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ORIGINAL ARTICLE

Melatonin inhibits spontaneous and oxytocin-induced contractions of rat myometrium *in vitro*

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Abstract

OBJECTIVE: The aim of this study was to investigate the effects of melatonin on spontaneous and oxytocin-induced contractility of pregnant and non-pregnant rat myometrium *in vitro*.

DESIGN: Myometrial strips were removed from virgin or late pregnant (21 days gestation) Wistar rats following decapitation and placed in an organ bath containing Krebs' solution at 37°C and pH 7.4, constantly bubbled with 95% oxygen-5% carbon dioxide and isometric contractions were recorded. Effects of cumulative concentrations of melatonin (0.1 to 10 μM) on spontaneous and oxytocin-induced contractions were studied. Possible involvement of Ca^{2+}-activated K^+ channels in inhibitory actions of melatonin was investigated by using apamin (100 nM).

RESULTS: Melatonin inhibited spontaneous and oxytocin-induced contractions of myometrium from both virgin and late pregnant rats in a dose-dependent manner. After inhibition of oxytocin-induced contractions by melatonin, application of prostaglandin $F_{2\alpha}$ (1 μM) but not high KCl (30mM) containing solution initiated contractile activity. Inhibitory response induced by melatonin (13μM) was not affected by apamin (100 nM).

CONCLUSIONS: Data from this study demonstrates that melatonin inhibits spontaneous and oxytocin-induced contractions of myometrium from pregnant and non-pregnant rats. Although the exact mechanism is not clear, melatonin-induced inhibition of myometrial contractions may results from its interactions with Ca^{2+} channels.

A portion of this work has been previously presented in an abstract form (Physiological Society Meeting, The Journal of Physiology 528 P, Aberdeen, 2000)

Abbreviations:

mm	Millimeter,
$PGF_{2\alpha}$	Prostaglandin F2 alpha,
g	Gram,
µg	micro gram,
µl	Micro liter
ml	Milliliter,
cm	Centimeter,
DMF	Dimethylformamide
mU/L	Milli unit/Liter,
ANOVA	Analysis of variance,
SEM	Standard error of mean,
UK	United Kingdom,
HRT	Hormone replacement therapy
ROS	Reactive oxygen species
APUD	Amine precursor uptake decarboxylation
USA	United States of America

Introduction

The pineal gland and its main hormone melatonin (N-acetyl-5-methoxytryptamine) is known to be involved in a variety of physiological processes including regulation of endocrine rhythms [1], antigonadotropic activity [2, 3], scavenging of free oxygen radicals [4], and neuroendocrine regulation of various immune functions [5]. Melatonin has also been reported to effect smooth muscle tone. There are remarkable species and tissue difference in the effects of melatonin on smooth muscle. Melatonin decreased the contraction of the small and large intestines in rats [6], whereas it caused contraction of the colonic smooth muscle from guinea pig [7]. Melatonin has been shown to induce vasoconstriction in rat caudal and cerebral arteries in a dose dependent manner [8, 9] but to inhibit the norepineprine-induced vasoconstriction [10] and to cause relaxation of porcine arterial smooth muscle [11]. In another study it was shown that melatonin alone had negligible effect on the contractility but markedly potentiated the block produced by succinylcholine with facilitation of the desensitization in phrenic nerve-hemidiaphragm preparations of rats suggesting that melatonin has calcium channel blocking effect [12].

Uterine smooth muscle is not an exception in the case of melatonin effect and there are conspicuous species differences in the effects of melatonin on myometrial contractility. Despite the high interest and widespread ongoing research about the effects of melatonin in other smooth muscles, the effect of melatonin on myometrial contractility is relatively poorly studied. Micromolar concentrations of melatonin have been shown to significantly inhibit spontaneous and oxytocin-induced contractions but not $PGF_{2\alpha}$-induced contractions of myometrium from ovariectomised rats [13]. In the same study the authors also demonstrated that melatonin diminished the release of prostaglandins from both uterus and medial basal hypothalamus. There are also reports about the inhibitory effect of melatonin on carbachol [14] and oxytocin-induced myometrial contraction in rats [15]. In contrast, melatonin in combination with noradrenaline potentiated contractions in human myometrium [16]. In an *in vivo* study conducted on pregnant sheep, melatonin infusion is reported to have no effect on myometrial contractility [17]. The inhibitory effect of melatonin on myometrial contractility has been attributed to melatonin-induced prostaglandin synthetase inhibition in the uterus. However, detailed mechanisms of the effect of melatonin on uterine smooth muscle contractility have not been clarified yet.

In this study, the effects of melatonin on spontaneous and oxytocin-induced contractility of pregnant and non-pregnant rat myometrim were investigated. The possible roles of prostaglandins and calcium-activated potassium channels in melatonin-induced inhibition of the rat myometrial contractility were also investigated.

Material and Methods

This study was approved by Firat University local ethics committee. Small (1 cm long, <2 mm wide, <1 mm thick) longitudinal strips of myometria were dissected from adult female virgin or late pregnant (21 day of gestation) Wistar rats after guillotine decapitation. Only one strip from each animal was used. Muscle strips were placed in a jacketed organ bath (25 ml volume) and tied at bottom end to a fixed metal hook and at top end to a force displacement transducer (Harvard Inst, Kent, UK) using a cotton thread. The force displacement transducer was coupled to an amplifier driving a direct writing oscillograph (Universal Oscillograph, Harvard Inst, Kent, UK). The organ bath contained Krebs solution of the following composition (mM): NaCl, 154; KCl, 5.4; $MgSO_4$, 1.2; glucose, 11.5; $CaCl_2$, 2; HEPES, 10; adjusted to pH 7·4 with 1 M NaOH at 37°C and continuously aired with 95% oxygen-5% carbon dioxide throughout the experimental period. Following a 30-minute equilibration period of the stabilization of myometrial strips with 1 g stretch tension and development of spontaneous contractile activity, contractile response to oxytocin was obtained by application of single dose of 800mU/L extracellular oxytocin. Most of the strips developed spontaneous contractions within 30-minute equilibration period and strips with no spontaneous activity in this period were removed.

The mean peak amplitude and frequencies of isometric contractions were evaluated by 10 minutes intervals before and after application of each dose of melatonin and comparisons were made between the

mean values. All mean peak amplitude values of contractions are given in gram (g).

Drugs used in the present study were melatonin, and $PGF_{2\alpha}$, indomethacin (Sigma Chemical Co., St. Louis, USA), oxytocin (5 IU/ml for injection, Eczaciba·ı, Turkey), apamin (Alomone Lab, Israel). Melatonin (1 mM) was initially dissolved in dimethylformamide (DMF, Sigma) and final concentration of melatonin was made up in Krebs' solution. Control studies were carried out with comparable volumes of DMF, which was used to dissolve melatonin. Different concentrations of melatonin were added to tissue bath cumulatively (0.1-10 μM).

Statistical analysis

The results are expressed as mean ±SEM. All statistical analysis were done using the statistical program SPSS for windows (version 6.0.1. SPSS Inc. Chicago, Illinois). For statistical analysis of amplitude and frequency values, analysis of variance (One way ANOVA) was performed. $P<0.05$ was considered statistically significant. Figures were made using Origin (version 5.0, Microcal Software Inc. Northampton, USA).

Results

Experiments were performed on myometrial strips from pregnant rats (21 day of gestation) that developed spontaneous contractions in 30-minute equilibration period. After the manifestation of spontaneous contractions, application of oxytocin significantly increased contractile activity of myometrium from both pregnant and non-pregnant rats compared to spontaneous activity (Figs. 1A, 3B, 4). The mean peak amplitude of oxytocin-induced myometrial contractions of pregnant rat myometrium was 2.74±0.22g (n=9). Melatonin inhibited these oxytocin-induced contractions in a dose dependent manner (Fig. 1A, Fig. 5). The mean peak amplitude of contractions was 2.69±0.23g (n=9; $p>0.05$), 2.53±0.25g (n=9; $p>0.05$), 2.05±0.34g (n=9; $p>0.05$) and 0.85±0.23g (n=9; $p<0.01$) after application of 0.1μM, 0.5μM, 1μM, and 5μM melatonin, respectively (Fig. 5).

Melatonin also inhibited the mean peak amplitude of oxytocin-induced contractions from non-pregnant rats (Fig. 1B) from mean control value of 2.00±0.18g (n=7) to 1.99±0.17g (n=7; $p>0.05$), 1.95±0.18g (n=7; $p<0.05$), 1.81±0.19g (n=6; $p>0.05$) and 1.11±0.16g (n=7; $p<0.01$), after application of 0.1μM, 0.5μM, 1μM, and 5μM melatonin, respectively (Fig. 5).

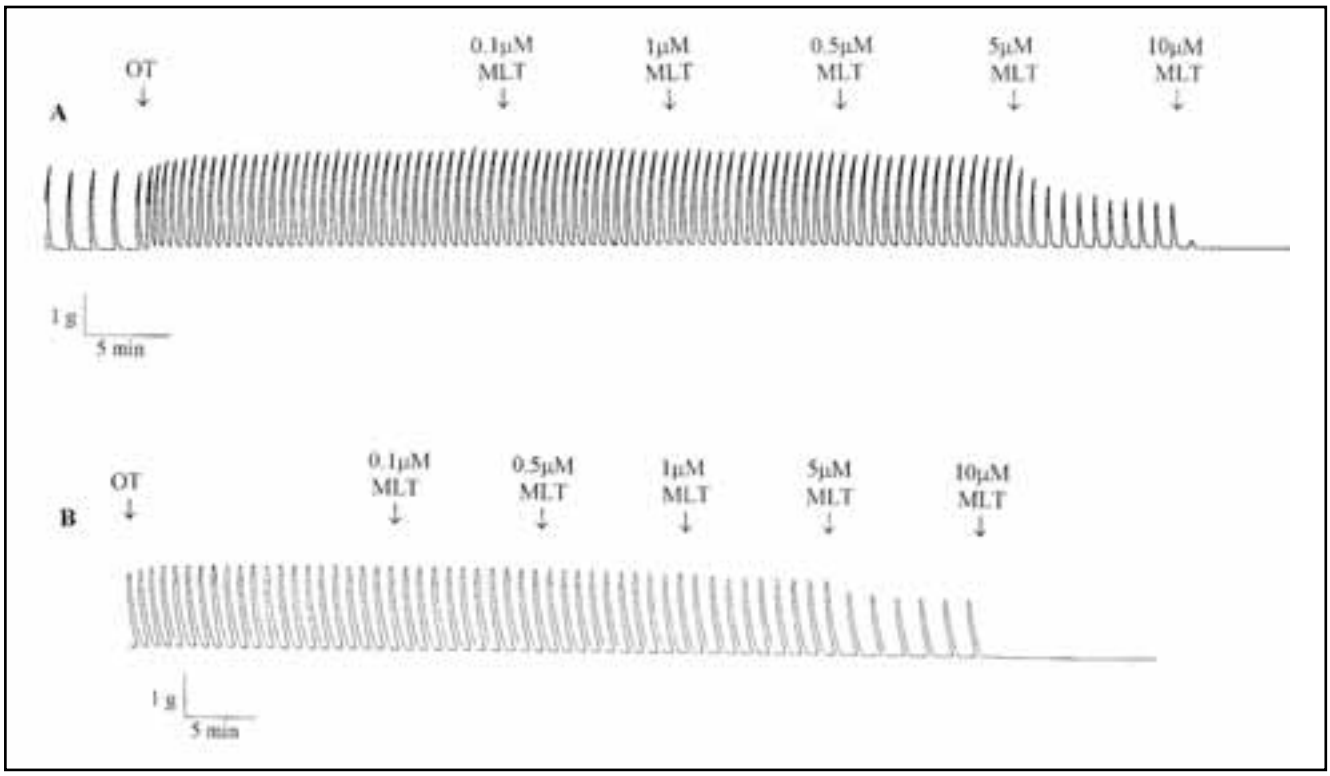

Fig. 1. The effects of melatonin on oxytocin-induced myometrial contractions. Original representative tracings showing the effects of different doses of melatonin on oxytocin induced contractions of myometrium from pregnant (A) and non-pregnant (B) rats. Oxytocin and subsequent doses of melatonin was applied cumulatively at points indicated by arrows. OT, oxytocin; MLT, melatonin; min, minute; g, gram.

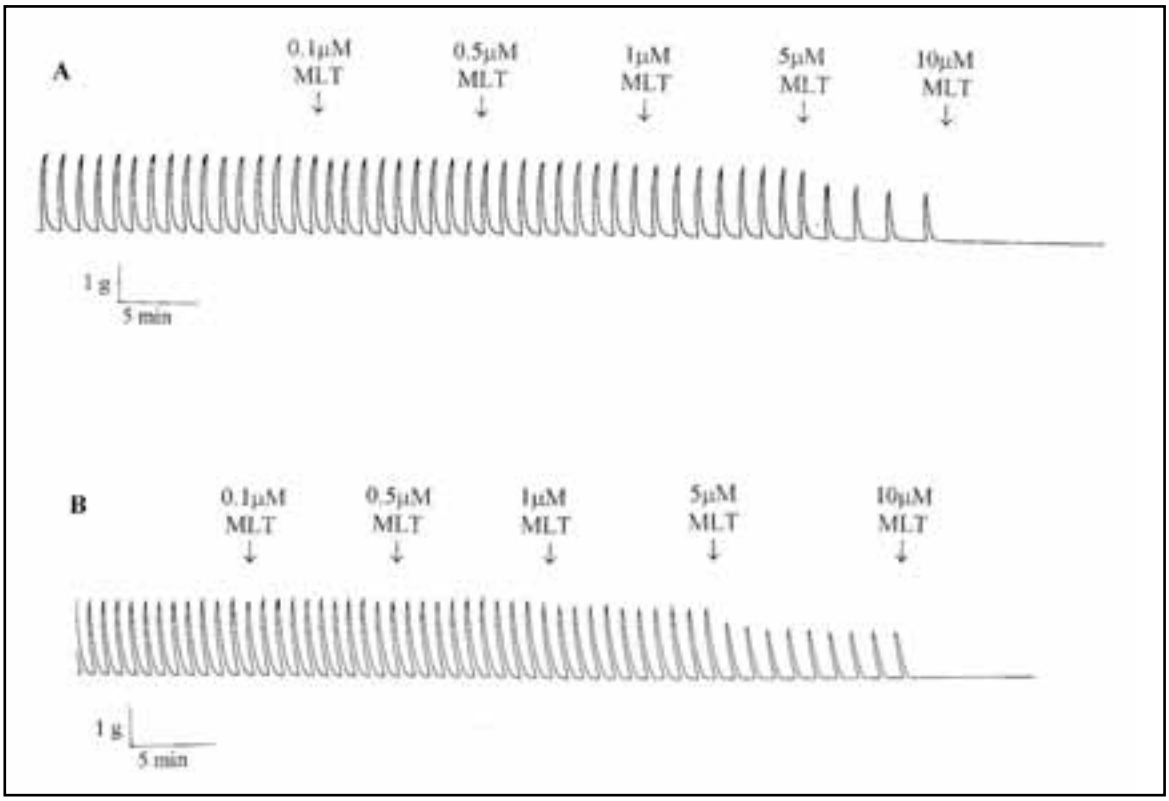

Fig. 2. The effects of melatonin on spontaneous myometrial contractions. Tracings showing the effects of different doses of melatonin on spontaneous contractions of myometrium from pregnant (A) and non-pregnant (B) rats. Different doses of melatonin was applied cumulatively at points indicated by arrows. MLT, melatonin; min, minute; g, gram.

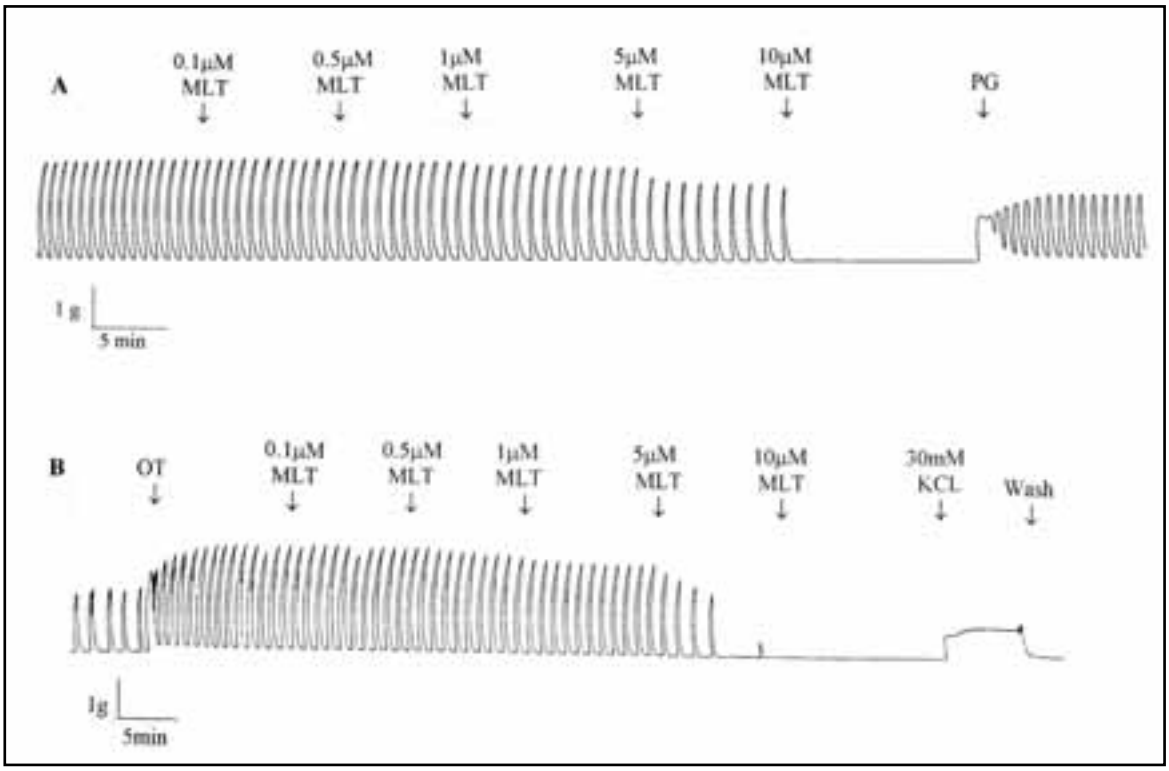

Fig. 3. Effects of prostaglandin $F_{2\alpha}$ and KCl after melatonin inhibition of contractions. (A) representative tracing showing the inhibitory effects of melatonin on spontaneous contractions. Prostaglandin $F_{2\alpha}$ (1μM) initiated contractions after complete inhibition of contractions with cumulative melatonin application. (B) representative tracing showing the effects of high KCl (30 mM) application after melatonin-induced complete block of oxytocin-induced contractions of myometrium from non-pregnant rats. Melatonin, prostaglandin $F_{2\alpha}$ and KCl were applied at indicated points and were continuously present throughout experiment. OT, oxytocin; MLT, melatonin; min, minute; g, gram; PG, prostaglandin $F_{2\alpha}$.

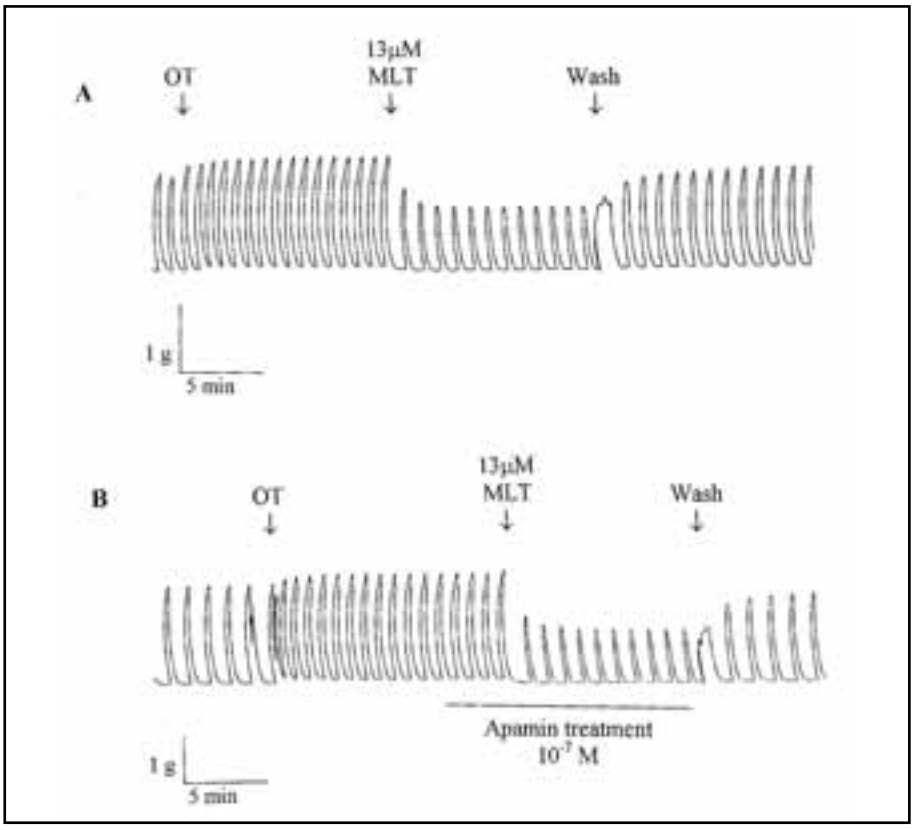

Fig. 4. The effects of apamin treatment on inhibitory effect of melatonin on oxytocin-induced myometrial contractions. Representative tracings showing the effects of 13μM melatonin on oxytocin-induced contractions of myometrium from non-pregnant rats. Single dose of 13 μM melatonin inhibited oxytocin-induced myometrial contractions in the absence (A) and presence (B) of apamin. Single dose of 13μM melatonin inhibited oxytocin-induced contractions by about 50% of the peak amplitude (A). When apamin pretreatment was performed (B) for 2.5 minute, subsequent application of 13μM melatonin caused similar inhibition of the oxytocin-induced contractions. Recovery was observed after wash with ordinary Krebs solution. Drugs were applied and washing was performed at indicated points. OT, oxytocin; MLT, melatonin; min, minute; g, gram.

Most of the (75 out of 84) myometrial strips from pregnant and non-pregnant rats developed spontaneous contractions with consistent amplitude and frequency after equilibration period and these spontaneous contractions could be recorded for several hours. Effects of melatonin on spontaneous contractions of myometrium from pregnant and non-pregnant rats were also investigated. Melatonin dose dependently inhibited these spontaneous contractions in all preparations (Figs. 2, 6). Mean peak amplitude of spontaneous contractions of myometrium from non-pregnant rats was 2.12±0.4 g (n=10) under control conditions, and 2.06±0.4 g (n=10, $p>0.05$), 2.02±0.4g (n=10, $p>0.05$), 1.88±0.4 g (n=10, $p>0.05$), and 1.20±0.07 g (n=10, $p<0.02$) after application of 0.1, 0.5, 1 and 5μM melatonin, respectively (Fig. 6). Melatonin also inhibited the mean peak amplitude of spontaneous contractions of pregnant rat myometrium from control values of 2.63±0.4 g (n=11) to 2.49±0.4 g (n=11, $p>0.05$), 2.27±0.5 g (n=11, $p>0.05$), 1.78±0.4 g (n=11, $p<0.05$), and 0.22±0.03 g (n=11, $p<0.001$) after application 0.1, 0.5, 1 and 5 μM melatonin, respectively (Fig. 6).

In order to determine the possible role of prostaglandins on the melatonin-induced inhibition of contractions, the effects of prostaglandin synthesis inhibitor indomethacin and prostaglandins were tested. 1 μg/ml indomethacin was added to prevent endogenous prostaglandin production [13]. After manifestation of spontaneous contractions, ordinary Krebs solution was replaced with indomethacin (1 μg/ml) containing Krebs solution and no significant change was observed in either amplitude (3.06±0.19g – 3.06±0.19g, n=6) or frequency (10.7±0.42 –10±0.45/10 minute period, n=6) of spontaneous contractions in myometrium from non-pregnant rats. Melatonin also inhibited spontaneous contractions in the presence of indomethacin, the amplitude of spontaneous contractions was 3.06±0.19g (n=6) under control conditions and 3.05±0.19g (n=6, $p>0.05$), 2.91±0.17g (n=6, $p>0.05$), 2.58±0.23g (n=6, $p>0.05$) and 1.70±0.01g (n=6, $p<0.01$) after applications of 0.1, 0.5, 1 and 5 μM melatonin, respectively. Experiments were also performed to determine the effect of melatonin on oxytocin-induced contractions in the presence of indomethacin (1 μg/ml) and it was found that melatonin similarly inhibited the amplitude of contractions

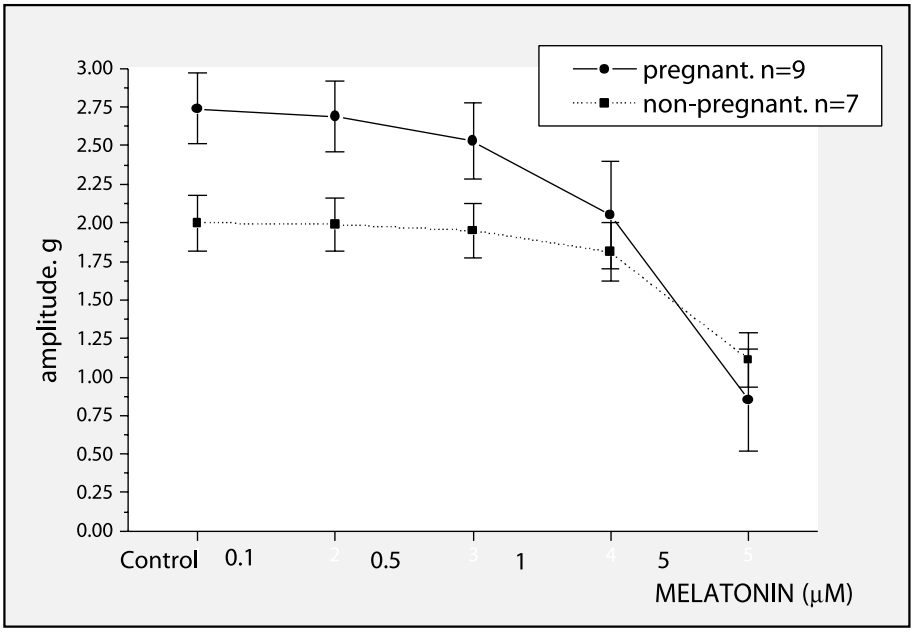

Fig. 5. Effects of different concentration of melatonin on mean peak amplitude of oxytocin-induced contractions of myometrium from pregnant and non-pregnant rats. (Mean ±SEM). Since 10 μM melatonin completely abolished the contractions data relating this period is not given.

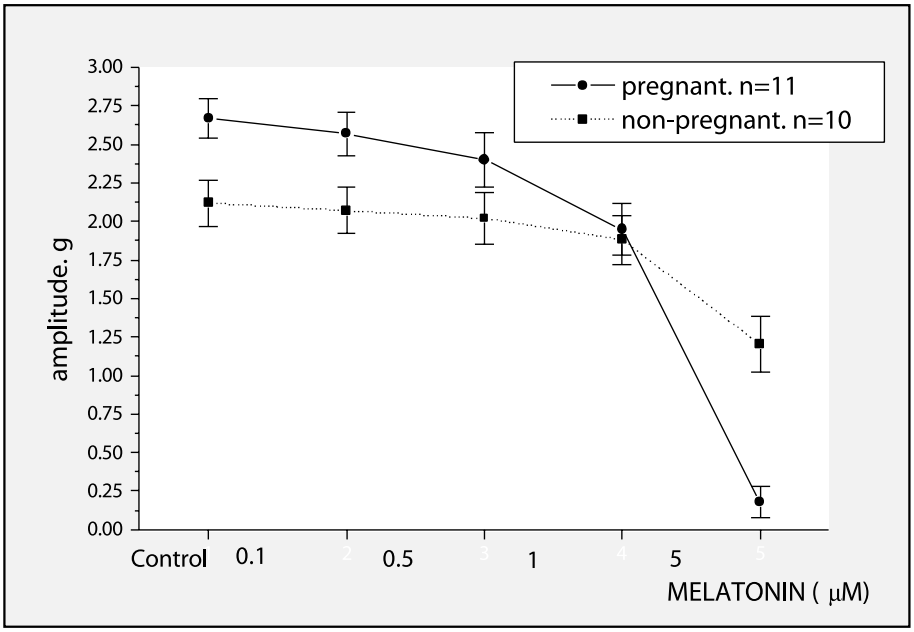

Fig. 6. Effects of different concentration of melatonin on mean peak amplitude of spontaneous contractions of myometrium from pregnant and non-pregnant rats. (Mean ±SEM). Since 10 μM melatonin completely abolished the contractions data relating this period is not given.

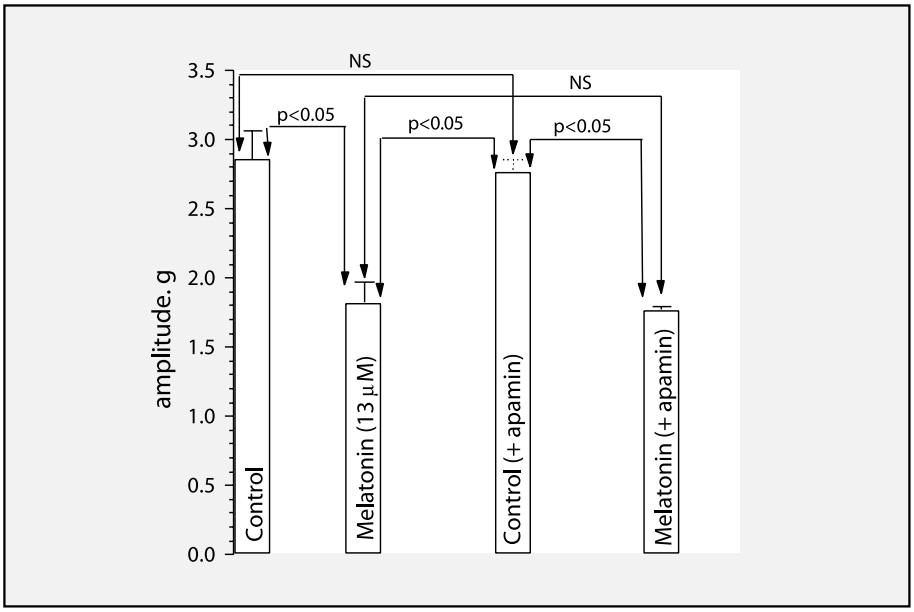

Fig. 7. Effects of apamin pretreatment (100 nM) on actions of melatonin on peak amplitude of oxytocin-induced contractions of myometrium from non-pregnant rats (mean ± SEM, n=6).

from mean control value of 3.23±0.25g (n=6) to 3.23±0.25g (n=6, p>0.05), 3.08±0.24g (n=6, p>0.05), 3.01±0.20g (n=6, p>0.05) and 2.23±0.28g (n=6, p<0.05) after application of its 0.1, 0.5, 1 and 5 μM concentrations, respectively.

When spontaneous and oxytocin-induced contractions are totally diminished after application of 10μM melatonin, contractions can be initiated with prostaglandin $F_{2\alpha}$ (1 μM) applications (n=6, Fig. 3A), but high KCl (30 mM) failed to induce similar contractions in the same myometrial strip, when applied after 10μM melatonin inhibition (n=5, Fig. 3B).

The last dose of cumulative melatonin application was 10μM and this high dose of melatonin completely abolished spontaneous and oxytocin-induced contractions in myometrial strips from both pregnant and non-pregnant rats, in all series of experiments conducted (Figs.1, 2, 3).

Additional experiments were performed to investigate the possible role of Ca^{2+}-activated K^+ channels on inhibitory actions of melatonin using apamin. For this experiment a single dose of melatonin (13μM) was applied on oxytocin-induced contractions of myometrium from non-pregnant rats and approximately 50% inhibition of amplitude was observed (n=6, Fig. 4A).

In a different set of experiments, after augmentation of contractions by oxytocin apamin (100 nM) pretreatment for 2.5 minutes was performed and no significant change was observed in inhibitory action of subsequently applied 13μM melatonin (Figs. 4B, 7). Melatonin (13μM) inhibited oxytocin-induced contractions of non-pregnant rat myometrium from 2.86±0.2g to 1.82±0.14g (p<0.05, n=6) and 2.77±0.09g to 1.78±0.08g (n=6, p<0.05) in the absence and presence of 100 nM apamin, respectively (Fig. 7). Melatonin (13μM) also inhibited spontaneous contractions of non-pregnant rat myometrium from 2.24±0.3g to 1.26±0.6g (p<0.05, n=5) in the presence of 100 nM apamin. Effects of apamin pre-treatment on oxytocin-induced contractions from pregnant rats were also investigated and it was found that application of a single dose of 13μM melatonin inhibited mean amplitude of oxytocin-induced contractions from 3.04±0.7g to 1.66±0.9g (n=4, p<0.05), when 100 nM apamin was applied for 2.5 minutes prior to application of melatonin. Apamin itself did not cause any change in oxytocin-induced (Fig. 4B) or spontaneous contractions (both amplitude and frequency).

In further series of experiments the melatonin solvent DMF was applied in comparable concentrations and even at 1.5 times higher concentrations DMF had no significant effect on either spontaneous or oxytocin-induced contractions of myometrium either from pregnant (n=5, data not shown), or from non-pregnant rats (n=5, data not shown).

All melatonin effects were reversible after a couple of washes (approximately in 30 minute) of myometrial strips with fresh Krebs solution (see Fig. 4).

Discussion

The results from this study indicate that melatonin inhibited spontaneous and oxytocin-induced contractions of rat myometrium from both pregnant and non-pregnant rats in a dose dependent manner. Addition of $PGF_{2\alpha}$ but not high KCl restored contractility of myometrial strips after melatonin-induced block and apamin treatment did dot make any change to inhibitory response to melatonin.

Our results are in agreement with the results of Gimeno *et al* [13]. Using myometrium samples from ovariectomised rats they showed that melatonin dose and time dependently inhibited spontaneous and oxytocin-induced contractions but not those induced by $PGF_{2\alpha}$. In our study inhibition of endogenous prostaglandin synthesis by treatment with indomethacin (1μg/mL) did not modify the response to melatonin but the addition of 1μM $PGF_{2\alpha}$ restored the contractions after melatonin-induced inhibition. Higher doses of indomethacin were not used because of its known inhibitory effect on spontaneous and oxytocin-induced myometrial contractions. In previous studies inhibitory actions of melatonin on myometrial contractions are attributed to its inhibitory effect on prostaglandin synthesis by the uterus. Indeed melatonin has been designated as a potent inhibitor of prostaglandin synthesis in various tissues including the uterus [13, 18–20]. The role of prostaglandins for parturition is well documented and although there are limiting site effects for clinical use due to their important roles in a wide variety of functions, inhibition of prostaglandin synthesis proved to decrease myometrial contractility [21].

Melatonin directly inhibited spontaneous contractions in our study in contrast to a previous study which demonstrated that melatonin did not have a direct effect on uterine contractility when used on its own but potentiated the contractile activity induced by norepinephrine in myometrium isolated from pregnant women [16]. This dissimilarity may be due to different effects of melatonin on species. It is also possible that the dose used in that study (up to 1 μM) may be insufficient to produce any inhibition.

Tissue- and species dependent variations of melatonin-induced responses in myometrium and in smooth muscle at large might be due to the presence of different melatonin receptor subtypes. Melatonin receptors have been shown to be expressed in endometrium of the rat uterus [22].

The melatonin effect may not always involve its interaction with specific receptors. It is shown that

melatonin produced concentration-dependent inhibition of porcine coronary artery, pulmonary artery and marginal artery of the colon pre-contracted with $PGF_{2\alpha}$ in a mechanism not involving currently known melatonin receptors and inhibition of cyclic GMP-specific phosphodiesterase [23].

Further studies for characterization of the role of melatonin receptors in inhibitory response to melatonin in rat myometrium using specific pharmacological receptor agonist and antagonists are necessary. But presently the lack of melatonin receptor subtype-selective agonists and antagonists prevents the full pharmacological characterization of these responses.

The controversial results obtained in the studies with melatonin may result from the use of different methods for dissolving melatonin. Ethanol has been used in other studies [15, 16] for dissolving melatonin. We found that ethanol caused inhibition o f contraction by its own but we found no effect of DMF on myometrial contractions. There are many reports relating to the effects of ethanol on uterine contractility [24]. Therefore; we used DMF which effectively dissolves melatonin and have no effect on uterine contractility at the concentration used in the present study.

The mechanism by which melatonin exerts its relaxing effect on myometrium remains to be clarified. Our finding that melatonin inhibited spontaneous contractions shows that melatonin may have direct effects on ion channels which are responsible for the pacemaker activity regulating contraction of myometrium or may change membrane potentials affecting the other ion channels.

Possible involvement of Ca^{2+}-activated K^+ channels in the inhibitory effect of melatonin on myometrial contractions was tested using selective small conductance Ca^{2+}-activated K^+ channel blocker apamin. It is known that in smooth muscles opening Ca^{2+}-activated K^+-channels may provide negative feedback to the excitation contraction coupling by hyperpolarizing the membrane potential and subsequently decreasing Ca-influx mediated by voltage gated calcium channels [25]. That apamin did not effect the inhibition by melatonin rules out the possibility of involvement of small conductance Ca^{2+} activated K^+ channels, which are sensitive to apamin. Ca^{2+}-cativated K^+ channels are highly selective to apamin which effectively blocks these channels at nanomolar concentrations [26]. In contrast to our findings apamin has reported to completely prevent melatonin-induced inhibition of ileal smooth muscle contractions [27] but only partially blocked the melatonin serotonin-induced contractions of rat gastric fundus [28].

Although we have no direct evidence, another possible mechanism of melatonin-induced inhibition of myometrail contractility is its interaction with calmodulin. Melatonin has been shown to bind with high affinity to Ca^{2+}-activated calmodulin [29]. Melatonin may prevent Ca-calmodulin complex from activation of myosin light chain kinase and thus, the contraction rate of myometrium may decrease. This hypothesis needs to be clarified by the studies regulating intracellular calcium and calmodulin.

As shown in the figures 5 and 6, the inhibitory action of melatonin on myometrium was found to be more effective in pregnant rats than non-pregnant ones. This effect may result from an increase in the sensitization of myometrium to melatonin by oestradiol near labor. Exogenous 17beta-estradiol augmented the effect of melatonin on MT2 receptors, resulting in vasodilatation of rat caudal artery [30]. A similar mechanism may play a role in uterus though MT2 receptors have not been shown to be present in uterus. There is additional evidence that estrogen modulates the effect of melatonin on smooth muscle contraction. Melatonin was found to be ineffective in untreated postmenopausal women whereas in hormone replacement therapy (HRT)-treated women, melatonin reduced systolic diastolic and mean blood pressure [31].

When high KCl (30 mM) was applied after melatonin-induced complete inhibition of contractions only modest contractions of myometrial strips, which were initially found to be responsive to high KCl (30 mM) application, were observed suggesting possible involvement of inhibition of voltage dependent Ca^{2+} channels in inhibitory effect of melatonin. Similar results were observed in rat ileal smooth muscle where Ca^{2+} channels were reported to be indispensable for melatonin-induced inhibitory response to occur [27]. To confirm the idea of the significant role of voltage dependent Ca^{2+} channels in melatonin-induced inhibition of myometrial contractility, electrophysiological evidence using isolated myometrial cells would be necessary.

Our finding that melatonin has been pharmacologically shown to inhibit myometrial contraction may have clinical importance. It has been proposed that the development of spontaneous abortions may result from a failure of the pineal gland to release melatonin sufficiently [32].

Melatonin may be useful for prevention of preterm labor. Melatonin is mainly synthesized in the pineal gland. It has been shown that melatonin is also synthesized in extrapineal tissues especially so-called APUD (amine precursor uptake decarboxylation) cells [33]. If melatonin is also synthesized in the uterus, it may have a more important role than estimated in pregnancy and labor. Melatonin may have a contribution to maintenance of pregnancy by directly relaxing myometrium.

In conclusion, melatonin inhibits spontaneous and oxytocin-induced contractions and this melatonin

action is may be due to inhibition of Ca^{2+} channels. Melatonin may have an important physiological role in control of pregnancy and in prevention of pre-term labor.

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