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Original article

The influence of melatonin on the heart rhythm – An *in vitro* simulation with murine embryonic stem cell derived cardiomyocytes

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ABSTRACT

Background: In healthy individuals, a major factor influencing the heart rate variability (HRV) is the circadian rhythm. The role of melatonin as an essential component of the circadian rhythm in the adult human organism and the beneficial effects of a treatment with melatonin during the fetal period is well described. Toxic effects of melatonin are discussed less frequently.
Since pharmacological studies cannot be carried out on pregnant women, the establishment of an equivalent *in vitro* model is important. We therefore tested whether melatonin can influence the beat rate variability (BRV) of spontaneously beating cardiomyocytes derived from murine embryonic stem cells (mESCs) and whether melatonin exhibits toxic effects in this *in vitro* model.
Methods: Microelectrode Arrays recorded extracellular field potentials of spontaneously beating cardiomyocytes. Melatonin was applied in a concentration range from 10^{-11} M to 10^{-5} M. The analysis of the BRV focused on time domain methods.
Results: In line with clinical observations, melatonin decreased the beating frequency and increased the BRV. The effect of melatonin up to a concentration of 10^{-6} M was reversible, whereas the application of higher concentrations induced an irreversible effect.
Conclusion: The study underlines the potential of this *in vitro* model to help explore the development of circadian rhythms and their modulation by melatonin in the embryonic phase. The results imply that melatonin influences the heart rhythm as early as during the embryonic heart development. Furthermore, the results indicate a potentially toxic effect of melatonin that has not been described in detail before.

1. Introduction

In recent decades, various *in vitro* models based on embryonic stem cell (ESC) derived tissue have contributed to a better understanding of physiological processes in humans and other mammals. In particular, these *in vitro* models can help to advance our knowledge about physiological processes during the embryonic developmental stages.

In previous studies we have focused on the beat rate variability (BRV) of spontaneously beating cardiomyocytes derived from murine and human embryonic stem cells (mESCs, hESCs) as well as human induced pluripotent stem cells (hiPSCs) [1,2]. The BRV can be considered as the *in vitro* equivalent of the heart rate variability (HRV) *in vivo* [1,3–5].

The HRV describes the physiological fluctuations of the time interval between successive heartbeats in humans [6]. It is primarily an expression of the uninterrupted influence of the autonomic nervous system on the heart rate, which continuously modulates the heart rate *via* vagal and sympathetic signals [7,8]. It is known from clinical studies that the HRV cannot only be influenced by physiological processes (*e.g.* respiration, blood pressure regulation) but also by certain cardiac and non-cardiac specific diseases, such as myocardial infarction and diabetic neuropathy [7–11]. Moreover, it is known that antiarrhythmic drugs can

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influence the HRV *in vivo*. In this context, we have previously analyzed the effect of antiarrhythmic drugs on the BRV *in vitro*. We observed remarkable similarities between the effect of these cardioactive drugs on the BRV of stem cell derived cardiomyocytes *in vitro* and the effect on the HRV of humans that has been described in clinical studies [1,2].

In healthy individuals, a major modulator of the HRV is the circadian rhythm. In fact, several clinical studies have described the effect of the day/night cycle on different cardiac specific parameters [12]. Bonnemeier et al. detected a marked day/night pattern of HRV parameters in healthy individuals aged 20–70 years with a peak at nighttime and a plateau at daytime [13]. The characteristic nocturnal peak and the day/night amplitude diminished with aging [13]. Massin et al. identified a circadian rhythm of the heart rate and HRV parameters in infants and children [14]. Their data confirmed a progressive maturation of the autonomic nervous system [14].

The ocular light plays a key role in the control of the circadian rhythm in humans by transmitting time of day information to the body. It starts a complex cascade of various physiological processes [15]. A key transmitter of the circadian rhythm is the hormone melatonin [15]. Endogenous melatonin is produced in the pineal gland and is generally secreted at night and declining back to baseline levels in the morning [15].

Essential components of the melatonin signaling pathway are the melatonin receptors MT1 and MT2. Both mammalian subtypes of the melatonin receptor are G-protein coupled and have been cloned and characterized by Reppert et al. in 1994 and 1995 [16,17]. The expression of these melatonin receptors has been verified in many different tissues; *e.g.* in various parts of the central nervous system and also in peripheral organs, such as heart, lung, liver and kidney [18–21]. The various intracellular effects mediated by melatonin receptors depend on the cellular milieu and comprise changes in intracellular cyclic nucleotides and calcium levels [21].

There is a strong correlation of HRV and circadian markers in humans (salivary cortisol and urinary 6-sulfatoxy-melatonin) [22]. Likewise, it has been shown that the administration of melatonin increased HRV parameters in healthy men [23].

It has been demonstrated *in vitro* that the melatonin release in a pineal cell culture from 13 and 14 day old chick embryos increased during the dark phase and decreased during the light phase of a 12 h light – 12 h dark cycle [24]. However, no rhythm of melatonin release was detected under constant dark conditions in a cell culture of 14 day old chick embryos, whereas the pineal cell culture of 18 day old chick embryos expressed a circadian rhythm of melatonin release under constant dark conditions [24]. This implies that circadian rhythms develop during the embryonic phase and are already modulated by melatonin.

The majority of studies describe positive effects of melatonin on the adult as well as fetal organism. Melatonin is believed to have antioxidant, protective effects and is considered to be neuroprotective [25,26]. It may also modulate the fetal cardiometabolic response to acute hypoxia, as it may occur during labor, by influencing blood pressure, vascular resistance and serum levels of catecholamines [27,28]. Due to its positive effects, exogenous melatonin is used in the prevention of perinatal complications as well as fetal and neonatal diseases. The therapeutic use of melatonin may positively influence intrauterine growth restriction and maternal preeclampsia [27].

Since the melatonin producing pineal gland develops after birth, the human fetus is dependent on maternal, transplacentally transmitted melatonin [26]. If the mother substitutes melatonin, *e.g.* to reduce jet lag, the fetus is also exposed to altered and potentially unphysiological levels of melatonin. Thus, negative consequences caused by modified melatonin concentrations could also occur, as placental dysfunctions that influence the fetal supply with melatonin are associated with different pregnancy disorders [25]. The suppression of maternal plasma melatonin, for example, may induce intrauterine growth retardation [26].

An animal study evaluated the maternal and developmental toxicity of melatonin applied to pregnant rats [29]. The authors noted mild sedation, reduced maternal food intake and reduced body weight gain [29]. There was no effect of melatonin on prenatal survival or fetal body weight [29]. Also there were no incidences of fetal malformations or variations [29].

There are only very few clinical studies that point to potential risks and adverse effects of melatonin usage in humans. Case reports concerning melatonin overdoses in adults describe clinical symptoms like lethargy, disorientation, blurred vision, drowsiness, dizziness and confusion [30,31]. There is no systematically collected data available that describes the effects of melatonin overdoses in pregnant humans.

Summarizing, age seems to play an important role in the maturation of circadian rhythms. Melatonin serves as a key mediator of the circadian rhythm to the human body, even at the embryonic phase. Due to ethical concerns, toxic effects of melatonin have not been ruled out clearly so far *in vivo*, especially during pregnancy. Thus, it would be of great interest to investigate whether the *in vitro* model based on ESC derived tissue would be a suitable physiological and pharmacological screening tool to study the maturation of circadian rhythms in the embryonic phase. As a suitable tool, it should allow to study effects of modulators of the developing system, such as melatonin, on established physiological parameters. As a first approach, the main focus of the present study was to evaluate the physiological and potentially toxic effect of exogenous melatonin on the BRV of spontaneously beating cardiomyocytes that are derived from mESCs.

2. Material and methods

2.1. Cell culture and differentiation

The cell culture of mESCs as well as the cell differentiation followed a well-established protocol that has been described in detail before [32,33].

Briefly, cells of the murine embryonic stem cell line D3 (αPig-44) were co-cultured on a monolayer of inactivated fibroblast feeder cells [32,33]. The culture medium consisted of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with fetal bovine serum (FBS), non-essential amino acids (NEA), 2-Mercaptoethanol, leukemia inhibitory factor (LIF, ESGRO®, Merck Millipore) and Geneticin. The mESCs were passaged three times per week.

In order to start the stem cell differentiation into embryoid bodies (EBs), 10^6 enzymatically dissociated mESCs were kept in suspension in bacterial dishes and placed on a horizontal shaker. Two days after the start of the differentiation process, 10^3 EBs were taken out of the suspension and transferred to separate bacterial dishes with fresh differentiation medium. These bacterial dishes were placed again on the horizontal shaker for further differentiation. The differentiation medium consisted of IMDM supplemented with FBS, NEA, 2-Mercaptoethanol and Penicillin combined with Streptomycin. The differentiation medium was exchanged on day 7 for the first time. The first beating EBs could usually be observed on day 8 of differentiation. If not stated otherwise, all media and reagents mentioned above were purchased from Gibco® (Invitrogen, Karlsruhe, Germany).

2.1.1. Ethics approval

The regulatory authorities at the Robert Koch Institute, Berlin, Germany, have approved the work with ESCs.

2.2. Experimental setup

The experimental setup has been described previously [1,2]. Standard MEA chambers (60 electrodes, 30 μm electrode diameter, 200 μm inter-electrode distance) in combination with the MEA1060-Inv-BC amplifier and data acquisition system from Multichannel Systems (MCS, Reutlingen, Germany) were used to record extracellular FPs of spontaneously beating cardiomyocytes. Measurements were performed

with a sampling rate of 2 kHz. A perfusion system based on a customized perfusor (B. Braun Melsungen AG, Germany) was used to ensure a continuous flow of 500 µL per minute and a constant concentration of melatonin within the MEA chamber. A heating plate as well as a heating cannula (PH01, MCS) ensured a constant temperature of 37 °C.

2.3. Experimental procedure

The experimental procedure was performed as described previously [1,2]. First, the electrode area of the MEA chambers was coated with 0.1 % gelatine and fibronectin (both Sigma Aldrich, Germany). Spontaneously beating EBs were then placed manually onto the electrode area and kept under incubator conditions for 48 h in order to ensure sufficient tissue attachment onto the electrodes. The spontaneously beating EBs were selected 13 days after the start of the differentiation. Consequently, all measurements were performed during the intermediate stage of development [34]. Prior to the start of an experiment, the differentiation medium in the MEA chambers was replaced by IMDM (without supplements) and the amount of medium was raised to 500 µL.

Experiments always started with the recording of a control measurement without melatonin. Then, measurements with hormonal/pharmacological intervention were recorded. The concentration of melatonin was increased after each recording. Experiments always ended with a recording after performing a washout (10 min perfusion with IMDM without supplements). The standard recording time period for the control measurements as well as each concentration of melatonin was five minutes.

2.4. Pharmacological substance

N-Acetyl-5-Methoxytryptamine (Melatonin) was used throughout the study (Sigma-Aldrich, Germany).

2.5. BRV analysis

The BRV analysis was performed as described recently [1,2]. The analysis was focused on time domain methods. Briefly, the standard deviation of normal-to-normal intervals (SDNN) was considered as a parameter describing the overall variability. The standard deviation of successive differences (SDSD) was rated as a parameter expressing the

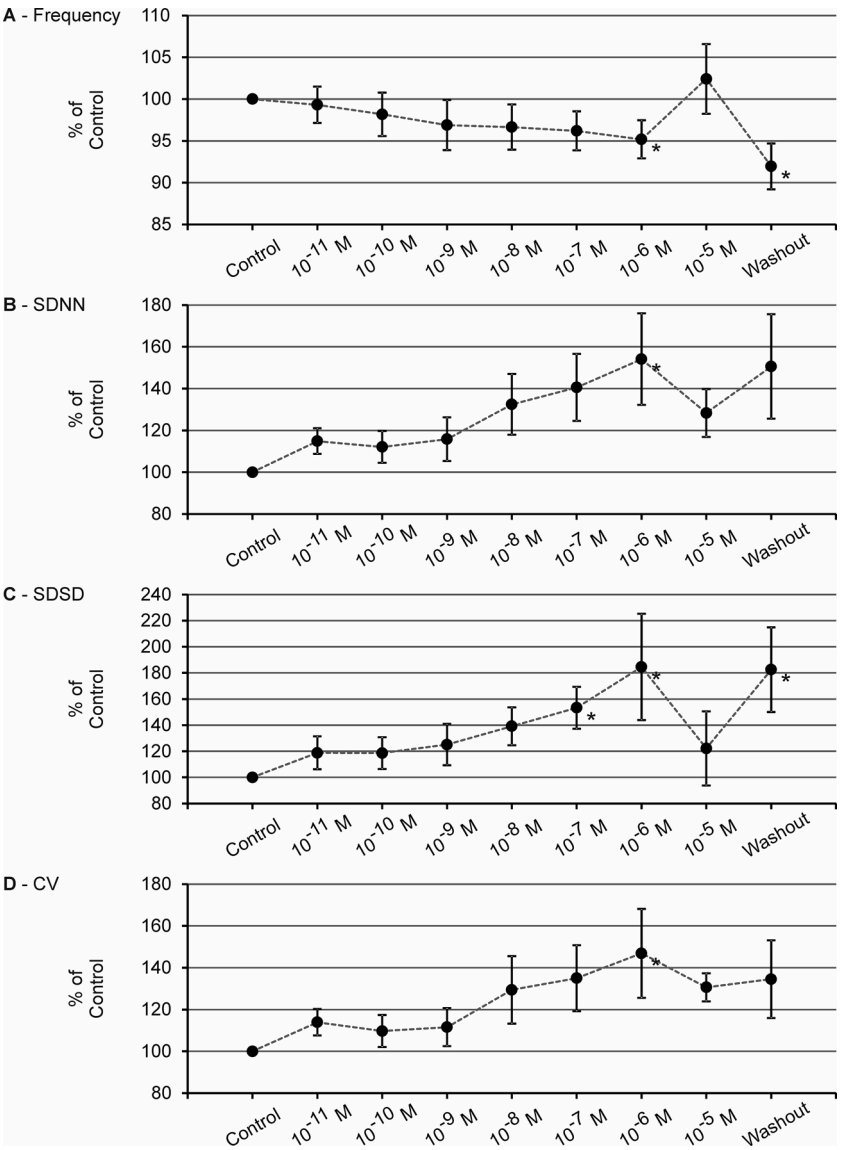


Fig. 1. Effect of melatonin on the BRV of mESC derived cardiomyocytes.
A-D: Beating frequency, SDNN, SDSD and CV (mean ± SEM). Melatonin decreased the beating frequency and increased the BRV parameters dose-dependently up to a concentration of 10^{-6} M (n = 8). Each EB was measured once. *p ≤ 0.05 (each concentration compared to control).

short-term variability. The coefficient of variation (CV, SDNN divided by the mean normal-to-normal interval) was included in the analysis of this study in order to remove the influence of the beating frequency on the calculation of BRV parameters.

2.6. Data analysis

A customized macro script for the Spike2-software (Cambridge Electronic Design, Cambridge, UK) was used for the analysis of the MEA data and the calculation of the BRV parameters. Artifacts and outliers were excluded from the analysis: The experiment with one EB was excluded from the analysis due to an arrhythmic control measurement. The measurement with a single concentration (10^{-9} M) of another EB was identified as an outlier and consequently also excluded from the analysis. The results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by the Wilcoxon-Test using SPSS software (IBM, USA). P-values ≤ 0.05 were considered statistically significant.

3. Results

Melatonin was used in a concentration range from 10^{-11} M to 10^{-6} M for five EBs (n = 5) and from 10^{-11} M to 10^{-5} M for three EBs (n = 3), respectively (see Fig. 1). The mean beating frequency (without pharmacological intervention) of all EBs (n = 8) was 6.35 ± 0.25 Hz (range 5.27 Hz – 7.51 Hz). The mean SDNN was 2.01 ± 0.28 ms, the mean SDSD was 2.16 ± 0.40 ms and the mean CV was 0.013 ± 0.002.

The application of melatonin induced a dose-dependent decrease of the beating frequency up to its minimum at a concentration of 10^{-6} M (95.2 % ± 2.3 SEM of the control, $p \leq 0.05$). The application of 10^{-5} M melatonin induced a slight increase of the beating frequency (102.4 % ± 4.2 SEM of control) without reaching significance.

The BRV parameters increased constantly with increasing concentration of melatonin up to a maximum level at 10^{-6} M (SDNN 154.1 % ± 21.9 SEM of control, $p \leq 0.05$ and SDSD 184.6 % ± 40.7 SEM of control, $p \leq 0.05$). The application of 10^{-5} M melatonin induced a decrease of BRV compared to the preceding concentration.

For the analysis of the washouts the EBs were split into two groups according to the applied concentration range (see Fig. 2). Group 1 included all EBs that were exposed to a maximum concentration of 10^{-6} M and group 2 included all EBs that were exposed to a maximum concentration of 10^{-5} M.

The beating frequency of the EBs in group 1 increased after the washout (92.5 % ± 2.5 SEM of control after the application of 10^{-6} M and 94.8 % ± 3.4 SEM after washout, $p > 0.05$) whereas the beating frequency of the EBs in group 2 decreased after the washout (102.4 % ± 4.2 SEM of control after the application of 10^{-5} M and 87.3 % ± 3.6 SEM of control after the washout, $p > 0.05$).

This observation becomes even more apparent when the BRV parameters of these two groups were compared. The overall variability (SDNN) of the EBs in group 1 decreased after the washout (159.3 % ± 36.0 SEM of control after the application of 10^{-6} M and 106.5 % ± 7.1 SEM after the washout, $p > 0.05$) whereas the overall variability of the EBs in group 2 increased after the washout (128.4 % ± 11.4 SEM of control after the application of 10^{-5} M and 224.1 % ± 36.9 SEM of control after the washout, $p > 0.05$).

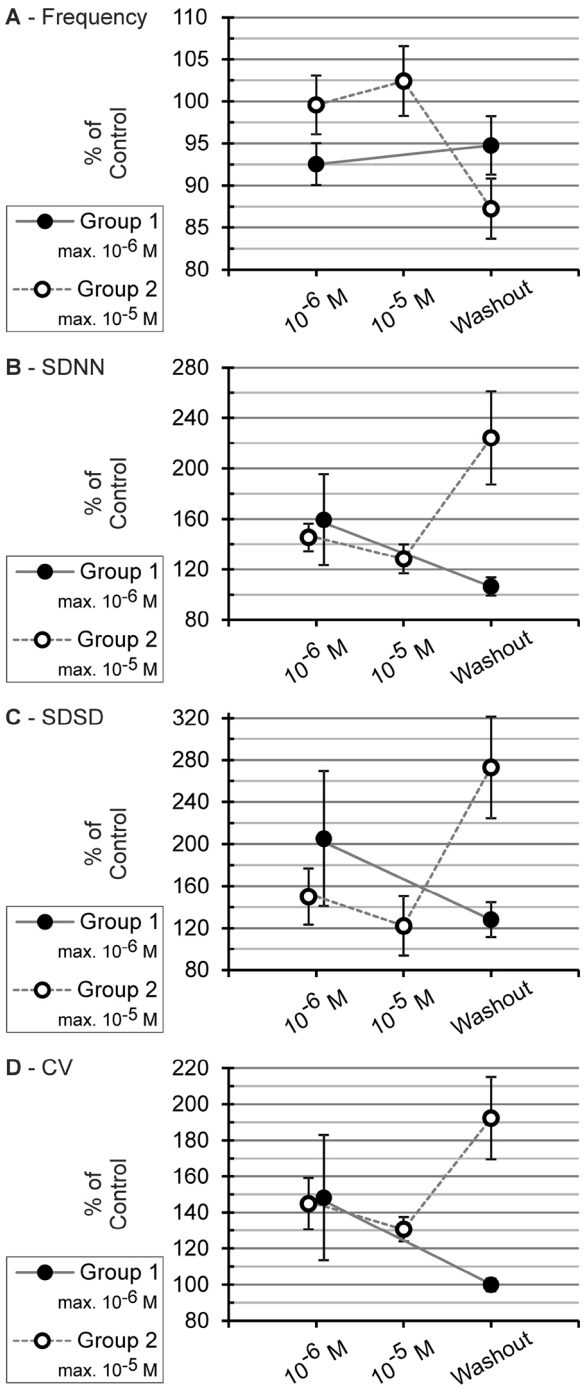


Fig. 2. Reversible effect of 10^{-6} M melatonin *versus* irreversible effect of 10^{-5} M melatonin.
A-D: Beating frequency, SDNN, SDSD and CV (mean ± SEM). Group 1 includes all EBs that were exposed to a maximum concentration of 10^{-6} M (n = 5). Group 2 includes all EBs that were exposed to a maximum concentration of 10^{-5} M (n = 3).

4. Discussion

The purpose of the present study was to examine the physiological and potentially toxic effects of melatonin on the beating frequency and the BRV of mESC derived cardiomyocytes.

Exogenously applied melatonin dose-dependently influenced the beating frequency and the BRV of mESC derived cardiomyocytes *in vitro*. This finding allows the conclusion that melatonin receptors are expressed by the cells within the EBs even at the embryonic developmental stage.

Melatonin induced a dose-dependent decrease of the beating frequency and a dose-dependent increase of BRV parameters up to a certain concentration (10^{-6} M). This finding is generally in accordance with the effect of melatonin *in vivo,* which leads to the assumption that the intracellular pathways that are linked to the melatonin receptors at this embryonic developmental stage operate in a similar manner compared to the intracellular pathways in the adult human heart.

In clinical trials reduced HRV, *e.g.* after myocardial infarction, is

generally associated with higher mortality [9]. Concurrently the increase of HRV, *e.g.* in patients with chronic congestive heart failure, decreases the risk of mortality [11]. In the literature melatonin is predominantly considered to have positive effects on the organism in the embryonic phase [25,27]. In view of the results of this study, the increase of the BRV induced by melatonin could also positively affect the HRV *in vivo* during the embryonic developmental stage.

From a pharmacological point of view, the analysis of the washouts is of great interest. 10^{-6} M melatonin induced a reversible effect, whereas 10^{-5} M melatonin led to an irreversible effect. Based on our experiments, we can only speculate about the underlying mechanisms. A possible explanation for this observation is that 10^{-5} M melatonin irreversibly damages the intracellular pathways that are linked to the melatonin receptors. High concentrations of melatonin could also induce a permanent and irreversible activation of the melatonin receptors. This would result in a dysfunction of the regulatory mechanisms.

The interruption of intracellular signaling pathways and/or regulatory mechanisms might also be responsible for the increased beating frequency and the decreased BRV after the application of 10^{-5} M melatonin (compared to the preceding concentration (10^{-6} M), see Fig. 2).

Keeping in mind that melatonin is generally believed to have predominantly positive effects on the human organism, this finding becomes particularly interesting. Case reports describing the symptoms of adult patients with melatonin overdoses report an uneventful and complete recovery without permanent damage [30,31]. However, there is no data on overdoses in human fetus and pregnant women. The results of this study point to a possibly toxic concentration of melatonin that could potentially lead to permanent damage.

Assuming that melatonin possibly damages the intracellular signaling pathways irreversibly and/or leads to permanent activation of the corresponding receptors, this effect may not only have a dramatic impact on the heart rhythm but also on other tissues expressing melatonin receptors, such as the central nervous system and different peripheral organs (*e.g.* lung, liver and kidney) [18–21].

The present study proved that the *in vitro* model based on cardiac tissue derived from mESC is a suitable model to characterize the effect of melatonin. Although previous studies have demonstrated strong similarities between the electrophysiological properties of murine and human cells, it would be interesting to transfer the observations of this study to an *in vitro* model based on cardiomyocytes derived from human ESCs [1,2]. Considering the clinical studies describing intrauterine growth retardation after suppression of maternal plasma melatonin, it would be equally interesting to study the effect of melatonin on the differentiation of embryonic stem cells [26]. Another question that could be addressed with this *in vitro* model is whether there are melatonin producing cells within murine EBs. In this case, the effects of a day/night cycle on the endogenous melatonin levels could be a study objective.

4.1. Conclusions and prospects

The present study allows the conclusion that the *in vitro* model based on mESC derived cardiomyocytes in combination with the MEA technique is a valuable tool to explore the development of cardio-specific circadian rhythms during the embryonic phase. Furthermore, our data underline the potential of this *in vitro* model to serve as a preclinical drug screening tool. Melatonin receptors and melatonin signaling pathways are already present at this stage of heart development and initiate the functional effects known from humans. We demonstrated dose-dependent effects of melatonin up to possibly toxic effects that have not been described before in embryonic heart tissue.

The effect of melatonin on cardiomyocytes from different developmental stages as well as on cardiomyocytes of other species could be addressed in future studies. Furthermore, the effect of a light/dark cycle on embryonic development could be studied with this *in vitro* model.

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Data availability statement

The datasets analyzed during this study are available from the corresponding author on request.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

References

[1] J. Niehoff, M. Matzkies, F. Nguemo, J. Hescheler, M. Reppel, Beat rate variability in murine embryonic stem cell-derived cardiomyocytes: effect of antiarrhythmic drugs, Cell. Physiol. Biochem. 38 (2) (2016) 646–658.
[2] J. Niehoff, M. Matzkies, F. Nguemo, J. Hescheler, M. Reppel, The effect of antiarrhythmic drugs on the beat rate variability of human embryonic and human induced pluripotent stem cell derived cardiomyocytes, Sci. Rep. 9 (1) (2019), 14106, https://doi.org/10.1038/s41598-019-50557-7.
[3] Y. Mandel, A. Weissman, R. Schick, L. Barad, A. Novak, G. Meiry, S. Goldberg, A. Lorber, M.R. Rosen, J. Itskovitz-Eldor, O. Binah, Human embryonic and induced pluripotent stem cell-derived cardiomyocytes exhibit beat rate variability and power-law behavior, Circulation 125 (7) (2012) 883–893, https://doi.org/10.1161/circulationaha.111.045146.
[4] M. Ben-Ari, R. Schick, L. Barad, A. Novak, E. Ben-Ari, A. Lorber, J. Itskovitz-Eldor, M.R. Rosen, A. Weissman, O. Binah, From beat rate variability in induced pluripotent stem cell-derived pacemaker cells to heart rate variability in human subjects, Heart Rhythm 11 (10) (2014) 1808–1818, https://doi.org/10.1016/j.hrthm.2014.05.037.
[5] O. Binah, A. Weissman, J. Itskovitz-Eldor, M.R. Rosen, Integrating beat rate variability: from single cells to hearts, Heart Rhythm 10 (6) (2013) 928–932, https://doi.org/10.1016/j.hrthm.2013.02.013.
[6] Task force of the European society of cardiology and the North American society of pacing, heart rate variability: standards of measurement, physiological interpretation and clinical use, Circulation 93 (5) (1996) 1043–1065, https://doi.org/10.1161/01.CIR.93.5.1043.
[7] P.K. Stein, M.S. Bosner, R.E. Kleiger, B.M. Conger, Heart rate variability: a measure of cardiac autonomic tone, Am. Heart J. 127 (5) (1994) 1376–1381, https://doi.org/10.1016/0002-8703(94)90059-0.
[8] J. Pumprla, K. Howorka, D. Groves, M. Chester, J. Nolan, Functional assessment of heart rate variability: physiological basis and practical applications, Int. J. Cardiol. 84 (1) (2002) 1–14, https://doi.org/10.1016/S0167-5273(02)00057-8.
[9] R.E. Kleiger, J.P. Miller, J.T. Bigger Jr., A.J. Moss, Decreased heart rate variability and its association with increased mortality after acute myocardial infarction, Am. J. Cardiol. 59 (4) (1987) 256–262, https://doi.org/10.1016/0002-9149(87)90795-8.
[10] C. Carpeggiani, A. L'Abbate, P. Landi, C. Michelassi, M. Raciti, A. Macerata, M. Emdin, Early assessment of heart rate variability is predictive of in-hospital death and major complications after acute myocardial infarction, Int. J. Cardiol. 96 (3) (2004) 361–368, https://doi.org/10.1016/j.ijcard.2003.07.023.
[11] K.C. Bilchick, B. Fetics, R. Djoukeng, S.G. Fisher, R.D. Fletcher, S.N. Singh, E. Nevo, R.D. Berger, Prognostic value of heart rate variability in chronic congestive heart failure (Veterans affairs' survival trial of antiarrhythmic therapy in congestive heart failure), Am. J. Cardiol. 90 (1) (2002) 24–28, https://doi.org/10.1016/S0002-9149(02)02380-9.
[12] H.V. Huikuri, K.M. Kessler, E. Terracall, A. Castellanos, M.K. Linnaluoto, R. J. Myerburg, Reproducibility and circadian rhythm of heart rate variability in healthy subjects, Am. J. Cardiol. 65 (5) (1990) 391–393, https://doi.org/10.1016/0002-9149(90)90308-N.
[13] H. Bonnemeier, U.K.H. Wiegand, A. Brandes, N. Kluge, H.A. Katus, G. Richardt, J. Potratz, Circadian profile of cardiac autonomic nervous modulation in healthy subjects, J. Cardiovasc. Electrophysiol. 14 (8) (2003) 791–799, https://doi.org/10.1046/j.1540-8167.2003.03078.x.
[14] M.M. Massin, K. Maeyns, N. Withofs, F. Ravet, P. Gérard, Circadian rhythm of heart rate and heart rate variability, Arch. Dis. Child. 83 (2) (2000) 179–182, https://doi.org/10.1136/adc.83.2.179.
[15] D.J. Skene, J. Arendt, Human circadian rhythms: physiological and therapeutic relevance of light and melatonin, Ann. Clin. Biochem. 43 (Pt 5) (2006) 344–353, https://doi.org/10.1258/000456306778520142.
[16] S.M. Reppert, D.R. Weaver, T. Ebisawa, Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses, Neuron 13 (5) (1994) 1177–1185, https://doi.org/10.1016/0896-6273(94)90055-8.
[17] S.M. Reppert, C. Godson, C.D. Mahle, D.R. Weaver, S.A. Slaugenhaupt, J.F. Gusella, Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor, Proc. Natl. Acad. Sci. U. S. A. 92 (19) (1995) 8734–8738, https://doi.org/10.1073/pnas.92.19.8734.

[18] M.L. Dubocovich, D.N. Delagrange, P. Fau - Krause, D. Krause Dn Fau - Sugden, D. P. Sugden D Fau - Cardinali, J. Cardinali Dp Fau - Olcese, J. Olcese, Nomenclature, classification and pharmacology of G protein-coupled melatonin receptors, Pharmacol. Rev. (1521–0081 (Electronic)) (2010).
[19] L. Naji, A. Carrillo-Vico, J.M. Guerrero, J.R. Calvo, Expression of membrane and nuclear melatonin receptors in mouse peripheral organs, Life Sci. 74 (18) (2004) 2227–2236, https://doi.org/10.1016/j.lfs.2003.08.046.
[20] P. Sallinen, S. Saarela, M. Ilves, O. Vakkuri, J. Leppäluoto, The expression of Mt1 and Mt2 melatonin receptor Mrna in several rat tissues, Life Sci. 76 (10) (2005) 1123–1134, https://doi.org/10.1016/j.lfs.2004.08.016.
[21] S.R. Pandi-Perumal, I. Trakht, V. Srinivasan, D.W. Spence, G.J.M. Maestroni, N. Zisapel, D.P. Cardinali, Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways, Prog. Neurobiol. 85 (3) (2008) 335–353, https://doi.org/10.1016/j.pneurobio.2008.04.001.
[22] P. Boudreau, G. Dumont, N.M. Kin, C.D. Walker, D.B. Boivin, Correlation of heart rate variability and circadian markers in humans, IEEE Eng. Med. Biol. Soc. 2011 (2011) 681–682, https://doi.org/10.1109/iembs.2011.6090153.
[23] K. Nishiyama, H. Yasue, Y. Moriyama, R. Tsunoda, H. Ogawa, M. Yoshimura, K. Kugiyama, Acute effects of melatonin administration on cardiovascular autonomic regulation in healthy men, Am. Heart J. 141 (5) (2001) 13A–17A, https://doi.org/10.1067/mhj.2001.114368.
[24] K. Akasaka, T. Nasu, T. Katayama, N. Murakami, Development of regulation of melatonin release in pineal cells in chick embryo, Brain Res. 692 (1–2) (1995) 283–286, https://doi.org/10.1016/0006-8993(95)00643-5.
[25] L. Sagrillo-Fagundes, E.M. Assuncao Salustiano, P.W. Yen, A. Soliman, C. Vaillancourt, Melatonin in pregnancy: effects on brain development and Cns programming disorders, Curr. Pharm. Des. 22 (8) (2016) 978–986.
[26] S.E. Voiculescu, N. Zygouropoulos, C.D. Zahiu, A.M. Zagrean, Role of melatonin in embryo fetal development, J. Med. Life 7 (4) (2014) 488–492.
[27] F. Rodrigues Helmo, R.M. Etchebehere, N. Bernardes, M.F. Meirelles, C. Galvão Petrini, L. Penna Rocha, M.L. Gonçalves dos Reis Monteiro, C. Souza de Oliveira Guimarães, V. de Paula Antunes Teixeira, M.A. dos Reis, J.R. Machado, R. R. Miranda Corrêa, Melatonin treatment in fetal and neonatal diseases, Pathol. - Res. Pract. 214 (12) (2018) 1940–1951, https://doi.org/10.1016/j.prp.2018.10.016.
[28] A.S. Thakor, B.J. Allison, Y. Niu, K.J. Botting, M. Seron-Ferre, E.A. Herrera, D. A. Giussani, Melatonin modulates the fetal cardiovascular defense response to acute hypoxia, J. Pineal Res. 59 (1) (2015) 80–90, https://doi.org/10.1111/jpi.12242.
[29] G. Jahnke, M. Marr, C. Myers, R. Wilson, G. Travlos, C. Price, Maternal and developmental toxicity evaluation of melatonin administered orally to pregnant Sprague-Dawley rats, Toxicol. Sci. 50 (2) (1999) 271–279, https://doi.org/10.1093/toxsci/50.2.271.
[30] J. Balentine, J. Hagman, More on melatonin, J. Am. Acad. Child Adolesc. Psychiatry 36 (8) (1997) 1013, https://doi.org/10.1097/00004583-199708000-00001.
[31] B.J. Holliman, P.A. Chyka, Problems in assessment of acute melatonin overdose, South. Med. J. 90 (4) (1997) 451–453.
[32] E. Kolossov, Z. Lu, I. Drobinskaya, N. Gassanov, Y. Duan, H. Sauer, O. Manzke, W. Bloch, H. Bohlen, J. Hescheler, B.K. Fleischmann, Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes, Fed. Am. Soc. Exp. Biol. 19 (6) (2005) 577–579, https://doi.org/10.1096/fj.03-1451fje.
[33] E. Kolossov, T. Bostani, W. Roell, M. Breitbach, F. Pillekamp, J.M. Nygren, P. Sasse, O. Rubenchik, J.W. Fries, D. Wenzel, C. Geisen, Y. Xia, Z. Lu, Y. Duan, R. Kettenhofen, S. Jovinge, W. Bloch, H. Bohlen, A. Welz, J. Hescheler, S. E. Jacobsen, B.K. Fleischmann, Engraftment of engineered Es cell-derived cardiomyocytes but not Bm cells restores contractile function to the infarcted myocardium, J. Exp. Med. 203 (10) (2006) 2315–2327, https://doi.org/10.1084/jem.20061469.
[34] J. Hescheler, B.K. Fleischmann, M. Wartenberg, W. Bloch, E. Kolossov, G. Ji, K. Addicks, H. Sauer, Establishment of ionic channels and signalling cascades in the embryonic stem cell-derived primitive endoderm and cardiovascular system, Cells Tissues Organs 165 (3–4) (1999) 153–164, https://doi.org/10.1159/000016695.