INTRODUCTION

The pineal gland is part of a photoregulatory system transducing photic input into neuroendocrine output, secreting melatonin (MEL), an important integrator of reproductive rhythms (1, 2). Changes in the duration of MEL secretion are evident in seasonally breeding animals, whose reproduction is influenced by seasonal variation in the duration of night and day (3). Maximum production occurs during nocturnal darkness and the duration and amplitude of MEL levels are negatively correlated with photoperiod length (3, 4). Melatonin is highly lipophilic and its release and circulating levels reflect the rate at which it is synthesized (5, 6). Light’s suppression of MEL production, whether during natural daytime or during interventional nocturnal exposure, is thought to be mediated by levels of arylalkylamine N-acetyltransferase (AANAT) (7, 8). Wehr et al. (9) have shown that patients with seasonal affective disorder exhibit seasonal variation in a physiological system that is known to regulate seasonal behavior in other mammals. The duration of the nocturnal period of active melatonin secretion in patients with SAD was longer in winter than in summer but in healthy volunteers there was no change. These findings suggested that neural circuits that have been shown to mediate seasonal behavior in mammals may also mediate pathogenesis of winter depression in humans.

The pineal gland of mammals, in contrast to lower vertebrates, does not respond directly to light, but is controlled by light via neuronal phototransduction originating in the retina. This signal travels through the optic nerve and is processed by the hypothalamic circadian pacemakers: the suprachiasmatic nuclei (SCN) providing time of day information (10-12). Melanopsin has been found to be the prime, but not sole, photoreceptor in this neuronal pathway (13, 14). A complementary theoretical model of humoral phototransduction proposes that tetrapyrrole-based molecules absorb photons and carry light’s time and energy signal via local blood circulation from eyes to brain (15). As the mechanism of light’s antidepressant effects remains unclear (16-19), this process may be of mechanistic importance. This model indicates a physiological function for light’s well-known capacities to stimulate heme-based enzymes to form reactive gases and to cause dissociation of gases such as nitric oxide (NO) and carbon.
domesticated pig (Sus scrofa domestica) exhibit strong seasonal behavioral and physiological changes from the winter solstice (second half of December) and short photoperiod days near the summer solstice (second half of June) and long photoperiod days near the summer day than during summer night (21). To explore this potential mechanism, we studied serum melatonin levels and related gene and protein expression in winter and summer in three conditions: control, ophthalmic venous infusion of plasma that was chemically enriched with CO, and ophthalmic venous infusion of blood that was enriched with CO by direct illumination. Specifically, we conducted in vivo experiments to determine the effect on serum MEL levels and gene expression of hydroxyindole-O-methyltransferase (HIOMT), arylalkylamine-N-acetyltransferase (AANAT) - the key enzymes in MEL synthesis - after two experimental imitations of the physiological increase in the CO concentration in the cavernous sinus of the mammalian perihypophysal vascular complex. In one condition we infused autologous blood plasma into the ophthalmic venous sinus with a chemically supplemented approximately 3-fold increase in the concentration of CO compared to the physiological state. In the other condition we infused autologous blood with a light-induced approximately 3-fold increase in the concentration of CO. CO dosing was based on the results obtained by Koziorowski and colleagues in which CO concentration in ophthalmic venous blood was approximately three times higher during the summer day than during summer night (21). To explore the chronic direct effect of these interventions, the experimental infusions were administered for 48 hours continuously. The animal model we used were mature males crossbred from a male wild boar (Sus scrofa) and a female domesticated pig (Sus scrofa domestica). The hybrid inherits strong seasonal behavioral and physiological changes from the boar and relative docility from the pig, creating a workable model for studies of seasonality. The experiments were performed during long photoperiod days near the summer solstice (second half of June) and short photoperiod days near the winter solstice (second half of December).

METHODS AND MATERIALS

Animals

These experiments were approved and conducted according to guidelines established by the Local Ethics Committee on Animal Experimentation in Lublin No. 8/2007. Mature males of a wild boar and pig crossbreed (age 12 months, body mass ~100 – 120 kg) from the Experimental Farm Branch Campus of the Faculty of Biotechnology, University of Rzeszow, Kolbuszowa, Poland were used. Animals were kept under natural illumination, assigned to individual pens for accommodation one week before experimental treatment, fed ad libitum and had free access to water.

During late June, the animals were maintained in an open-sided shed and exposed ad libitum to approximately 30,000 lx of daytime natural illumination. The mean ambient temperature was 24°C during the light phase and 12°C during the nocturnal phase. During late December, the animals were housed in a windowed room and exposed to between 40 and 50 lx of daytime natural illumination. Mean temperature during the day and night was 12°C.

Experimental schedule

36 animals were randomly assigned to three experimental groups.

Experiment 1 (Control). Animals were kept in natural photoperiodic conditions, one group (n = 6) during the longest days of summer (June) and another (n = 6) during the shortest days of winter (December). The animals received autologous blood infused into the ophthalmic venous sinus (OphVS) for 48 hours at a rate of 8.3 ml/h.

Experiment 2. Animals received autologous blood plasma infused into the OphVS for 48 hours with an experimentally induced increase in CO concentration (see below). The identical procedure was performed during the longest days of summer (June; n = 6) and during the shortest days of winter (December; n = 6).

Experiment 3. The animals received autologous blood infused into the ophthalmic venous sinus for 48 hours with an experimentally-induced increase in the CO concentration achieved by two-hour bright light exposure (see below). This experimental treatment was repeated during the longest summer days (June; n = 6) and during the shortest winter days (December; n = 6).

Preparation of autologous plasma with elevated concentration of carbon monoxide and its infusion in this experimental group

Systemic venous blood was repeatedly collected under sterile conditions from each animal. Heparinised blood (10 IU/ml, Polfa, Poland) was centrifuged (1000 g, 20 min) and plasma was transferred to a sealed glass container (50 ml). Plasma concentration of CO in the ophthalmic venous blood (OphVB) was estimated (22). The average concentration was 1.2 nmol/ml and 0.9 nmol/ml in June and December, respectively. Plasma was supplemented with chromatographically pure CO (0.8 cm³ to each portion) and stirred with a roller for 30 min, and the concentration of CO was measured again. The autologous plasma, with increased concentration of CO, up to 4.5 nmol/ml in June and 3.1 nmol/ml in December (Table 1), was infused at a rate of 8.3 ml/h with the use of a pump (SEP 21S, Ascor, Poland) for 48 hours into the ophthalmic sinus (OphS), from

Table 1. Mean ± S.E.M. values of carbon monoxide concentration (nmol/ml) in autologous blood (control) and in autologous plasma supplemented with chromatographically pure CO, ***P ≤ 0.001.

<table>
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<th>Concentration of CO (nmol/ml)</th>
<th>Concentration of CO (nmol/ml)</th>
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<tr>
<td></td>
<td>in autologous blood (control)</td>
<td>in autologous plasma supplemented with chromatographically pure CO</td>
</tr>
<tr>
<td>Summer</td>
<td>1.2 ± 0.1</td>
<td>4.5 ± 0.5***</td>
</tr>
<tr>
<td>Winter</td>
<td>0.9 ± 0.1</td>
<td>3.1 ± 0.1***</td>
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which the venous blood flowed into the venous cavernous sinus (VCS) of the perihypophyseal vascular complex (PVC). The autologous blood cells remaining after the collection of plasma were mixed with Ringer’s solution in a volume equivalent to the collected plasma. The suspension was stirred with a roller for 30 min and then continuously infused into the external jugular vein (JV) as a protection against anemization.

**Preparation of autologous blood with elevated concentration of carbon monoxide, achieved by bright light exposure and its infusion in this experimental group.**

Systemic venous blood was repeatedly collected under sterile condition from each animal and heparinised (10 IU/ml, Polfa, Poland). Concentration of CO in blood was estimated using a standard addition method (21). The average concentration was 1.51 nmol/ml and 0.89 nmol/ml in June and December, respectively. This autologous blood was pumped at 8.8 ml/hour through a syringe into a spiral clear plastic cannula wrapped around a standard 2.5 cm diameter illuminated white fluorescent bulb (Narva LT-T8 Standard). The spiral cannula was placed approximately 20 cm between two lamps with white light-emitting diodes (LEDs) (Lumie Desklamp). Blood exiting the spiral cannula drained via an external nasal vein into the animal’s ophthalmic venous sinus (Fig. 1). Measured illuminance at the surface of the cannula was approximately 10,700 lux. This illuminance is comparable to that used to treat winter depression and was intended to represent the natural summertime increase in light that we have observed associated with elevated CO in ophthalmic venous blood in these animals. After two-hours bright light exposure the concentration of CO was measured again. The autologous blood, with increased concentration of CO, up to 5.1 nmol/ml in June and 2.1 nmol/ml in December (Table 2), was infused at rate of 8.3 ml/h with use of a pump (SEP 21S, Ascor, Poland) for 48 hours into OphS.

**Surgical procedures and blood sample collection.**

Infusion of solutions to experimental animals and collection of blood samples was by catheterization. The animals were not fed for 12 hours before surgery. They were pre-medicated with 0.05 mg/kg LM of atropine (Biowet, Gorzow Wielkopolski, Poland) followed 10 min later by 2 mg/kg LM. of azaperone (Janssen Pharmaceutica, Beerse, Belgium). Once sedation had occurred, anesthesia was induced by intravenous administration of about 10 mg/kg of thiopental (Sandoz GmbH) into an ear vein. Silastic catheters (o.d., 2.4 mm; i.d., 1.8 mm) were inserted: 1) into the external jugular vein for thiopental administration to maintain a deep level of anesthesia during surgery and collection of systemic blood samples during the experimental procedure for analysis of MEL; 2) into the dorsal nasal vein in a cephalic direction through the angular vein of the eye to reach the OphS, from which the venous blood flowed into the VCS of the PVC, for infusion of autologous blood or autologous blood plasma. Catheters were fixed to the skin on the back of the animals to collect blood samples with minimal stress for them.

All animals were sampled at 2-h intervals for 48 hours. Blood samples (10 ml) were obtained via external jugular venous catheters and collected into heparinized tubes. Samples were immediately centrifuged at room temperature and blood plasma was decanted and stored at ~20°C until analyzed for MEL content. To facilitate sampling during the night, dim red penlights were used only during passing through the barn, and direct light exposure of the animals’ eyes was avoided.

**Pineal gland tissue.**

In order to analyze the effect of CO on Hiomt and Aanat mRNA and HIOMT and AANAT proteins levels after the end of experiments, the animals of the control (Control) and experimental groups (CO, Light) were sacrificed during the summer day (n = 3), during the summer night (n = 3), during the winter day (n = 3) or during the winter night (n = 3). Pineal gland tissue was immediately dissected and frozen (in liquid nitrogen) prior to RNA isolation and protein extraction.

![Fig. 1. Schematic drawing of blood-illumination apparatus showing 1 - pump, 2 - syringe, 3 - cannula, 4 - light sources, and 5 - nasal vein drainage to the ophthalmic venous sinus (illustration by Jeanette Kuvin Oren).](image)

**Table 2.** Mean ± S.E.M. values of carbon monoxide concentration (nmol/ml) in autologous blood (control) and in autologous blood after white light illumination; **P ≤ 0.01; ***P ≤ 0.001.

<table>
<thead>
<tr>
<th></th>
<th>Concentration of CO (nmol/ml) in autologous blood (control)</th>
<th>Concentration of CO (nmol/ml) in autologous blood after illumination with white light</th>
</tr>
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<tbody>
<tr>
<td><strong>Summer</strong></td>
<td>1.5 ± 0.1</td>
<td>5.1 ± 0.4**</td>
</tr>
<tr>
<td><strong>Winter</strong></td>
<td>0.9 ± 0.1</td>
<td>2.1 ± 0.1**</td>
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**Table 3.** Primer sequences for genes encoding the key enzymes of melatonin (Aanat and Hiomt) and Gapdh for real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer forward (5’→3’)</th>
<th>Primer reverse (5’→3’)</th>
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<tbody>
<tr>
<td>HIOMT</td>
<td>CATGCGTGTCCCGAGGTTCTCT</td>
<td>CAGCTTCAAGGACACACAGA</td>
</tr>
<tr>
<td>AANAT</td>
<td>TGGAATTGACACGCAATAGA</td>
<td>TGGAACGCTTGCTCATATTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGTCCTGAGACAGATGTTG</td>
<td>CCCGATGCAGCCAAAT</td>
</tr>
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</table>
Hormone analyses

Plasma melatonin was analyzed by radioimmunoassay (Buhlmann Laboratories AG, Schoenbuch, Switzerland). Duplicate aliquots (400 µl) of standards, extracted controls and extracted plasma samples were pipetted into the tubes, followed by 100 µl of anti-melatonin antiserum (Kennaway G280); caprine against melatonin conjugated to bovine thyroglobulin, and 100 µl of the 125I-melatonin tracer. The tubes were then incubated for 20 h at 2 – 8°C. While stirring the second antibody, 100 µl of the suspension was added to the tubes, after which they were incubated at 2 – 8°C. After 15 min 1 ml of cold, distilled water was added to the tubes, which were then centrifuged at 2 – 8°C. After 15 min the supernatant was removed and the radioactivity of the tubes was counted in a gamma counter for 2 min. Selected samples were reanalyzed on a later occasion, in order to ensure assay repeatability.

Total RNA extraction and reverse transcription

Total RNA was extracted from samples/pineal organs using the TRI Reagent (Invitrogen) procedure (23), and precipitated with ethanol. Each RNA pellet was dissolved in RNase-free water and the quantity and quality of RNA was assessed spectrophotometrically at 260 and 260/280 nm, respectively (NanoDrop, Thermo Scientific).

Extracted RNA (1 µg) was reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems).

Real time RT-PCR

Messenger RNA (mRNA) levels of Hiomt and Aanat were determined by real-time quantitative PCR using the SYBR Green method (Power SYBR Green Master Mix, Applied Biosystems). Oligonucleotide primer pairs (Table 3) were constructed based on the sequence of the GenBank using Primer3 software. The results for the genes were analyzed on the StepOnePlus System (Applied Biosystems) and normalized against the housekeeping gene - glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (24).

Western blot analysis of proteins

Total tissue proteins were acetone precipitated from the interphase and organic phenol-chloroform phase remaining after RNA isolation. Protein concentration in the supernatant was determined using bicinchoninic acid (BCA Protein Assay Kit, Pierce Biotechnology). Samples containing 50 µg of protein were fractioned by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Non-specific binding was blocked for 30 min with 10% BSA (bovine serum albumin) in Tris-buffered saline with 1% Tween 20 (TBST). Then membranes were incubated overnight at 4°C with polyclonal rabbit anti-HIOMT and anti-AANAT antibodies (1:200 dilution, Santa Cruz Biotechnology). The membranes were then washed with TBST and incubated for 60 min at room temperature with goat horseradish peroxidase-conjugated anti-rabbit IgG (1:10000 dilution, Santa Cruz Biotechnology). Antibody detection was performed with an enhanced chemiluminescence reaction (BM Chemiluminescence Western Blotting Kit, Roche). HIOMT and AANAT signals were visualized and quantified with the Fusion FX7 System and Fusion-Capt software (Vilber Lourmat). To confirm equal loading of the samples on the gel, the blots were incubated for 30 min with mouse anti-GAPDH antibody (1:1000 dilution, Santa Cruz Biotechnology) and then processed as described above. The density of each HIOMT and AANAT protein band was normalized to the density of GAPDH band.

Statistical analysis

All the results were plotted as mean ± S.E.M. Two-way ANOVA was used to compare groups regarding the factors „season”, treatment (control, CO, Light) and the interaction between them. When no significant interaction between factors existed, each factor was analyzed separately by one-way ANOVA. Post hoc analysis was performed using the Bonferroni multiple comparison test. Linear regression analyses were performed on the duration of elevated hormone concentrations relative to the amount of total AANAT protein in pineal glands. Effects were considered statistically significant if P ≤ 0.05. All statistical tests and graphics were done using GraphPad Prism version 5.01 for Windows. Melatonin profiles were fitted with the following equation:

\[ y = Y_0 + \left( \frac{Y_{max}}{1 + \exp\left( A \times (x - DT50) \right)} \right) \times \left( 1 + \exp\left( B \times (x - DT50) \right) \right) \]

where \( y \) was the \( n \)th data point, \( x \) the time point of the \( n \)th point, \( Y_0 \) basal level measured during daytime, and \( Y_{max} \) the maximum of the nocturnal peak. IT50 was defined as the time point at which 50% of the maximal increase in MEL level was reached. Similarly, the time at which 50% of the decrease was reached was called DT50. A and B corresponded respectively to the slope values at IT50 and DT50 of the fitted curve through the data points. IT50 and DT50 were used to characterize timing of the onset and offset of the MEL peak respectively. The duration and amplitude of the MEL peak were defined as the differences between DT50 and IT50 and Ymax and Y0, respectively (25).

RESULTS

Analysis of day/night and summer/winter rhythms of plasma melatonin

Seasonal changes of mean serum MEL concentration in animals are presented in Fig. 2. All animals exhibited a clear rhythm in MEL secretion with high MEL concentration occurring during the dark phase. In the control group no difference in nocturnal MEL amplitude between summer and winter was found. The mean duration of increased nocturnal MEL secretion in summer and winter was 8.2 ± 0.2 and 16.5 ± 0.3 h, respectively (difference between the duration of the MEL peak 8.3 ± 0.2 h; P < 0.001). Significant differences were detected between control and two experimental group (CO and Light) for its 24-h profile of melatonin secretion (two-way ANOVA, P < 0.001); also, a significant interaction between treatment (CO, Light) and time of the year was found (two-way ANOVA, P < 0.001). After infusion of autologous plasma with increased CO we observed significant changes in MEL amplitude dependent on photoperiod. During the winter’s long nights, mean MEL levels decreased in association with CO infusion (control group 59 ± 2 versus CO infusion group 36 ± 2 pg/ml; P < 0.01). (Winter control amplitude was 39 ± 5 pg/ml; winter CO amplitude was 18 ± 3 pg/ml; difference between means was 21 ± 4 pg/ml; P < 0.05). Conversely, from the same experimental procedure during the summer’s short nights, mean MEL levels increased in association with CO infusion (control group 52 ± 2 versus CO infusion group 61 ± 3 pg/ml; P < 0.05). During the day we did not observe significant seasonal differences in MEL concentration associated with CO infusion. The spectral variation after infusion of irradiated autologous blood, the duration of the MEL peak during the winter night was shortened versus the control group (winter control 16.5
± 0.3 h; winter illuminated 14.1 ± 0.4 h; difference between means was 2.3 ± 0.4 h; P < 0.05). During the summer we did not observe significant difference in MEL concentration and peak duration after infusion of illuminated autologous blood.

Analysis of day/night and seasonal rhythms of pineal Aanat and Hiomt mRNA and protein

Expression of the enzymes AANAT and HIOMT responsible for the generation of MEL was detected in the pineal glands in both day and night samples in both seasons. Both seasonal and treatment (CO, Light) variation in AANAT and HIOMT expression were revealed by two-way ANOVA analysis of the data obtained. In untreated animals a day/night rhythm in Aanat mRNA was detected (Fig. 3). A significant interaction between season and treatment was found (two-way ANOVA, P < 0.001). After infusion of autologous plasma with increased CO concentration, Aanat mRNA significantly increased during the winter day (two-way ANOVA, winter day CO 1.69 ± 0.03 versus winter day 1.00 ± 0.09 RQ; P < 0.01); day/night rhythm in Aanat mRNA was detected (winter day CO 1.69 ± 0.03 versus winter night CO 1.99 ± 0.06; P < 0.01
and summer day CO 1.11 ± 0.12 versus summer night CO 1.70 ± 0.01; P < 0.01) (Fig. 3). In the third experimental group, after 48 hour infusion of light-irradiated autologous blood, Aanat mRNA decreased during the summer day (two-way ANOVA, control 1.33 ± 0.10 versus irradiated 0.69 ± 0.05 RQ; P < 0.01) and at night both in summer (two-way ANOVA, control 1.82 ± 0.08 versus irradiated 0.37 ± 0.06 RQ; P < 0.001) and winter (two-way ANOVA, control 1.57 ± 0.17 versus irradiated 0.58 ± 0.05 RQ; P < 0.001); a rhythm in Aanat mRNA was detected with day level approximately twofold greater than night level (Fig. 3). In contrast to the pattern of Aanat mRNA, a significant seasonal variability in AANA T immunoreactive protein was evident, with winter values approximately 2-fold greater than summer values in the pineal (winter day 1.80 ± 0.21/winter night 2.53 ± 0.21 versus summer day 0.99 ± 0.09/summer night 1.27 ± 0.11 OD). A day/night difference in AANAT was observed only during the winter (winter day 1.80 ± 0.02 versus winter night 2.53 ± 0.21 OD; P < 0.05) (Figs. 4 and 5). No interaction was found between two factors (season and treatment) (two-way ANOVA, P > 0.05). Total pineal AANAT protein decreased during the winter day (winter day 1.80 ± 0.21 versus winter day CO 1.01 ± 0.09 OD; P < 0.01) and night (winter night 2.53 ± 0.21 versus winter night CO 1.20 ± 0.11 OD; P < 0.01) and increased in summer day (summer day 0.99 ± 0.09 versus summer day CO 1.97 ± 0.34 OD; P < 0.05) and night (summer night 1.27 ± 0.11 versus summer night CO 1.92 ± 0.11 OD; P < 0.01); day and night AANAT protein content were similar (Figs. 4 and 5). Results for AANAT protein levels in the „Light“ experimental group were accidentally lost.

**Fig. 4.** Western blot analysis of AANAT isolated from pineal glands collected either at midday (Short-Day; Long-Day) or at midnight (Long-Night; Short-Night) in control group and after a 48 h infusion to the ophthalmic venous sinus with autologous blood plasma with approximately 3-fold increased of CO concentration, performed by supplementation of autologous blood plasma with gaseous CO, during the Winter (A) and Summer (B). Data are shown as the mean and S.E.M., *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA analysis, Bonferroni post hoc. N = 6 animals per group.

**Fig. 5.** Immunoblots of AANAT (23 kDa), HIOMT (38 kDa) and GAPDH (37 kDa) from eight representative subjects used in the analysis. Each well was loaded with 50 µg of total protein.
Day/night rhythm in pineal *Hiomt* mRNA was detected in summer with night level greater than day level (summer night 1.76 ± 0.07 versus summer day 1.20 ± 0.03 RQ; P < 0.05) (Fig. 6). A significant interaction between season and treatment was found (two-way ANOVA, P < 0.001). After infusion of autologous plasma with increased CO concentration *Hiomt* mRNA level decreased during the summer day (two-way ANOVA, summer day 1.20 ± 0.03 versus summer day CO 0.83 ± 0.02 RQ; P < 0.01) and night (two-way ANOVA, summer night 1.76 ± 0.07 versus summer night CO 1.21 ± 0.06 RQ; P < 0.001) and similar to the control group, the summer rhythmic pattern of *Hiomt* mRNA was maintained (summer day CO 0.83 ± 0.02 versus summer night CO 1.21 ± 0.06 RQ; P < 0.05) (Fig. 6). In the third experimental group a difference in *Hiomt* mRNA was observed; *Hiomt* mRNA level decreased in summer day (two-way ANOVA, control 1.20 ± 0.03 versus irradiated 0.75 ± 0.03 RQ; P < 0.001) and night (two-way ANOVA, control 1.76 ± 0.07 versus irradiated 1.32 ± 0.07 RQ; P < 0.001) but a rhythmic pattern of *Hiomt* mRNA was maintained (Fig. 6). Day and night HIOMT protein values were similar; summer/winter differences were apparent only during the night (winter night 2.12 ± 0.22 versus summer night 1.48 ± 0.19; P < 0.05) (Figs. 7 and 5). No interaction for HIOMT protein values

![Hydroxyindole-O-methyltransferase (Hiomt) mRNA](image1)

*Fig. 6. Changes in the pineal gland in the circadian expression at the transcript level of *Hiomt* in control group, after a 48 h infusion to the ophthalmic venous sinus with autologous blood plasma with approximately 3-fold increased of CO concentration, performed by supplementation of autologous blood plasma with gaseous CO and after a 48 h infusion to the ophthalmic venous sinus with autologous blood with approximately 3-fold increased CO concentration, performed by white light illumination of autologous blood by white light, during the Winter (A) and Summer (B). Data are shown as the mean and S.E.M., **P < 0.01; ***P < 0.001, two-way ANOVA analysis, Bonferroni post hoc. n = 6 animals per group.*

![Hydroxyindole-O-methyltransferase (HIOMT) protein](image2)

*Fig. 7. Western blot analysis of HIOMT isolated from pineal glands collected either at midday (Short-Day; Long-Day) or at midnight (Long-Night; Short-Night) in control group and after a 48 h infusion to ophthalmic venous sinus with autologous blood plasma with approximately 3-fold increased of CO concentration, performed by supplementation of autologous blood plasma with gaseous CO, during late December (A) and late June (B). Data are shown as the mean and S.E.M.*
was found between two factors (season and treatment) (two-way ANOVA, P > 0.05). After infusion of autologous plasma with increased CO concentration HIOMT protein level did not change during summer; in winter we observed increase in day (winter day 1.32 ± 0.24 versus winter day CO 2.20 ± 0.18 OD; P < 0.05) and night (winter night 2.12 ± 0.22 versus winter night CO 3.01 ± 0.21 OD; P < 0.05) protein levels. A day/night rhythm in pineal HIOMT protein content was detected only during the winter (winter day CO 2.20 ± 0.18 versus winter night CO 3.01 ± 0.21 OD; P < 0.05) (Figs. 7 and 5). Results for HIOMT protein levels in third experimental group were accidentally lost.

In the control group and the experimental group after infusion of autologous plasma with increased concentration of CO during the winter (short-day and long-night) and during the summer short-night there was a positive correlation between total AANAT protein in pineal glands and circulating MEL (r = 0.86, P < 0.05).

**DISCUSSION**

Prior results indicate that the gasotransmitter CO is released from the eye into ophthalmic venous blood depending on the intensity of sunlight (21). This study was designed to determine whether an increased concentration of CO in ophthalmic venous blood affects the synthesis of MEL, and therefore, whether CO released from the eye under ambient light can be a carrier of a light signal, a form of humoral phototransduction (15). As the experimental infusions were administered for 48 hours continuously and did not vary over 24 hour periods as do normal environmental light conditions, the effects that we have demonstrated represent physical changes observed, but are not necessarily representative of normal physiological changes. Paradoxically, the effects of the administration of CO or illumination of blood upon MEL and levels of its producing enzymes AANAT were not consistent between winter and summer. Whether this is due to the non-physiologically timed administration of CO or light is unclear. It is also possible that the use of different individual animals between the summer and winter studies may have contributed to variability in the results. Our data replicate the prolonged release of free CO in extracorporeal blood by bright light observed by Haldane and Smith (26) and confirm that such increases in free CO levels occur in blood alone and do not require retinal tissue.

All experimental groups exhibited a robust circadian serum MEL rhythm with high concentrations occurring during scotophase (period of darkness). We have noted the difference in the scotophase MEL response in term of mean concentration of increased MEL levels after 48 hours infusion of autologous blood plasma with an experimentally induced approximate 3-fold increase in the concentration of CO into the ophthalmic venous sinus (Fig. 2). During winter this treatment limited the nocturnal MEL rise. During the summer this same treatment enhanced the nocturnal MEL rise. An increase in nocturnal MEL amplitude was shown by Takasu et al. (27) after exposure to daytime bright light for 7 days of male subjects (20 – 29 year old). They proposed that daytime bright light, through the retinohypothalamic tract and SCN, exerted prolonged stimulation of the sympathetic nerves and activated β-adrenergic receptors of the pineal cells to increase MEL secretion. In our model, during the summer, the animals were stimulated by at least two overlapping signals: natural light acting on the retina and stimulating the retinohypothalamic tract and CO flowing into the ophthalmic venous sinus, which we interpret as carrying information about light intensity and timing. Support for our hypothesis that CO can participate in this regulation is provided by overexpression of inducible HO (HO-1) and increase in HO-1 protein in the retina in response to intensive light in vivo (28).

In the third experimental group, where an approximate 3-fold CO concentration increase in infused autologous blood was achieved by illumination with white light, during the summer we did not observe changes in nocturnal MEL amplitude. We can speculate that the period of infusion of illuminated autologous blood (48 hour) was too short to affect the nocturnal MEL amplitude. For example, Takasu et al. (27) observed an increase in the MEL level after seven days of repeated daytime bright light stimulation. An additional factor that differentiates this illuminated light experimental system from the infusion of CO in blood plasma is that this *ex vivo* irradiation may well have caused other changes in the blood besides the presumed CO production. Interestingly, in this light-treated blood group the level of Aanat mRNA was significantly lowered in comparison to the control and CO-treated groups both in June and December. This condition had little effect on nocturnal MEL amplitude, suggesting that the remaining level of Aanat mRNA was still sufficient to allow AANAT activation and MEL synthesis.

In the control group, we did not observe a marked summer/winter variation in the mRNA levels of Aanat (Fig. 3) and Hiomt (Fig. 6). Also changes in the amplitude of the MEL peaks after CO infusion did not correlate with the expression of these enzymes (Figs. 2, 3 and 6). Instead, changes in MEL levels were always associated with parallel changes in AANAT protein levels (Figs. 2 and 4). HIOMT protein level was elevated during the December night after CO infusion but this variation did not appear to modify MEL synthesis in the crossbred animal model (Figs. 2 and 7). This indicates that the photoperiodic variation in the MEL synthesis in these animals might be driven by AANAT rather than HIOMT and that regulation of MEL production occurs by cAMP inhibition of degradation by proteasomal proteolysis of AANAT protein (7).

Mammalian pineal gland synthesizes and releases melatonin with daily and seasonal rhythms under the control of the master biological clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus (29). A study performed in an identical model has shown that CO can affect the clock gene expression. Elevated concentration of CO in OphVB influenced the expression level of clock gene in the preoptic area and dorsal hypothalamus. The experimental animals after CO treatment had their master clock machinery deregulated which could cause chronodisruption (30). Also most clock genes are expressed in structures outside of the SCN in central and peripheral structures (31-34). One of these structures is the pineal gland which expresses Per1, Per2, Clock and Bmal1 genes (35-37). Nuclear receptors REV-ERBs that control the rhythmic transcription of BMAL1 are highly dynamic receptors that are responsive to heme, redox and gases, as carbon monoxide (30, 38-40). Oscillation in REV-ERBs ligands may affect the phase and amplitude of circadian rhythms and physiological outputs of the circadian system (41) suggesting new mechanisms for the systemic coordination of molecular clock and metabolism.

The circadian effects of melatonin appear to be mediated by melatonin receptors in the hypothalamic suprachiasmatic nucleus, the site of a circadian clock (42), and reproductive effects mediated by melatonin receptors in the hypothyalamic pars tuberalis (43). It is well-known that an inverse relationship exist between stress and reproductive functions. Neurons located within the parvicellular part of the paraventricular nucleus (PVN) of the hypothalamus release both corticotrophin-releasing hormone (CRH) and vasopressin (AVP), the neuropeptides that initiate the endocrine response to stressors stimulating the release of pituitary adrenocorticotropin hormone (44). Heme oxygenase-derived CO was shown to reduce the hypothalamic release of both CRH and AVP stimulated by depolarizing and immuno-inflammatory stimuli in the rat (45-47). The study conducted by Juszczak et al. (48) was shown that
subtype MT, membrane melatonin receptor may contribute to the inhibitory effect of physiological concentration of melatonin on functional regulation of vasopressinergic neurons in the rat. Taken together these data suggest a possible role for CO in the crosstalk among hypothalamic-pituitary-gonadal (HPG) and stress axis.

Increased carbon monoxide serum CO level is related to hypoxia during sleep in obstructive sleep apnea syndrome (OSAS) patients (49). OSA patients have an abnormal melatonin secretion pattern; show a later peak of melatonin in the night toward the morning hours. Zirklik et al. (50) reported that in OSA patients showed a later peak of melatonin at 6:00 a.m. after continuous positive airway pressure therapy (CPAP), melatonin peak returned to 2.00 a.m. The improvement in the oxidative condition by CPAP therapy is associated with the normalization of serum CO levels (49).

This work demonstrates that non-physiological changes in CO concentration in ophthalmic venous blood can have an acute impact on systemic MEL levels. In order to determine the physiological relevance of this finding, it would be useful to study the effect of normal variation of CO levels in ophthalmic venous blood upon MEL and to study the same animals in different seasons. If confirmed, it would support the concept that the effect of light in MEL secretion is mediated, at least in part, via CO.

Abbreviations: AANAT, arylalkylamine N-acetyltransferase; AVP, vasopressin; CO, carbon monoxide; CPAP, continuous positive airway pressure therapy; CRH, corticotrophin-releasing hormone; HIOMT, hydroxyindole-O-methyltransferase; HPG, hypothalamic-pituitary-gonadal axis; JV, external jugular vein; MEL, melatonin; NO, nitric oxide; OphS, ophthalmic sinus; OphVB, ophthalmic venous blood; OphVS, ophthalmic venous sinus; OSAS, obstructive sleep apnea syndrome; PVC, venous cavernous sinus


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