## Liver antioxidant and aerobic status improves after metformin and melatonin administration in a rat model of high-fat diet and mammary carcinogenesis

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Liver antioxidant and aerobic status improves after metformin and melatonin administration in a rat model of high-fat diet and mammary carcinogenesis

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Short title: Melatonin and metformin as antioxidants in liver
Abstract

Oxidative stress is involved in the development of various cancers. In the present study the effect of long-term administration of peroral antidiabetic metformin and pineal hormone melatonin on liver antioxidant and aerobic status in female Sprague-Dawley rats carrying mammary tumors induced by N-methyl-N-nitrosourea was evaluated. Both substances were administered in a preventive/curative manner (12 days before and 16 weeks after the carcinogen application).

Carcinogen administration induced oxidative stress: the level of thiobarbituric acid reactive products (TBARS) as a marker of reactive oxygen species (ROS) generation in liver increased as well as the level of oxidatively modified protein content (OMP, aldehyde and ketone derivates). Metformin administration restored succinate dehydrogenase and lactate dehydrogenase activity and associated ROS production and OMP content to the level of intact rats, with predominant activation of superoxide dismutase (SOD) and glutathione reductase (GR). Melatonin alone and in combination with metformin decreased TBARS content too. OMP content decreased in all groups receiving chemoprevention. The rise in total antioxidant capacity after melatonin and particularly metformin and melatonin combination might result from the initiation of anaerobic metabolism and increasing SOD, GR and glutathione peroxidase (GPx) activity.

Long-term administration of metformin and melatonin exerts antioxidant properties in liver, especially in combination.

Keywords: metformin, melatonin, liver, high-fat diet, oxidative stress, mammary carcinogenesis


Introduction

Breast cancer is the most common malignancy globally (Global Burden of Disease Cancer Collaboration, et al. 2013) and in Europe accounts for 13.5% of all cancer cases (Ferlay et al. 2013). Among many factors, reactive oxygen species (ROS) are involved in etiology and progression of breast cancer (Liou and Storz 2010). ROS may control antioxidant gene expression (Carpentieri et al. 2012; Sekkin et al. 2015) and high levels of oxidative stress biomarkers, including lipid peroxidation products (e.g. malondialdehyde or isoprostanes), protein oxidative products (e.g. carbonyls and diene-conjugates) and DNA adducts are frequently identified in breast cancer patients (Ray et al. 2000; Kedzierska et al. 2012). Alternatively, ROS can act as tumor suppressors too, thus, fighting cancer may include either ROS-depleting or ROS-elevating strategies and therapy choice depends on a complete understanding of cancer cell redox state (Yang et al. 2013) which is an important component of basic research.

Chemoprevention is one of the strategies to decrease cancer incidence and cancer death rates. Numerous substances, both natural and synthetic proved their preventive and/or oncostatic properties both in vitro and in vivo. However, in human population, it is not possible to control factors which may have an impact on cell proliferation to such an extent as in experimental studies. The risk of malignant transformation may increase due to various factors related to lifestyle, shift work, insomnia, diet, including excessive consumption of fat. All this makes the extrapolation of experimental data to human population more difficult. In addition, chemoprevention is not a short term “fix” as the risk is ongoing and lifelong prevention may be required to provide adequate protection.

The interconnection between metabolic pathways and cell growth regulation is known but not yet fully understood. Some metabolic disorders including diabetes (particularly diabetes type 2) are associated with higher cancer risk related to impaired insulin receptor and
insulin-like growth factor signaling and subsequent cell proliferation enhancement. Importantly, cancer patients often develop glucose intolerance (Goodwin et al. 2012; Leone et al. 2014). From this point of view, peroral antidiabetics with pleiotropic properties that showed antitumor activity draw more and more attention as diabetes mellitus type 2 incidence as well as cancer incidence rises. By far, anti-tumor properties were reported in two groups of peroral antidiabetics: biguanides, particularly metformin (MF) (Pollak 2013; Anisimov 2015) and thiazolidinediones (Bojková et al. 2014; Monami et al. 2014). Inhibitory effect of biguanides in cancer has been reported many years ago by Dilman group (Dilman and Anisimov 1980). However, due to lactic acidosis risk only metformin is now used in clinical practice. Oncostatic effects of MF were reported in numerous neoplasms including mammary cancer (Anisimov 2015; Zhu et al., 2015). The key mechanism of MF action arises from both AMPK-dependent (Dowling et al. 2007) and AMPK independent (Sahra et al. 2011) inhibition of mTOR, a key pathway that regulates cell proliferation. Other MF actions that may modulate malignant transformation include IGF-1 pathway (Liu et al. 2011) and reduction in ROS and associated DNA damage (Algire et al. 2012). Our group recorded partial oncostatic effect of MF in a rat breast cancer model using a standard diet type (Bojková et al. 2009). Clinical studies showed lower cancer incidence in diabetic patients treated with MF in comparison with those treated with other hypoglycemic drugs (Goodwin et al. 2012; Leone et al. 2014; Anisimov 2015).

MF efficacy in breast cancer may be enhanced in combination with various other chemopreventives (Wang et al. 2014; Guo et al. 2015; Falah et al. 2017), including pineal hormone melatonin (MEL). MEL is one of the oldest signaling molecules and acts as chronobiotic and reproduction regulator. In addition to pineal gland MEL is synthesized in other sites including immune cells (Carrillo-Vico et al. 2004). MEL is a potent antioxidant (Pieri et al. 1995; Carpentieri et al. 2012; Kurhaluk et al. 2017, 2018) and exerts also
antiproliferative, proapoptotic, anti-inflammatory, immunomodulative, and antiangiogenic effects (Hardeland et al. 2011). MEL also suppresses mTOR pathway (Jung et al. 2013; Ferreira et al. 2014; Prieto-Domínguez et al. 2017). These properties point to its oncostatic effect which was firstly reported in MCF-7 mammary adenocarcinoma line (Hill and Blask 1988). Our group reported inhibitory effects of MEL (Kubatka et al. 2002, 2014; Orendáš et al. 2009, 2014) and also MEL and MF combination (Bojková et al. 2018) in mammary carcinogenesis and the results indicate that preventive-curative way (i.e. MEL administration before and after carcinogenesis induction) is the most suitable as proved by increased latency and survival time; combination with other oncostatic substances is preferable.

N-methyl-N-nitrosourea (NMU) is a direct alkylating compound which has been mostly used as a mammary carcinogen but may induce tumors in various organs (e.g. skin, lung, gastrointestinal tract, ovary, uterus, prostate and hematopoietic system), depending on species and strain, dose, route and age at administration (Faustino-Rocha et al. 2015). Our working group has been using a model of NMU-induced mammary carcinogenesis in female Sprague-Dawley rats (for detailed description see Materials and Methods section) in chemopreventive studies for more than 20 years and we did not observe frequent malignancies (<10% of group size) on sites other than the mammary gland. However, apart from DNA alkylation damage, NMU may induce oxidative stress (Somanah et al. 2016), thus, enhancing antioxidant capacity may have a positive impact on carcinogenesis.

The aim of this work was to evaluate the effect of peroral antidiabetic MF, and MEL administered alone and in combination on oxidative stress parameters, antioxidant enzyme activity, enzymatic biomarkers of hepatotoxicity and aerobic/anaerobic metabolism markers in a rat model of high-fat diet and mammary carcinogenesis. We used a high-fat diet in order to better mimic situation in human population. Increased exposure of hepatocytes to free fatty acids leads to higher ROS production and steatosis (Kohli et al. 2007). High-fat diet is also
associated with inflammation (Tarantino et al. 2011; Zhang et al. 2015; Masi et al. 2017) which may lead to liver fibrosis and cirrhosis and as such is a risk factor for hepatocellular carcinoma. Therefore, we intended to evaluate the efficacy of both substances against hepatocellular damage induced by NMU, a well-established mammary carcinogenic agent.

2. MATERIALS and METHODS

Ethical research approval

The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic (accreditation No. Ro-2054/13-221). The animals were treated and sacrificed in a humane manner according to the principles provided in the Law No. 289/2003, 489/2003, and 23/2009 of Slovak Republic for the Care and Use of Laboratory Animals. The animals were cared for in accordance with Guide to the Care and Use of Experimental Animals (Vol. 1, 2nd ed., 1993, and Vol. 2, 1984, available from the Canadian Council on Animal Care (CCAC), 190 O’Connor St, Suite 800, Ottawa, ON K2P 2R3, Canada).

Mammary carcinogenesis model.

Female rats of sensitive Sprague-Dawley strain (Velaz, Prague, Czech Republic) aged 30 days were used in the experiment. The animals were adapted to standard vivarium conditions with a temperature of 23 ± 2°C, a relative humidity of 60–70% and an artificial regimen light-dark cycle of 12: 12 h (lights on from 7 a.m., light intensity 150 lx/cage). During the experiment, the animals (6/cage) were fed the high-fat diet (10% total fat, 2.5% from lard, 7.5% from palm olein; Biofer, Slovak Republic) and tap water or MEL solution, respectively, ad libitum.
Mammary carcinogenesis was induced by N-methyl-N-nitrosourea (NMU, cat. no. N4766; Sigma-Aldrich, Deisenhofen, Germany) administered intraperitoneally (50 mg/kg body weight) on the 42nd postnatal day. NMU solution was freshly prepared before administration by dissolving NMU in 0.9% NaCl (the average volume dose per rat was 0.5 ml).

Chemoprevention and experimental groups

Chemoprevention with MF and MEL was initiated 12 days prior to carcinogen application and lasted until experiment termination. MF (Actos; Lilly S.A., Alcobendas, Spain) was administered in a diet at a concentration of 2000 ppm. MEL (cat. no. M5250, Sigma-Aldrich, Deisenhofen, Germany) was administered in tap water at a concentration of 20 mg/L daily from 3 p.m. to 8 a.m. (only water from 8 a.m. to 3 p.m.). Animals were assigned randomly to one of five experimental groups (n= 18/group, except the intact group, n= 10): (1) CONT, control group without chemoprevention; (2) MF, chemoprevention with metformin; (3) MEL, chemoprevention with melatonin; (4) MF+MEL, chemoprevention with combination of metformin and melatonin; and (5) INT, intact group. Animals were weighed weekly and palpated to register the presence, number, location and size of each palpable tumor. Food and water intake over the 24-h period was monitored within the 4th, 9th and 14th week of the experiment (dated from NMU administration). During the experiment, the average daily intake of MF ranged from 28 to 32 mg/rat/day, which equals to ca 880 mg/m² (considering a body surface of 0.034 m² in 245 g rat). To compare, the recommended daily dose of MF in diabetic patients is 1700 mg, which equals to ca 940 mg/m² (considering a body surface of 1.8m²). The average daily intake of MEL ranged from 0.36 to 0.48 mg/rat/day. The MEL dose we used was supraphysiological, about 4-fold higher than a usual dose used for treatment of sleep disorders and jet lag disorder in humans (considering body
surface). In the last – 16th week of the experiment, the animals were killed by quick
decapititation and mammary tumors and selected organs and tissues were removed for further
evaluation.

**Liver homogenate preparation**

The liver specimens were weighed, washed in ice-cold buffer, and minced. The
minced tissue was rinsed with cold isolation buffer to remove blood and homogenized in a
glass Potter-Elvehjem homogenizer with a motor-driven Teflon pestle on ice. The isolation
buffer consisted of 120 mM KCl, 2 mM K$_2$CO$_3$, 10 mM HEPES and 1 mM EDTA; pH of 7.2
was adjusted with KOH.

Liver homogenate was used for the determination TBARS and OMP levels, total
antioxidant capacity (TAC) as well as CAT and SOD (1:1000), glutathione reductase (GR)
and glutathione peroxidase GPx (1:20) activities. For the quantification of proteins the
Bradford method with bovine serum albumin as a standard was used. Absorbance was
recorded at 595 nm.

**Biochemical assays**

**2-Thiobarbituric acid reactive substances assay.**

TBARS products were measured by the method of described by Kamyshnikov (2004)
according to the following principle: distilled water, 20% trichloracetic acid and 0.8% 2-
tiobarbituric acid (TBA) reagent were added to liver homogenate and boiled in a water bath at
100°C for 10 min. TBARS products were then cooled in ice-cold water, centrifuged at 3,000g
for 10 min.; and measured by spectrophotometry at 540 nm. TBARS level was expressed in
µmol of MDA per mg protein for liver tissue.
**Total antioxidant capacity assay.**

The TAC in liver was estimated by measuring the TBARS level according the oxidation of Tween 80. This level was determined colorimetrically according to the method of described by Galaktionova et al. (1988). The method is based on the reaction of Fe²⁺/ascorbate-induced oxidation of Tween 80, resulting in a stable 2-thiobarbituric acid reactive substances level – whose maximum absorbance was measured at 532 nm. The absorbance of the blank was defined as 100%. The TAC of the sample (%) was calculated with respect to the absorbance of the blank.

**Carbonyl derivatives of protein assay.**

The OMP level was analyzed by method described by Levine et al. (1990) and modified by Dubinina et al. (1995). The method is based on the reaction of carbonyl derivatives of amino acids with 2, 4-dinitrophenylhydrazine after adding liver homogenate. The absorbance of solution was measured at 370 nm (aldehyde derivates, OMP AD) and 430 nm (ketonic derivatives, OMP KD) against an ethanol-ethylacetate blank. Carbonyl groups content was expressed in nanomoles per mg of protein.

**Superoxide dismutase activity assay.**

SOD activity of the tissues samples in the supernatant were performed according to Kostiuk et al. (1990). It is a colorimetric method based on the principle of measuring absorbance of the coloured quercetin autoxidation complex in an alkaline medium at 406 nm against water blank. SOD activity was determined in units of SOD per mg of protein.

**Catalase activity assay.**
CAT activity was determined using the Koroliuk et al. (1988) method. Activity of the enzyme was estimated by the decrease in absorbance of hydrogen peroxide at 410 nm. The reaction mixture contained liver homogenate diluted in incubation medium (1:10), \( \text{H}_2\text{O}_2 \) and ammonium molybdate. One unit of catalase activity was expressed in nmol \( \text{H}_2\text{O}_2 \) per minute per mg of protein.

**Glutathione reductase activity assay.**

Liver glutathione reductase (GR) activities was determined metric according to the colourimetric method of Glatzle et al. (1974) based on the NADPH oxidation in the presence of GSSG in sodium phosphate buffer (pH 6.6). The method based on the measurement of changes in absorbance at a wavelength of 340 nm, caused by the oxidation NADPH. A blank without NADPH was used, and the GR activity was expressed in nanomoles of NADPH per minute per mg of protein.

**Glutathione peroxidase activity assay.**

GPx activity was evaluated by using the nonenzymatic utilization of GSH after incubation of liver homogenate with 5,5-dithiobis-2-nitrobenzoic acid according by the method of Moin (1986). The absorbance was measured at 412 nm. The assay mixture contained 0.1 M Tris–HCl buffer, 6 mM EDTA, 12 mM sodium azide (pH 8.9), 4.8 mM GSH, liver homogenate sample, 20 mM t-butylhydroperoxide, and 0.1 mL of 0.01 M 5, 5-dithiobis-2-nitrobenzoic acid. GPx activity was expressed in nanomoles of GSH per minute per mg of protein.

**Alanine aminotransferase and aspartate aminotransferase activities assay**
ALT and AST activity was analyzed spectrophotometrically by a standard enzymatic method described by Reitman and Frankel (1957). The substrates in the reaction for AST were as follows: α-ketoglutaric acid plus L-aspartate, and for ALT as follows: α-ketoglutaric acid plus L-alanine. The intensity of the colour was related to enzymatic activity of appropriate enzymes. Pyruvate salt was used as the standard for calibrating the graph composition in the measurement of both AST and ALT activities. One unit per L of AST or ALT is defined as the liberation of 1 mmol of pyruvate per hour at 37°C incubation per L of plasma or 1 mg protein.

**Lactate dehydrogenase activity assay**

For the determination of LDH activity the colorimetric method of Sevela and Tovarek (1959) was used. The principle of the method is based on pyruvate to lactate reduction catalyzed by LDH in the presence of NAD\(^+\). The reduction of NAD\(^+\) is coupled to the reduction of L-lactate. One unit per L of LDH is defined as the formation of 1 mmol of pyruvate per hour at 37°C incubation per L of plasma or 1 mg protein in liver or heart tissues.

**Succinate dehydrogenase (SDH) activity**

SDH activity was analyzed spectrophotometrically by method of Eschenko and Volski (1982). The enzyme assay mixture consisted of 1 mL 0.1M phosphate buffer (pH 7.8), 0.1 mL of 0.1M succinic acid (pH 7.8), 25 mM EDTA (pH 7.8), 150 mM sodium aside. The absorbance of the obtained supernatant was measured at 420 nm and was compared with the absorbance of the blank. One unit of SDH activity is defined as the amount of enzyme required for decomposition of 1 nmol succinic acid per min per mg of protein.

**Statistical analysis**
Results are expressed as means ± SEM. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors tests (P>0.05). Homogenity of variance was checked using the Levene’s test. Significant differences between groups were evaluated by GraphPad Prism (version 5.01; GraphPad Software Inc., California, USA).

Results

During the experiment, no significant permanent changes were recorded in food and water intake (data not shown). MF and MEL administration had no significant effect on mammary tumor growth, but MF administration reduced cumulative tumor mass by 51% and MEL administration decreased tumor incidence by 22% vs CONT group. No effect was recorded after combined treatment (data not shown).

We evaluated also triglycerides concentration in serum and liver. Serum concentration of triglycerides was within physiological range defined for rat in all groups. Liver concentration of triglycerides did not differ among groups, but was about twice as high as the concentration of triglycerides in the liver of intact rats fed with standard diet type (compared to results from our previous relevant experiments (Table 1).

The level of total antioxidant capacity (TAC) in the liver tissue was determined at first part of our investigation. The results obtained have shown no significant differences in TAC levels (Fig.1) in CONT group compared to INT group. Single administration of chemopreventive substances had no effect but there was a significant increase in TAC in the liver in MF+MEL group compared to CONT and also MEL group.
Fig. 2 presents the lipid peroxidation estimated by TBARS products level. There was a significant increase in TBARS level in the liver of CONT group compared to INT group. MEL administration (single and in combination with MF) resulted in a significant decrease in the TBARS level.

The level of OMP (Fig. 3) in the liver was higher in CONT group when compared to INT group. Chemoprevention significantly decreased OMP AD level but only a non-significant decrease in all groups receiving chemoprevention was found regarding OMP KD level.

Antioxidant enzyme activity is summarized in Table 2. CONT group showed increase in CAT activity compared to INT group. MF and MEL administration increased SOD activity compared to CONT group but no such effect was found in MF+MEL group. MF decreased also CAT activity and GPx activity in comparison with CONT group; GR activity was higher when compared both to CONT and INT group. MEL administration resulted in decrease of GR activity in comparison with MF group; GPx activity was higher when compared to both INT and MF group. Combined chemoprevention decreased SOD activity compared to MF group, GR activity was higher compared to CONT group, and GPx activity was lower in comparison with both CONT and MEL group.

The next step in our investigation involved the measurement of alanine (ALT) and aspartate aminotransferase (AST) activity as enzymatic biomarkers of hepatotoxicity, and succinate dehydrogenase (SDG) and lactate dehydrogenase (LDG) activity as cell markers of aerobic and anaerobic metabolism. Higher ALT activity was found in control and MEL group
in comparison with INT group. No significant changes were recorded regarding AST activity (Fig. 4).

Liver SDG activity increased significantly in CONT group (by 61.7%, $P<0.001$). The treatment with MF and MEL modified this value. The SDG activity was lower by 44.4% ($P<0.001$) and 29.4% ($P<0.01$), respectively, compared to CONT group (Fig. 5). NMU administration also increased LDG activity (by 34.7%, $P<0.01$). Overall, enhancement of advantageous aerobic metabolism may be associated with the above mentioned increase in the production of ROS and damage of cellular proteins identified by the content of their modified forms. MF treatment decreased LDG activity compared to CONT group MEL administration led to increase in LDG activity when compared both to INT and MF group. Combination of chemopreventives decreased it when compared to MEL group.

**Discussion**

In the present work, we focused on the effect of MF, MEL and their combination on the enzymatic and substrate biomarkers of aerobic and anaerobic metabolic pathways associated with oxidative stress in liver in a rat model of breast cancer.

Due to a high number of analyses, we did not assess of the effects exerted by a high-fat diet alone. Nevertheless, such evaluations are sufficiently covered in the literature. Also, we eliminated this factor by giving a high-fat diet to all the rats investigated. Using such approach we obtained the data on the influence of metformin and melatonin on oxidative stress in the liver tissue of female Sprague-Dawley rats remaining on a high-fat diet and subjected to the mammary carcinogenic process induced by NMU; the data that have not been yet reported.
Free radical homeostasis in cells ensures coordinated work between enzymatic and non-enzymatic systems of ROS generation on the one hand, and ROS elimination on the other. Oxidative stress plays a role in a number of pathologies including liver fibrosis (Poli 2000) and has been also proposed as one of the modes of actions of carcinogens. Experimental data showed the sequence of cell events in carcinogenesis related to generation of ROS (Klaunig et al. 2010; 2011). Oxidative stress in cancer cells promotes tumor development, but it can also be useful in the search for new therapeutic strategies. Breast cancer patients show dysregulation of ROS homeostasis, as detected by various indicators in plasma, various blood cells, and tissues (Kedzierska et al. 2012; Sosa et al. 2013; Kurhaluk et al. 2018). A defense against ROS includes both enzymatic and non-enzymatic antioxidant defenses. Enzymatic defense, such as SODs, CATs and peroxidases, protect cells by directly scavenging superoxide radicals and hydrogen peroxide. These enzymes convert ROS to less reactive species. At the tissue level, enzymes of glutathione antioxidant defense play a particularly important role. SODs catalyze the dismutation reaction of superoxide anion to hydrogen peroxide. CAT and other peroxidases initiate reduction of H$_2$O$_2$ to 2H$_2$O. Accordingly, SOD and CAT serve in tandem, often considered as front-line antioxidant defenses.

Our study demonstrated oxidative stress induction in the liver of Sprague-Dawley rats administered with mammary carcinogen NMU. We found that the NMU treatment was accompanied by generation of ROS estimated by increased level of TBARS products and higher OMP content level. Increased CAT activity was not sufficient to ameliorate these parameters. MF and MEL were reported to induce changes in antioxidant gene expression and basic antioxidant enzymes activities (Carpentieri et al. 2012; Martin-Montalvo et al. 2013; Chukwunonso et al. 2016) and thus contribute to their anticancer properties. Chemopreventive substances in our experiment attenuated ROS generation and induced antioxidant enzymes,
combined treatment was more effective as showed by increased TAC. Our results demonstrate ability of MF and MEL to decrease oxidative stress which was reported before (Onken et al. 2010; Esteghamati et al. 2013; Sekkin et al. 2015). Although enhancement of antioxidant defence did not result in significant mammary tumor inhibition, attenuation of oxidative stress and reported inhibition of inflammation and fibrosis by both MF (Kita et al., 2012; Martin-Montalvo et al. 2013) and MEL (Hu et al. 2009; Lebda et al. 2018) clearly indicate their preventive potential against hepatocellular carcinoma.

A very important role in reaction to different oxidative stress (hypoxia, cancer, psychoemotional stress, diabetes mellitus etc) is played by the ALT and AST in α-ketoglutarate formation, which is next supplied to the Krebs cycle. Thus, the reactions of α-ketoglutarate and pyruvate formation may be considered as a very important link between different (aerobic and anaerobic) metabolic pathways. In addition, these enzymes serve as biomarkers of hepato- and cardiotoxicity. Our results do not imply liver damage; ALT activity was higher after NMU administration but AST activity remained unaltered and AST/ALT ratio was approximately 1:1.

Biomarkers of aerobic and anaerobic metabolic pathways play an important role in the pathogenesis of different pathologic states, cancer mainly. The SDG and LDG activity in the liver after NMU treatment demonstrated their importance for understanding the relation of aerobic and anaerobic metabolic pathways to carcinogenesis. Our results suggest that these changes were associated with enhanced oxidative stress. MF, alone and in combination restored SDG/LDG activity, MEL enhanced anaerobic metabolism through LDG activation which may attenuate ROS production and subsequently oxidative stress. The results of this study provide evidence that administration of MF and MEL in NMU-induced mammary carcinogenesis resulted in different metabolic pathways of oxidative stress initiation.
**Conclusion**

1. Female Sprague-Dawley rats carrying NMU-induced mammary tumours showed markers of oxidative stress in liver (increased TBARS and OMP content), induction of liver antioxidant defence system (increased CAT activity) and induction of advantageous aerobic metabolism type (increased SDG activity).

2. Administration of MF restored markers of aerobic/anaerobic metabolism (SDG, LDG activity) to the level of intact animals and inhibited ROS production (decreased TBARS and OMP content), though total antioxidant capacity remained unchanged. We assume that attenuation of oxidative stress was achieved predominantly through SOD and GR activation.

3. Administration of MEL increased total antioxidant capacity through a substantial reduction of ROS formation (decreased TBARS and OMP content. This may be due to the initiation of anaerobic type of metabolism (increased LDG activity) and increasing liver antioxidant defense system through SOD, GR and GPx initiation.

4. Combined treatment with MF and MEL showed best results in attenuation of oxidative stress through substantial increase of total antioxidant capacity, decrease of TBARS and OMP content and GR induction. Aerobic/anaerobic metabolism markers were restored to the level of intacts.

We assume that adding melatonin supplements to antidiabetic treatment may attenuate oxidative stress and subsequently modify cancer risk.
Acknowledgements

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Conflicts of interest

The authors declare that there are no conflicts of interest.
References


Table 1. Triglycerides concentration in the serum and liver

<table>
<thead>
<tr>
<th>Parameters</th>
<th>INT</th>
<th>NMU</th>
<th>MF</th>
<th>MEL</th>
<th>MF+MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (mmol/l)</td>
<td>0.718±0.206</td>
<td>0.949±0.304</td>
<td>0.881±0.295</td>
<td>1.12±0.707</td>
<td>1.13±0.308*</td>
</tr>
<tr>
<td>Liver (µmol/g)</td>
<td>64.0±20.4</td>
<td>56.5±18.8</td>
<td>55.1±20.0</td>
<td>63.0±27.7</td>
<td>53.6±22.3</td>
</tr>
</tbody>
</table>

Legend

INT – intact animals, NMU – control group (no chemoprevention), MF – chemoprevention with metformin, MEL – chemoprevention with melatonin, MF+MEL – chemoprevention with metformin and melatonin. Significant differences between groups are designated as follows: vs INT: a (P<0.05)
Table 2. Antioxidant defense in the liver.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>INT</th>
<th>NMU</th>
<th>MF</th>
<th>MEL</th>
<th>MF+MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/min mg protein)</td>
<td>600±84.4</td>
<td>563±109</td>
<td>761±88.4</td>
<td>722±119b</td>
<td>570±96.2c</td>
</tr>
<tr>
<td>Catalase (µmol H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;/min mg protein)</td>
<td>10.2±0.48</td>
<td>13.6±1.78b</td>
<td>10.1±1.82b</td>
<td>11.8±1.99</td>
<td>12.5±2.76</td>
</tr>
<tr>
<td>GR (nmol NADPH/min mg protein)</td>
<td>9.55±2.76</td>
<td>5.73±1.97</td>
<td>19.1±7.64abbb</td>
<td>11.2±4.26cc</td>
<td>13.9±4.94bb</td>
</tr>
<tr>
<td>GPx (µmol GSH/min mg protein)</td>
<td>11.3±4.62</td>
<td>22.4±11.9</td>
<td>11.1±4.04b</td>
<td>26.3±9.59aacc</td>
<td>9.97±5.23bbddd</td>
</tr>
</tbody>
</table>

**Legend:**

INT – intact animals, NMU – control group (no chemoprevention), MF – chemoprevention with metformin, MEL – chemoprevention with melatonin, MF+MEL – chemoprevention with metformin and melatonin.

Values are expressed as means ± SD. Significant differences between groups are designated as follows: vs INT: a (P<0.01), aaa (P<0.001); vs NMU: b (P<0.05), bb (P<0.01), bbb (P<0.001); vs MF: c (P<0.05), cc (P<0.01); vs MEL: ddd (P<0.001).
Figures

Fig.1. The total antioxidant capacity (%). Values are expressed as means ± SD.

Legends
INT – intact animals, NMU – control group (no chemoprevention), MF – chemoprevention with metformin, MEL – chemoprevention with melatonin, MF+MEL – chemoprevention with metformin and melatonin. Significant differences between groups are designated as follows:
vs NMU: bb (P<0.01); vs MF: c (P<0.05); vs MEL: d (P<0.05)

Fig.2. The level of lipid peroxidation (TRARS products content). Values are expressed as means ± SD.

Legends
INT – intact animals, NMU – control group (no chemoprevention), MF – chemoprevention with metformin, MEL – chemoprevention with melatonin, MF+MEL – chemoprevention with metformin and melatonin.
Significant differences between groups are designated as follows: vs INT: aa (P<0.01); vs NMU: bb (P<0.01)

Fig. 3. Oxidatively modified proteins (OMP) content (aldehyde derivates, AD; ketonic derivates, KD).
Values are expressed as means ± SD.

Legend
INT – intact animals, NMU – control group (no chemoprevention), MF – chemoprevention with metformin, MEL – chemoprevention with melatonin, MF+MEL – chemoprevention with metformin and melatonin.
Significant differences between groups are designated as follows: vs INT: aa ($P<0.01$), aaa ($P<0.001$); vs NMU: b ($P<0.05$), bb ($P<0.01$)

**Fig. 4.** Alanine- and aspartate aminotransferase activity in the liver. Data are expressed as means ± SD.

**Legend**

INT – intact animals, NMU – control group (no chemoprevention), MF – chemoprevention with metformin, MEL – chemoprevention with melatonin, MF+MEL – chemoprevention with metformin and melatonin.

Significant differences between groups are designated as follows: vs INT: a ($P<0.05$), aa ($P<0.01$)

**Fig. 5.** Succinate dehydrogenase activity (A) and lactate dehydrogenase activity (B). Values are expressed as means ± SD.

**Legend**

INT – intact animals, NMU – control group (no chemoprevention), MF – chemoprevention with metformin, MEL – chemoprevention with melatonin, MF+MEL – chemoprevention with metformin and melatonin.

Significant differences between groups are designated as follows:

vs INT: aa ($P<0.01$), aaa ($P<0.001$); vs NMU: bb ($P<0.01$), bbb ($P<0.001$); vs MF: ccc ($P<0.001$); vs MEL: ddd ($P<0.001$)
Figure 1
Figure 2
Figure 3
Figure 4

AlAT AsAT

mmol/h mg protein

INT NMU MF+ NMU MEL+ NMU MF+MEL+NMU

a aa
Figure 5