Experimental Gerontology 110 (2018) 151–157

Contents lists available at ScienceDirect

Experimental Gerontology

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

The influence of ageing on the extrapineal melatonin synthetic pathway

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ARTICLE INFO

Section Editor: Holly M. Brown-Borg

Keywords:
AA-NAT and ASMT
SOD
Catalase
Cu
Zn
Mn

ABSTRACT

Ageing affects various physiological and metabolic processes in a body and a progressive accumulation of oxidative damage stands out as often used explanation. One of the most powerful scavenger of reactive oxygen species (ROS) in all organs is melatonin. A majority of melatonin supplied to the body via blood originates from the pineal gland. However, we have been interested in a locally produced melatonin.

We have used 2.5- and 36-months-old Wistar rats. Tissues were collected and gene expression of AA-NAT and ASMT, the two key enzymes in a synthesis of melatonin, was determined in brain, liver, kidney, heart, skin, and intestine. Since melatonin can influence antioxidant enzymes, the activity of superoxide dismutase (SOD) and catalase (CAT), and the level of GSH were measured in liver. In addition, Copper (Cu), Zinc (Zn), and Manganese (Mn) were also determined in liver since these microelements might affect the activity of antioxidant enzymes.

The expression of AA-NAT and ASMT was increased in liver and skin of old animals. A positive correlation in AA-NAT and ASMT expression was observed in liver, intestine and kidney. Moreover, the activity of CAT enzyme in liver was increased while SOD activity was decreased. SOD and CAT were probably affected by the observed decreased amount of Cu, Zn, and Mn in liver of old animals.

In our model, extrapineal melatonin pathway in ageing consisted of complex interplay of locally produced melatonin, activities of SOD and CAT, and adequate presence of Cu, Zn and Mn microelements in order to defend organs against oxidative damage.

1. Introduction

Ageing is unstoppable, complex biological process that affects various physiological and metabolic processes in a body, resulting in a decrease of their function (Barton et al., 2016). However, ageing is not a uniform process, since different organs, tissues and cell types may age at different rates (Hamezah et al., 2017; Bonomini et al., 2010). There are a few suggested mechanisms of ageing, and progressive accumulation of oxidative damage stands out as probably the most used explanation (Flores et al., 2012). Not all organs are equally exposed to the damaging factors, such as to the reactive oxygen species (ROS), since different organs have different functions. In that respect, liver and skin are, in general, more exposed to possible oxidative damaging factors than some other organs.

A defense mechanism against ROS consists of antioxidant enzymes and various scavenging molecules. One of the most powerful scavenger of ROS in all organs, with more effective properties than classical antioxidant, is melatonin (Baydas et al., 2002). A majority of melatonin supplied to the body via blood originates from the pineal gland. However, we have here focused on a locally produced melatonin in various organs. A predominant melatonin synthesis in both animals and humans occurs via serotonin-*N*-acetylserotonin-melatonin pathway (Tan et al., 2016) and is regulated by the two key enzymes: arylalkyamine-*N*-acetyltransferease (AA-NAT) and acetylserotonin *O*-methyltransferase

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https://doi.org/10.1016/j.exger.2018.06.010
Received 27 April 2018; Received in revised form 8 June 2018; Accepted 11 June 2018
Available online 12 June 2018
0531-5565/

(ASMT) / hydroxyindole-*O*-methyltransferase (HIOMT). In addition to a dominant role of AA-NAT in acetylation of serotonin, alternative enzymes, arylamine-*N*-acetyltransfereases (NAT-1, NAT-2), were detected in rodent (C57BL/6 mouse) and human skin (Debiec-Rychter et al., 1996; Slominski et al., 2003; Kawakubo et al., 2000). An inactive AA-NAT enzyme, due to a specific gene insertion or deletion, is functionally replaced by NAT-1 enzyme during acetylation of serotonin (Slominski et al., 2005). Higher activity of these enzymes could suggest higher production of melatonin.

It has been reported that melatonin levels were much higher in peripheral organs, especially in skin and liver, compared to the serum melatonin (Slominski et al., 2008; Bubenik, 2002). Apart from its important role in protection against ultraviolet and X-ray radiation-induced oxidative stress, melatonin and its metabolites in the skin may have other numerous functions, such as: induction of DNA repair and anticancer activity, blocking apoptosis, maintaining mitochondrial membrane potential and ATP synthesis, anti-inflammatory and immunostimulatory actions, regulation of melanin pigmentations, thermoregulations, and wound healing (Slominski et al., 2018; Slominski et al., 2017a). The melatonin activity in the skin is determined by a topography, health status of the skin, involved cell type, and the animal strain (Slominski et al., 2017a). Moreover, as it was first noticed in rat liver and later in other organs, melatonin can be metabolized by cytochrome 450 (CYP) into powerful antioxidant factors (AFMK and AMK), which have a role in attenuating of ROS and RNS (reactive nitrogen species) elements in mitochondria (Semak et al., 2008; Slominski et al., 2017b).

It has been shown that activity of both enzymes, AA-NAT and ASMT, is in correlation with the expression of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (Rodriguez et al., 2004). Also, certain essential microelements may affect the activity of antioxidant enzymes. Namely, manganese (Mn), zinc (Zn), and copper (Cu) inhibit activity of AA-NAT (Zhan-Poe and Craft, 1999) and they are also cofactors of CuZnSOD/MnSOD complexes. Moreover, Cu and Zn block CAT activity (Xianyong et al., 2017) and Cu and Mn may possess pro-oxidative properties (Valko et al., 2005).

During ageing, many processes are affected, leading to diminished expression of antioxidative factors (Reiter et al., 2002; Karasek, 2004) and changed regulation of the essential microelements like Cu, Zn, and Mn (Rotter et al., 2015). Therefore, it could be assumed that these events are related to the disorders of many systems. Since not all members of the same community have the same life span and all organs in an individual do not age at the same rate (Acuña-Castroviejo et al., 2014), we decided to use rats as old as possible to compare the expression of the AA-NAT and ASMT, the activity of SOD, CAT, and GSH-Px, and the level of Mn, Zn, and Cu between young adult and very old animals in various tissues. The role of serum melatonin in advance age is well known (Hardeland et al., 1995), but the role of locally produced melatonin is not firmly established. The goal of this work was to better understand antioxidant processes related to the melatonin pathway in various organs of very old individuals.

2. Materials and methods

2.1. Animals

Wistar rats were obtained from the Military Medical Academy Animal Research Facility (Belgrade, Serbia). Experimental animals were divided into two groups, younger animals (7 rats) that were 2.5 months (10 weeks) old, and older ones (7 rats) that were 36 months (3 years) old. In each group, each animal was housed in its own cage and kept under controlled room condition (temperature of 20 °C, humidity of 70%, light-dark cycle of 12–12 h). Food (Veterinary Institute, Subotica, Serbia) and water were available ad libitum. Experiments were conducted in accordance with the procedures described in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Washington, DC, USA).

At appropriate time, animals were sacrificed between 12 a.m. to 3 p.m. The parts of organs were quickly removed, transferred into liquid CO_2 and then stored at −80 °C. Organ samples were used for a) RNA extraction, followed by real-time PCR analysis, b) estimation of enzymatic activity, and c) determination of Cu, Zn, and Mn concentration.

2.2. RNA extraction and cDNA synthesis

Cellular RNA for each sample, was isolated from 50 mg of extrapineal tissues (liver, skin, kidney, brain cortex, heart, and intestine), using Trisol Isolation Reagent (Invitrogen, Carlsbad, CA, USA) and chloroform (Serva, Heidelberg, Germany) as a denaturing solution. Following denaturation, extraction of RNA was conducted by a precipitation with isopropanol (Serva, Heidelberg, Germany). The obtained RNA pellet was dissolved in 10 µl of RNAase-free water, and the samples were proceeded to quantification by spectrophotometer (Eppendorf, Hamburg, Germany) at 260 nm. RNA samples were stored at −80 °C. To reduce a risk of DNA contamination, isolated RNA was purified before cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reaction volume (10 µl) for a purification consisted of: 1 µg of RNA, 1 µl of 10× Reaction buffer with $MgCl_2$, 1 µl of DNase I-RNase-free, and a nuclease-free H_2O. Following incubation at 37 °C for 10 min, 1 µl of 50 mM EDTA was added into mix that was incubated at 65 °C for 10 min in order to inactivate enzymes. The prepared RNA was then used for reverse transcription.

Single-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit. The components added to total volume of 20 µl were as follows: 1 µg of total RNA, 1 µl of random hexamer primer, 4 µl of 5× Reaction Buffer, 1 µl of RiboLock RNase inhibitor, 2 µl of 10 mM dNTP, 1 µl of RevertAid M-MuLV RT, and 12 µl a nuclease-free H_2O. Reaction was conducted at 25 °C for 5 min, followed by incubation at 42 °C for 60 min, and terminated at 70 °C for 5 min. Reaction products were stored at −20 °C until they were used in PCR reaction. To verify the results of the first strand cDNA synthesis, positive (GAPDH amplification) and negative control (RT- and NTC) reactions were performed.

2.3. Quantitative real time-qRT-PCR of AA-NAT and ASMT in extrapineal tissues

To estimate the expression level of AA-NAT and ASMT genes, real time quantitative PCR was performed using a Line Gene PCR machine (Hangzhou Bioer Technology, Shanghai, China). The cDNA amplification was conducted by adding the following components: 12.5 µl of 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 µM of forward and 0.2 µM of reverse primers (AA-NAT, ASMT), or 0.1 µM primers of housekeeping gene (HPRT), 2 µl of the first strand cDNA synthesis reaction, and a nuclease-free H_2O to 25 µl. Primer sequences were employed from the previously published paper by Sanchez-Hidalgo et al., 2009. The temperature profile was as follows: initial denaturation at 95 °C for 10 min, then 40 cycles at 95 °C for 10 s; 57 °C for 20 s (AA-NAT) or 58 °C for 45 s (ASMT); 72 °C for 20 s, and finally extension at 72 °C for 5 min. In each reaction, tested samples were amplified in two replicates, and the NTC control was included for the assessment of a possible contamination. The specificity of amplification for each target was confirmed by melting curve analysis. A level of AA-NAT and ASMT expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Data of 36-months-old group were normalized to data of 2.5-months-old animals and represented by fold change values.

2.4. Determination of SOD and CAT enzymatic activity in liver

The total superoxide dismutase (SOD) activity was determined by

measuring the ability of crude synaptosomal fraction to inhibit the radical-mediated autoxidation of epinephrine (Sigma Aldrich, Sent Luis, SAD) (Sun and Zigman, 1978). For determination of mitochondrial SOD2 activity, samples were treated with 8 mM KCN (Sigma Aldrich, Sent Luis, SAD) and then analyzed according to a previously described method (Sun and Zigman, 1978). The cytosolic SOD1 activity was calculated as the difference between a total and a mitochondrial SOD activity. The activities were expressed as U/mg protein. For the catalase (CAT) activity, a formation of stable complex of hydrogen peroxide with ammonium molybdate was measured and its absorption at 405 nm was determined by a spectrophotometer (3000 Evolution, Biochemical Systems International, Areko, Italy) (Góth, 1991). Results were expressed as units of enzymatic activity per milligram of protein (U/mg protein).

2.5. Determination of reduced GSH in liver

The content of reduced glutathione (GSH) was determined spectrophotometrically using DTNB (Sigma-Aldrich, Sent Luis, SAD). DTNB reacts with aliphatic thiol compounds at pH 8.0 by producing a yellow p-nitrophenol anion (Ellman, 1959) whose absorption was measured at 412 nm. Results were expressed as nmol of reduced glutathione per milligram of proteins (nmol/mg).

2.6. Determination of Cu, Zn, and Mn concentration in liver

The concentrations of Cu, Zn, and Mn were measured in the liver of younger and older animals. Following the treatment of the liver tissue (500 mg) with 8 ml of HNO_3 (Zorka Pharma, Šabac, Serbia) and 2 ml of 30% H_2O_2, tissue digestion was conducted in a microwave (ETHOS TC, Milestone S.r.l., Sorisole, Italy) from room temperature (20 °C) to 180 °C, and then continued at 180 °C for 10 min. Following a digestion step, liver tissue was cooled and placed into a flask with deionized water. A microelement concentration was determined by atomic absorption spectrometer “SpectrAA 220” (Varian, Palo Alto, California, USA) according to Varian Atomic Absorption Spectrometers (AAS) Analytical Methods. Concentrations were expressed as mg of the analyzed microelement per kg of analyzed tissue (mg/kg of tissue).

2.7. Statistical analysis

Using descriptive statistics, a level of AA-NAT and ASMT expression is presented as median with interquartile range (IQR) value, while statistical significance between tested groups was evaluated by non-parametric analysis (Mann-Whitney-*U* test), for each tissue separately. A coefficient of correlation between AA-NAT and ASMT was determined by Spearman's test. The level of antioxidants (SOD, CAT, and GSH), and microelements (Cu, Zn, and Mn) was expressed as mean ± SD, and analyzed using the *t*-test. Statistically significance was set at $p < 0.05$. Statistical analysis was performed using the SPSS 17.0 software (Chicago, IL, USA).

3. Results

3.1. Gene expression of AA-NAT and ASMT enzymes

AA-NAT expression was analyzed in six tissues: brain, liver, kidney, heart, skin, and intestine. The median values and IQR of AA-NAT in 2.5-months-old animals were following: brain (1.0 fold, IRQ = 0.9), liver (0.58 fold, IRQ = 0.9), kidney (1.0 fold, IRQ = 1.9), heart (1.03 fold, IRQ = 0.95), skin (0.83 fold, IRQ = 0.4), and intestine (1.0 fold, IRQ = 0.7). The median values and IQR of AA-NAT in 36-months-old animals were following: brain (0.69 fold, IRQ = 0.5), liver (1.41 fold, IRQ = 2.2), kidney (1.22 fold, IRQ = 59.2), heart (0.24 fold, IRQ = 13.7), skin (1.85 fold, IRQ = 8.7), and intestine (0.24 fold, IRQ = 1.2). Among analyzed peripheral organs, a statisticaly

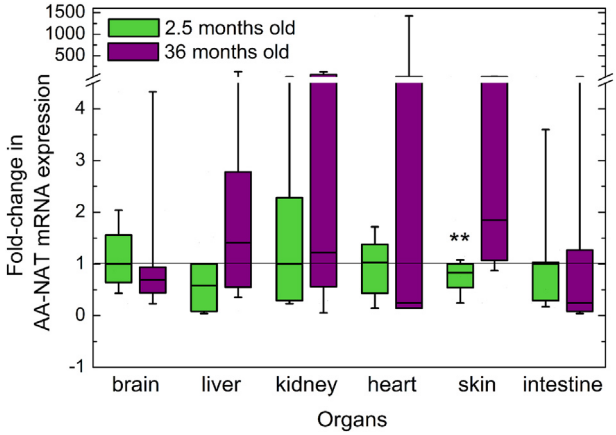

Fig. 1. Analysis of AA-NAT gene expression by real time PCR in various rat organs. The data are presented as median and IRQ values in 2.5- and 36-months-old rats. Both groups were of size $n = 7$. Statistically significant increase of aged compared to young rats was obtained for skin with $p < 0.01$ (**).

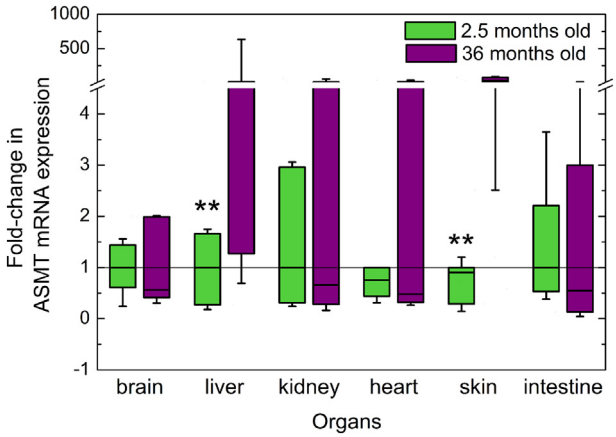

Fig. 2. Analysis of ASMT gene expression by real time PCR in various rat organs. The data are presented as median and IRQ values in 2.5- and 36-months-old rats. Both groups were of size $n = 7$. Statistically significant increase of aged compared to young rats was obtained for liver and skin with $p < 0.01$ (**).

significant higher expression of AA-NAT, was present in the skin of older animals when compared to younger ones ($p < 0.01$), and a trend of increasing value was also noted in liver, Fig. 1.

Similar pattern of expression was obtained for ASMT enzyme, Fig. 2. The median and IQR values of ASMT in 2.5-months-old animals were following: brain (1.0 fold, IRQ = 0.8), liver (1.0 fold, IRQ = 1.4), kidney (1.0 fold, IRQ = 2.6), heart (0.7 fold, IRQ = 0.5), skin (0.9 fold, IRQ = 0.7), and intestine (1.0 fold, IRQ = 1.6). The median values of ASMT and IQR in 36-months-old animals were following: brain (0.56 fold, IRQ = 1.5), liver (4.6 fold, IRQ = 10.4), kidney (0.66 fold, IRQ = 6.9), heart (0.48 fold, IRQ = 12.5), skin (29.8 fold, IRQ = 64), and intestine (0.55 fold, IRQ = 2.8). Analyzing ASMT expression in six peripheral organs, a statistically significant increase was registered in the skin ($p < 0.01$) and the liver ($p < 0.01$) of older animals when compared to the group of younger animals.

We have found a positive coefficient of correlation of AA-NAT and ASMT in 2.5-months-old group in intestine ($r = 0.791$, $p < 0.05$) and kidney ($r = 0.882$, $p < 0.01$). In the group of 36-months-old animals, we have discovered a positive coefficient of correlation of AA-NAT and ASMT genes expression in liver ($r = 0.929$, $p < 0.05$), intestine ($r = 0.964$, $p < 0.001$), and kidney ($r = 0.964$, $p < 0.001$). Linear interpolation through the data points is illustrated in Fig. 3.

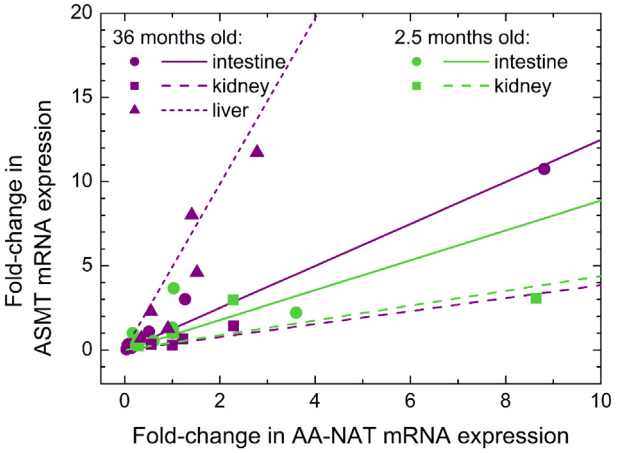

Fig. 3. Relation between AA-NAT and ASMT gene expression. The data points along with the corresponding linear interpolation are shown for intestine, kidney, and liver of 36-months-old rats as well as for intestine and kidney of 2.5-months-old rats; namely, in those cases with detected correlation between the two gene expressions. It was assumed that AA-NAT and ASMT gene expressions were proportional; therefore, all linear interpolations originate in the coordinate system origin. Note that three data points are outside the graph limits.

3.2. The activity and level of antioxidant enzymes SOD and CAT and reduced GSH

We have measured the enzymatic activity of SOD and CAT, and a level of reduced GSH in liver tissue of 2.5-months-old and 36-months-old animals. As shown in Fig. 4, we have detected a statistically significant decrease of SOD in older animals when compared to the younger ones (790.8 ± 54.1 U/mg vs. 1015.3 ± 56.1 U/mg, $p < 0.01$). In contrast to SOD value, we have found a statistically significant increase of CAT in the older group when compared to the younger group (55.5 ± 2.2 U/mg vs. 50.4 ± 0.4 U/mg, $p < 0.05$). However, there was no statistically significant change in reduced GSH content between tested groups (young, 62.3 ± 8.3 nmol/mg; old, 60.5 ± 16.4 nmol/mg).

3.3. Microelement concentration of SOD cofactors Cu, Zn and Mn in the liver

A concentration of constitute SOD cofactors, namely Cu, Zn, and Mn was determined. A significantly lower level of all three microelements was found in the 36-months-old when compared to the 2.5-months-old animals. This can be seen in Fig. 5. Namely, Cu 4.96 ± 0.15 mg/kg vs. 6.69 ± 0.39 mg/kg, $p < 0.01$; Zn 34.96 ± 1.02 mg/kg vs. 39.62 ± 1.31 mg/kg, $p < 0.01$; and Mn 2.33 ± 0.12 mg/kg vs. 3.27 ± 0.14 mg/kg, $p < 0.001$.

4. Discussion

This study was performed in order to provide a better understanding of melatonin synthetic pathway in relation to the ageing processes. Based on the obtained results, significant differences existed in the expression of the AA-NAT and ASMT genes, in the activity of SOD, CAT and GSH-Px, and the level of Mn, Zn and Cu between young and old individuals.

We have used 3-years-old and 2.5-months-old rats in our experiments. To the best of our knowledge, this is the first time that melatonin synthetic pathway was analyzed in such old rats. Although the role of the serum melatonin in many age-related diseases of advanced age is well known, the role of locally present melatonin in ageing tissue is not clearly determined. The expression of AA-NAT and ASMT, the key

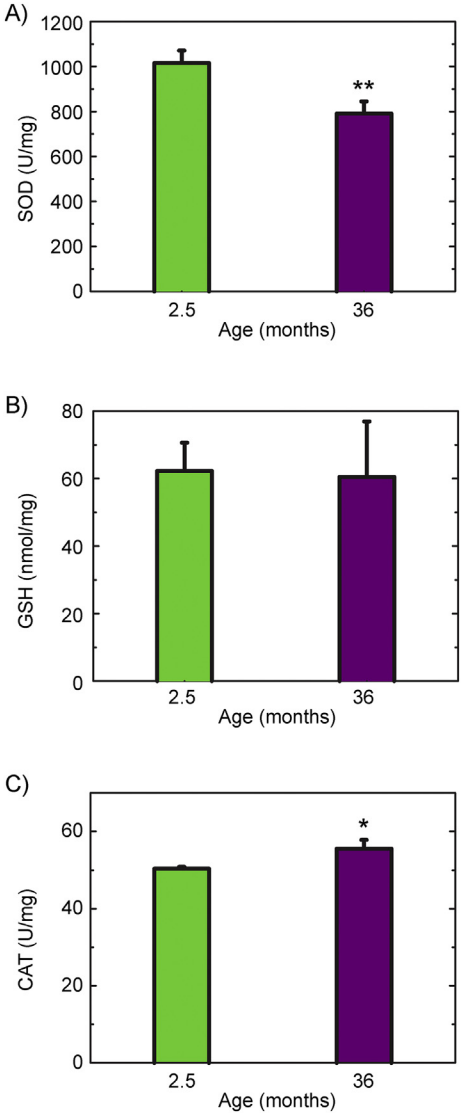

Fig. 4. The activity of SOD and CAT enzymes, and the level of GSH in the rat liver of 2.5- and 36-months-old rats. The data are presented as means ± standard deviations (SDs). Statistically significant decrease of SOD and CAT activity in older rats was obtained with $p < 0.01$ (**) and $p < 0.05$ (*), respectively.

enzymes of melatonin synthesis, has been previously evaluated in various tissues (Stefulj et al., 2001; Uz et al., 2002; Slominski et al., 2002; Hong and Pang, 1995; Konturek et al., 2007) and it is mainly influenced by the exposure to different oxidative stress factors (Jaworek et al., 2003). Here, we investigated age-related melatonin production in peripheral tissues by measuring AA-NAT and ASMT gene expression.

We have shown a statistically significant increase in expression of AA-NAT in skin, and a trend of its increase in liver in older animals when compared to the younger ones. Our gene expression results are in agreement with the AA-NAT increased enzymatic activity in liver and kidney of 12-month-old animals when compared to 3-month-olds (Sanchez-Hidalgo et al., 2009). We have also found a statistically significant increase of ASMT gene expression in liver and skin of older rats. Our result with increased ASMT gene expression in liver differs from ASMT liver enzymatic activity reported by Sanchez-Hidalgo et al. This difference could be due to the age of animals (12 vs. 36 months). In our study, a liver was functional in the advanced age and we assumed that the observed increase in ASMT gene activity was rather a consequence of an extra need for elimination of deleterious agents generated during

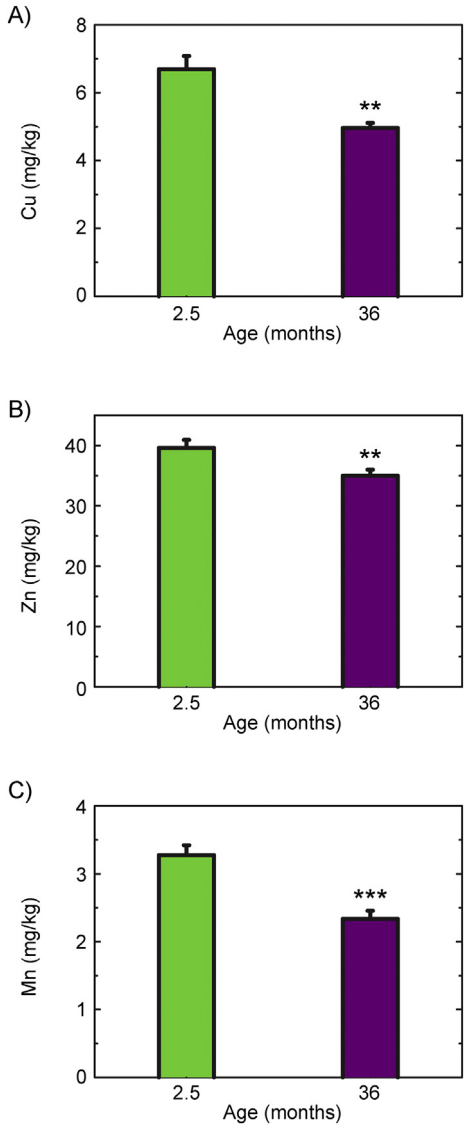

Fig. 5. Concentrations of micro-elements Cu, Zn, and Mn in a liver of 2.5- and 36-months-old rats. The data are presented as means ± standard deviations (SDs). Statistically significant decrease of Cu, Zn, and Mn in older rats was obtained with $p < 0.01$(**), $p < 0.01$(**), and $p < 0.001$ (***), respectively.

ageing than an insufficient ASMT activity in the early period. This possible extra need for elimination of harmful molecules has been reflected in the positive correlation of AA-NAT and ASMT gene activity in the liver of older, but not younger, animals. Also, our results with AA-NAT/ASMT expression in skin support a key role of melatonin in antioxidant defense in this tissue of older animals. Similar results with antioxidative defense in a human skin (Slominski et al., 2005) and in a skin of young adult rats (Semak et al., 2004) has been shown. As far as we know, we believe that a statistically significant increased expression of AA-NAT/ASMT in skin of older rats was registered for the first time in this study.

We have found an increased gene expression of functionally related AA-NAT and ASMT enzymes in skin and liver of very old animals. The result may reflect an increased defense against oxidative stress in organs that due to their function may be more exposed to the oxidative damage. Moreover, since AA-NAT and ASMT mRNAs were detected in all studied tissue, and assuming that the expression of AA-NAT and ASMT does reflect the activity of the enzymes (Xu et al., 2018), their amount could be used not only for a determination of locally produced melatonin, but also as an estimate of oxidative stress and a local tissue defense (Acuña-Castroviejo et al., 2014). Since we were interested into locally present melatonin, we used a brain cortex instead of a whole brain. We did not observe a statistically significant difference in the brain gene expression of AA-NAT and ASMT between the young and the old group. These results suggested that a sufficient activity of AA-NAT and ASMT genes was present in a brain cortex throughout the lifespan. The same conclusion about sufficient gene activity we applied to the intestine results since no statistically significant difference was observed between young and old animals. Moreover, we have detected a positive correlation between gene expression of AA-NAT and ASMT in intestine of both, young and old animals. In some tissues, such as heart and brain, no change of expression and no correlation of AA-NAT or ASMT genes were found. We have assumed that in these tissues there were no need for such an action of AA-NAT and ASMT since these organs have not been involved in increased neutralization or elimination of toxic molecules. In contrast, in kidney, which is involved in clearance of various molecules from the body, we have detected a positive correlation of AA-NAT and ASMT gene activity. Since, AA-NAT expression had a tendency to increase and ASMT expression had a tendency to decrease in kidney, a possibility of *N*-acetylserotonin accumulation was present. *N*-acetylserotonin is the immediate precursor of melatonin, has 5–20 times stronger antioxidant activity (Oxenkrug, 2005) than melatonin in some tissue, and its role does not always overlap with a melatonin function (Ben Shahar et al., 2016). Knowing that kidney is involved in elimination of many unwanted molecules from a body and that a possible accumulation of *N*-acetylserotonin (NAS) occurs in this organ, a further examination of a melatonin pathway in ageing kidney is needed to fully understand the observed processes.

A local production of melatonin, outside pineal gland and brain, was shown for the first time in cultured hamster skin (Slominski et al., 1996). Melatonin is rapidly metabolized in the skin, mainly by the indolic pathway to 6(OH)M (6-hydroxymelatonin) or 5MT (5-methoxytryptamine), and less by the kynuric pathway to AFMK (N^1-acetyl-N^2-formyl-5-methoxykynuramine) and AMK (N^1-acetyl-5-methoxykynuramine) (Slominski et al., 2017b). These melatonin metabolites are involved in the antioxidative defense. A level of 6(OH)M in the skin is higher than levels of other metabolites, and 6(OH)M can further be converted to *N*-acetylserotonin (NAS) by cytochrome 450, through microsomal and mitochondrial metabolic pathway (Slominski et al., 2017b). Moreover, NAS can be both a precursor and a metabolite of melatonin. On the other hand, the skin inflammatory reactions and an exposure to UV radiation may activate the non-enzymatic melatonin conversion into potent oxygen scavenger, AFMK and AMK. When comparing a production of melatonin and its metabolites in the human skin, it was reported much higher level of 6(OH)M and 5MT than melatonin, and slightly less AFMK than melatonin (Kim et al., 2015).

The results of our study that showed a high AA-NAT and ASMT gene expression in the skin and the liver, could also lead to the assumption that a production of NAS is increased in very old animals, if enzymes' activities are proportional to the gene expression (Xu et al., 2018). As both enzymes (AA-NAT and ASMT) are involved in melatonin synthesis, their levels may be considered as significant parameters in indirect estimation of cell capacity to maintain local homeostasis, especially in the skin, which is the largest organ.

Classic melatonin synthesis by key enzymes, AA-NAT and ASMT, had been identified in all vertebrates in pineal glands and various extrapineal tissues (Hardeland et al., 2006; Slominski et al., 2002). It has been reported that the expression of AA-NAT and ASMT in peripheral organs leads to 10- to 1000-fold higher amount of melatonin in local tissue than in plasma (Fischer et al., 2006; Bubenik, 2002; Kobayashi et al., 2005). Since AA-NAT and ASMT were expressed in all tissues we studied and a difference in expression of these genes between young and old in some tissue was present, there is a possibility for a use of AA-NAT and ASMT gene expression as a predictive factor of tissues defense against oxidative damage during ageing. It has been reported that AA-NAT and ASMT gene expression could be associated to the amount of

both protein enzymes, as well as to the level of melatonin (Xu et al., 2018). However, that correlation has not been always present when comparing our results with the results of others (Sanchez-Hidalgo et al., 2009), but it is present for example in the case of AA-NAT and ASMT gene expression and melatonin level in a liver.

Knowing that melatonin influences the antioxidant enzymes activities (Rodriguez et al., 2004; Reiter et al., 2000), we have also decided to monitor enzymatic activities of superoxide dismutase (SOD) and catalase (CAT), as well as the level of reduced glutathione (GSH) in the liver. We have found in older animals a statistically significant increase of CAT activity, a decrease of SOD enzymatic activity, and no change in GSH in ageing. Decreased values of not only SOD, but also of CAT and GSH were observed in 12- and 24-month old rats when compared to the 3-month old (Manikonda and Jagota, 2012). A difference between their and our results could be due to a different experimental set up and a different age of animals. However, their results (Manikonda and Jagota, 2012) and results of others (Molpeceres et al., 2007, Kireev et al., 2013) with a restoration of antioxidant enzymes activities upon supplementation with melatonin support our findings. Due to different results with CAT and SOD in our experiments, we have also determined the level of Cu, Zn, and Mn in the liver and have found their amounts to be decreased in advanced age. Since Cu and Mn serve as cofactors for SOD, their lack might be responsible for the lower activity of SOD. In addition, a possible positive regulation of SOD by AA-NAT and ASMT via melatonin would not be feasible if insufficient quantities of Cu and Zn were present. Moreover, it is also possible that a reduced level of certain microelements in the liver could lead to the absence of their inhibitory effect to AA-NAT expression (Zhan-Poe and Craft, 1999). For example, enhanced AA-NAT gene expression, released from microelements blocking action could have stimulated SOD and CAT production. However, less Cu and Zn will lead to decreased activity of SOD and increased activity of CAT in older animals (Xianyong et al., 2017). In addition, we have found a significantly lower concentration of Cu, Zn, and Mn in kidney of older animals when compared to the younger ones (data not shown). Overall, our results have supported a role of microelements in regulation of antioxidant enzymes, especially in liver during ageing. However, a complex relation between microelements and antioxidative defense system exists that requires further studies. For example, melatonin forms complexes with copper and zinc (Limson et al., 1998) and can protect against free radical damage in rat liver (Parmar et al., 2002).

The expression of AA-NAT and ASMT, the two key genes involved in a synthesis of melatonin, was increased in liver and skin of old animals. A correlation in AA-NAT and ASMT expression was also observed in liver, intestine and kidney. Moreover, the activity of CAT enzyme in a liver of old animals was increased while SOD activity was decreased. The activity of these enzymes, known to be influenced by the melatonin, was probably affected by the decreased amount of Cu, Zn, and Mn in liver of old animals. We concluded that, in our model, extrapineal melatonin pathway in ageing consisted of complex interplay of locally produced melatonin, activities of SOD and CAT, and adequate presence of Cu, Zn and Mn microelements in order to defend organs against oxidative damage.

Acknowledgements

This study was supported by the grants No 175075, No III-41019, and No 45003 from the Ministry of Education, Science and Technological Development, Government of Serbia.

Disclosure statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Funding information

No funding body had any involvement in the preparation or content of this article or in the decision to submit it for publication.

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