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Melatonin Reverses Bupivacaine-Induced Cardiotoxicity in Human Cardiomyocytes by Attenuating TRPV1-Mediated Apoptosis, ROS Production, and Mitochondrial Depolarization

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ABSTRACT

Bupivacaine, a widely used local anesthetic, induces cardiotoxicity through mitochondrial dysfunction, oxidative stress, and apoptosis. However, the role of TRPV1 channels in this process and the potential protective effect of melatonin remain elucidated. We hypothesised that melatonin mitigates bupivacaine-induced cardiotoxicity by attenuating TRPV1-mediated apoptosis, ROS production, and mitochondrial depolarization in human cardiomyocytes.

Human cardiomyocytes were divided into the following experimental groups: control, bupivacaine (1 mM, 24 h), bupivacaine+melatonin (50 µM), bupivacaine +melatonin+capsazepine (a TRPV1 antagonist, 0.1 mM), bupivacaine+capsazepine, and capsaicin (a TRPV1 agonist) groups. Apoptosis was quantified using the APOPercentage assay, while intracellular ROS levels and mitochondrial membrane potential were assessed using Dihydrorhodamine 123 and JC-1 staining, respectively.

Bupivacaine administration significantly increased apoptosis, ROS levels, and mitochondrial depolarization compared to control group ($p<0.001$). Co-treatment with melatonin markedly attenuated all three parameters ($p<0.001$ vs bupivacaine). The TRPV1 antagonist capsazepine further enhanced the protective effect, whereas the TRPV1 agonist capsaicin exacerbated bupivacaine-induced injury ($p<0.001$).

Our findings demonstrate that bupivacaine activates TRPV1 channels, leading to calcium overload, ROS burst, mitochondrial depolarization, and apoptosis. Melatonin mitigates these effects by modulating TRPV1-dependent pathways, which is consistent with previous reports on digoxin-induced cardiotoxicity and melatonin's antioxidant properties. Melatonin effectively protects human cardiomyocytes against bupivacaine-induced cardiotoxicity by attenuating TRPV1-mediated apoptosis, ROS production, and mitochondrial depolarization. This novel mechanism suggests that melatonin may serve as a potential adjunctive therapy to prevent local anesthetic-induced cardiac toxicity. Further in vivo studies are warranted to confirm these findings.

Keywords: Bupivacaine, Melatonin, Apoptosis, Cardiotoxicity, Cardiomyocyte

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INTRODUCTION

Bupivacaine is a widely used, potent, long-acting amide local anaesthetic that provides effective perioperative and regional anaesthesia. However, its clinical utility is limited by a well-documented potential for systemic toxicity, particularly cardiotoxicity, which may manifest as severe arrhythmias, contractile dysfunction, and even cardiac arrest, especially after accidental intravascular injection or with high plasma concentrations [1]. The mechanisms underlying bupivacaine-induced cardiotoxicity are multifactorial and extend beyond the classic blockade of voltage-gated sodium channels. Emerging evidence indicates that bupivacaine also inhibits voltage-gated potassium and calcium channels, disrupts mitochondrial energy metabolism, and alters intracellular calcium homeostasis, thereby exacerbating its deleterious effects on cardiomyocytes [2,3]. In particular, bupivacaine has been shown to induce mitochondrial dysfunction, as evidenced by reduced oxygen consumption, increased mitochondrial swelling, and decreased mitochondrial membrane potential in cardiomyocytes [4].

Reactive oxygen species (ROS) overproduction and subsequent oxidative stress are pivotal in the pathogenesis of local anaesthetic-induced cardiac injury. Bupivacaine treatment has been consistently associated with increased ROS levels, lipid peroxidation, and compromised antioxidant defences, leading to cellular damage and apoptosis. Apoptosis in this context is often triggered through the mitochondrial pathway, involving the loss of mitochondrial membrane potential, the release of pro-apoptotic factors, and the activation of caspases. Therefore, targeting mitochondrial integrity and oxidative stress represents a promising strategy to mitigate bupivacaine-induced cardiotoxicity [5].

Melatonin, an endogenous indoleamine produced by the pineal gland, possesses potent antioxidant, anti-inflammatory, and anti-apoptotic properties. It has been shown to protect against myocardial ischemia-reperfusion injury, doxorubicin-induced cardiotoxicity, and other forms of cardiac damage by scavenging free radicals, enhancing endogenous antioxidant enzymes, and preserving mitochondrial function. Importantly, recent studies have demonstrated that melatonin can exert cardioprotective effects by modulating TRPV1 channels. For instance, in a model of digoxin-induced cardiac damage, melatonin reduced oxidative stress, calcium influx, and apoptosis by inhibiting TRPV1 channel overactivation, thereby improving cell viability [6].

Despite these insights, the specific role of TRPV1 channels in bupivacaine-induced cardiotoxicity and the potential protective effect of melatonin via TRPV1 modulation remain largely unexplored. We hypothesise that bupivacaine-induced cardiotoxicity involves TRPV1-mediated ROS production, mitochondrial depolarisation, and apoptosis in human cardiomyocytes, and that melatonin may reverse these toxic effects by regulating TRPV1 channels. To test this hypothesis, the present study will investigate the effects of bupivacaine on apoptosis, ROS levels, and mitochondrial membrane potential in human cardiomyocytes and evaluate whether melatonin can counteract these changes, with a focus on the involvement of TRPV1 channels.

METHODS

Reagents/Stains

Human Cardiomyocyte Complete Media with Serum, Human Cardiomyocyte Serum Free Media (Celprogen, Torrance, CA, USA), Trypsin–EDTA, dimethyl sulfoxide, Dihydrorhodamine-123 (DHR 123) were procured from Sigma Aldrich (St. Louis, MO). Caspase 3 (AC-DEVD-AMC) and 9 (AC-LEHD-AMC) substrates were procured from Enzo (Lausanne, Switzerland). APO percentage assay with a release buffer was procured from Biocolor (Belfast, Northern Ireland). Fura 2 (AM) florescent calcium stain was procured from Calbiochem (Darmstadt, Germany), Pluronic® F-127 was procured from Biovision (San Francisco, USA). Probenecid, and a mitochondrial stain 5.50, 6.60-tetrachloro-1.10.3.30-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was procured from Santa Cruz (Dallas, Texas, USA).

Cell Culture

Human cardiomyocyte cells were obtained from Celprogen Inc. (Cat. Number: 36044-15; Celprogen, Torrance, CA, USA). Cells were cultured in Human Cardiomyocyte Complete Media with Serum (Commercial form includes serum and antibiotics). Cultures were maintained in T25 flasks (Human Cardiomyocyte Primary Cell Extracellular Matrix, Cat. Number E36044-15-T25, Celprojen, Torrance, CA, USA) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. At 75–85% confluence, cells were treated with the compounds described in the experimental groups section. Following treatment, cells were washed, detached using 0.25% trypsin–EDTA, collected into 15-mL tubes, and centrifuged at 100 × g for 5 min. After washing with fresh medium, cells were prepared for subsequent experiments.

Study Groups

HCCs (Human Cardiomyocyte Cells) were cultured at 37°C. The cells were divided into five main groups.

Group 1 (Control): The HCCs were not incubated with Bupivacaine, melatonin and capsazepine (TRPV1 channels antagonist, Cpz) but were kept in a flask containing the same cell culture medium and conditions.

Group 2 (Bupi+Mel): The HCCs in the group were incubated with 1 mM Bupivacaine and 50 µM melatonin for 24 hours. [7,8].

Group 3 (Bupi+Mel+Cpz): The HCCs in the group were incubated with 1 mM Bupivacaine and 50 µM melatonin for 24 hours. Then, it was incubated with capsazepin (TRPV1 channels antagonist, Cpz, 0.1 mM, 30 min).

Group 4 (Bupi): The HCCs in the group were incubated with 1 mM Bupivacaine for 24 hours. [7].

Group 5 (Bupi+Cpz): The HCCs in the group were incubated with 1 mM Bupivacaine for 24 hours and then incubated with capsazepin (TRPV1 channels antagonist, Cpz, 0.1 mM, 30 min).

For apoptosis, intracellular reactive oxygen species, and mitochondrial depolarization, the HCCs in all groups were further treated with the TRPV1 channel agonist Capsaicin (Cap, 0.1 mM, 10 min) to activate the TRPV1 channel prior to the related analyses.

Apoptosis and Intracellular ROS Production

Apoptosis was assessed using the APOPercentage™ assay (Biocolor Ltd., Belfast, Northern Ireland) according to the manufacturer's instructions. This dye-uptake assay selectively stains apoptotic cells due to phosphatidylserine externalization. Following treatment, apoptotic HeLa and human cardiomyocyte cells were quantified spectrophotometrically at 550 nm using a microplate reader (Synergy™ H1, Biotek, USA) as described previously. Intracellular ROS production was evaluated using Rhodamine 123 (Rh123). Fluorescence intensities were measured at excitation/emission wavelengths of 488/543 nm using the same microplate reader [9,10].

Mitochondrial Membrane Potential (JC-1) Analysis

Mitochondrial membrane potential (ΔΨm) was assessed using the JC-1 dye (1 µM). HeLa and cardiomyocyte cells were incubated with JC-1 at 37 °C for 45 min. Green fluorescence was measured at 485/535 nm and red fluorescence at 540/590 nm using a fluorescence spectrophotometer (Synergy™ H1, Biotek, USA). Mitochondrial depolarization was expressed as the red/green fluorescence ratio (590/535) and quantified relative to control values [10,11].

Statistical Analyses

All results were expressed as means±standard deviation (SD). Significant values in the groups were assessed with one-way ANOVA. Statistical Analyses were calculated using GraphPad Prism version 7.04 for Windows (GraphPad Software, San Diego, California, USA). P <0.05 was considered significant.

RESULTS

Effects of Bupivacaine and Melatonin on Apoptosis, ROS and Mitochondrial Depolarization Levels in Human Cardiomyocyte Cells (HCCs)

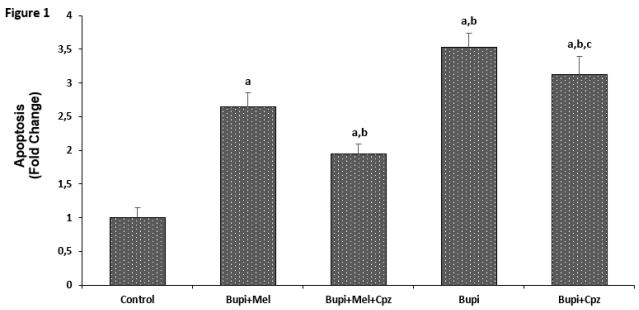

Figure 1. The effect of Bupivacaine (Bupi, 1 mM, 24 hours) and Melatonin (Mel, 50 µM, 24 hours) on apoptosis levels in the cardiomyocyte cells. Cells are stimulated by Capsaicin (Cap 0.1 mM, 10 min.), but they were inhibited by Capsazepine (Cpz 0.1 mM for 30 min) (mean ± SD and n=10). [a]p<0.001 vs control, [b]p<0.001 vs Bupi+Mel, [c]p<0.001 vs Bupi.

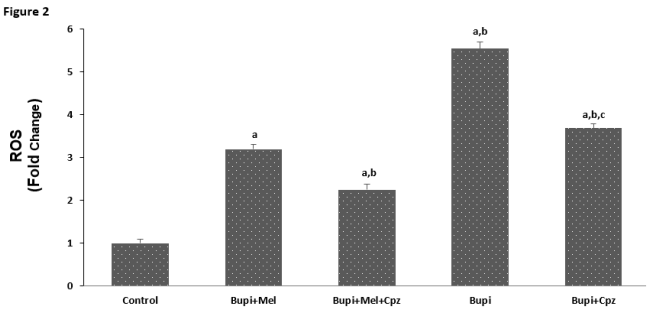

Figure 2. The effect of Bupivacaine (Bupi, 1 mM, 24 hours) and Melatonin (Mel, 50 µM, 24 hours) on reactive oxygen species levels in the cardiomyocyte cells. Cells are stimulated by Capsaicin (Cap 0.1 mM, 10 min.), but they were inhibited by Capsazepine (Cpz 0.1 mM for 30 min) (mean ± SD and n=10). [a]p<0.001 vs control, [b]p<0.001 vs Bupi+Mel, [c]p<0.001 vs Bupi.

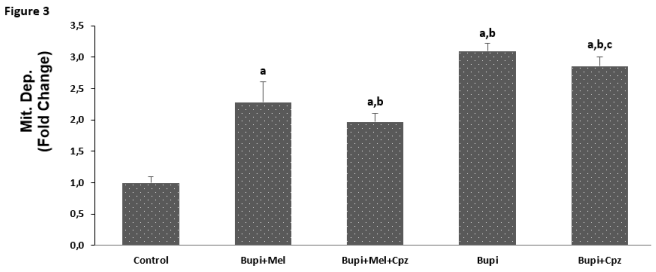

Figure 3. *The effect of Bupivacaine (Bupi, 1 mM, 24 hours) and Melatonin (Mel, 50 µM, 24 hours) on mitochondrial depolariation levels in the cardiomyocyte cells. Cells are stimulated by Capsaicin (Cap 0.1 mM, 10 min.), but they were inhibited by Capsazepine (Cpz 0.1 mM for 30 min) (mean ± SD and n=10).* $^{a}p<0.001$ *vs control,* $^{b}p<0.001$ *vs Bupi+Mel,* $^{c}p<0.001$ *vs Bupi.*

The effect of bupivacaine and melatonin on apoptosis (Fig. 1), ROS (Fig. 2), and mitochondrial depolarization (Fig. 3) levels in Human Cardiomyocyte Cells (HCCs). The TRP Vanilloid 1 channel stimulator capsaicin and blocker capsazepin were used to evaluate apoptosis, ROS and mitochondrial depolarization levels through TRPV1 channels in the human cardiomyocyte cells. As shown in Figures 1, 2, and 3, the apoptosis, ROS and mitochondrial depolarization levels in HCCs were greater in the bupivacaine and bupivacaine+melatonin groups in comparison to the control group ($p<0.001$). The apoptosis, ROS and mitochondrial depolarization levels were lower in the bupivacaine+melatonin groups in comparison to the bupivacaine group ($p<0.001$).

When bupivacaine+melatonin was compared with bupivacaine+melatonin+capsazepine and bupivacaine was compared with bupivacaine+capsazepine groups the groups using capsazepine channel inhibitor had lower cytosolic calcium levels than the groups not using capsazepine in apoptosis, ROS and mitochondrial depolarization levels ($p<0.001$).

DISCUSSION

In the present study, we have demonstrated for the first time that melatonin effectively reverses bupivacaine-induced cardiotoxicity in human cardiomyocytes by attenuating TRPV1-mediated apoptosis, ROS overproduction, and mitochondrial depolarisation. Our results show that exposure to bupivacaine significantly increased apoptosis, intracellular ROS levels and loss of mitochondrial membrane potential in HCCs. These detrimental effects were markedly reduced by co-treatment with melatonin. Importantly, the protective action of melatonin was further enhanced by the TRPV1 antagonist capsazepine, whereas the TRPV1 agonist capsaicin aggravated the bupivacaine-induced injury. These findings indicate that TRPV1 channels play a key role in bupivacaine cardiotoxicity and that melatonin exerts its cardioprotective effects, at least in part, through modulation of TRPV1-dependent pathways.

The cardiotoxicity of bupivacaine has long been attributed to its blockade of voltage-gated sodium channels. Still, growing evidence points to a more complex, multifactorial pathogenesis involving mitochondrial dysfunction and oxidative stress [1]. Our results are consistent with those of Hiller et al., who demonstrated that bupivacaine accumulates in the myocardium and causes reversible mitochondrial swelling, reduced oxygen consumption, and downregulation of energy metabolism [1]. In line with these observations, our results show a profound increase in mitochondrial depolarisation and ROS generation in bupivacaine-treated HCCs. The close association between mitochondrial membrane potential loss and the activation of the intrinsic apoptotic pathway has been well established; indeed, dissipation of mitochondrial membrane potential leads to the release of pro-apoptotic factors and the activation of caspase-9 and caspase-3. Our study further supports this by showing that bupivacaine-induced apoptosis is significantly attenuated by melatonin.

The involvement of intracellular calcium increase in bupivacaine-induced apoptosis was first reported by Kim et all. [2]. It has been reported that bupivacaine induced cell death in a dose-responsive and time-dependent manner in primary cultured rat cardiomyocytes, with apoptotic characteristics such as DNA fragmentation, chromatin condensation, decreased precursor caspase-3 protein level, increased cleaved Poly-ADP-ribose polymerase (PARP), and cytochrome C release into the cytoplasm [3]. Our findings extend these observations by showing that TRPV1 channels mediate at least part of the calcium influx triggered by bupivacaine, as capsazepine significantly reduced the bupivacaine-induced apoptotic response. More recent work by Plakhotnik et al. [5] has further elucidated that bupivacaine differentially perturbs cardiomyocyte calcium dynamics and that calcium may mitigate bupivacaine cardiotoxicity, suggesting that the risk of bupivacaine cardiotoxicity may be higher than that of ropivacaine in a calcium-deficient context [5]. Our results are in full agreement with this concept, as we observed that bupivacaine-induced TRPV1-mediated calcium overload (as evidenced by capsaicin-induced aggravation of injury) is a critical determinant of toxicity.

Several studies support our finding that bupivacaine significantly increases ROS production and mitochondrial depolarisation. It has been reported that bupivacaine depresses mitochondrial metabolism by inhibiting the respiratory chain

and, in turn, enhances ROS production [6]. Moreover, bupivacaine uncouples mitochondrial oxidative phosphorylation, inhibits respiratory chain complexes I and III, and enhances ROS production [7]. The ATP synthesis-synchronised ROS, especially the abnormally increased ROS production induced by bupivacaine, can lead to oxidative damage to mitochondrial proteins, membranes, and DNA, impairing the mitochondria's ability to synthesise ATP [8,22]. Our results corroborate these findings, as bupivacaine treatment led to a marked increase in ROS levels and loss of mitochondrial membrane potential, both of which were reversed by melatonin. Notably, the TRPV1 antagonist capsazepine also reduced these parameters, indicating that TRPV1 activation is upstream of the mitochondrial dysfunction.

The role of TRPV1 channels in cardiomyocyte injury has remained controversial. While TRPV1 activation on sensory neurons triggers the release of cardioprotective neuropeptides such as calcitonin gene-related peptide (CGRP), direct activation of TRPV1 channels on cardiac myocytes appears to be detrimental [9]. Sun et al. showed that TRPV1 activation by capsaicin exacerbated hypoxia/reoxygenation-induced apoptosis in H9C2 cells through calcium overload and mitochondrial dysfunction, and that this effect was blocked by capsazepine or TRPV1 siRNA [10]. Our study extends these observations to bupivacaine toxicity: we found that bupivacaine alone significantly increased TRPV1-mediated apoptosis, ROS and mitochondrial depolarisation, and that the TRPV1 agonist capsaicin further aggravated these parameters. Conversely, pharmacological inhibition of TRPV1 with capsazepine not only reduced the bupivacaine-induced damage but also potentiated the protective effect of melatonin. These data strongly support the concept that overactivation of myocardial TRPV1 channels is a critical mediator of bupivacaine cardiotoxicity.

The expression and functionality of TRPV1 channels in adult mouse cardiomyocytes have been demonstrated, and studies have shown that TRPV1 activation can have both protective and detrimental effects depending on the context [11]. In the context of ischemic conditioning, TRPV1 activation has been linked to cardioprotection by increasing the release of calcitonin gene-related peptide and substance P [12]. However, in the setting of direct cardiotoxic insult, such as bupivacaine exposure, TRPV1 overactivation appears to exacerbate injury, a concept that aligns with the “double-edged sword” nature of TRPV1 in the cardiovascular system [13].

Melatonin is a multitargeting molecule with potent antioxidant and anti-apoptotic properties. It scavenges both reactive oxygen and reactive nitrogen species, stimulates the activity of endogenous antioxidant enzymes, and chelates transition metals, thereby reducing the formation of the highly toxic hydroxyl radical. Moreover, melatonin accumulates in mitochondria, where it protects the electron transport chain from oxidative damage and maintains mitochondrial membrane potential [14, 15, 23]. Our results are in full agreement with these known actions: melatonin treatment significantly lowered ROS levels, restored mitochondrial membrane potential and reduced the percentage of apoptotic HCCs after bupivacaine exposure. Importantly, the degree of protection afforded by melatonin was comparable to, and additive with, TRPV1 antagonism. This suggests that melatonin may act not only as a direct free-radical scavenger but also as a negative modulator of TRPV1 channel activity.

Previous studies by Ovey and Oncel, on digoxin-induced cardiac damage, demonstrated that TRPV1 channels are overactivated during digoxin toxicity and that melatonin exerts cardioprotection by modulating TRPV1 channels [16, 17]. In that study, apoptosis values were significantly lower in the melatonin and digoxin+melatonin groups than in the digoxin group of cardiomyocytes, and cell viability values were higher in the digoxin+capsazepine, digoxin+melatonin, and digoxin+melatonin+capsazepine groups than in the digoxin group [18]. Our findings provide the first evidence that the same mechanism operates in bupivacaine-induced cardiotoxicity, thereby extending the applicability of melatonin-TRPV1 modulation to a different cardiotoxic agent.

The relationship between melatonin and TRPV1 has also been explored in other cell types. In a study on doxorubicin-induced toxicity in breast cancer cells, melatonin supported the effects of doxorubicin by activation of TRPV1 and apoptosis, as well as by inducing cell death [19]. However, it should be noted that the context is different: in cancer cells, increased apoptosis is desired, whereas in cardiomyocytes, inhibition of excessive TRPV1-mediated apoptosis is protective. This dichotomy highlights the importance of context-specific modulation of TRPV1.

In a recent study on cisplatin-induced cervical cancer cells, the combined application of cisplatin and melatonin led to a significant increase in apoptosis, with the effect mediated through TRPV1 channels: melatonin elevated cytosolic Ca^{2+} levels, promoted apoptosis, and increased intracellular ROS levels through TRPV1 [20, 21]. These findings further support TRPV1's role as a key mediator of melatonin's effects. However, the direction of modulation depends on the cellular context and the nature of the insult.

CONCLUSIONS

In summary, our study demonstrates that bupivacaine induces cardiotoxicity in human cardiomyocytes by activating TRPV1 channels, which, in turn, promote ROS overproduction, mitochondrial depolarisation, and apoptotic cell death. Melatonin effectively reverses these toxic effects by attenuating TRPV1-mediated pathways, as evidenced by enhanced protection with combined melatonin and capsazepine treatment and by aggravation of injury with the TRPV1 agonist capsaicin. These findings provide a novel mechanistic insight into bupivacaine-induced cardiac injury and highlight the therapeutic potential of melatonin as a cardioprotective agent in the setting of local anaesthetic toxicity. Further in vivo studies and clinical trials are warranted to evaluate the translational value of this strategy.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

Disclosure

The authors have reported no conflicts of interest in preparing and publishing this article.

Ethics committee approval

Ethics committee approval is not required in this study. The study was conducted following the international declaration, guidelines, etc.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Referee Evaluation Process

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