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Melatonin modulates glucocorticoid receptor mediated inhibition of antioxidant response and apoptosis in peripheral blood mononuclear cells

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ABSTRACT

Pineal melatonin is known for its immunomodulatory and anti-stress properties. It modulates stress condition by regulating antioxidant responses and apoptosis in the immune cells. Stress causes increased glucocorticoid level that acts through glucocorticoid receptor (GR) and is translocated into nucleus under regulation of HSP90 based chaperone machinery. Melatonin influences glucocorticoid and GR mediated stress condition in rodents, but till date there are no reports which could suggest the effect of melatonin treatment on GR mediated apoptosis and inhibition of Nrf-2/hemeoxygenase-1 (HO-1) induced antioxidant status in immunocompetent cells (peripheral blood mononuclear cells; PBMCs). Therefore, in the present study, we considered GR mediated inhibition of Nrf2 and HO-1 along with anti-apoptotic Bcl-2 expression in PBMCs. The PBMCs were treated with synthetic glucocorticoid; dexamethasone (Dex) and melatonin (Mel), to explore the effect of melatonin treatment in regulation of GR mediated apoptosis and inhibition of antioxidant status in immune cells. It was noted that melatonin treatment retained GR into cytoplasm by inhibiting the dissociation of HSP90 from GR-HSP90 complex and enhanced expression of Nrf2/HO-1 and Bcl-2 expression. This led to increased HO-1 expression and elevated Bcl-2 led to increased Bcl-2/Bax ratio that might ultimately enhanced the cellular antioxidant response and survival under glucocorticoid mediated stress condition. Our observations suggest that the declined GR nuclear translocation upon melatonin treatment might be responsible for the up-regulation of Nrf2 mediated HO-1 activity and increased Bcl-2/Bax ratio in PBMCs to maintain the immune homeostasis under stress condition.

1. Introduction

Stress is a constellation of events that acts as stimulus (stressor) to activate stress response in the physiological system (Dhabhar and McEwen, 2001). Stress has been reported to suppress or dysregulate immune function (Glaser and Kiecolt-Glaser, 2005) and increase susceptibility to various infections (Cohen et al., 1991). The detrimental effect of stress causes imbalance of the physiological homeostasis by declining the immune function (such as the reduction of immune cells activity, lymphocyte population, proliferation and NK cell activity) (Webster Marketon and Glaser, 2008) along with decreased antioxidant response that leads to immunocompromised state (Dohms and Metz, 1991). The increased glucocorticoids (GC) in response to stress via HPA axis (Saplosky et al., 2000) exerts its effects via intracellular GC receptor (GR) and modulates the expression of different target genes (Stahn and Buttgereit, 2008). The high GC and GR level has been associated with declined immune responses i.e. stress mediated immunosuppression (Ashwell et al., 2000). The stress induced immunosuppression might be a result of decline antioxidant response and increased apoptosis in immunocompetent cells/organs (Kannan and Jain, 2000). The GR activation induces apoptosis by suppressing anti-apoptotic proteins Bcl-2 and enhancing Bax expression (Distelhorst, 2002; Lepine et al., 2005). GR activation has also been reported for declining antioxidant response (Kratschmar et al., 2012). GC binding to GR in the cytoplasm and translocates GR into the nuclear compartment. The GR nuclear translocation is tightly regulated by HSP90 based chaperone machinery in which HSP90 has been suggested to play an imperative role (Grad and

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Picard, 2007). HSP90 binds with GR and form HSP90-GR complex that regulates functional activation and inactivation of GR (Freeman and Yamamoto, 2002). It has also been reported that alteration in HSP90/GR ratio and increased nuclear HSP90 attenuates activity of GR activity (Ouyang et al., 2012).

Melatonin has been suggested to down-regulate GC and GR mediated inhibition of immune responses (Gupta and Haldar, 2013). Seasonal variation in melatonin concentration influences GR expression in human mononuclear leucocytes (Blackhurst et al., 2001). The activation of GR has been associated with suppressed antioxidant response signaling via Nrf2 and down-regulates an array of antioxidative enzymes like superoxide dismutase (SOD), hemeoxygenase-1 (HO-1), catalase (CAT) etc. (Kratschmar et al., 2012). The seasonal melatonin levels positively influences antioxidative enzymes activity (SOD, CAT) in daily manner (Singh et al., 2013). The down-regulation of Nrf2 signaling has been suggested to increase apoptosis by influencing apoptotic proteins (Pan et al., 2013). However, there is no report available which could suggest that how melatonin modulates GR mediated inhibition of Nrf2 and thereby regulating antioxidant response and induction of apoptosis in PBMCs in any ruminant species like goat. We hypothesized that melatonin might be repressing the nuclear translocation of GR to modulate antioxidative enzyme activity and to down regulate apoptosis under glucocorticoid induced stress condition in PBMCs of goat *Capra hircus*.

Therefore, in the present study, we considered GR mediated inhibition of Nrf2 dependent HO-1 expression along with anti-apoptotic Bcl-2 expression in the PBMCs. The PBMCs were treated with dexamethasone (Dex) and melatonin (Mel) alone and in combination to explore the effect of melatonin treatment in regulation of GR mediated inhibition of antioxidative enzymes activity and apoptosis. The expression of GR; complete, nuclear and cytoplasmic fraction was noted along with nuclear HSP90 expression. To note Nrf2 mediated antioxidant response, HO-1 and Bcl-2 expression was noted upon melatonin and dexamethasone treatment *in vitro*. The successive effect of dexamethasone and melatonin treatment was checked on apoptotic markers like Bax, Caspase-3 in PBMCs along with Bcl-2/Bax ratio. The cell mediated immune responses of PBMCs were noted by measuring IL-2 secretion and proliferative responses to mitogen Concanavalin-A challenge in goat *Capra hircus*.

2. Material and method

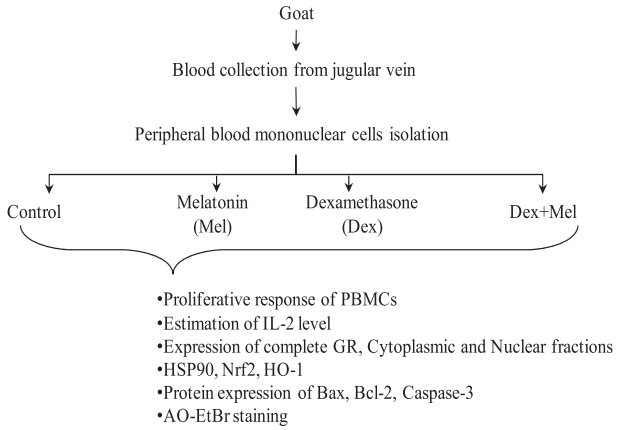
All the experiments were performed in accordance with institutional practice and within the framework of the animals Act of 2007 of Govt. of India on animal welfare (Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA).

2.1. Animal maintenance

In the present study, 7 adult goats (4–5 years; BW 35 ± 2 kg) were procured from Institute of Agricultural Sciences, Banaras Hindu University, at Varanasi (latitude 25° 18′ N and longitude 83° 1′ E). The goats were maintained as per farm rule under ambient condition (mean temp. 30 ± 2 °C; humidity 85 ± 5%; day length ~12L:12D; for the months of August–September). They were fed on green leaves and grasses as per seasonal availability along with usual roughages (hay, protein grains, mineral cake) and water *ad libitum*. The health was monitored by recording the body temperature (normal rectal temperature: 102.5–103 °F) and rumen movement by authorized veterinarian doctor. Proper prophylactic measures were adopted in terms of vaccination against enterotoxemia, foot and mouth diseases, and peste des petits ruminant (PPR). The goats were treated with antihelmintics twice per year and 0.5% solution of malathion (acaricidal baths) was sprayed externally at 7–20 days as described by Chowdhury et al. (2002).

2.2. Experimental plan

The blood was collected from jugular vein to isolate PBMCs from all the goats. The PBMCs were cultured in four groups, i.e. 1) control, 2) melatonin (Mel), 3) Dexamethasone (Dex), and 4) Dexamethasone (Dex) along with melatonin (Mel). The dexamethasone, a synthetic glucocorticoid was used to mimic the glucocorticoid induced immunosuppression under *in vitro* condition. Immunosuppressive dose of dexamethasone (2 μM) and an immunoproliferative dose of melatonin (500 pg/ml) were used for *in vitro* treatment to PBMCs. To check the proliferative response and apoptotic markers cells were incubated for 48 h, for GR nuclear translocation experiments cells were treated for 4 h at 37 °C in CO_2 incubator. The proliferative response of PBMCs, IL-2 secretion, expression of GR, HSP90, Nrf2, HO-1 and apoptotic marker proteins (Bcl-2, Bax, Caspase-3) were checked after the treatments.**(Schematic representation)**.



2.3. Blood collection

The blood samples were collected from the jugular vein in pre-heparinized tube from all the goats (n = 7) by applying a minimal stress after 2 h of sunset (at 20:00 h; IST) under dim red light (less than 1 lx). The blood samples were processed for isolation of peripheral blood mononuclear cells.

2.3.1. Isolation of peripheral blood mononuclear cells

The PBMCs were isolated by density gradient centrifugation (Boyum, 1968) and published elsewhere Singh et al. (2013). Lymphocyte separation media was used according to manufacturer's instruction (HiSep™ LSM 1084, HiMedia, Mumbai, India). Briefly, the white band at the plasma-ficoll (Hisep, Himedia) interphase was collected and washed twice with PBS and finally suspended in complete media RPMI 1640 (Himedia, India) supplemented with 10% fetal bovine serum (FBS) and 100 units of penicillin and streptomycin (Sigma Aldrich, USA).

2.3.2. MTT assay peripheral blood mononuclear cells (PBMCs) proliferation

Cell-mediated immune function was assessed by measuring PBMCs proliferation in response to T-cell specific mitogen, Concanavalin-A (Con-A) by using a colorimetric assay based on the reduction of tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-