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Research Article

Regulatory Effect of Melatonin on the Proangiogenic Factors in Rat Retina in Experimental Oxygen-Induced Retinopathy

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Abstract

The oxygen-induced retinopathy was modeled in Wistar rat pups.

Experimental groups: Rats with untreated retinopathy, rats with retinopathy treated with melatonin, healthy controls. The pups received 0.1mg of melatonin per day intraperitoneally during 14 days after birth. Vitreous body and retinas were collected on 7, 14 and 21 postnatal days. Total protein and antioxidant activity were measured in vitreous. Homogenates of retinas were studied for the HIF-1α, VEGF-A and angiotensin II levels. These parameters can adequately characterize the permeability of hematoophthalmic barrier, oxidative stress and intensity of pathologic retinal neovascularization.

The development of oxygen-induced retinopathy was accompanied with a dramatic increase of protein and antioxidant activity in the vitreous body that indicate the pathologically increased permeability of the hemato-ophthalmic barrier. Concentrations of HIF-1α, VEGF-A and angiotensin II in retina on the 7 postnatal day were significantly higher than in healthy pups. This can be a trigger for the future aberrant angiogenesis in retina. In the melatonin treated group protein concentration and antioxidant activity in the vitreous body was equal to that in healthy rats. Retinal HIF-1α and VEGF-A levels on the 7 postnatal day in this group were significantly lower than in untreated animals.

These data demonstrate that melatonin effectively stabilize the hemato-ophthalmic barrier and suppress the aberrant retinal neovascularization that makes it a prospective agent for the therapy of the retinopathy of prematurity.

Keywords: Retinopathy of rematurity; Melatonin; HIF-1α; VEGF; Angiotensin II

Abbreviations

ROP: Retinopathy of Prematurity; OIR: Oxygen-Induced Retinopathy; VEGF: Vascular; Endothelial Growth Factor; HIF: Hypoxia-Inducible Factor; AT II: Angiotensin II; HOB: Hemato-Ophthalmic Barrier; AOA: Antioxidant Activity.

Introduction

Retinopathy of Prematurity (ROP) is a severe vasoproliferative eye disease and one of the leading causes of irreversible bilateral visual loss in preterm infants all over the world [1]. Today we see the increasing incidence of premature burths. Advances in neonatal care allow smaller and younger preterm newborns to survive, while very immature infants are predisposed to the most severe and therapy resistant ROP with poor functional outcome [2,3].

The ROP development is associated with a disturbance of normal retinal vascular development. The pathogenesis of ROP is divided into two discrete phases: phase of the arrest of vascular growth and phase of vasoproliferation. After burth a preterm infant is exposed to the relative hyperoxia compared with the intrauterine environment. Supplemental oxygen given to premature infants can lead to abnormally high oxygen saturation. Hyperoxia suppress the expression of proangiogenic factors and retinal vessel growth. Further increase of metabolic requirements of the developing but poorly vascularized retina and growing relative tissue hypoxia stimulate the expression of growth factors and hypoxia-mediated vasoproliferation. This is the start of the phase of abnormal retinal vascularization. Further it can regress spontaneously, but in severe causes it leads to the ingrowth of immature blood vessels into the vitreous body, preretinal membrane formation and traction retinal detachment [4,5,6].

The key role in the development of ROP is assigned to the imbalance of factors regulating retinal angiogenesis such as a number of growth factors, cytokines, hormones and intracellular matrix components forming a complex system [4,6,7]. The retinal vascularization of a preterm infant has to complete in the conditions of abnormal biochemical and immune homeostasis. Role of certain factors in different phases of ROP may vary considerably, that must be taken into account while searching for the new treatment approaches.

It is well known that Vascular Endothelial Growth Factor(VEGF) is critical for the retinal vascular development [7]. Phase I of ROP involves relative hyperoxia and decreased VEGF levels, whereas phase II involves relative hypoxia and increased VEGF levels [8]. The

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use of VEGF-neutralizing antibodies or VEGF receptor 2 (VEGFR-2) inhibitors in the rat model of oxygen-induced retinopathy showed that VEGF signaling through VEGFR-2 caused disordered divisions of endothelial cells and contributed to tortuosity and dilatation of retinal vessels, as in severe human ROP [5]. This fact gives a pathogenetic basis for the use of anti-VEGF agents for the treatment of ROP [6,9].

The main regulator of VEGF gene expression is Hypoxia-inducible factor-1 (HIF-1). It is a key transcription factor that mediates increased expression of hypoxia-regulated genes [10]. Active HIF-1 consists of two subunits: HIF-1α expression of which is increased in hypoxic tissue, and HIF-1β which is constitutively expressed. In normoxic conditions HIF-1 is unstable and degrade immediately while hypoxia leads to its stabilization, migration to the nucleus where it binds to the hypoxia response elements in the regulatory regions of many genes including VEGF gene [11].

Local retinal renin-angiotensin system, particularly angiotensin II (AT II), is one more important regulator of retinal angiogenesis in normal and pathologic conditions [12]. It stimulates pericyte migration, and endotheliocytes and smooth muscle cell proliferation [13]. In vivo and in vitro studies suggest, that AT II may act by stimulating VEGF synthesis and potentiating VEGF related effects [14].

It is impossible to study pathogenetic pathways of ROP in preterm infants. Because many newborn mammals complete their retinal vascularization postnatally they are widely used for modeling of ROP. The most widely used model of oxygen fluctuations is in the rat, in which oxygen levels fluctuate between 50% and 10% every 24 hours [15]. It is known as Oxygen-Induced Retinopathy (OIR) and leads to the pathologic changes in retinal vascularization similar to those in human ROP [16].

Melatonin is an endogenously produced indolamine synthesized in the pineal gland from the neurotransmitter serotonin. It plays a key role in a variety of important physiological functions, including regulation of circadian rhythms, visual, reproductive, cerebrovascular, neuroendocrine, and neuroimmunological actions [17]. As melatonin is normally produced by retinal cells [18], it must be well endured and have minimal side effects.

Lately we see the increasing interest for the antiangiogenic effect of melatonin in different tumors [19], and neovascular eye diseases, particularly age-related macular degeneration [20] and diabetic retinopathy [21]. It was demonstrated, that melatonin can decrease the retinal VEGF-A and HIF-1α levels in adult rats with experimental diabetic retinopathy [20], and rat pups with OIR [22].

Oxygen-induced oxidative stress is strongly associated with ROP, because retinal antioxidant reserve in preterm infants is not sufficient to provide protection against reactive oxygen species [5,23]. Many studies established the relationship between a high-oxygen saturation and abnormalities of retinal blood vessel development [24]. Melatonin is a potent direct free radical scavenger protecting tissues from damage by oxidative stress. Several its metabolites also have antioxidant properties [25]. Moreover, melatonin can stimulate expression of endogenous antioxidant enzymes superoxide dismutase, catalase, and glutation-peroxidase [26].

The proliferative phase of ROP is associated not only with the increase of expression of growth factors, but also with active synthesis of Tumor growth factor α, Interleukin 1βand other proinflammatory cytokines. All these genes are regulated by the transcription factor NF-κB. Melatonin can effectively suppress their expression via inhibiting of NF-κB activation [27], and this is one more pathway for the prevention of ROP development.

On this background we designed a study to investigate the association between oxidative stress, vascular growth factors, AT II, and melatonin in the rat model of OIR to achieve an experimental basis for using melatonin for the ROP therapy.

Materials and Methods

Animal care and induction of OIR

A study was conducted using 45 Wistar rat pups. All experiments were carried out in strict accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. All animals were maintained under standard laboratory conditions of temperature 22-24°C, relative humidity 60-70%, and 12 h light-dark cycle. They were fed a standard commercial pellet diet and water ad libitum.

We used the modified Penn model of OIR [15,28]. Just after birth, litters of Wistar rats and their mothers were exposed to environments that cycled between 60 and 15% oxygen every 12 h for 14 days. Then all rats were exposed to room air for 7 days. Control group was raised at room conditions for 21 day.

Grouping and melatonin treatment

All pups were divided into 3 batches, and each batch into 3 groups 5 animals each: healthy controls, untreated rats with OIR, melatonin treated rats with OIR. Melatonin (Sigma-Aldrich, USA) was dissolved in sterile 0,05M phosphate buffer solution pH7.4 containing 10% of dimetilsulfoxide and injected intraperitoneally daily for 14 days after burth. Each animal received 0.1 mg of melatonin per day. Controls received the equal amount of vehicle.

Sample preparation

On the 7, 14, and 21 day after burth one batch of pups (15 animals, 30 eyes) were euthanized. Eyes were enucleated immediately, dissected in the limb region, and liqud fraction of the vitreous body was aspirated with filter paper strips that were weighted before and after sampling. Soaked strip parts were placed in cold 0,05M PBS pH7.4 (20-fold volume) for 30 min for the eluation of vitreous body components, then centrifugated at 3000 g for 10 min, and the supernatants were stored at -70°C until biochemical analyses were done.

Retinas were extracted, placed in a cold lysis buffer (Lysis buffer-3, Cloud-Clone corp., USA) 200 µl per each retina, homogenized with a ultrasound homogenizator UP50H (Hielsher, Germany) for 10 sec (amplitude 122 µm, 125 V/sm^2), centrifugated at 3000 g for 10 min, and the supernatants were stored at -70°C until biochemical analyses were done.

Biochemical analysis

Antioxidant Activity (AOA) was estimated in vitreous eluate via chemiluminescent method in the hemoglobin-luminol-H_2O_2 model

system, with trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, Sigma-Aldrich, USA) as standard [29]. Results were expressed in equivalent trolox concentrations (μM trolox).

Enzyme-Linked Immunosorbent Assay (ELISA) was performed for determinations VEGF, HIF-1α, and AT II (ELISA kit for hypoxia inducible factor 1 alpha, ELISA kit for VEGF-A, ELISA kit for angiotensin II, Cloud-Clone Corp, USA) in retinal homogenates. The results for HIF-1α were expressed in nanograms per milligram protein (ng/mg protein) and for VEGF and AT II in picograms per milligram protein (pg/mg protein).

Total protein by Lowry [30] was measured in vitreous eluate, and retinal gomogenates.

Statistics

Data were analysed using Stastistica for Windows (version 10.0, Stat.Soft. Inc, USA). Mann-Witney test was applied with $p \leq 0.05$ considered statistically significant.

The data are expressed as the mean ±standard error of the mean (SEM).

Results

Total protein in vitreous

Total protein in vitreous of healthy rats decreased 1.5-fold from 7 to 14 postnatal day ($p<0.01$) and remained unchanged up to 21 day (Figure 1). The OIR development lead to the dramatic increase of vitreous total protein (1.5-3-fold) at all 3 time points, compared with healthy animals ($p<0.01$). The decrease of protein level from 7 to 21 postnatal day in this group was insignificant. Melatonin treatment caused statistically significant decrease of total protein level up to the one in healthy rats ($p<0.01$ against OIR group).

Antioxidant activity in vitreous

In healthy rats AOA in vitreous increased gradually from 7 to 21 postnatal day reaching finally 2-fold initial level ($p<0.01$) (Figure 2). In OIR group it rose dramatically on the 14 day ($p<0.01$) and then decreased on the 21 day remaining still 2 times higher than in healthy rats ($p<0.01$). AOA in the melatonin-treated group did not differ from that in healthy rats at all time points.

HIF-1α in retinal homogenate

Concentration of HIF-1α in retinas of healthy rats decreased gradually by 10% per 7 days. The decrease was small, but statistically significant: $p<0.05$ on the 14 postnatal day, and $p<0.01$ on the 21 day, compared to the initial level. Retinal HIF-1α in OIR group was significantly higher on the 7, and 14 day ($p<0.01$) (Figure 3). It decreased gradually from 7 to 21 day as in healthy animals ($p<0.01$ on the 21 day compared with 7 day). In the melatonin-treated group on the 7 day HIF-1α was higher than in controls ($p<0.02$), but lower than in OIR group ($p<0.01$). On the 14 day it did not differ from that in healthy animals. On the 21 postnatal day all groups had equal retinal HIF-1α concentration.

VEGF-A in retinal homogenate

In healthy animals VEGF-A level decreased from 7 to 14 postnatal day by 22% ($p<0.02$) and remained unchanged up to 21 day (Figure 4). In rats with OIR it was 1,5-fold higher on the 7 day ($p<0.01$) and normal on the 14 and 21 days. Melatonin-treated rats showed normal

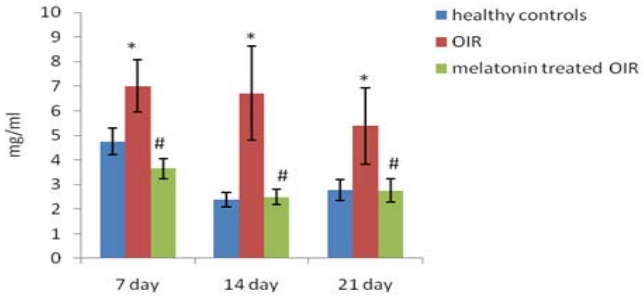

Figure 1: Total protein concentration in vitreous body of healthy rat pups, untreated rat pups with OIR, and pups with OIR receiving intraperitoneal injections of melatonin (mg/ml). * $p<0.01$ compared with healthy controls, # $p<0.01$ compared with untreated OIR group. OX axis -days after burth. The data are expressed as the mean ±standard error of the mean.

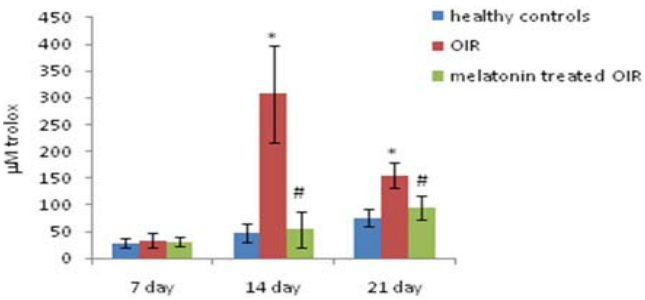

Figure 2: Antioxidant activity in vitreous body of healthy rat pups, untreated rat pups with OIR, and pups with OIR receiving intraperitoneal injections of melatonin (μM trolox). * $p<0.01$ compared with healthy controls, # $p<0.01$ compared with untreated OIR group. OX axis -days after burth. The data are expressed as the mean ± standard error of the mean.

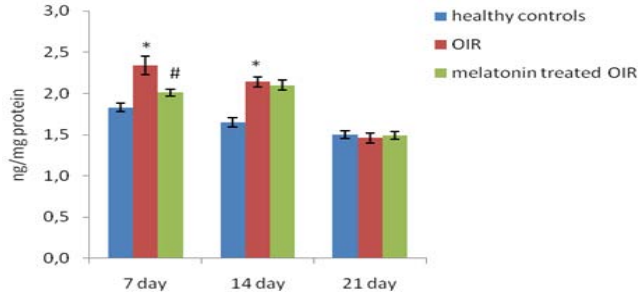

Figure 3: Concentration of HIF-1α in homogenates of retina of healthy rat pups, untreated rat pups with OIR, and pups with OIR receiving intraperitoneal injections of melatonin (ng/mg protein). * $p<0.01$ compared with healthy controls, # $p<0.01$ compared with OIR group. OX axis-days after burth. The data are expressed as the mean ± standard error of the mean.

VEGF-A level during all experiment.

Angoitensin II in retinal homogenate

Concentration of AT II in retinas of healthy rats increased gradually by 50% per 7 days ($p<0.01$ compared with the 7 day level) (Figure 5). In OIR group AT II level on the 7 postnatal day was significantly higher than in healthy rats ($p<0.01$). It remained unchanged on the 14 day and increased only by 25% on the 21 day ($p<0.02$ compared with 7 day level). Melatonin treatment lead to the 16% decrease of retinal AT II on the 7 day, but it was statistically insignificant. On the 21 day AT II concentrations in all the 3 groups

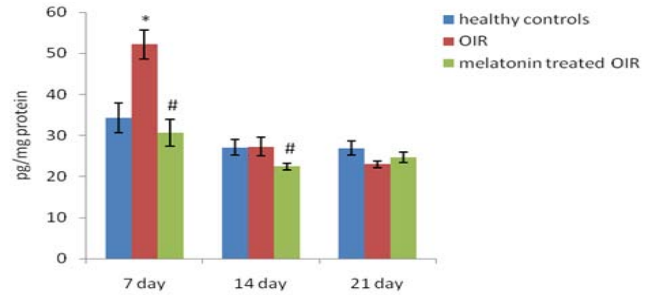

Figure 4: Concentration of VEGF-A in homogenates of retina of healthy rat pups, untreated rat pups with OIR, and pups with OIR receiving intraperitoneal injections of melatonin (pg/mg protein). * p<0.01 compared with healthy controls, # p<0.01 compared with untreated OIR group. OX axis-days after burth. The data are expressed as the mean ± standard error of the mean.

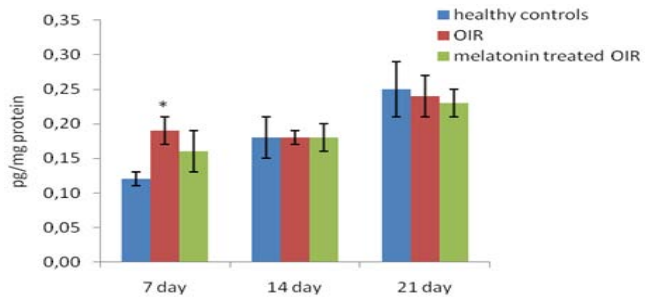

Figure 5: Concentration of angiotensin II in homogenates of retina of healthy rat pups, untreated rat pups with OIR, and pups with OIR receiving intraperitoneal injections of melatonin (pg/mg protein). * p<0.01 compared with healthy controls. OX axis -days after burth. The data are expressed as the mean ± standard error of the mean.

were similar.

Discussion

Experimental OIR pathogenesis involves two studies as human ROP: hyperoxia-induced suppression of retinal angiogenesis leading to the formation of avascular zones, and the stage of increasing hypoxia and pathological growth of aberrant blood vessels with immature Hemato-Ophthalmic Barrier (HOB) [31].

The present experiment, as several previous studies, using rat OIR model revealed, that developing retinopathy is accompanied with a significant increase of total protein in the vitreous body on the 7, 14, and 21 postnatal days. It has been already shown, that vitreal protein concentration in OIR increase prior to the beginning of neovascularization [32], and our histological study [28] proved, that it can reliably characterize the severity of retinopathy. Normal protein concentration in vitreous is very low, and such a significant increase can be due to the retinal vascular leakage.

Histological study showed, that on the 10 postnatal day rats with OIR have aberrant differentiation of retinal cells layers, and avascular zones in the posterior patina. On the 14 day there are apparent signs of the increased functional activity of retinal vessels endothelium, and expression of Proliferating Cells Nuclear Antigene in endothelial cells. On the 21 day we saw the pathologic ingrowth of new vessels through the inner border membrane into the vitreous [28].

The aberrant vessels leakage may be the cause of the dramatic increase of AOA in vitreous on the 14, and 21 days. On the 7 postnatal day groups do not differ in this parameter, but on the 14 day, after the start of active angiogenesis, AOA increase in spite of the hypoxia-induced oxidative stress. We suppose that a substantial amount of plasma protein antioxidants could transsudate through the imperfect HOB.

In the present study we saw a substantial increase of vascular growth factors levels in retina of rats with OIR on the 7 postnatal day-that is in the period of avascular zone formation [28], corresponding to the preclinical stage of human ROP. This is a noteworthy moment, when protein level in vitreous is already significantly higher than normal, but native antioxidants reserve is still sufficient to suppress the oxidative stress.

Melatonin has a complex of properties, that make it a potent blocker of pathologic angiogenesis. One of the main regulatory pathways of the process is one involving HIF-1α and VEGF. Hypoxia increases HIF-1α levels by inhibiting its ubiquitination and degradation by the proteasome, and HIF-1 complex upregulates the VEGF gene expression. But there are non-hypoxic stimuli inducing VEGF expression in vascular smooth muscle cells, and one of them is AT II that activates the HIF-1α expression via the NF-κB transcription factor [33]. The same pathway takes place in the case of HIF-1α upregulation by reactive oxygen species [34], and several proinflammatory cytokines like TNF-α and Interleukine-1 [35].

Melatonin can prevent the increase of HIF-1α expression due to direct inhibiting of of NF-κB nuclear translocation [27], and also by blocking of HIF-1α nuclear translocation that is necessary for the active HIF-1 formation [36]. It is well known as a potent reactive oxygen species scavenger, inhibitor of NO production, and activator of antioxidant enzyme expression [17,37], thus it can reduce HIF-1α upregulation via reducing oxidative stress.

The present study demonstrate, that melatonin decrease retinal HIF-1α, VEGF-A, and AT II levels on the 7 postnatal day i.e. in the preclinical phase of OIR. On the 14, and 21 days melatonin-treated rats showed no pathologic increase of protein concentration in vitreous, that indicate stabilization of GOB and normalization of vascular wall permeability.

Previously made histological study revealed the reduction of morphologic signs of OIR: on the 10 postnatal day we saw normal blood vessels in post equatorial retina, normal differentiation of retinal layers, on the 21 day there were no signs of extraretinal vasoproliferation [28].

The ability of melatonin to stabilize the blood-brain barrier was demonstrated on the cerebral injury model in mice [38]. The HOB has similar structure. One of the key factors influencing its permeability is the matrix metalloprotease-9 (MMP-9) activity, that degrade vascular endothelial cadherin and occluding-main components of adherent and tight junctions between endothelial cells, and disrupt the integrity of the endothelial barrier resulting in the increase of vascular permeability [39]. Melatonin stabilize tissue barriers suppressing the MMP-9 expression by NF-κB-dependent pathway, and upregulating the expression of tissue metalloprotease inhibitor [39,40]. Kaur et al. showed that melatonin administration reduced the abnormal retinal

vascular permeability induced by hypoxia [41]. Our study revealed the statistically significant decrease of vitreous protein in melatonin-treated rats, that indicate the stabilization of GOB in this group.

The 7 postnatal day was noteworthy for retinal AT II concentration also. It was apparently increased in rats with OIR, and significantly lower in the melatonin-treated group, although not normal. AT II is one of the angiogenesis regulators [13, 33] and such an increase may be in response for the avascular zone formation.

Local ocular renin-angiotensin system plays an important role in the pathogenesis of neovascularization in the ROP [13]. Treatment of rats with OIR angiotensin-converting enzyme inhibitors and AT1 receptor antagonists prevented retinal neovascularization [42], while 2 week administration of AT II resulted in 2-fold increase of VEGF expression in rat retina [43].

Thus we can suppose, that injections of melatonin in ROP human can prevent development of severe complications by normalizing retinal angiogenesis via the downregulation of proangiogenic factors.

Melatonin is important for the normal retinal vascularization and glial maturation [24,44]. Human fetus does not secrete melatonin, but receives melatonin from mother's blood [45]. Normally melatonin secretion commences at about 12 weeks after burth, but in preterm infants it is delayed 3-6 weeks, and even 52 weeks after burth they have significantly lower melatonin level than full-term infants of the same age [46].

Conclusion

The OIR development in rats is accompanied by the statistically significant increase of vascular growth factors in retina on the 7 postnatal day. Abnormal retinal vascularization leads to the dramatic increase of total protein concentration and AOA in vitreous body due to the increased permeability of HOB.

Intraperitoneal injections of melatonin during the first 14 postnatal days prevent aberrant retinal vascularization via the downregulation of HIF-1α, VEGF-A and AT II in retina, suppress oxidative stress, and stabilize the HOB.

Our findings provide evidence, that melatonin can be effective for the preventing of ROP development in preterm infants. Since the most pronounced decrease of vascular growth factors levels in retina was detected on the 7 postnatal day, we suppose, that melatonin therapy will be more effective in the early postnatal period.

It is noteworthy that melatonin is a natural hormone, and it has been proven to be safe for neonates [47]. Because of its lipophilic properties it can easily cross most physiological barriers including the fetal-placentar barrier [45], that extend the spectrum of its administration ways.

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