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Increased melatonin levels after hemorrhagic shock in male and female C3H/HeN mice

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Abstract. Although hemorrhagic shock leads to significant alterations of several hormones, e.g. ACTH, corticosterone and β-endorphin, it is not known whether plasma melatonin levels are affected under this condition and if so, whether the effects are comparable in males and females. Using a radioimmunoassay, it was found that plasma melatonin levels were significantly increased in male and proestrus female C3H/HeN mice immediately after hemorrhagic shock. However, in male mice, by two hours after hemorrhage and resuscitation, plasma melatonin returned to levels comparable to those seen in control and sham-operated animals. Proestrus female mice, on the other hand, showed significantly increased plasma melatonin levels at two hours after surgery when compared to unoperated control animals. Although the significance and biological role of the transient increased plasma melatonin levels after hemorrhagic shock remain to be determined, it appears that the pineal gland and/or an extrapineal source of melatonin, of both male and proestrus female mice responds to severe hypotension by increased release of melatonin.
Key words. Plasma melatonin level; hemorrhagic shock; male mice; female mice; resuscitation.

The pineal gland and its major secretory product melatonin have been reported to be involved in the immunoneuroendocrine network and it has been shown that exogenous melatonin administered in the evening counteracts the depression of antibody production, thymus cellularity and antiviral resistance induced by acute restraint stress and/or administration of corticosteroids and/or cyclophosphamide in mice[1,2]. On the other hand, different types of stressful procedures, such as immobilization, forced swimming, and hypoglycemic shock, which are all known to induce immune depression, have been reported to increase daytime melatonin production[3,4].

With respect to hemorrhagic shock, it is well established that major blood loss leads to endocrine alterations, which include the increased release of adrenocorticotrophic hormone (ACTH), corticosterone and β-endorphin[5]. Furthermore, hemorrhagic shock is known to cause a marked depression in both specific and nonspecific immunity[6,7]. These alterations in various immune functions are apparent immediately after hemorrhage (starting as early as 30 min after induction of hypotension) and they persist for a prolonged period of time (5–7 days)[6,7]. However, it remains unknown whether circulating levels of melatonin, the primary hormone produced by the pineal gland, are also altered after hemorrhagic shock.

Gender differences in immune responses to various adverse conditions have been observed and epidemiological studies have revealed that after hemorrhagic shock, females are less susceptible to sepsis than males[8]. The hormones of the endocrine system are thought to be intimately involved in this immunological dimorphism[9]. Profound changes in endogenous hormone levels occur during the proestrus stage when estrogen levels reach their cyclic peak in female mice[10]. Since there is no information concerning the response of the pineal gland to hemorrhage, we attempted to determine whether hemorrhagic shock affects melatonin release. Furthermore, since different levels of sex hormones, especially ovarian estrogen[10], in females versus males could affect endocrine and immune functions, the second aim of this study was to compare female (e.g. proestrus females as the estrogen levels peak during this stage of the estrous cycle) and male endocrine response of the pineal gland following major blood loss.

Materials and methods

Animals. Inbred male and proestrus female C3H/HeN mice (Charles River Laboratories, Portage, MI), 9 to 11

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weeks old (24–26 g BW) were used in this study. The stage in the estrous cycle of female mice was determined by regular examination of the vaginal smear. The vaginal smear was classified according to the definitions of Rugh[11]. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. This project was approved by the Institutional Animal Care and Use Committee of Michigan State University.

Male and female animals each were randomized into one of five groups. Group one consisted of unoperated control animals (Unop.). Group two were sham-operated animals (Sham), which were sacrificed immediately after the procedure. Group three consisted of hemorrhaged animals which were sacrificed immediately after hemorrhage (Hem.) without the shed blood being returned and without receiving fluid resuscitation, group four were sham-operated animals which were sacrificed 2 h after the procedure. Group five consisted of hemorrhaged animals, which were sacrificed 2 h after the return of shed blood and fluid resuscitation (2× the shed blood volume Ringer's lactate).

Animals in hemorrhage groups were lightly anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL) and restrained in a supine position. Both femoral arteries were aseptically cannulated with polyethylene 10 tubing (Clay-Adams, Parsippany, NJ) using a minimal dissection technique. The animals were then heparinized [two U beef lung heparin (Upjohn Labs., Kalamazoo, MI)/25 g body weight] and blood pressure was constantly monitored by attaching one of the catheters to a blood pressure analyzer (Digi-Med™, Louisville, KY). Upon awakening, the animals were bled through the other catheter, to a mean blood pressure (BP) of 35 ± 5 mmHg (prehemorrhage BP was ~95 ± 5 mmHg), which was maintained for 60 min. At the end of the hypotensive period, the shed blood was returned to the animals in group five and Ringer's lactate was infused to provide adequate fluid resuscitation. The catheters were removed, vessels ligated and groin incisions were closed. Animals in group three were sacrificed immediately after the hypotensive period by methoxyflurane overdose. Animal surgery was scheduled such that all animals were sacrificed at ~2:00 pm, in order to avoid artifacts due to marked circadian fluctuation of melatonin levels. There was no mortality with this hemorrhage model.

Plasma collection and storage. After methoxyflurane anesthesia, blood was obtained by cardiac puncture and placed in prechilled EDTA-containing microcentrifuge tubes (Microtainer Becton Dickinson and Co., Rutherford, NJ), which were kept on crushed ice. After the blood was withdrawn, animals were sacrificed with methoxyflurane overdose. The tubes were then centrifuged at 16,000 × *g* for 15 min in a refrigerated (4 °C) room. Plasma was separated, placed in pyrogen-free microcentrifuge Eppendorf-tubes, immediately frozen, and stored (−80°C) until the time of assay.

Radioimmunoassay (RIA). Plasma melatonin levels were quantified using the Bühlmann Melatonin RIA kit (Bühlmann Laboratories AG, Allschwil, Switzerland), which measures melatonin by a double-antibody radioimmunoassay based on the Kennaway G280 anti-melatonin antibody[12]. Each sample was assayed in duplicate, using 125 µl aliquots of extracted plasma. The minimum detectable dose of melatonin was calculated to be 0.3 pg/ml. The specificity of the assay was determined at 50% binding and was found to be as follows: melatonin 100%, serotonin <0.001%, 5-hydroxy-indoleacetic acid <0.001%, N-acetylserotonin 0.027%, 5-methoxytryptamine 0.003%, 5-methoxytryptophan 0.001%, 6-sulfatoxymelatonin 0.002%, 5-methoxytryptophol 0.001%. Melatonin levels of the unknowns were assigned by interpolation against a melatonin standard curve plotted from 5 premeasured synthetic melatonin standards (0.5–50 pg/ml). The antibody used in this RIA has been used by others to detect melatonin levels in rodent species[12].

Statistical analysis. Results are presented as mean ± SEM. Statistical analysis was performed using a one-way ANOVA and multiple comparison procedures (Bonferroni's method, Student-Newman-Keuls method). Statistical significance was recognized when $p < 0.05$.

Results

Effect of hemorrhagic shock on melatonin release in male mice. Immediately after hemorrhagic shock, plasma melatonin concentration increased significantly compared to sham-operated or unoperated control animals. However, at 2 h after hemorrhage and fluid resuscitation plasma melatonin was comparable to unoperated control and sham levels (fig. 1A).

Effect of hemorrhagic shock on melatonin release in proestrus female mice. Proestrus female mice showed a significant increase of plasma melatonin levels immediately after hemorrhagic shock compared to either sham-operated or unoperated control animals (fig. 1B). Two hours after hemorrhage and fluid resuscitation, melatonin levels were comparable to sham-operated animals. Comparing sham-operated and hemorrhaged animals at two hrs after surgery to unoperated control animals revealed significantly higher plasma melatonin levels in the surgery groups (fig. 1B).

Comparison of the effects of hemorrhagic shock on melatonin release in male and proestrus female mice. The results indicate that there were no significant differences in baseline melatonin release or in the melatonin release at any point after shock between male and proestrus female mice.

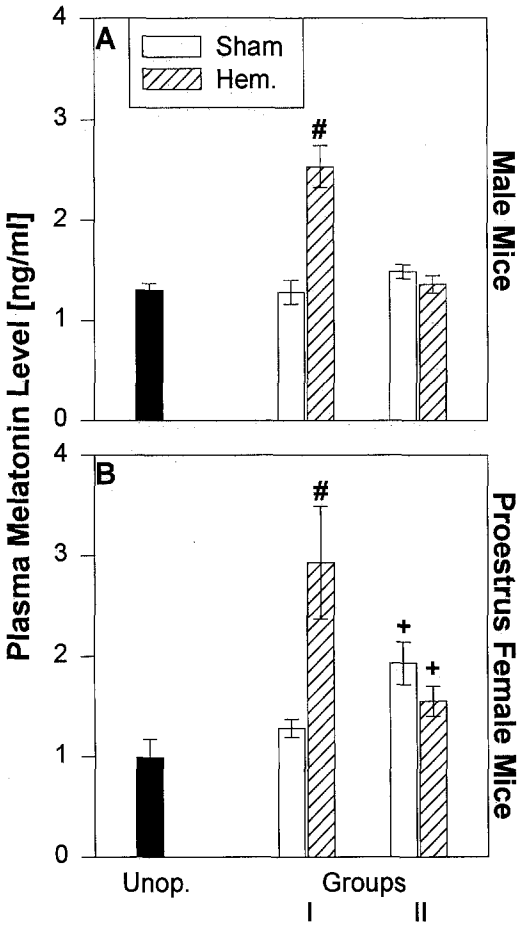

Figure 1. Circulating plasma Melatonin levels [pg/ml] in male (*A*) N = 7/group) and proestrus female (*B*) N = 6/group) C3H/HeN mice as determined by specific radioimmunoassay (Bühlmann Labs.). Unop.: unoperated control animals; Sham: Controls; Hem.: Hemorrhage; Group I: sacrificed immediately after hemorrhage; Group II: sacrificed at 2 h after hemorrhage and resuscitation.
#$p < 0.05$ hemorrhaged versus corresponding sham-operated animals, +$p < 0.05$ versus unoperated control animals

Discussion

Research on the involvement of melatonin in the immunoneuroendocrine network has shown immunoenhancing effects of this major secretory product of the pineal gland after stress- and drug-induced immune depression[13]. So far, effects of stressful conditions on the release of melatonin have been studied after restraint, forced swimming, hypoglycemic shock, and electroconvulsive shock[3,4,14,15]. Alternatively, to study the effect of melatonin on depressed immune function, immunosuppression was induced prior to melatonin treatment by administration of glucocorticoids and/or cyclophosphamide[1,2]. Although considerable work has been done and is being carried out dealing with melatonin during and after stress and immune suppression, no information is available concerning the effect of circulatory shock and/or trauma on melatonin release. In this respect, we believe this is the first study to report the effects of hemorrhagic shock on plasma melatonin levels. Our data indicate that the pineal gland (and/or an extrapineal source of melatonin e.g. the gut), through the release of its hormone melatonin, is involved in the early endocrine response to hemorrhagic shock. Our data show no gender difference in the release of melatonin following hemorrhagic shock, when comparing plasma melatonin levels at different time points after hemorrhagic shock and sham-operation. This finding is interesting as female sex-steroids are reported to influence hormone release and endocrine functions[9,16]. Nevertheless, proestrus female mice display significantly higher plasma melatonin levels in sham-operated and hemorrhaged animals at 2 h after surgery, when compared to unoperated control animals. This is of interest, considering the effects of sex-steroids on hormone release[9,16] and it remains to be determined whether this increased release of melatonin is due to a different endocrine stress-response in proestrus female mice following the hemorrhage procedure and sham-operation.

Our findings of increased plasma melatonin levels immediately after hemorrhagic shock are in accordance with observations of others who have reported increased daytime melatonin levels following various kinds of stress, such as immobilization, forced swimming, and hypoglycemic shock[3,4]. On the other hand, our results with hemorrhagic shock are not in accordance with findings in which electroconvulsive shock was used as a stressor and increased melatonin levels were not found[14,15]. However, in one of those studies melatonin levels were measured 9 h after shock, which might have obscured the effect of that stress on melatonin production[15]. In another study, the animals were killed at 30 to 240 min after. The authors explained the lack of stimulation of the pineal gland as the result of a catecholamine reuptake mechanism, which protects the pinealocytes from stimulation[14]. Since melatonin levels immediately after shock were not included, it remains unknown whether or not there was a transient (e.g. between 0 and 30 min after electroconvulsive shock) increase in that study.

Since plasma melatonin levels at 2 h after hemorrhage and resuscitation were comparable to levels in sham-operated animals, we propose that these changes occurred due to: 1) prevention of further melatonin release as soon as the stress of hypotension is counteracted by fluid resuscitation; and/or 2) depletion of the pineal gland of melatonin productive capacity leading to the impaired ability to maintain increased plasma levels for a prolonged period of time. The reason for significantly increased plasma melatonin levels in proestrus female mice at 2 h after surgery (compared to unoperated control animals) remains to be determined. Nevertheless, it appears that female sex-steroids, especially

ovarian estrogen, play a role in this reaction to surgical stress, which differs from that observed in male mice.

As hemorrhage induces an early systemic inflammatory response[6], indicated by the early (0 to 4 h) elevation of circulating tumor necrosis factor (TNF) and interleukin-6 (IL-6) levels, which are thought to contribute to host immunodysfunction, melatonin might be seen as a hormone which acts to amplify inflammatory mediator release leading to immunosuppression. However, in view of the reported immunoenhancing effects of melatonin (for a review see ref. 13), it can alternatively be proposed that the early increase in plasma melatonin level may be an effort of the organism to cope with the adverse circulatory condition and its deleterious effects. Nonetheless, it remains to be determined if melatonin plays a major role in regulating metabolism and/or host immune response following hemorrhagic shock.

The animals used in the present study (i.e. C3H/HeN mice), had relatively low plasma melatonin levels compared to other mouse strains[17]. This may be due to the collection of the plasma by cardiac puncture in the present study, whereas in the other study blood was collected from the retro-orbital plexus and pooled before assaying[17]. Nevertheless, C3H/HeN mice are able to produce melatonin[18] and such mice also show pineal melatonin rhythms synchronized by environmental lighting cycles[19]. These observation[17,18] lead us to conclude that the threefold increase in plasma melatonin levels after hemorrhagic shock appears to be due to increased synthesis and release from the pineal gland. Nevertheless, we cannot exclude the possibility of extrapineal melatonin release from the gut or other sources. In summary, our results indicate that plasma melatonin levels increase significantly during hemorrhagic shock in both males and females. Following resuscitation, however, melatonin levels return to normal. The exact significance and consequences of the elevated melatonin levels during severe hypotension remain to be determined.

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