. Z drugiej strony, LPO indukowana przez KIO_3, podobnie jak LPO indukowana przez substraty reakcji Fentona [18], była wyższa w jajniku niż w homogenatach tarczycy. Ta obserwacja potwierdza więc hipotezę, że w warunkach fizjologicznych poziom stresu oksydacyjnego w tarczycy (wynikający głównie z reakcji oksydacyjnych niezbędnych do syntezy hormonów tarczycy) jest znacznie wyższy niż w innych tkankach. Jednocześnie ten fizjologicznie wysoki poziom stresu oksydacyjnego w tarczycy sprawia, że gruczoł tarczowy jest mniej podatny na działanie prooksydantów, takich jak jodan lub żelazo (stosowane w reakcji Fentona).

Prawdopodobne jest, że wywołana przez KIO_3 peroksydacja lipidów w homogenatach tarczycy wieprzowej jest efektem bezpośredniego działania oksydacyjnego tej substancji na błony komórkowe. Należy jednak podkreślić, że prawdopodobnie również inne makrocząsteczki w komórkach tarczycy mogą podlegać bezpośredniemu działaniu KIO_3, co zostało potwierdzone dla nDNA i mtDNA [19].

Przeprowadzano badania mające na celu sprawdzenie potencjalnej toksyczności jodanu, ale jego szkodliwość nie została do tej pory potwierdzona w badaniach u ludzi. Jednak biorąc pod uwagę właściwości chemiczne jodanu i udowodnione w powyższych badaniach jego działanie prooksydacyjne, nie można wykluczyć, że związek ten może być potencjalnie niebezpieczny. Z tego powodu wskazane jest poszukiwanie nowych substancji potencjalnie chroniących przed prooksydacyjnymi efektami KIO_3.

W obecnym badaniu zaobserwowano, że nie tylko melatonina, ale także IPA obniżają poziom peroksydacji lipidów wyindukowany przez KIO_3. Jednak najważniejszą obserwacją było to, że melatonina użyta łącznie z IPA wykazywała jeszcze silniejsze działanie niż każdy z antyoksydantów zastosowany osobno. Należy podkreślić, że efekty ochronne zarówno melatoniny, jak i IPA oraz obu tych substancji stosowanych jednocześnie, wykazano jedynie dla stężeń KIO_3 odpowiadających fizjologicznemu stężeniu jodu w tarczycy (będącego skutkiem zalecanej podaży jodu).

Należy zaznaczyć, że ochronne działanie melatoniny zaobserwowano we wszystkich badanych tkankach (przy zastosowaniu KIO_3 w stężeniach 10 mM, 7.5 mM i 5.0 mM), ale

najważniejszą obserwacją jest to, że melatonina wykazywała najsilniejsze działanie ochronne w gruczole tarczowym — była to jedyna tkanka, w której zaobserwowano korzystne działanie melatoniny przy wyższym stężeniu KIO_3, tj.15 mM. Dodatkowo poziom LPO po inkubacji w obecności KIO_3+melatonina był istotnie niższy w gruczole tarczowym niż w innych badanych tkankach, ale różnice te mogą wynikać ze słabszego szkodliwego działania KIO_3 na tarczycę.

W obecnych badaniach wykazano, że melatonina użyta w stężeniach zwykle stosowanych w warunkach *in vitro* (tj. 1.0-5.0 mM) istotnie obniżała poziom peroksydacji lipidów indukowany przez KIO_3, gdy ten związek został zastosowany w dawkach odpowiadających fizjologicznym stężeniom jodu w tarczycy. Na podstawie obecnych wyników należy uznać, że wskazane jest utrzymywanie wysokiego stężenia melatoniny w organizmie, aby zapobiec oksydacyjnym uszkodzeniom w gruczole tarczowym. Z tego powodu sugeruje się unikanie czynników obniżających stężenie melatoniny, takich jak korzystanie z silnego światła w godzinach nocnych. Ponadto korzystne może być stosowanie egzogennej melatoniny przez osoby starsze, ponieważ fizjologiczne stężenie melatoniny obniża się wraz z wiekiem.

Melatonina została wybrana do obecnych badań, ponieważ jej właściwości antyooksydacyjne są znane od dawna [9,10]. Efekty te były obserwowane zarówno w warunkach *in vivo*, jak i *in vitro*. Na przykład w gruczole tarczowym melatonina obniżała poziom LPO wywołany przez substraty reakcji Fentona (Fe^{2+}+H_2O_2) lub $KBrO_3$. Mechanizmy, dzięki którym melatonina skutecznie chroni przed peroksydacją lipidów, obejmują bezpośrednie i pośrednie efekty antyoksydacyjne oraz działanie jako zmiatacza wolnych rodników [11]. Melatonina stymuluje enzymy antyoksydacyjne (tj. peroksydazę glutationową, reduktazę glutationową, dysmutazę ponadtlenkową i katalazę), pobudza syntezę glutationu oraz współdziała ze zmiataczami wolnych rodników. Ponadto zarówno melatonina, jak i jej metabolity [(N1-acetylo-N2-formylo-5-metoksykynuramina (AFMK), N-acetylo-5-metoksykynuramina (AMK) i cyklo-3-hydroksymelatonina (c3OHM)] są w stanie neutralizować praktycznie wszystkie wolne rodniki. IPA, podobnie jak melatonina, jest endogennym donorem elektronów, który neutralizuje RFT (takie jak $^{\cdot}OH$ i $O_2^{\cdot-}$) i działa synergistycznie z glutationem. Jego łańcuch boczny nie ulega dekarboksylacji, a zatem, w przeciwieństwie do innych substancji indolowych, nie jest przekształcany w reaktywne związki pośrednie o właściwościach prooksydacyjnych.

Obie substancje, melatonina i IPA, są uznawane za bezpieczne i nie wykazują istotnych działań niepożądanych [12,13].

Egzogenna melatonina jest podawana w celach terapeutycznych w dawkach 2-10 mg. W dostępnej literaturze najwyższa dawka melatoniny zastosowana w badaniach klinicznych wynosiła 25 mg [20]. W wyniku dożylnego podania melatoniny w dawce 25 mg odnotowano jej stężenie we krwi ~7.52 x 10^5 pg/mL. W innym badaniu melatonina zastosowana w dawce 10 mg dożylnie pozwalała uzyskać stężenie we krwi ~3.9 x 10^5 pg/mL, a po podaniu doustnym – stężenie ~3.5 x 10^3 pg/mL [21]. Odnosząc te stężenia do stosowanych w obecnym badaniu (5.0 mM melatoniny odpowiada ~1.16 x 10^9 pg/mL) należy zauważyć, że stężenia zastosowane w naszych eksperymentach przekraczają dawki standardowe o kilka rzędów wielkości. Niestety nie przeprowadzono podobnych badań z IPA. Należy podkreślić, że na podstawie otrzymanych przez nas wyników dotyczących ochronnego działania melatoniny stosowanej razem z IPA w warunkach *in vitro* nie można bezpośrednio wnioskować o ewentualnych efektach w warunkach *in vivo*.

W odniesieniu do powyższych wyników warto przypomnieć, że zarówno melatonina, jak i IPA są związkami o korzystnych właściwościach, dzięki którym możemy rozpatrywać ich zastosowanie w wielu obszarach medycyny. Melatonina jest regulatorem rytmu okołodobowego i układu odpornościowego, a także bierze udział w regulacji ciśnienia krwi i autonomicznej regulacji układu sercowo-naczyniowego. Jej działanie terapeutyczne stwierdzono w badaniach dotyczących niektórych nowotworów (np. raka piersi, raka jajnika i endometrium, raka prostaty, raka wątroby czy guzów jelita), chorób układu krążenia bądź zaburzeń psychicznych [22]. Obecnie trwają badania oceniające zastosowanie melatoniny w leczeniu COVID-19 [23]. Odnośnie IPA, substancję tę można uznać za potencjalną opcję terapeutyczną w chorobie Alzheimera [13].

W obecnej pracy porównywano potencjalne działanie ochronne 17ß-estradiolu (znanej endogennej substancji antyoksydacyjnej) stosowanego w stężeniu 1.0 mM (najwyższe stężenie możliwe do uzyskania w warunkach *in vitro*) z ochronnym działaniem melatoniny zastosowanej w tym samym stężeniu. Ponieważ 17β-estradiol nie wykazał działania protekcyjnego w tym modelu, można wnioskować, że melatonina jest lepszym potencjalnym czynnikiem ochronnym, przynajmniej przed prooksydacyjnym efektem KIO_3.

Istotną obserwacją było również to, że substancje indolowe wykazywały efekt ochronny jedynie przy stężeniach KIO_3 10 mM i 7.5 mM (praca oryginalna 1) lub 18.75 mM – 8.75 mM (praca oryginalna 2), które odpowiadają fizjologicznemu stężeniu jodu w tarczycy. Chociaż stężenie jodu w gruczole tarczowym różni się w zależności od wieku, różnice te prawdopodobnie nie są duże; można zatem stwierdzić, że powyższe stężenia KIO_3

odpowiadają fizjologicznemu stężeniu jodu w tarczycy w każdym wieku. Efekty prooksydacyjne KIO_3 nie były redukowane przez melatoninę lub IPA, gdy ten prooksydant był stosowany w wyższych lub niższych stężeniach niż wymienione powyżej.

Trudno jednoznacznie uzasadnić te dość nieoczekiwane wyniki. Można postawić hipotezę, że podczas rozwoju filogenetycznego u ssaków wykształciły się mechanizmy obronne chroniące przed dobrze poznanymi czynnikami toksycznymi, na które organizmy mogły być potencjalnie narażone przez długi czas. Być może dlatego melatonina zmniejszała peroksydację lipidów indukowaną przez KIO_3 w stężeniach odpowiadających fizjologicznemu stężeniu jodu w tarczycy. Chociaż tarczyca (jak i cały organizm) może być narażona na znacznie wyższe stężenia jodu (np. w wyniku leczenia farmakologicznego), nie jest to powszechna sytuacja epidemiologiczna, lecz raczej rzadkie zdarzenie kliniczne. Można więc postawić hipotezę, że w toku ewolucji nie powstały mechanizmy ochronne przeciwko takim rzadkim, niefizjologicznym warunkom.

WNIOSKI

4. **Melatonina i kwas indolo-3-propionowy bardzo wyraźnie obniżają poziom oksydacyjnych uszkodzeń lipidów błon komórkowych spowodowanych działaniem jodanu potasu (KIO_3) użytego w stężeniach odpowiadających fizjologicznym stężeniom jodu w tarczycy.**

5. **Melatonina i kwas indolo-3-propionowy wywierają kumulacyjny efekt ochronny przed oksydacyjnymi uszkodzeniami lipidów błon komórkowych tkanki tarczycy wywołanymi przez KIO_3 użyty w stężeniach odpowiadających fizjologicznym stężeniom jodu w tarczycy; sugeruje to, że te dwie substancje indolowe powinny być stosowane jednocześnie w celu uzyskania lepszego efektu ochronnego przed stresem oksydacyjnym.**

6. **W porównaniu z innymi tkankami, gruczoł tarczowy jest mniej wrażliwy na prooksydacyjne działanie KIO_3. Z drugiej strony, najsilniejsze działanie ochronne melatoniny wykazano właśnie w tkance tarczycy, co sugeruje, że gruczoł ten skuteczniej odpowiada na antyoksydacyjne działanie melatoniny.**

WNIOSEK OGÓLNY

Melatonina i kwas indolo-3-propionowy, w szczególności przyjmowane jednocześnie, powinny być rozważane w celu zapobiegania możliwym uszkodzeniom oksydacyjnym w gruczole tarczowym (a także w innych tkankach) wywołanym przez związki jodu stosowane w profilaktyce jodowej.

6. Komentarz do cyklu prac w języku angielskim – Commentary

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 [1].

The thyroid gland is an organ of "oxidative nature", in which oxidative processes are necessary for example for thyroid hormone biosynthesis [2]. 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 [3-5]. 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 [6]. 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 [7]. Programs of salt iodization are based on the use of either potassium iodide (KI) or potassium iodate (KIO_3) [7]. 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) [8]. 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 [9-13]. Both are safe and it is generally accepted that they do not reveal side effects [12,13].

Melatonin has been shown to prevent experimentally-induced oxidative damage to macromolecules in different tissues, among others in the thyroid gland [9]. 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).

<u>Original paper 2</u>

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.

<u>Original paper 3</u>

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.

Discussion

The total body iodine content in humans was estimated to be 12-25 mg, of which 5-15 mg is stored in the thyroid [14]. Whereas the thyroid contains about 80% of the total body iodine, internal organs and blood contain 14%, muscle and fat – 5%, and bones – 1% [15]. Compared to the thyroid gland, in some of extrathyroidal tissues only traces of iodine are found. The ratio of the iodine concentration in kidney, liver, muscle and skin to that in the thyroid gland was calculated as 1 to 100,000. However, even in tissues with a low level of iodine concentrations such as the gastrointestinal tract, kidneys or liver, high doses of KIO_3 have shown potential toxicity.

The concentration of inorganic iodine in human or rat thyroid was calculated to be approx. 9 mM [3-5]. Due to similarity between human and porcine thyroid (volume, hormone synthesis), it may be estimated that iodine concentration in porcine thyroid is at similar level.

Currently, despite the worldwide strategies for the prevention and control of iodine deficiency, it is still a widespread public health issue, especially in pregnant women. Severe iodine deficiency may be associated with many adverse effects, such as the increased risk of pregnancy loss and infant mortality, neonatal hypothyroidism, cretinism and neuropsychomotor retardation. Moreover iodine deficiency may lead to goiter – a risk factor for thyroid cancer [6].

Programs of salt iodization are based on the use of either potassium iodide (KI) or potassium iodate (KIO_3) [7]. Because iodate is more stable than iodide (iodide is easily oxidized to I_2 and then lost by evaporation), the former compound is preferentially recommended by some health authorities as an additive to salt for correcting iodine deficiency. On the other hand, the superiority of KI over KIO_3 may rely on its stronger protective effects against oxidative damage to mtDNA. Although iodate has been conferred

GRAS (“generally recognized as safe”) status by the Food and Drug Administration, available publications show “dual nature” of KIO_3.

Considerations concerning potential toxicity of KIO_3 are as follows: iodic acid (HIO_3), together with chloric acid ($HClO_3$) and bromic acid ($HBrO_3$), belongs to the class of oxohalogen acids. Halogenate salts are stable under most conditions, but due to their oxidative properties they may react rapidly with easily oxidisable substances. As previously mentioned, KIO_3 belongs to the group of GRAS, but due to its similarity to $KBrO_3$ (known potential carcinogen belonging to the group 2B according to IARC) it is justified to check their mutagenic and carcinogenic potential. On the other hand, iodate has a lower oxidative potential than bromate has, and it did not induce toxic effects under conditions in which bromate did [8].

It is worth emphasizing that the highest lipid peroxidation caused by KIO_3 was observed in present studies for the concentration of around 15 mM, which is of the same order of magnitude as physiological concentration of iodine in the thyroid. It should be stressed that at this concentration of iodine, KI did not increase the level of lipid peroxidation in porcine thyroid homogenates[16].

Although concentrations of iodine in all other tissues are much lower than in the thyroid gland, damaging effects of KIO_3 were observed in all examined tissues (i.e., the thyroid, the ovary, the spleen, the liver, the brain, the small intestine, and the kidney).

When compared the damaging effects of KIO_3 in different tissues, LPO induced by this compound was significantly lower in the thyroid gland than in any other examined porcine tissue (except kidney). This observation illustrates the fact that the thyroid gland has adapted to maintain large concentrations of iodine. As the thyroid constitutes an organ, in which oxidative processes are indispensable for proper functioning and thyroid hormone synthesis, some protective mechanisms have been developed to protect this gland against the huge amount of iodine. One of the thyroidal adaptations to iodine excess is the Wolff–Chaikoff effect. This effect, still not completely explained, was observed in rats exposed to high amounts of iodide, which resulted in transient reduction in the thyroid hormone synthesis; the block lasted approx. 24h. This adaptation is associated with a decrease in expression of the sodium-iodide symporter (NIS), resulting in reduced intrathyroidal iodine concentration; thus, this is the next mechanism contributing to maintain proper thyroid function. NIS is an intrinsic membrane protein, found mainly in the basolateral membrane of thyroid follicular cells; its regulator is not only TSH, but also iodine itself [17].

Also a significantly lower LPO level induced by KIO_3 has been observed in the kidney compared to other tissues. This observation may be justified by the following reason. As it was mentioned above, KIO_3 is one of halogenate salts, therefore it is similar to $KBrO_3$. The latter is used to experimentally induce renal tumors and it has been classified as possibly carcinogenic to humans (group 2B according to IARC). Although KIO_3 has been conferred GRAS status by the FDA, it was not listed as a carcinogen with IARC, and it did not induce toxic effects under conditions in which bromate did, kidney tissue is presumably more resistant to iodate than other tissues.

The basal level of LPO was lower in the ovary than in the thyroid homogenates. On the other hand, LPO induced by KIO_3, similarly to LPO induced by Fenton reaction substrates [18], was higher in the ovary than in the thyroid homogenates. This observation also confirms the hypothesis, that under physiological conditions oxidative stress in the thyroid (resulting mostly from oxidative reactions indispensable for thyroid hormone synthesis) is at a substantially higher level than in other tissues. At the same time this physiologically high level of oxidative stress in the thyroid makes this organ less vulnerable to pro-oxidative agents, such as iodate or iron (used in the Fenton reaction).

It is probable that KIO_3-caused lipid peroxidation in porcine thyroid results from direct oxidative effects of this compound on cellular membranes. However, it should be stressed that probably also other macromolecules in thyroid cells can be directly affected by KIO_3, as it has been documented for nDNA and mtDNA [19].

Iodate was tested for its potential toxicity, but that was not confirmed till now in humans. However, taking into account chemical properties of iodate and its prooxidative effects documented in present studies, it cannot be excluded that this compound is potentially dangerous. For this reason it is advisable to search for new potential protective tools against pro-oxidative nature of KIO_3.

In the present study it has been observed that not only melatonin but also IPA decreased lipid peroxidation induced by KIO_3. The most important observation is, however, that melatonin used together with IPA revealed even stronger protective effects than each of these antioxidants used separately. It should be stressed that the protective effects of either melatonin or IPA as well as of both indole substances used simultaneously were observed

only when KIO_3 was applied in concentrations corresponding to physiological iodine concentration in the thyroid (which obviously result from recommended iodine supply).

It should be noted that protective effects of melatonin were observed in all examined tissues (when KIO_3 was applied in concentrations of 10 mM, 7.5 mM and 5.0 mM), but the most important observation is that melatonin revealed the strongest protective action in the thyroid gland — it was the only tissue, in which beneficial effects of melatonin were observed against as high KIO_3 concentration as 15 mM. Additionally, LPO levels resulting from KIO_3+melatonin exposure were lower in the thyroid compared to other tissues, but these differences may be due to a weaker damaging effect of KIO_3 in the thyroid.

In the present studies we have shown that melatonin, in concentrations usually used in *in vitro* experiments (1.0-5.0 mM), significantly reduced lipid peroxidation induced by KIO_3, when this compound was used at doses corresponding to physiological concentrations of iodine in the thyroid. On the basis of the present results it is still advisable to maintain high concentrations of melatonin to prevent oxidative damage in the thyroid gland. It is suggested to avoid factors, which decrease melatonin concentrations in organisms, such as strong light at night. Furthermore, it may be beneficial to use exogenous melatonin by elderly, because physiological concentrations of melatonin decrease with age.

Melatonin was chosen for this research, because protective effects of this compound against oxidative stress have been known for a long time [9,10]. These effects were observed both *in vivo* and *in vitro* experiments. In the thyroid gland for example, melatonin reduced lipid peroxidation caused by Fenton reaction substrates (Fe^{2+}+H_2O_2) and by $KBrO_3$. The mechanisms by which melatonin protects against lipid peroxidation involve direct or indirect antioxidative effects and free radical scavenging activities of this indoleamine [11]. Melatonin stimulates antioxidative enzymes (i.e. glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase), upregulates synthesis of glutathione and cooperates with free radical scavengers. Moreover, melatonin and its metabolites (N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), N-acetyl-5-methoxykynuramine (AMK), and cyclic-3-hydroxymelatonin (c3OHM)) are able to detoxify practically all free radicals and reactive species. IPA, similar to melatonin, is an endogenous electron donor that detoxifies ROS (such as the $^{\bullet}OH$ and the $O_2^{\bullet -}$) and acts synergistically with glutathione. Its side chain cannot be decarboxylated, and thus, unlike other indoles, it cannot be converted to a reactive prooxidant intermediate.

Both substances, melatonin and IPA, are recognized as safe and do not reveal any adverse effects [12,13].

Exogenous melatonin is applied therapeutically in doses between 2 and 10 mg. In available studies the highest dose of melatonin used in clinical trials was 25 mg [20]. The intravenous administration of melatonin in a dose of 25 mg resulted in blood concentration of ~7.52×10^5 pg/mL. In another study melatonin used in a dose of 10 mg intravenously resulted in blood concentration of ~3.9×10^5 pg/mL and when used orally – in concentration of ~3.5×10^3 pg/mL [21]. Relating these concentrations to those used by us (5.0 mM of melatonin is equivalent to ~1.16×10^9 pg/mL) it can be concluded that the concentrations used in our experiments exceed the standard doses by several orders of magnitude. Unfortunately, similar studies with IPA have not been performed. It should be stressed, that these results concerning protective *in vitro* effects of melatonin used together with IPA cannot be directly extrapolated into *in vivo* conditions.

In the context of our results, it is worth recalling that both melatonin and IPA are regarded as interesting chemical compounds with potential properties for use in many fields of medicine. Melatonin is a regulator of the circadian rhythm and immune system and is also involved in blood pressure and autonomic cardiovascular regulation. Its therapeutic effects have been reported in certain tumors (e.g. breast cancer, ovarian and endometrial carcinoma, prostate cancer, hepatoma or intestinal tumors), cardiovascular diseases or psychiatric disorders [22]. The research currently under way evaluates potential protective effects of melatonin against COVID-19 [23]. Concerning IPA, this substance may be regarded as a potential treatment option for Alzheimer's disease [13].

In the present study we compared potential protective effects of 17ß-estradiol (a well known endogenous antioxidant) used in the concentration of 1.0 mM (the highest achievable concentration) with protective effects of melatonin used in the same concentration. Because 17β-estradiol was not protective at all in this model, it can be concluded that melatonin is a better potential protective agent, at least against prooxidative effects of KIO_3.

The observation, which should be also discussed, is that indole substances were effective only when KIO_3 was used at concentrations of 10 mM and 7.5 mM (original paper 1) or from 18.75 mM to 8.75 mM (original paper 2); these concentrations correspond to physiological iodine concentration in the thyroid. Although the iodine concentration in the thyroid differs depending on the age, such differences are presumably not huge; therefore it can be stated that these effective concentrations of KIO_3 correspond to physiological iodine

concentration in the thyroid at any age. Pro-oxidative effects of KIO_3 were not reduced by melatonin or IPA when this pro-oxidant was used either in higher or in lower concentrations than mentioned above.

It is hard to present clear explanation of these rather unexpected results. However, it can be hypothesized that during phylogenetical development in mammals, protective mechanisms have been developed to protect against well recognized toxic agents, to which organisms are potentially exposed for a long period of time. That can be the reason why melatonin reduced lipid peroxidation induced by KIO_3 in concentrations corresponding to physiological concentration of iodine in the thyroid. Although the thyroid and the whole organism can be exposed to much higher concentrations of iodine (e.g. resulting from pharmacological treatment), that is not a common epidemiological or any other individual situation. Thus it can be hypothesized that protective mechanisms have not been developed against these rare conditions.

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.

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7. Oświadczenia współautorów

Łódź, 27.02.2023r.
(miejscowość i data)

lek. Paulina Iwan

(dane osobowe)

OŚWIADCZENIE

Jako pierwszy autor publikacji:

Iwan P., Stępniak J., Karbownik-Lewińska M. Melatonin reduces high levels of lipid peroxidation induced by potassium iodate in porcine thyroid. Int J Vitam Nutr Res. 2021; 91: 271-277

oświadczam, iż w wyżej wymienionej pracy mój wkład w powstanie publikacji polegał na: zaplanowaniu doświadczeń, przeprowadzeniu doświadczeń, udziale w opracowaniu statystycznym wyników, przygotowaniu pierwszej wersji manuskryptu

Mój udział w realizacji pracy szacuję na 60%.

Paulina Iwan

Podpis

Łódź, 27.02.2023 r.
(miejscowość i data)

Prof. dr hab. n. med.
Małgorzata Karbownik-Lewińska

(dane osobowe)

OŚWIADCZENIE

Jako pierwszy lub równorzędny autor publikacji:

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; 91: 271-277

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(stworzeniu konceptu pracy, zebranie materiału badawczego, wykonaniu analiz, obliczeniu statystycznym, interpretacji wyników, krytycznej ocenie treści artykułu, zaakceptowaniu ostatecznej treści artykułu). Mój udział w realizacji pracy szacuję na 30 %.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy przedłożonej przez lek. Pauline Iwan, jako części cyklu publikacji do przeprowadzenia przewodu doktorskiego.

Małgorzata Karbownik-Lewińska
Podpis

Łódź, 27.02.2023r.
(miejscowość i data)

dr n.med. Jan Stępniak

(dane osobowe)

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Jako pierwszy lub równorzędny autor publikacji:

Iwan P, Stępniak J, Karbownik-Lewińska M. Melatonin reduces high levels of lipid peroxidation induced by potassium iodate in porcine thyroid. Int J Vitam Nutr Res. 2021; 91: 271-277

oświadczam, iż w wyżej wymienionej pracy mój wkład w powstanie publikacji polegał na: udziale w opracowaniu statystycznym wyników i w edytowaniu manuskryptu

(stworzeniu konceptu pracy, zebranie materiału badawczego, wykonaniu analiz, obliczeniu statystycznym, interpretacji wyników, krytycznej ocenie treści artykułu, zaakceptowaniu ostatecznej treści artykułu). Mój udział w realizacji pracy szacuję na 10 %.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy przedłożonej przez lek. Paulinę Iwan, jako części cyklu publikacji do przeprowadzenia przewodu doktorskiego.

Stępniak J.
Podpis

Łódź, 27.02.2023r.
(miejscowość i data)

lek. Paulina Iwan
....
....
(dane osobowe)

OŚWIADCZENIE

Jako pierwszy autor publikacji:

Iwan P, Stępniak J, Karbownik-Lewińska M. Cumulative Protective Effect of Melatonin and Indole-3-Propionic Acid against KIO_3-Induced Lipid Peroxidation in Porcine Thyroid. Toxics. 2021; 21; 9:89

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Mój udział w realizacji pracy szacuję na 60 %.

Paulina Iwan
Podpis

Łódź, 27.02.2023r.
(miejscowość i data)

Prof. dr hab. n.med.
Małgorzata Karbownik-Lewińska

(dane osobowe)

OŚWIADCZENIE

Jako pierwszy lub równorzędny autor publikacji:

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nadzorze nad wszystkimi etapami badania

(stworzeniu konceptu pracy, zebranie materiału badawczego, wykonaniu analiz, obliczeniu statystycznym, interpretacji wyników, krytycznej ocenie treści artykułu, zaakceptowaniu ostatecznej treści artykułu). Mój udział w realizacji pracy szacuję na 30 %.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy przedłożonej przez lek. Pauline Iwan, jako części cyklu publikacji do przeprowadzenia przewodu doktorskiego.

Małgorzata Karbownik-Lewińska
Podpis

Łódź, 27.02.2023r.
(miejscowość i data)

dr n.med. Jan Stępniak

(dane osobowe)

OŚWIADCZENIE

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Stępniak Jan
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