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The influence of melatonin on apoptosis of human neutrophils*

Wpływ melatoniny na apoptozę ludzkich neutrofilów

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Summary

Aim: Melatonin (Mel) besides its main role in circadian and seasonal rhythm coordination, plays a role in immunoregulation and inflammatory responses. The melatonin's ability to modulate apoptosis is one of its important roles related to its effect on immune system but the exact effect of its action and the mechanisms of apoptosis control by melatonin remain still unclear. The goal of our study was to examine the involvement of melatonin in the apoptosis of human neutrophils *in vitro* and possible mechanisms of this action.

Material/Methods: We measured the effect of melatonin on the spontaneous and TNF-α-induced apoptosis of human neutrophils using propidium iodide and Annexin-V and on caspase-3 activation, apoptosis-related surface antigen expressions, intracellular reactive oxygen species (ROS) generation and cytochrome c release using flow cytometry and commercial reagents.

Results: Melatonin does not affect spontaneous apoptosis of human neutrophils and mitochondrial cytochrome c release but protects the cells from the significant rise of TNF-α-induced apoptosis and cytochrome c release. Intracellular ROS generation in PMA-stimulated neutrophils did not change after the influence of melatonin but the significant drop of ROS generation in neutrophils stimulated with TNF- α was upregulated to the control level after preincubation of the neutrophils with melatonin. Melatonin did not change significantly Fas, Fas-L and active caspase-3 expressions in neutrophils.

Conclusions: Melatonin does not affect the spontaneous apoptosis, however, inhibits TNF-α-induced apoptosis of human neutrophils. Our findings suggest that the intrinsic pathway of the process is a result of the melatonin induced mitochondrial alterations.

Keywords: **melatonin • apoptosis of neutrophils • TNF-α**

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Abbreviations:

DHR 123 – dihydrorhodamine 123, **fMLP** – N-formyl-L-methionyl-L-leucyl-L-phenylalanine, **mel** – melatonin, **OZ** – opsonized zymosan, **PMA** – phorbol 12-myristate 13-acetate, **PMNL** – polymorphonuclear leukocytes, **ROS** – reactive oxygen species, **TNF-α** – tumor necrosis factor-α.

INTRODUCTION

Melatonin (Mel) is a hormone synthesized primarily by the pineal gland, as well as by retina, skin, gastrointestinal tract and immune cells including: lymphocytes, bone marrow and mast cells, affecting these cells through paracrine signaling [31, 36]. Its main role is to coordinate circadian and seasonal rhythms, especially sleep-wake cycles, moreover, melatonin participates in regulation of blood pressure, retinal and gastrointestinal functions and plays a role in immunoregulation and inflammatory responses [36]. The influence of melatonin on the immune system is indisputable but ambiguous, it depends on many factors (e.g., sex, age, time of day or season, dose of melatonin administration) [6]. Melatonin stimulates innate and specific immune responses and production of inflammatory mediators, both under physiological and immunosuppressive conditions [7, 16, 29]. Some authors describe also its anti-inflammatory properties, which are primarily a result of antioxidant and anti-apoptotic actions [23, 32, 33].

Melatonin is a powerful scavenger of reactive oxygen species (ROS), such as $OH^{\bullet}$, $O_2^{\bullet-}$, $NO^{\bullet}$. Its interaction with ROS leads to formation of melatonin metabolites such as cyclic 3-hydroxymelatonin (3-OHM), N^1-acetyl-N^2-formyl-methoxykynuramine (AFMK) and N^2-acetyl-5-methoxykynuramine (AMK), which also are potent scavengers and protect against ROS and oxidative stress [21]. In addition to this direct effect, melatonin acts as an indirect antioxidant through various complex mechanisms including induction of the activity of antioxidant enzymes, suppression of pro-oxidant enzymes and maintenance of mitochondrial homeostasis [36], although some recent experiments suggest that melatonin may have pro-oxidant effect in some cells [43, 44, 49]. Mechanism of this action is the ability to elicit the activation of some ROS-generating enzymes such as lipoxygenases, cyclooxygenases, NO-synthase, and NADPH-oxidase [2, 44].

There is growing evidence that ROS play an important role in the process of apoptosis. This is confirmed by the studies, in which mediators of apoptosis induce intracellular ROS production or apoptosis is inhibited by the addition of antioxidants or anti-oxidative drugs. In another system, ROS can activate anti-apoptotic pathway, resulting in increased cell survival [47].

Apoptosis, or programmed cell death, plays an important role in inflammatory processes and in resolution of inflammatory reactions. It occurs through the mitochondrial (intrinsic) and death-receptor (extrinsic) pathway. The first is initiated by intracellular stress and the second by the cell-surface receptor-specific ligand engagement [9]. Melatonin plays a role in inhibiting apoptosis in immune cells, on the other hand increases apoptotic cell death in cancer cells [12, 17, 42, 49]. Although the effect of melatonin on apoptosis is ambiguous, it seems that melatonin's ability to modulate apoptosis is one of its more important roles related to its effect on the immune system. The exact mechanisms of the apoptosis control by melatonin still remain unclear, however, it is believed that the effect is the result of modulation of the redox state of cells and the balance between pro- and anti-apoptotic bcl-2 family members [11, 13, 42].

The goal of our study was to examine the involvement of melatonin in apoptosis of human neutrophils *in vitro* and possible mechanisms of this action, including influence on caspase-3 activation, apoptosis-related surface antigen expressions, intracellular ROS generation and cytochrome c release. To induction of apoptosis, TNF-α, a pleiotropic cytokine and an important mediator in the immune response, was used. Due to the fact that it can both induce and inhibit apoptosis in a time- and concentration manner [45], based on our previous study the concentration and time of incubation needed for induction of apoptosis has been selected [data unpublished]. In some pathological conditions, where an increased plasma concentrations of inflammatory mediators, especially TNF-α is observed, the concentration of melatonin is reduced [38, 40]. To demonstrate the possibility role of melatonin in the treatment of immune diseases linked to increased apoptosis, the effect of melatonin on apoptosis of neutrophils at pharmacological doses was investigated.

MATERIALS AND METHODS

Reagents

Melatonin, HEPES solution 1M, fetal calf serum (FCS), penicillin/streptomycin solution, phorbol 12-myristate 13-acetate (PMA), dihydrorhodamine 123 (DHR), propidium iodide (PI), human recombinant TNF-α were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol and DMSO were acquired from POCh (Gliwice,

Poland). Phosphate-buffered saline (PBS; without Ca^{2+} and Mg^{2+}) and RPMI 1640 medium were obtained from Biomed (Lublin, Poland). Polymorphprep was bought from Alexis-Shield PoC AS (Oslo, Norway). Monoclonal anti-human CD-95 (Fas)/FITC and anti-human CD-178 (Fas-L)/FITC were purchased from Ancell Corporation (Bayport, MN, USA). Annexin V-FITC apoptosis detection kit I and FITC rabbit anti-active caspase-3 were acquired from BD Pharmingen (San Diego, CA, USA). Innocyte flow cytometric cytochrome c release kit was obtained from Calbiochem (San Diego, CA, USA). 24-well tissue culture plates were from Nunc (Roskilde, Denmark).

PMNL isolation

Venous blood was drawn from healthy male volunteers aged 29-46 years under informed consent according to the procedure approved by the Ethics Committee for Research Studies of the Medical University of Lodz (RNN 558/09/KB of 14.07.2009) and in accordance with the Declaration of Helsinki. Human polymorphonuclear leukocytes (PMNL) were isolated using Polymorphprep according to the manufacturer's instructions. After centrifuging (450-500 g) for 30 minutes, the lower PMNL-rich interphase was collected and washed with phosphate-buffered saline (PBS). Residual erythrocytes were lysed with a hypotonic NH_4Cl solution and then cells were washed twice with PBS. Cell viability was routinely determined by the trypan blue exclusion test.

Neutrophil culture

Isolated PMNL were suspended at a density of $1x10^6$/ml in RPMI 1640 containing 10% of heat-inactivated fetal calf serum (FCS), 1M HEPES, 100U of penicillin and 100 µg/ml of streptomycin. Melatonin was dissolved in absolute ethanol and diluted with PBS (10 mM stock solution) and then added to the medium at a concentration of 20 µg/ml.

Neutrophils were incubated in humidified atmosphere with 5% CO_2 at 37°C in the presence of 100 ng/ml TNF-α and 20 µg/ml melatonin. For the experiments, melatonin was added 3 hours before TNF-α treatment. Control-cells were incubated with RPMI medium only. The total time of incubation was 6 hours. PMNL were harvested from cultures by washing off the cells with cold PBS and then washed twice. After transferring the cells to FACS tubes, they were assayed using the procedures described below.

Measurement of neutrophil apoptosis by Annexin-V binding to cell surface

Apoptosis was measured using FITC Annexin V Apoptosis Detection Kit according to the manufacturer's specifications. PMNL harvested from cultures were suspended in 1X Binding Buffer at a concentration of $1x10^6$/ml. 100 µl of the solution ($1x10^5$/ml) was transferred to a FACS tube and 5µl Annexin-V-FITC and 5µl PI was added. After gently vortexing, cells were incubated for 15 minutes at room temperature in the dark. Then, 400 µl of 1X Binding Buffer was added to each tube and cells were immediately analyzed with a flow cytometer (FACScan, Becton Dickinson, San Diego, CA, USA). We evaluated the percentage of cells in early apoptosis (ANXV+/PI-) and in late apoptosis or necrotic cells (ANXV+/PI+). Additionally, the percent of all death cells (sum of ANXV+/PI- and ANXV+PI+) was analyzed.

Analysis of DNA content by PI staining

PMNL (10^5/200µl) were permeabilized using 1 ml of ice-cold 70% ethanol and stored at -20°C for one day. Then, neutrophils were washed twice with PBS and stained with 100 µl PI (from 50 µg/ml stock solution) in the dark at room temperature for 15 minutes. The fluorescence of individual nuclei was measured using a FACScan equipped with Cell Quest software for cell acquisition and data analysis. The percentage of apoptotic neutrophils was calculated as a hypodiploid DNA peak in the FL-2 fluorescence channel.

Quantification of apoptosis-related surface antigens and caspase-3 expression

The expression of CD95 (Fas) and CD178 (Fas-L) was assessed using commercial monoclonal antibodies. PMNL (10^5/100 µl) were incubated on ice with 80 µl of mouse anti-human CD95/FITC or anti-human CD178/FITC diluted 50-fold in PBS, respectively. After 45 minutes of incubation, cells were analyzed by FACS.

To measure the active form of caspase-3, FITC-labeled rabbit monoclonal antibodies were used. PMNL (10^6) were incubated with 1 ml of ice-cold 70% ethanol and stored at -20°C for one day. Then, cells were washed twice with PBS and incubated with 10 µl of monoclonal antibodies in the dark for 1 hour. Analysis was performed using a flow cytometer (FACScan). The mean fluorescence intensity (MFI) was used as a measurement of antigen and caspase-3 expressions.

Measurement of intracellular reactive oxygen species (ROS) levels

We tested the influence of melatonin and TNF-α, alone or in combination, on intracellular oxygen potential of neutrophils non-stimulated and stimulated with PMA. The generation of ROS was assessed using dihydrorhodamine-123 (DHR123). It is an uncharged and non-fluorescent indicator of ROS production that easily enters most of the cells. ROS induced oxidation transforms DHR 123 into rhodamine-123 (Rh-123), which exhibits green fluorescence and localizes to mitochondrial membranes.

In this method, the stock solution of dihydrorhodamine 123 was made by dissolving 1.0 mg DHR in 1.0 ml DMSO and stored at -70°C. For flow cytometry, 10 µl of

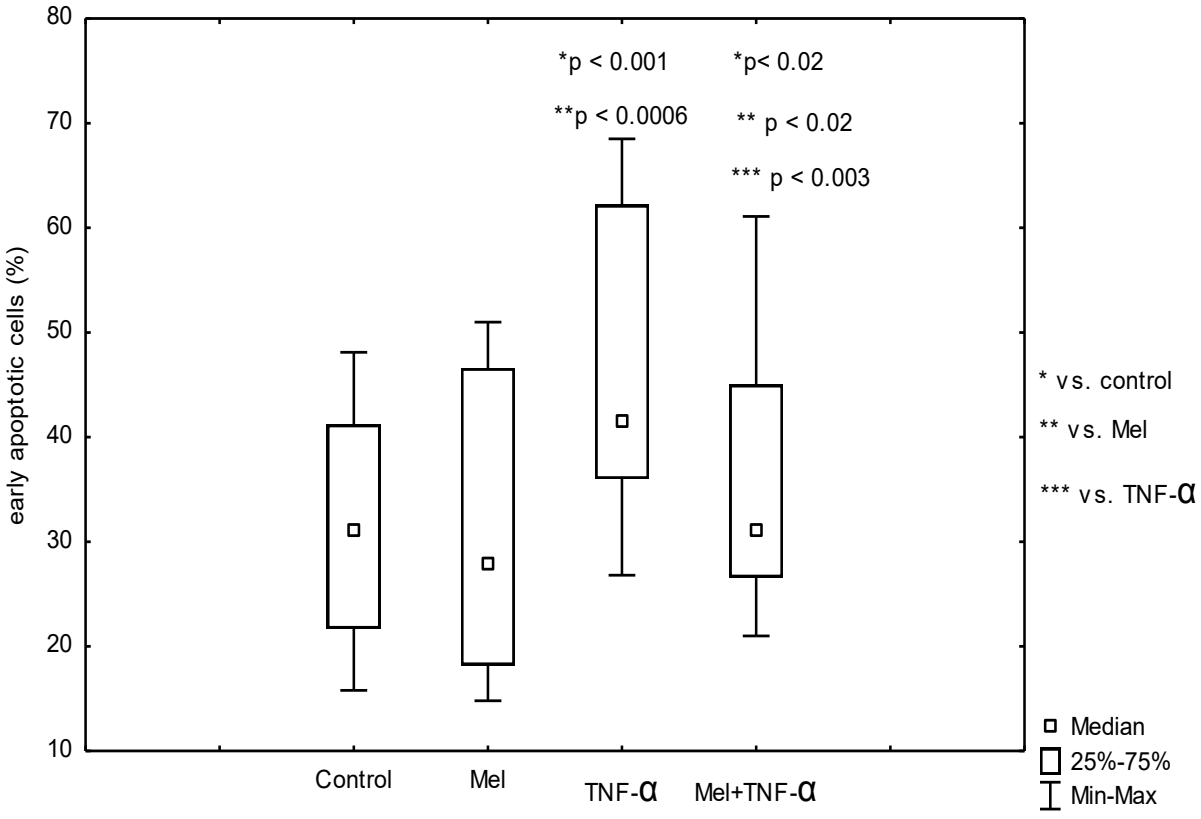

Fig. 1. The percentage of cells in early apoptosis in different experimental conditions. Neutrophils were incubated with Mel (20 µg/ml), TNF-α (100 ng/ml) or untreated (control) for 6 hours. Additionally, cells were pre-incubated with Mel for 3 hours and subsequently stimulated with TNF-α for additional 3 hours. Apoptosis was determined as described in "Materials and Methods". Data are expressed as the percentage of the ANXV+/PI- cells for 15 separate experiments

the stock was dissolved in 325 µl in PBS to give a working solution of 30 µg/ml. Phorbol myristate acetate (PMA) was dissolved in DMSO to form a 1.6 mM stock solution. Before flow cytometry, a 5 µM working solution was made by dilution with PBS.

Cells harvested from cultures (10^5/100 µl) were placed in a water bath (37^0C). Then, 12.5 µl of 5 µM PMA solution was added to the stimulated and 12.5 µl of PBS to the unstimulated cells. Following the 5- minute incubation, 12.5 µl of the working DHR123 solution was added to all tubes and they were incubated for additional 10 minutes at 37^0C. Afterwards, cells were put on ice for 5 minutes, to stop the reactions. Then neutrophils were washed once with ice cold PBS. The supernatant was discarded and the cells were resuspended in 1ml of 1% paraformaldehyde in PBS and analyzed by FACS, immediately. Fluorescence was measured on the FL-1 green channel.

Cytochrome c release by flow cytometry

The relocalization of cytochrome c from the mitochondria to the cytoplasm was detected using InnoCyte flow cytometric cytochrome c release kit according to the instructions. Cultured neutrophils ($1x10^6$)were incubated with permeabilization buffer for 10 minutes on ice and fixed with 1% paraformaldehyde for 20 minutes at room temperature. After washing, cells were blocked in blocking buffer for 1 h followed by incubation with anti-cytochrome c and anti-IgG FITC antibodies. Staining of the cells was performed at room temperature in the dark for 1h, every time. Finally, analysis was made within 1 hour by flow cytometry and the cytochrome c release was determined on the basis of MFI in FL-1 channel.

Statistical analysis

Data are expressed as median and interquartile range (Med [25%; 75%]) in the text and additionally minimum and maximum are shown in figures. To compare the different treatments of the same samples, statistical significance was calculated using Wilcoxon test. Values $p \leq 0.05$ were considered significant. As statistical software package, STATISTICA 12 (StatSoft, Poland) was used.

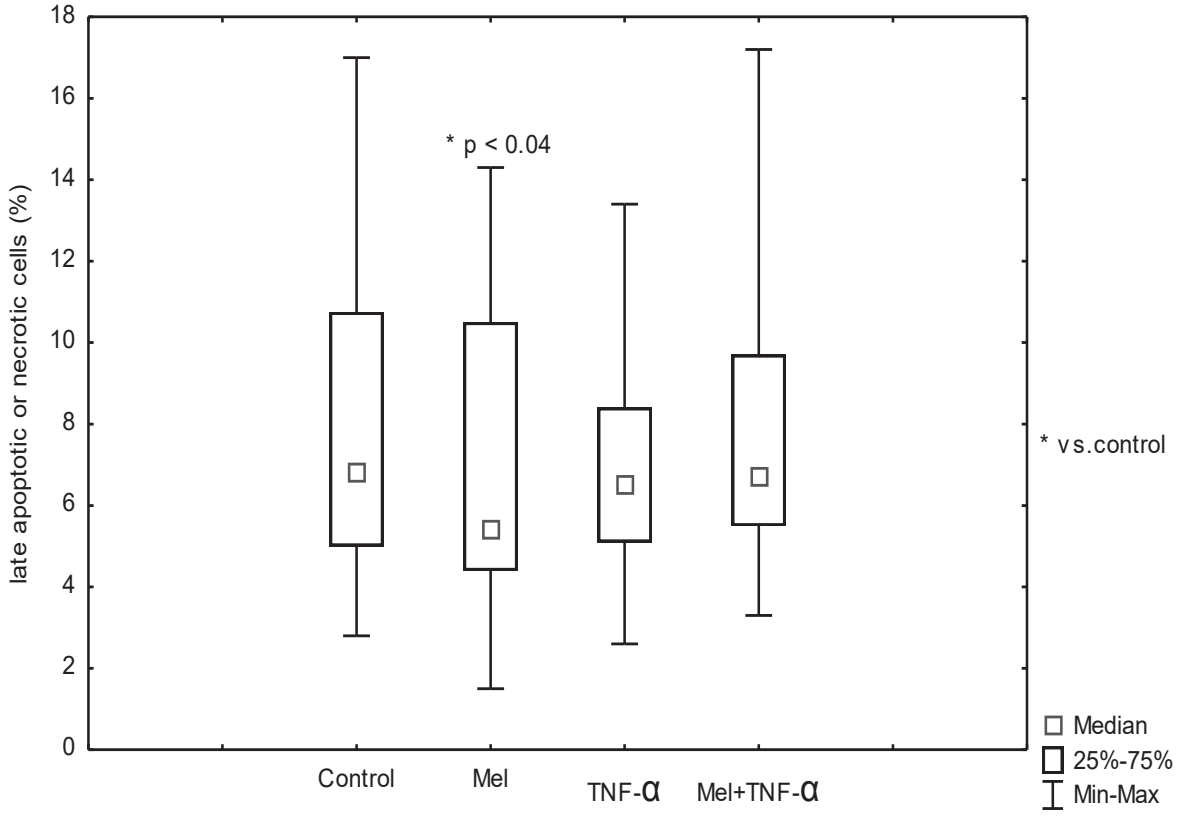

Fig. 2. The percentage of necrotic cells or cells in the late apoptosis in different experimental conditions. Neutrophils were incubated with Mel (20 μg/ml), TNF-α (100 ng/ml) or untreated (control) for 6 hours. Additionally, cells were pre-incubated with Mel for 3 hours and subsequently stimulated with TNF-α for additional 3 hours. Apoptosis was determined as described in "Materials and Methods". Data are expressed as the percentage of the ANXV+/PI+ cells for 15 separate experiments

RESULTS

Apoptosis of neutrophils was evaluated *in vitro* by measuring externalization of phosphatidylserine by Annexin-V-FITC binding and cellular DNA content by PI staining. Analysis of data obtained by flow cytometry was made using WinMDI 2.9.

The percentage of early apoptotic cells (ANXV+/PI-) after 6 h of culture treated with melatonin alone (27.8% [18.2%; 46.6%]) did not differ significantly as compared to the control group (31% [21.7%; 41.2%]). Neutrophils incubated in the presence of TNF-α (100 ng/ml) had a significantly higher percentage of ANXV+/PI- cells (41.6% [36%; 62.2%]) than controls ($p < 0.001$) and cells cultured with melatonin alone ($p<0.0006$). In the samples pre-treated with melatonin before addition of TNF-α, the percentage of early apoptotic cells was significantly reduced compared with TNF-α (31% versus 41.6%; $p < 0.003$) and also significantly increased in comparison with control and cells treated with melatonin alone ($p < 0.02$ in both cases) (Figure 1).

The percentage of late apoptotic or necrotic neutrophils (ANXV+/PI+ cells) was significantly lower in samples treated with melatonin alone in comparison with control (5.4% [4.4%; 10%] versus 6.8% [5%; 11%]; $p<0.04$). In cultures treated with TNF-α, separately or in combination with melatonin, the percentage of ANXV+/PI+ cells did not differ (Figure 2).

Total apoptotic neutrophils were determined as the sum of ANXV+/PI- and ANXV+/PI+ cells measured by ANXV-FITC and PI binding. Melatonin alone did not change the percentage of total apoptotic cells compared with control (38.9% versus 43.2%). TNF-α caused a significant increase in the neutrophil apoptosis in comparison with control (48.1% [42.7%; 71.8%] versus 43.2% [28.5%; 50.3%]; $p < 0.004$). In our experiment, melatonin pre-treatment significantly reduced rates of apoptosis (37.7% [32.7%; 52%]) in comparison with TNFα alone. Additionally, apoptosis in cultures treated with melatonin in combination with TNF-α was significantly lower than in cultures with melatonin alone ($p < 0.02$). Figure 3 shows the percentage of the sum of cells binding Annexin V-FITC (ANXV+/PI- and ANXV+/PI+) in all experimental conditions.

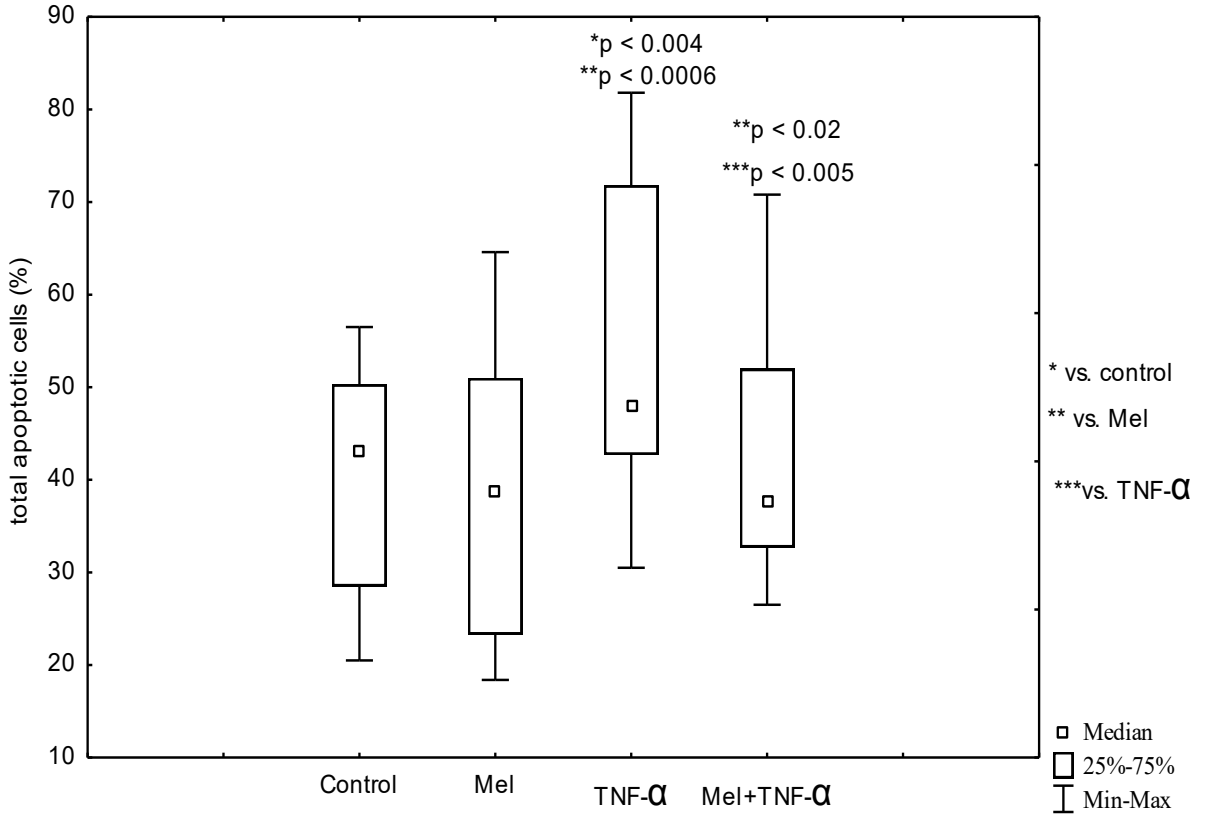

Fig. 3. Effect of melatonin on spontaneous and TNF-α – induced apoptosis of human neutrophils *in vitro* measured by ANX V-FITC and PI binding. Neutrophils were incubated with Mel (20 μg/ml), TNF-α (100 ng/ml) or untreated (control) for 6 hours. Additionally, cells were pre-incubated with Mel for 3 hours and subsequently stimulated with TNF-α for additional 3 hours. Apoptosis was determined as described in "Materials and Methods". Data are presented as the percentage of the sum of cells binding Annexin V-FITC (ANXV+/PI- and ANXV+/PI+) for 15 separate experiments

The evaluation of cellular DNA staining as the second marker of apoptosis, showed similar effects of melatonin on spontaneous and TNF-α–induced human neutrophil apoptosis (Figure 4). There was no significant difference between the percentage of apoptotic cells (as measured by the appearance of sub-diploid DNA peak) in melatonin treated and control samples (22.5% [9%; 28%] versus 16.4% [10%; 26%]) after 6 hours of culture. The percentage of cells with fractional DNA content was significantly increased in neutrophils treated with TNFα in

Table 1. Fas, Fas-L and caspase-3 expressions in all experimental conditions. Data are presented as the median and interquartile range – Me [25%; 75%] for 15 experiments

	Experimental conditions			
	Control	Mel	TNF-α	Mel + TNF-α
Fas [MFI]	58 [35; 66]	64 [44; 70]	62 [47; 72]	60 [54; 72]
Fas-L [MFI]	132 [97; 165]	138 [81; 205]	138 [105; 180]	136 [108; 196]
Active caspase-3 [MFI]	45 [23; 80]	42 [23; 96,5]	46 [16; 96,5]	48 [25; 75]

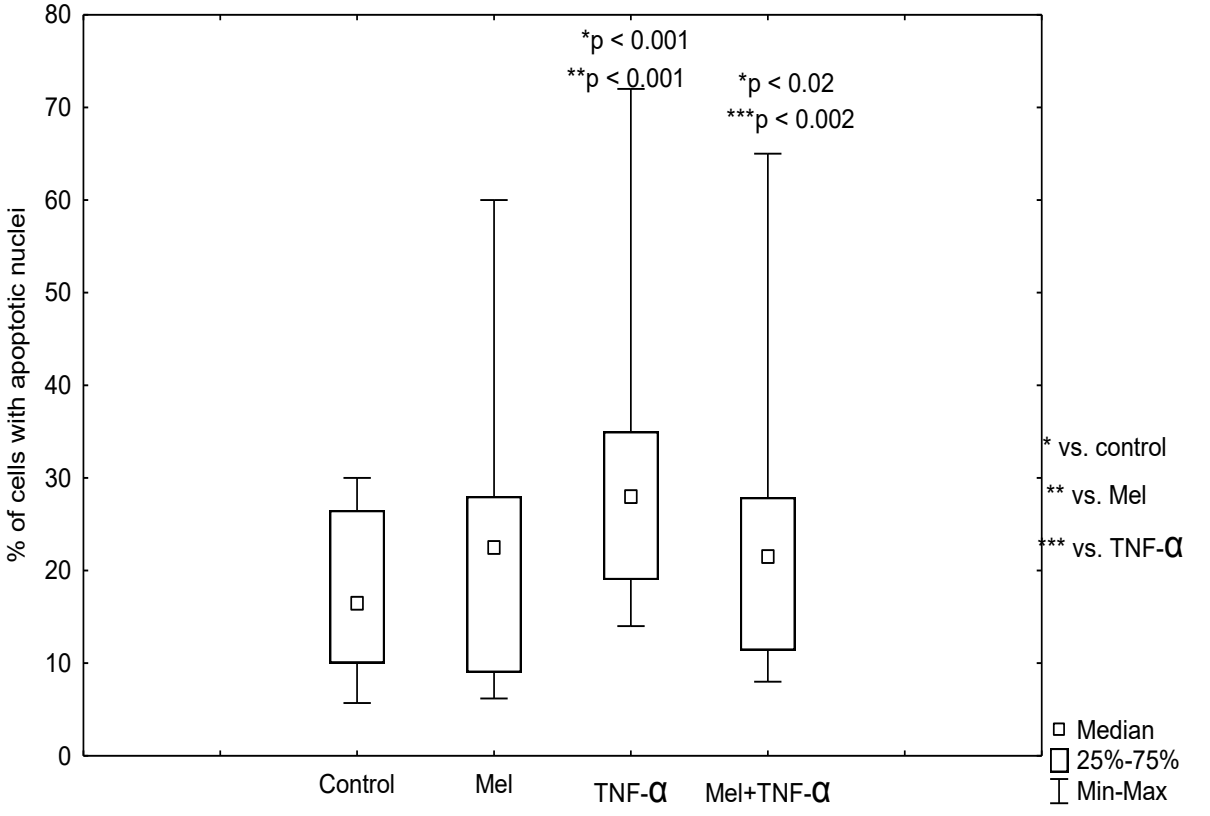

Fig. 4. Effect of melatonin on spontaneous and TNF-α–induced neutrophil apoptosis *in vitro* measured by DNA content. Neutrophils were incubated with Mel (20 μg/ml), TNF-α (100 ng/ml) or untreated (control) for 6 hours. Additionally, cells were pre-incubated with Mel for 3 hours and subsequently stimulated with TNF-α for additional 3 hours. Apoptosis was determined as described in "Materials and Methods". Data are expressed as the percentage of apoptotic neutrophils for 15 separate experiments

comparison with control (28% versus 16.4%; $p < 0.001$). Addition of melatonin to the culture before TNF-α stimulation decreased the number of apoptotic cells when compared with TNF-α–treated samples (21.5% versus 28%; $p < 0.002$) however, increased in comparison with control (21.5% versus 16.4%; $p < 0.02$).

We did not observe any changes in expressions of Fas, Fas-L or active caspase- 3 after melatonin or TNF-α treatment (alone or in combination) in comparison with control group. Exact results of our experiment are shown in Table 1.

The production of ROS (measured as MFI) was studied in resting conditions and after stimulation of the receptor independent pathway with PMA using DHR 123.

Baseline ROS generation was 73 MFI [63 MFI; 85 MFI] in control group and did not differ from other experimental conditions. There was no change in spontaneous ROS production between cells cultured in the presence of melatonin (116 MFI [64 MFI; 124 MFI]), TNF-α (78 MFI [64 MFI; 142 MFI]) or both reagents in combination (69 MFI [55 MFI; 115 MFI]).

Figure 5 presents the impact of melatonin, TNF-α, and both reagents on intracellular ROS generation in PMA--stimulated neutrophils. Under PMA stimulation, a significant increase in ROS generation was observed in comparison with spontaneous conditions for each treatment of cultured neutrophils ($p < 0.008$). TNF-α decreased ROS production in PMA-stimulated neutrophils (425 MFI [257 MFI; 514 MFI]) compared with control (632 MFI [390 MFI; 1200 MFI]), while pre-treatment of cells with melatonin significantly intensified this process (1055 MFI [750 MFI; 1810 MFI], $p < 0.008$ vs. TNF-α-stimulated cells). Melatonin alone had no impact on PMA-stimulated intracellular ROS generation.

To investigate mitochondrial relationship with spontaneous and TNF-α-induced apoptosis, an analysis of cytochrome c release was carried out. As indicated in Figure 6, neutrophils treated with TNF-α released a lot more cytochrome c from the mitochondria into cytosol as compared to control group (6.2% versus 3.4%; $p<0.01$). A significant decrease in the cytochrome c release in cultures treated with melatonin alone (4% [2%; 5%]) or in combination with TNF-α (2.9% [2.1%; 3.6%]) was observed in comparison with TNF-α condition (6.2% [4.8%; 7.9%]).

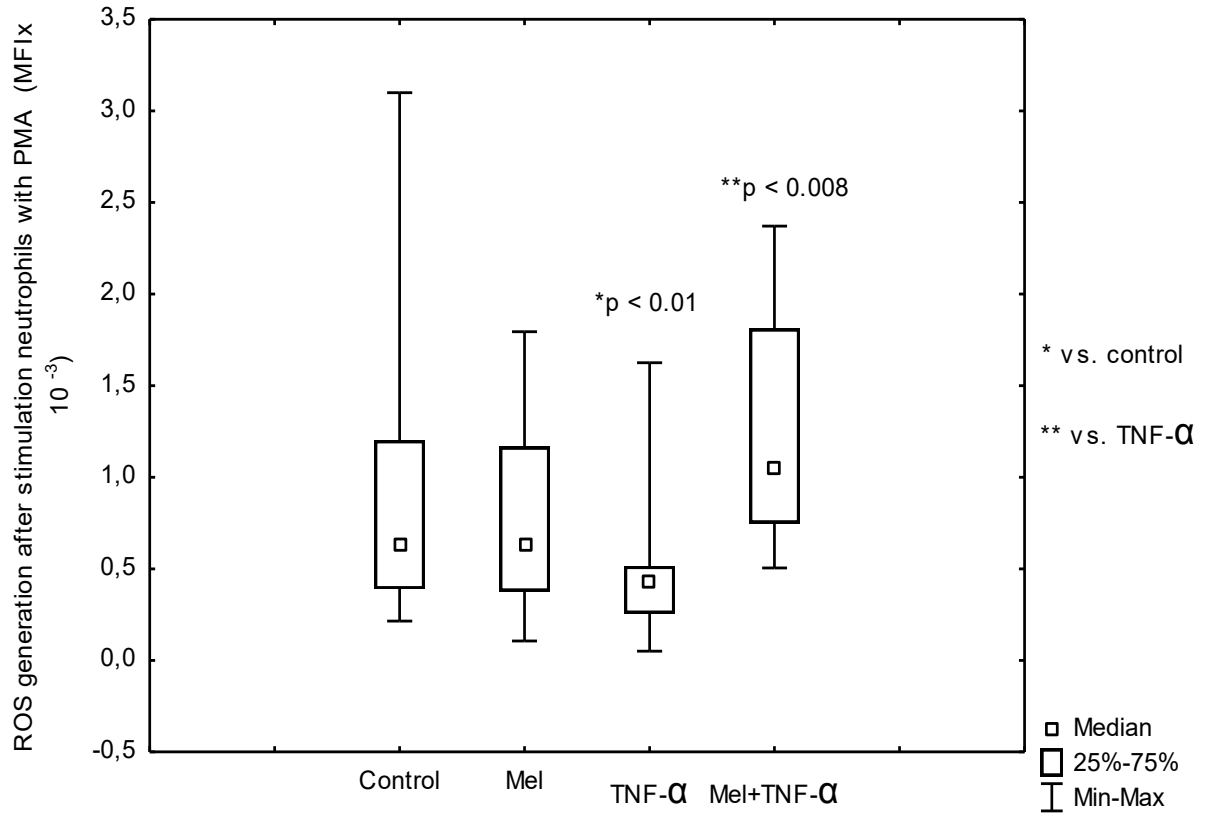

Fig. 5. Intracellular ROS generation in PMA-stimulated neutrophils in all experimental conditions. Neutrophils were incubated with Mel (20 μg/ml), TNF-α (100 ng/ml) or untreated (control) for 6 hours. Additionally, cells were pre-incubated with Mel for 3 hours and subsequently stimulated with TNF-α for additional 3 hours. ROS generation was determined as described in "Materials and Methods". Data are expressed as the mean fluorescence intensity (MFI) of DHR 123 for 10 experiments

DISCUSSION

According to many authors, melatonin (Mel) downregulates cell apoptosis in healthy cells by the intrinsic pathway, which is mediated by mitochondrial alterations [13, 42]. Based on the evidence that apoptosis induced by various factors (e.g., UVB, inflammatory cytokines) is dependent on intensified accumulation of ROS, it is believed that the anti-apoptotic action of melatonin results partially from its radical scavenging and antioxidant properties [11, 12, 14]. The idea is supported by experiments in which the oxidative conditions are the direct cause of apoptosis, Mel exerting its radical scavenging ability counteracts cell death [10, 24, 51]. Because some studies demonstrated that the extrinsic pathway of apoptosis may be linked to intracellular ROS generation [18], we addressed this issue and observed that neither Mel nor TNF-α (alone or in combination) affects baseline ROS generation in cultured neutrophils. The results different from those presented in our study were described by Espino et al., who demonstrated that TNF-α treatment stimulates ROS production in human neutrophils and pre-incubation with Mel decreases this process evoked by TNF-α [14], though the unlike effects might be a result of different time of cell culturing. ROS, both intra- and extracellular, can indirectly activate spontaneous and Fas-mediated apoptosis [46], but their effect on TNF-α-induced apoptosis is ambiguous. The vast majority of studies indicate that ROS play a crucial role in TNF-α-induced apoptosis, however several authors claim otherwise [18, 25, 26, 45]. The most common evidence of its role in TNF-α-induced apoptosis is based on the observation of the effect of antioxidants or antioxidant enzymes on TNF-α-induced apoptosis and the ROS generation in response to TNF-α is rarely examined. For instance, glutathione, a potent antioxidant, abrogates TNF-α-induced apoptosis, however, superoxide dismutase (SOD) does not inhibit this process [25, 27]. There are results in literature that TNF-α stimulation induces a rapid rise (within 1-2 hours) of intracellular ROS in neutrophils [14, 18]. Because the increase in ROS is detected in the early stages of TNF-α-induced apoptosis and is short-lived, therefore the longer time of incubation may be the cause of our result demonstrating no effect of TNF-α on ROS generation.

In our experiment, TNF-α decreased ROS generation in PMA-stimulated neutrophils compared with untreated cells. These results are in accordance with the report performed by Yamashita et al. The authors observed that

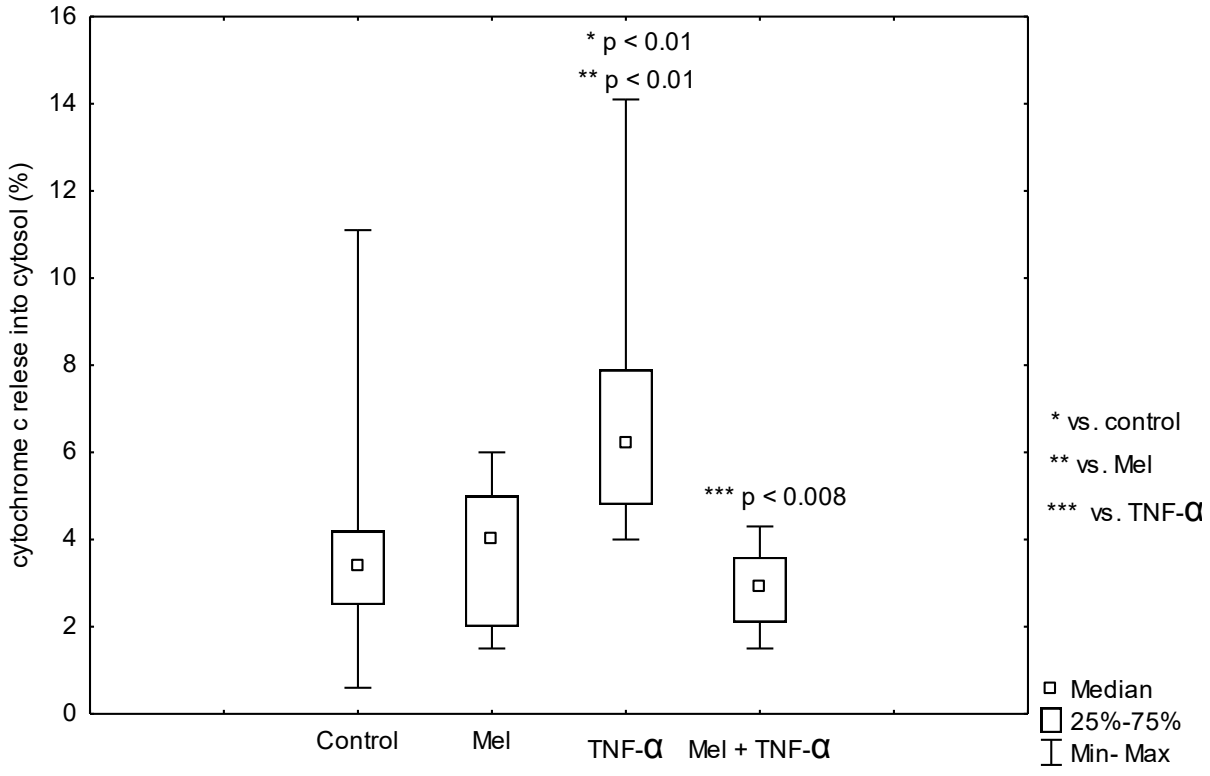

Fig. 6. Percentage of mitochondrial release of cytochrome c into cytosol in experimental conditions was detected using Innocyte cytochrome c release kit as described in "Materials and Methods". Neutrophils were incubated with Mel (20 μg/ml), TNF-α (100 ng/ml) or untreated (control) for 6 hours. Additionally, cells were pre-incubated with Mel for 3 hours and subsequently stimulated with TNF-α for additional 3 hours. Data are presented for 10 separate experiments

already after 2 hours, ROS generation was suppressed in neutrophils stimulated with TNF-α together with fMLP, OZ or PMA in parallel with the appearance of apoptotic changes. Additionally, after blockade of caspase no reduction in ROS levels was observed. Authors suggest that TNF-α introduces a priming signal for enhanced oxygen radical production that is usually masked by TNF-α-induced apoptotic processes [50]. Other authors have also shown that apoptotic neutrophils exhibit impaired ability to synthesize ROS [48]. We have indicated that pre-treatment of cells with melatonin before TNF-α addition normalizes ROS generation, despite the widely reported scavenging properties of Mel [11, 12].

Many experiments proved the leading role of caspase-3 in the extrinsic or intrinsic pathway of the activation of apoptotic process [41, 50]. Several studies have shown, that accelerated rate of neutrophil apoptosis induced by TNF-α results from activation of caspase-3 [8, 18,39,50].This evidence is in contrast to the results obtained in our experiment, in which no differences in expression of this enzyme in cells undergoing spontaneous and TNF-α stimulated apoptosis were found. Our previous study also did not reveal any change in the caspase-3 expression after neutrophil stimulation with TNF-α, although we demonstrated the presence of the active caspase-3 using fluorescence microscopy [30]. Neutrophil pre--treatment with melatonin does not affect caspase-3 expression, which is contrary to experiments, where the caspase-3 activity was downregulated by Mel in apoptosis induced by TNF-α or other factors [14, 42].

Among the all cell lines of the immune system, neutrophils are the fastest cells, which undergo spontaneous apoptosis as a result of high expression of Fas and Fas-L [1]. There is little information about the effect of Mel on this apoptosis-related surface antigen expression. No wonder that without the effect of the molecule expression Mel does not influence the spontaneous apoptosis of neutrophils. *In vivo* experiments have shown that anti-apoptotic effect of melatonin could involve the control of Fas, Fas-L and p53 pathways [37].

Based on our results, it appears that the promotion of TNF-α-induced apoptosis is not associated with the Fas/Fas-L system. Similar conclusions, despite a much shorter time of incubation with TNF-α, were reached by Salamone et al., who demonstrated that blocking antibodies directed to Fas and Fas-L did not impair enhanced apoptosis induced by TNF-α [45].

It is widely accepted that the changes in mitochondrial function are involved in apoptosis induction and the release of cytochrome c is a central event in apoptotic signaling. Cytochrome c and other molecules released from mitochondria can activate downstream apoptosis signals via activation of caspase-3 [28]. In our study, we have examined the involvement of mitochondria in spontaneous and TNF-α-induced apoptosis and the role of melatonin in mitochondrial dysfunctions. We demonstrated that TNF-α induced cytochrome c release into the cytoplasm and that the melatonin treatment before the TNF-α stimulation effectively reduced this process. Treatment of cells with TNF-α alters mitochondrial membrane permeability, induces mitochondrial swelling and clustering, what leads to cytochrome c release and activation of caspase cascade [3]. Some authors, demonstrating no changes in mitochondrial potential or no release of cytochrome c after TNF-α stimulation, suggest that TNF-α-induced neutrophil apoptosis is independent of the mitochondrial death pathway [18]. It is an interesting fact that despite no changes in the active caspase-3 expression observed in our experiments, the release of cytochrome c after TNF-α stimulation was detected. Some authors showed that cytochrome c release was observed before the caspase-3 activation in TNF-α-induced apoptosis, suggesting that cytochrome c release can be important prior to or at the same time with the caspase-3 activation, which has a leading role in the apoptotic process [4, 34].

Relatively recent literature data has raised the possibility that TNF-α might trigger apoptosis through pathway involving lysosomes with the participation of cathepsins [15, 19, 22].This enzymes can directly induce cytochrome c release from mitochondria and subsequent activation of caspase -9 and -3 [20, 22, 52].In some studies have been shown that melatonin prevents lysosomal enzyme disruption and downregulates the activities of cathepsins [5, 35]. Based on the literature data mentioned above, it seems likely that hampering effects of melatonin on TNF-α-induced apoptosis of neutrophils can result from its inhibitory influence on cathepsin activity.

CONCLUSIONS

The results of our study indicate that melatonin does not affect the spontaneous neutrophil apoptosis but protects TNF-α-induced apoptosis of human neutrophils. Our findings suggest that the intrinsic pathway of the process is a result of the melatonin induced mitochondrial alterations. Further analysis of the molecular target is a goal of ongoing and future research.

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