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Short-term *in situ action* of melatonin on ion transport in mice kept at restraint stress

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Summary

Melatonin, a pleiotropic hormone, is involved in many physiological functions including combating oxidative stress. However, its role in ion transport during stress response is not yet understood. The dose-dependent effect of *in situ* melatonin was examined in Swiss albino mice. Perfusion of melatonin at 10^{-7} M for 20 minutes produced a significant decrease in Na^+, K^+-ATPase activity in the kidney, liver, stomach and intestinal tissues. A dose-responsive decrease in cytosolic and mitochondrial H^+ ATPase activity was found in these tissues after melatonin perfusion. Likewise, the cytosolic and mitochondrial Ca^{2+} ATPase activities decreased in the kidney, liver, stomach and intestine. The mitochondrial Mg^{2+} ATPase activity decreased in the tested tissues in a dose-responsive manner. Subjecting mice to restraint stress for seven days increased the Na^+, K^+-ATPase, H^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase activities to significant levels in kidney, liver, stomach and intestinal tissues. On the contrary, *in-situ* perfusion of melatonin to stressed mice at 10^{-9} M caused decrease in the stress-induced hyperactivity of these transmembrane ion transporters. These results provide evidence for a role of melatonin in ion transporter activity and point to a protective role of melatonin in ion transport during stress response in mice.

Key words: ATPase, melatonin, mice, stress

Introduction

In the vertebrate endocrine network, the pineal hormone melatonin has emerged as a multifunctional hormone and its distribution emphasizes its key role as an intracellular neuroendocrine regulator and coordinator of many complex and interrelated biological processes (Bubenik et al*.,* 1999; Messner et al*.,* 2001; Kvetnoy et al*.,* 2002; Bubenik, 2008). Melatonin prevents oxidative stress in diabetic subjects as well (Nishida, 2005) and protects endothelial cells from hypoxia (Matějovská et al*.,* 2008). Melatonin released into the bloodstream acts as a hormone and controls biological functions associated with circadian rhythms. Melatonin also appears to be a powerful cytostatic drug (Blask, 1993; Conti and Maestroni, 1995), one of the most powerful scavengers of free radicals (Reiter et al*.,* 2003, 2007) and an effective antihypertensive drug (Kawashima et al., 1987). It has been reported that melatonin stimulation of antioxidative enzymes, such as superoxide dismutase and glutathione reductase, contributes to its protection of gastric mucosa against damage caused by ischemia–reperfusion (Cabeza et al., 2001). In another study, melatonin reduced plasma levels of malondialdehyde, a lipid peroxidation product (Akbulut et al., 1997).

Stress as a disruption in the normal homeostatic functions causes dysfunction of physiological processes and leakage of cytoplasmic enzymes into the blood of mice (Stinnett and Seasholtz, 2010: Peter, 2013). An increase in the activities of these enzymes in the plasma or serum of mice by acute emotional stress, such as restraining, have been shown to be due to the damage of various tissues (Highman and Altland, 1962; Pearl et al*.,* 1966; Meltzer, 1971; Sánchez et al*.,* 2002). The stress response is a series of physiological and behavioral changes involving the HPA axis and the sympathetic nervous system that helps the organism to cope with these challenges (Ruszymah and Khalid, 1999; Sterling and Eyer, 1988). Physical restraint or immobilization has been used as a stressor for the induction of stress response in animals (Pare and Glavin, 1986). As a stress model

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physical restraint causes the stress-associated changes in the physiological, immunological, and neurobiological status of mammals (Glavin et al., 1994; Shi et al., 2003). Moreover, a number of studies have reported that immobilization or restraint stress is accompanied by disturbance in antioxidative capacity of the organism (Kovacheva-Ivenova et al., 1994; Oishi et al., 1999).

ATPases are membrane pumps widely used as indicators of osmoregulatory indices in many animals including mammals (Peter et al., 2000; Fe´ Raille and Doucet, 2001). In mammals, Na^+, K^+-ATPase activity is responsible for regulation of membrane polarization (Guerra et al., 1981) and thermogenesis (Ismail Beigi, 1988). Cellular Na^+ and K^+ gradients are essential for nutrient transport and Na^+, K^+-ATPase is precisely regulated according to the cell's demand for Na^+ and K^+ in fishes (Evans, 1998). V-type H^+-ATPases located in cell surface regulate cytosolic pH to ~7.0, the optimal physiological pH, whereas within intracellular membrane they are involved in cellular processes such as receptor-mediated endocytosis, membrane trafficking, protein processing or degradation and nutrient uptake (Nishi and Forgac, 2002). Cytosolic Ca^{2+}-ATPases are responsible for the expulsion of Ca^{2+} from the cytosol of all eukaryotic cells. Mitochondria take up calcium through an uptake pathway due to its low-Ca^{2+} affinity (Calì et al., 2012). Mitochondrial Ca^{2+} accumulation has role in satisfying energy demands by increasing the ATP production through activation of mitochondrial enzymes, and modulation of the dynamics of calcium signals in cell functions (Brini, 2003). Mg^{2+} ATPase, a membrane transporter ubiquitous in animal cells, is involved in the transport of Mg^{2+} and utilizes it as a substrate for its functioning. It can also operate in the reverse direction, hydrolyzing ATP and pumping protons under certain conditions. Although these ATPases have varied ion transporting roles, its mechanism of actions may have several features in common (Graham and Rega, 1998; Dean et al., 1984; Hediger et al., 2004). Extensive studies have shown that these transporters are regulated by a number of hormones including thyroid hormone (Peter et al., 2000), cortisol (Babitha and Peter, 2010), prolactin and insulin (Peter et al., 2014), corticosteroids (Fe´ Raille and Doucet, 2001) and calcitonin (Friedman and Gesek, 1995).

However, the functional relationship between melatonin and ion transporter activity and its probable role during stress response have not been elucidated in mammals so far. The present study, therefore, examined the dose-dependent response of short-term *in situ* melatonin perfusion on the activity of ion transporters in various tissues of mice kept in either non-stressed or restraint-stress condition.

Materials and Methods

Animal Holding Conditions

Twelve weeks old healthy adult female Swiss albino mice (*Mus musculus*) born and reared in the animal house (University of Kerala), were used as the test species. Animals were kept in groups of four each in polypropylene cages (Size: 29 x 22 x 14 cm) with stainless steel–wire mesh top. All animals were maintained under a 12 h L;D photoperiod at room temperature of 24 ± 4° C and relative humidity of 70 ± 10% with minimum noise levels and limited handling. Animals were allowed *ad libitum* access to standard pelleted maintenance diet (Sri Sai Durga Feeds and Products, Bangalore, India) and purified tap water. Cage bedding was changed once in every two days. The experiment was carried out under the same environmental conditions as those in the animal's house, with the animals being brought to the experimental room in their home cages. The Guidelines of Institutional Animal Ethics Committee (IAEC) of the university [IAEC-KU-31/2011-12-ZOO-MCSP (2)] were followed.

Experimental Protocol

The dose-dependent effect of melatonin in mice

In this experiment the dose-responsive in *situ* effects of melatonin on ion transporter activities were studied in the kidney, liver, stomach and intestine of mice to understand the short-term actions of this hormone. The effects of varied concentrations (10^{-9}, 10^{-8}, and10^{-7} M) of *in situ*

melatonin (SRL, India) perfusions were done in the test species.

The effect of melatonin in restraint-stressed mice

In the second set of experiments a selected dose melatonin (10^{-7} M) was perfused to normal mice. Likewise, melatonin was perfused to the mice which were exposed to the psychosocial stressor in the form of intermittent restraint stress for seven days.

Perfusion of varied doses of melatonin to normal mice

Animals were assigned to four groups (total n= 16). The mice were anaesthetized with chloroform (Sisco Research Laboratories, Mumbai, India). Then they were swabbed with 70% alcohol to wet the fur and the abdominal skin was shaved. A midline abdominal incision was made and abdominal aorta and vena cava were exposed. These blood vessels were cleared of connective tissue and fat with cotton swabs. A #23 needle was inserted in the aorta and arterial blood was removed with the help of a canula. Then, without any interruption of pressure/flow, desired concentration of the melatonin dissolved in the perfusion medium was perfused for 20 min. Krebs Ringer bicarbonate Buffer (KRB saline) (Sigma-Aldrich, USA) was used as perfusion medium. The control mice were perfused with KRB saline at the rate of 1 ml/min for 20 min. Similarly, the remaining mice were perfused with 10^{-9}, 10^{-8}, and 10^{-7} M melatonin for 20 min. After perfusion the mice were sacrificed and tissues such as stomach, intestine, kidney and liver were collected in SEI buffer and stored at -80^{o} C for further analysis.

Perfusion of melatonin to restrained mice

Animals (total n= 16) were assigned to two sets. The first set comprised of two groups which were normal and served as non-stressed mice. The second set of mice were kept under restraint stress for 60 min daily between 11 am-12 noon for one week as described by Brunton et al. (2007). The non-stressed mice were maintained undisturbed for the same duration. At the end of the seventh day all the animals were anaesthetized and melatonin or vehicle was perfused. The first group in non-stressed mice was perfused with KRB saline for 20 min whereas the second group in non-stressed mice were perfused with 10^{-7} M melatonin for 20 min. On the other hand, the first group in the second set of stressed mice was perfused with KRB saline for 20 min whereas the second group of stressed mice was perfused with 10^{-7} M melatonin. After perfusion the mice were sacrificed and tissues such as stomach, intestine, kidney and liver were collected in SEI buffer and stored at -80^{o} C for further analysis.

Isolation of mitochondria

Isolation of mitochondria from tissues was done as described by Peter et al. (2014). Mitochondria were isolated from kidney, liver, stomach and intestine tissues. Briefly, the tissues were kept in ice-cold 0.25M sucrose. A 10% tissue homogenate was prepared and subjected to differential centrifugation at 4^{0} C (Eppendorf R3435). First, it was centrifuged at 700 x g for 10 min to separate the cell debris and nuclei. The supernatant was spun at 10,000 x g for 10 min and the pellets were washed twice by repeating the centrifugation. This mitochondrial pellet was later suspended in fresh ice-cold 0.25 M SEI buffer containing 0.25 M sucrose, 10 mM Na_2EDTA, and 0.1 M imidazole. The supernatant collected first was taken as post-mitochondrial supernatant (PMS) and it served as the source for cytosolic transporter assays. The protein concentrations in mitochondria as well as in cytosol were determined using modified Biuret assay (Alexander and Ingram, 1980) with bovine serum albumin as the standard.

Quantification of ion-specific ATPases

Na^+, K^+-ATPase specific activity

A portion of the tissue homogenized in SEI buffer (pH 7.4) and centrifuged at 700 x g for 10 min was used for analyzing the Na^+, K^+-ATPase activity. The ouabain-sensitive Na^+, K^+-ATPase-specific activity in the kidney, liver, stomach and intestinal

tissue homogenates was quantified adopting the method of Peter et al. (2000). Saponin (0.2 mg protein^{-1}) was routinely added to optimize substrate accessibility. The samples in duplicate were added to a 96-well microplate containing 100 mM L^{-1} NaCl, 30 mM L^{-1} immidazole (pH 7.4), 0.1 mM L^{-1} EDTA and 5 mM L^{-1} $MgCl_2$ with or without ouabain and incubated at 37^0 C. The reaction was initiated by the addition of ATP and was terminated with addition of 8.6% TCA. The liberated inorganic phosphate was determined in Autoreader 4011 (Spam Diagnostics Ltd., Surat, India) at 700 nm and expressed in µM P*i* h^{-1} mg protein^{-1}.

H^+-ATPase specific activity

The baffilomycin-sensitive H^+-ATPase activity in the kidney, liver, stomach and intestinal cytosolic and mitochondrial fractions was measured as described earlier (Peter et al., 2014) using baffilomycin A as inhibitor. The samples in duplicate were added to a 96-well microplate containing baffilomycin A. The reaction was initiated by addition of ATP and terminated by adding 8.6% TCA and the inorganic phosphate content was determined as above and expressed in µM P*i* h^{-1} mg protein^{-1}.

Ca^{2+}-dependent ATPase specific activity

The vanadate-dependent Ca^{2+}-ATPase activity in the kidney, liver, stomach and intestinal cytosolic and mitochondrial fraction was determined as described earlier (Peter et al., 2014) using vanadate as inhibitor. Samples in duplicate were added to a 96-well microplate containing either $CaCl_2$ or vanadate. The inorganic phosphate content released was determined as above and expressed in µM P*i* h^{-1} mg protein^{-1}.

Mg^{2+}-dependent ATPase specific activity

The specific activity of oligomycin-sensitive Mg^{2+}-ATPase in the kidney, liver, stomach and intestinal mitochondrial fraction was determined following the method already described (Peter et al., 2014) and oligomycin was used as inhibitor. Mitochondrial samples in duplicate were added to a 96-well microplate with or without oligomycin. The inorganic phosphate content released was measured and expressed in µM P*i* h^{-1} mg protein^{-1}.

Statistical analysis

Data were collected from four animals in each group. Statistical difference among groups was tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between groups was analyzed with the help of Graphpad Software (Graphpad Instat-3, San Diego) and the level of significance was accepted if $P < 0.05$.

Results

Effect of varied doses of *in situ* melatonin on ion transporters in mice

Perfusion of 10^{-7} M melatonin produced a significant ($P< 0.05$) decrease in the Na^+/K^+-ATPase activity in the kidney and liver tissues when compared to saline perfused mice whereas 10^{-8} M and 10^{-9} M melatonin had little effect on Na^+/K^+-ATPase activity in these tissues (Fig.1). Melatonin perfusion resulted a significant reduction in the Na^+/K^+-ATPase activity in the stomach and intestine at 10^{-7},10^{-8} and 10^{-9} M concentrations when compared to saline-perfused control mice (Fig.1).

The cytosolic H^+-ATPase activity showed a significant decrease in the kidney ($P<0.001$) and liver tissues after 10^{-7}, 10^{-8} and 10^{-9} M melatonin perfusion in mice compared to saline-perfused normal mice (Fig.1). In the stomach ($P<0.01$) and intestine ($P<0.001$) the cytosolic H^+-ATPase activity decreased after melatonin perfusion at 10^{-7}, 10^{-8} and 10^{-9} M compared to saline-perfused normal mice (Fig.1). Mitochondrial H^+-ATPase activity decreased significantly in the kidney ($P<0.01$), liver ($P<0.01$), stomach ($P<0.05$) and intestinal ($P<0.001$) tissues of mice following 10^{-7}M melatonin perfusion, compared to control mice. Melatonin perfusion at 10^{-8} and 10^{-9} M also caused decrease in the mitochondrial H^+-ATPase activity (Fig.2).

Cytosolic Ca^{2+} ATPase activity decreased after 10^{-7}, 10^{-8} and 10^{-9} M melatonin perfusion

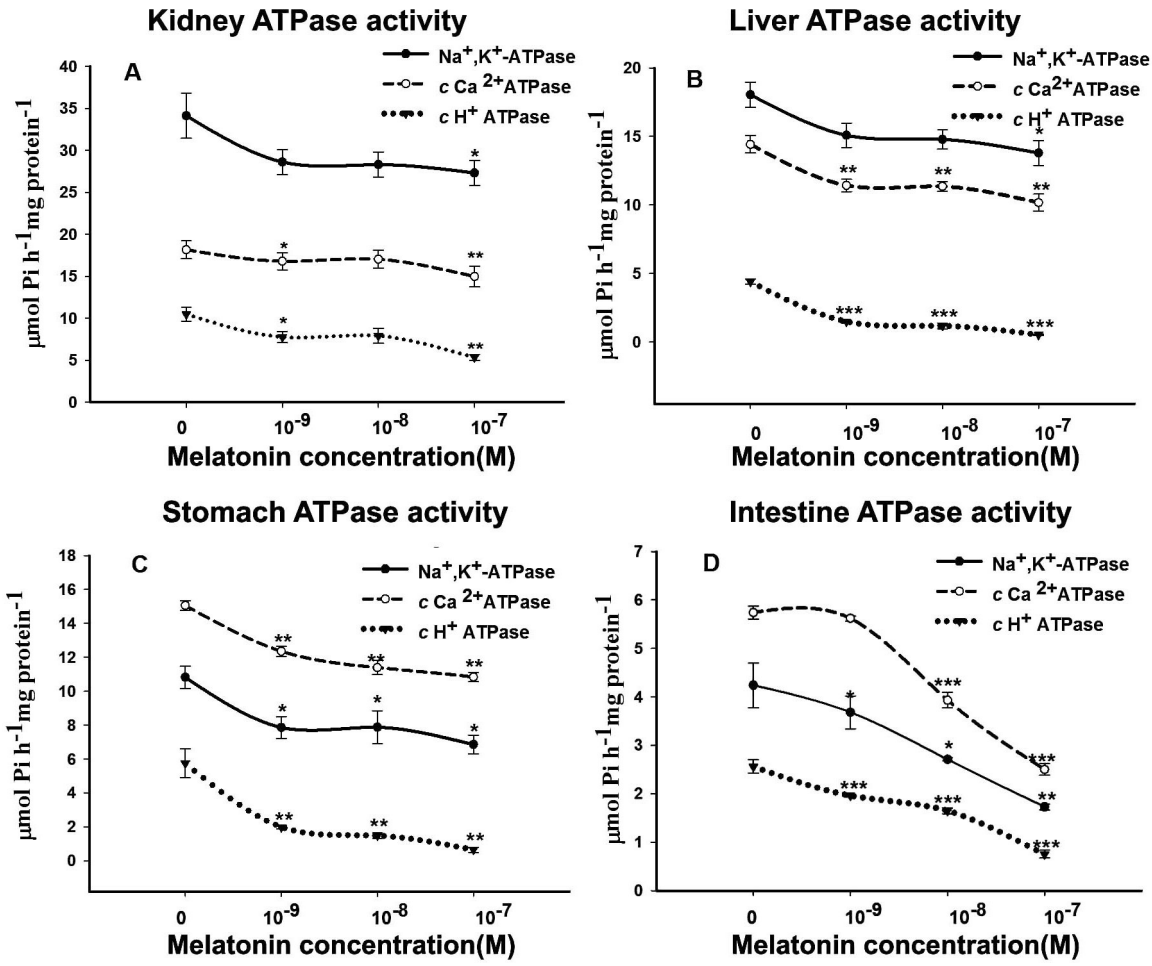

Fig.1 Dose-dependent effect of *in situ* melatonin for 20 min on the ATPase activities in the cytosol of kidney (A), liver(B), stomach(C) and intestine(D) of mice. Each point represents mean ± SEM for four mice. ***P< 0.001, **P< 0.01 and *P< 0.05 denote significant difference from that of control.

(Fig.2) in kidney, liver, stomach and intestine. A significant (P<0.01) reduction in cytosolic Ca^{2+}ATPase was observed in the kidney, liver, stomach and intestine (P<0.001) following 10^{-7} M melatonin whereas 10^{-9} M produced little change in the intestinal cytosolic Ca^{2+}ATPase activity (Fig.1). The mitochondrial Ca^{2+}ATPase activity significantly decreased after 10^{-7} M concentration of melatonin perfusion in all tissues whereas 10^{-8} and 10^{-9} M doses produced little effect (Fig.2).

The mitochondrial Mg^{2+}ATPase activity decreased significantly in the liver (P<0.05), kidney (P<0.05), stomach (P<0.01) and intestine (P<0.01) after melatonin perfusion at 10^{-7} M concentration (Fig.2). In the stomach and intestine the lower doses of melatonin perfusion also produced significant reduction in mitochondrial Mg^{2+}ATPase activity (Fig.2).

Effect of *in situ* melatonin on ion transporters in stressed mice

The Na^+/K^+ATPase activity in the stomach and intestine showed significant (P<0.05) reduction in normal mice groups after 10^{-9} M melatonin infusion whereas kidney and liver tissues tended to show a slight decline (Fig.3). Restraint stress caused a significant (P<0.001) increase in Na^+/K^+ATPase activity in the kidney, liver, stomach and intestinal

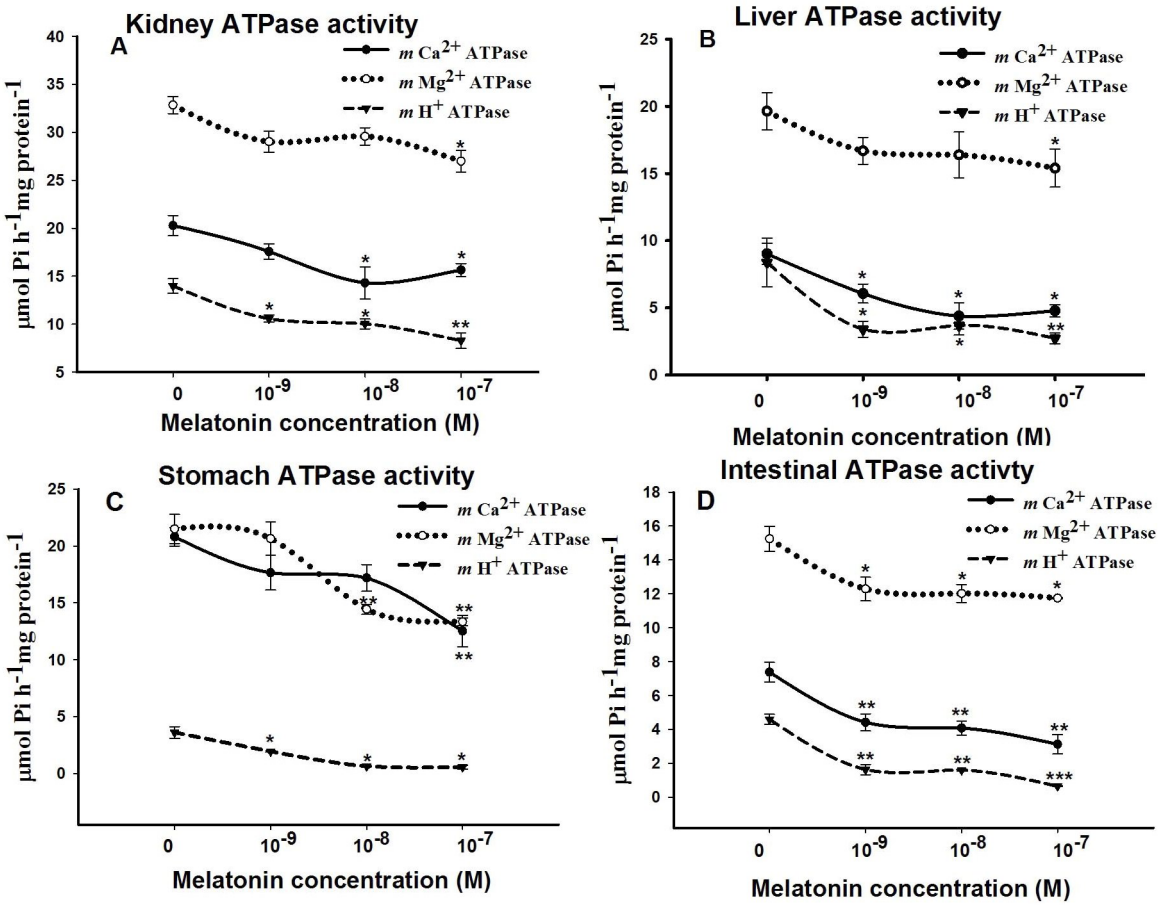

Fig.2 Dose-dependent effect of *in situ* melatonin for 20 min on ATPase activities in the mitochondria of kidney (A), liver (B), stomach (C) and intestine (D) of mice. Each point represents mean ± SEM for four mice. ***P< 0.001, **P< 0.01 and *P< 0.05 denote significant difference from that of control.

tissues of mice perfused with saline (Fig.3). However, the Na^+/K^+ATPase activity in kidney, liver and intestine decreased significantly (P<0.05) after 10^{-9} M melatonin perfusion in stressed mice compared to stressed mice perfused with saline (Fig.3).

The cytosolic H^+ATPase activity in the melatonin perfused normal mice decreased significantly in the liver (P< 0.01), stomach and intestine (P<0.05) (Fig.3). However, restraint stress increased the cytosolic H^+-ATPase activity in kidney (P<0.001), liver (P<0.001) and intestine (P<0.05) in mice (Fig.3). On the contrary, in stressed mice the cytosolic H^+-ATPase activity decreased significantly in the kidney (P< 0.01), liver (P< 0.01) and stomach (P<0.05) after 10^{-9} M melatonin perfusion when compared to stressed mice perfused with saline (Fig.3). The mitochondrial H^+-ATPase activity also followed similar pattern in all the tested tissues of stressed mice (Fig.3). Infusion of melatonin caused decrease (P<0.05) in stress-induced rise in activity of mitochondrial H^+-ATPase in liver and intestine when compared to stressed mice perfused with saline (Fig.4).

The cytosolic Ca^{2+}ATPase activity in the liver and stomach decreased (P<0.05) with 10^{-9} M melatonin perfusion (Fig.3). Restraint stress caused significant increase (P<0.001) in the cytosolic Ca^{2+}ATPase in all the tissues when compared to non-stressed mice perfused either with saline or with melatonin (Fig.3). But 10^{-9} M melatonin perfusion produced significant (P<0.05) decrease in the cytosolic Ca^{2+}ATPase activity in the kidney, liver,

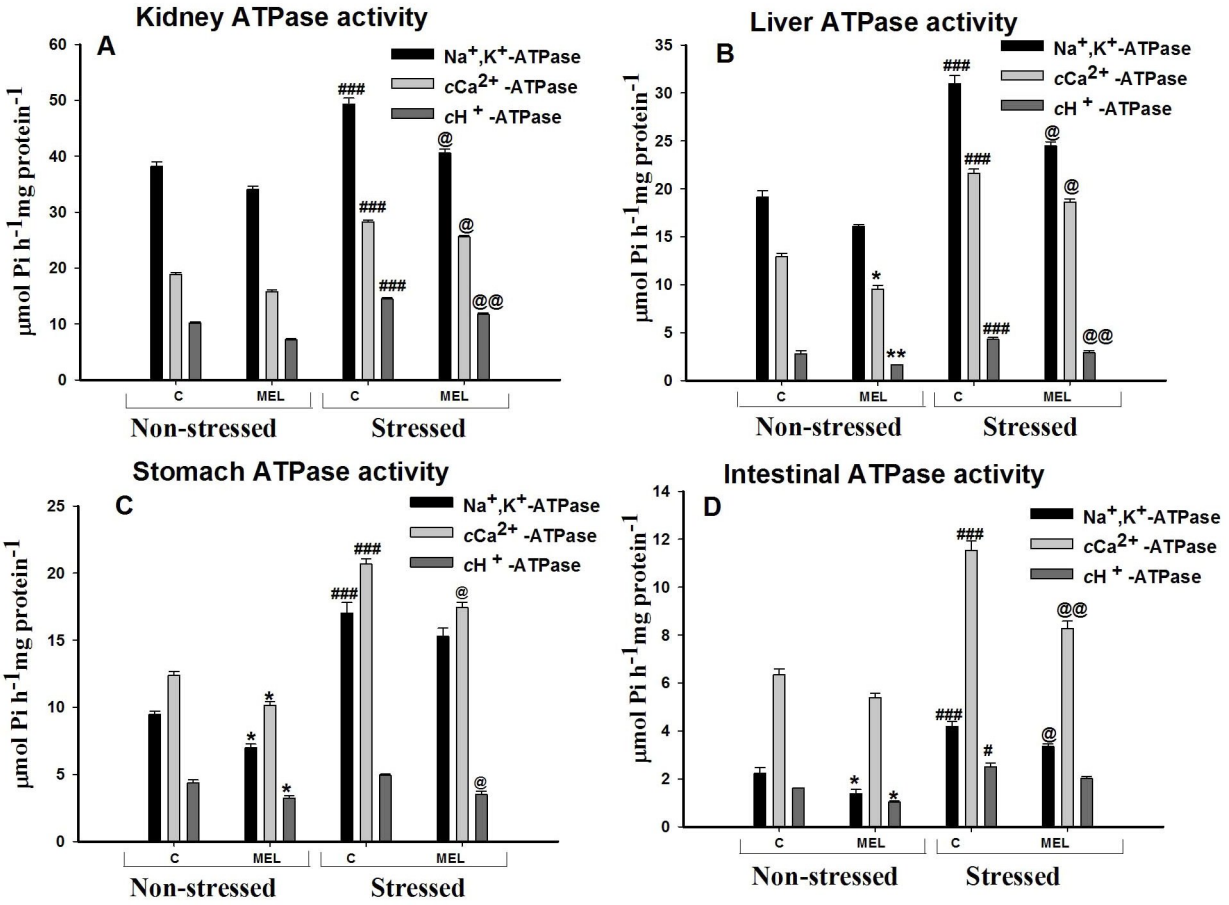

Fig.3. Effect of *in situ* melatonin for 20 min on the ATPase activity in the cytosol of kidney (A), liver (B), stomach (C) and intestine (D) of mice kept at non-stressed or restraint stressed condition. Each column represents mean ± SEM for four mice. $^{**}P< 0.01$ and $^{*}P< 0.05$ from saline perfused normal mice. $^{###}P< 0.001$ and $^{#}P< 0.05$ denotes significant difference between stressed and non-stressed mice. $^{@@}P< 0.01$ and $^{@}P< 0.05$ denote significant difference from melatonin perfused and saline perfused stressed mice.

stomach and intestine ($P<0.01$) of stressed mice (Fig.3). The mitochondrial Ca^{2+}ATPase activity decreased ($P<0.05$) in the liver, stomach and intestine after melatonin infusion in non-stressed mice. Restraint stress caused a significant ($P<0.001$) increase in mitochondrial Ca^{2+}ATPase activity in the kidney, liver, stomach and intestine (Fig.4). Infusion of melatonin decreased the stress-induced increase in the activity of mitochondrial Ca^{2+}ATPase to significant levels in the kidney, stomach and intestine (Fig.4).

The mitochondrial Mg^{2+}ATPase activity decreased to significant levels ($P<0.05$) in the liver and stomach after 10^{-7} M melatonin perfusion (Fig.4). Stress produced significant increase in the mitochondrial Mg^{2+}ATPase activity in all the tested tissues. On the contrary, infusion of melatonin decreased ($P<0.05$) the stress-induced change in the mitochondrial Mg^{2+}-ATPase in the respective tissues (Fig.4).

Discussion

Melatonin, a powerful free radical scavenger, acts ubiquitously as an intracellular antioxidant. Being lipophilic, melatonin has access to cytosolic, mitochondrial and nuclear compartments. The effects of melatonin may result from binding to high affinity receptors in the plasma membrane, cytosol or the nucleus, leading to changes in gene expression or altering second messenger levels such as cAMP and Ca^{2+} (Reiter et al., 2003). Melatonin is known to

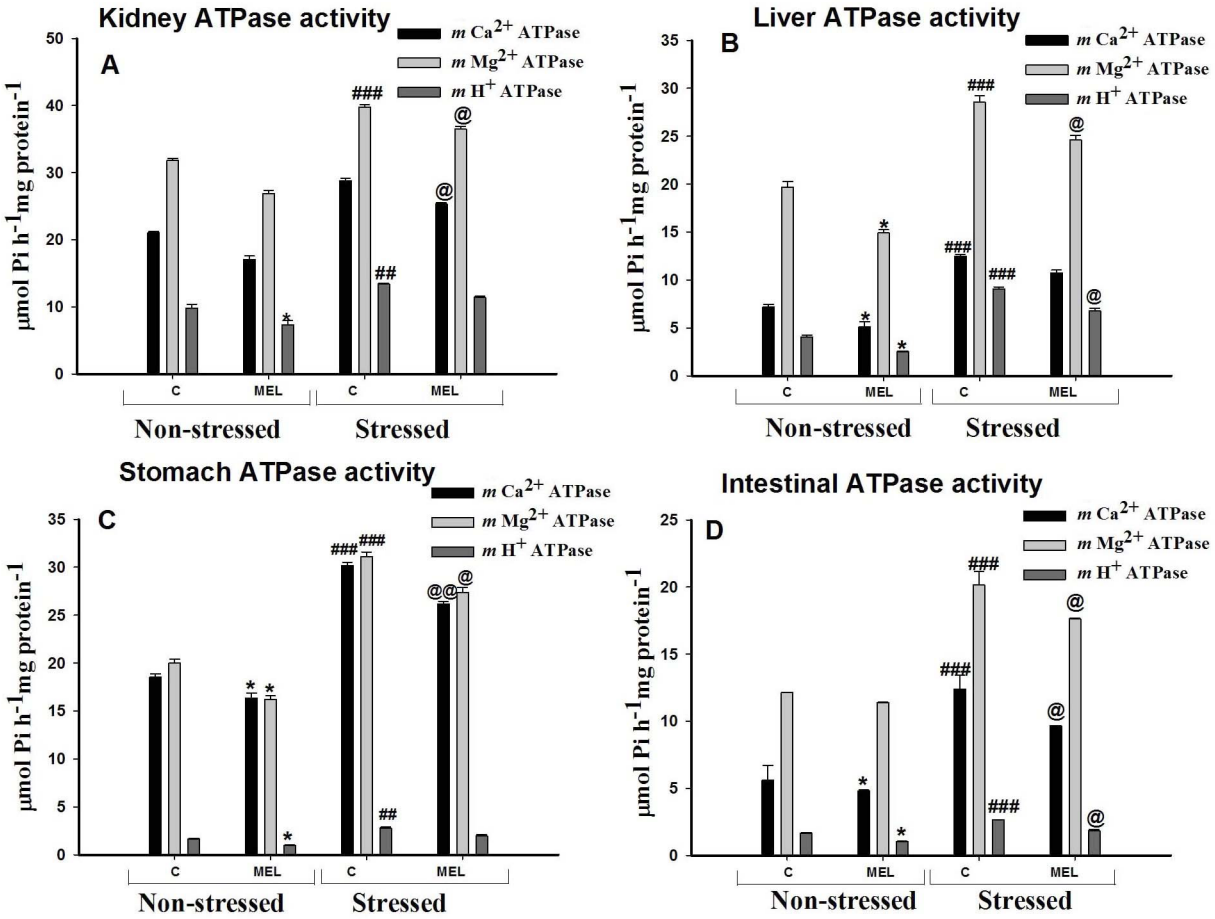

Fig.4. Effect of *in situ* melatonin for 20 min on the ATPase activity in the mitochondria of kidney (A), liver (B),stomach(C) and intestine (D) of mice kept at non-stressed or restraint stressed condition.. Each column represents mean ± SEM for four mice. $^{*}P< 0.05$ from saline perfused normal mice. $^{\#\#\#}P< 0.001$ and $^{\#\#}P< 0.01$ denotes significant difference between stressed and non-stressed mice. $^{@@}P< 0.01$ and $^{@}P< 0.05$ denote significant difference from melatonin perfused and saline perfused stressed mice.

maintain the efficiency of oxidative phosphorylation and ATP synthesis by increasing the activity of the respiratory complexes I, III, and IV (Reiter et al., 2007). These effects were attributed to the intra-mitochondrial presence of melatonin, thus showing melatonin's participation in the physiological regulation of mitochondrial homeostasis (L´opez et al., 2009). There is evidence of melatonin involvement in complex relationships between the nervous and endocrine systems (Guerrero and Reiter, 2002).

ATPases are lipid-dependent membrane-bound enzymes. Any perturbation in the activities of ATPases affects membrane status by inflicting changes in electrophysiological energetics and normal homeostasis (Mahendravarma and Surendrakumar, 1996). The effect of melatonin on ion transporter activities was tested in various tissues. A dose-dependent effect of *in situ* melatonin was observed in the ATPase activity of the liver, kidney, intestine and stomach tissues of non-stressed mice. A progressive decline in tissue's ATPase activity from its basal level was observed when mice were given short-term melatonin perfusion. The higher the dose perfused, the greater was the reduction in ATPase activity in all the tissues examined. 10^{-7} M melatonin produced significant reduction in Na^+,K^+-ATPase, *c* H^+-ATPase, *m* H^+-ATPase, *c* Ca^{2+}- ATPase, *m* Ca^{2+}- ATPase and

mMg^{2+}-ATPase activities in tissues followed by 10^{-8} M melatonin. Melatonin perfusion at 10^{-9} M appeared to be effective in maintaining optimum ATPase activity in most of the tissues.

As an essential sodium ion transporter generating transmembrane Na/K gradient across cell membrane, Na^+/K^+-ATPase is involved in the transport of many ions and it regulates many cellular functions (Evans, 1998). The Na^+/K^+-ATPase activity increased to significant levels in the renal, hepatic, gastric and hepatic tissues of restrained mice. Since Na^+/K^+-ATPase is critically involved in the maintenance of the intracellular Na^+ and K^+ concentrations and participates in the maintenance of cell volume and electrochemical gradients (Benziane et al., 2012), the increased transporter may point to ion regulatory action of melatonin in these tissues. Activation of Na^+/K^+-ATPase especially in kidney by catecholamines and corticosteroids like aldosterone and corticosterone have been reported earlier (Abdulnour et al.,1994; Ahmad and Medford,1995; Aperia et al., 1994). Kidney being the major organ in maintaining homeostasis, its activity is greatly mediated through the luxuriant distribution of Na^+/K^+-ATPase enzyme throughout the different parts of individual nephron (Beron and Verrey, 1994). The requirement for modulators of the Na^+/K^+-ATPase is likely to be greatest in tissues like kidney, in which perturbations of the intracellular alkali cation content underlie their specialized functions (Verrey et al., 2003). The Na^+/K^+-ATPase is subjected to both short- and long-term regulation by a variety of hormones. Short-term regulation involves either direct effects on the kinetic behavior of the enzyme or translocation of sodium pumps between the plasma membrane and intracellular stores (Aperia et al., 1994). On the other hand, long-term regulatory mechanisms generally affect de novo Na^+/K^+-ATPase synthesis or degradation (Ahmad and Medford, 1995).

Liver is the organ of immense importance due to its peculiar anatomy and metabolic functions which are exactly meant for efficient removal of the toxins. The ATPase establishes the Na^+ gradient needed for the transport of bile salts in liver (Erlinger, 1982) and drives the activity of many other transporters. Liver cell maintains its cellular Na/K gradient and directs cellular biochemical functions including nitrogen handling with the help of Na^+/K^+-ATPase activity (Babitha and Peter, 2010). The GI tract and liver are responsible for a disproportionately high fraction of whole-body energy utilization. The energetic cost of Na^+/K^+-ATPase, protein synthesis and degradation, substrate cycling and urea synthesis contribute substantially to energy expenditure (McBride and Kelly, 1990). Na^+/K^+-ATPase-dependent respiration is a major component of cellular energy expenditure in the small intestine of the rat (Liberman et al., 1979). Restraint stress induced irregularities in gastrointestinal motility which has been reported previously from various studies in rodent models (Tache, 1989; Koob and Heinrichs 1999; Maillot et al., 2000). All these energy-consuming processes account for a rise in pump activity.

Restraint stress also induced an increase in H^+-ATPase activity similar to Na^+/K^+-ATPase activity. Stress invariably evoked the activation of hypothalamic–pituitary–adrenal axis and the hormonal outputs of the respective glands and subsequent cellular changes. The level of cation stimulation of ATP hydrolytic activity may also be another reason for hyper H^+-ATPase activity during stress. Stress mobilizes endocrine, sympathetic, and behavioral systems to promote physiological adaptation and maintain homeostasis (Kathol et al., 1989; Marti et al., 1993; Blanchard et al., 1995; Herman et al., 1995). Moreover, V-type H^+-ATPase activity and F-ATPases share structural and functional similarities (Nishi and Forgac, 2002).

Ca^{2+} serves as a signal transducer, mediating signals from activated plasma membrane receptors, to carry out a variety of functions such as hormone secretion, neurotransmission and kinase phosphorylation (Wasserman, 2005). The cytosol uses the strategic placement and dynamic properties of Ca^{2+} transporters and channels to generate spatially and temporally complex Ca^{2+} fluctuation (Lee et al., 1997; Wilson et al., 1998; Kasai, 1999). The restraint stress increased Ca^{2+} mobilization which induced

hyper-Ca^{2+}ATPase activity in cytosol and mitochondria. Kultz (2003) reported that a Ca^{2+} transporting ATPase as part of the minimal stress proteome may be required to restore cytosolic calcium levels after the initial stress signal has been perceived, which is consistent with the present results. In stressed mice melatonin perfusion could lower the elevated Ca^{2+}-ATPase activity to baseline levels in cytosol as well as in mitochondria. It is suggested that melatonin undoubtedly plays a physiological role in restoring Ca^{2+} stores in cytosol and cell compartments during stress response in mice.

Mg^{2+}-ATPase are membrane transporters ubiquitous in animal cells that utilize ATP as a substrate for their functioning. Mg^{2+}-ATPase has abundant distribution and dual localization in mitochondria and cytosol and also acts as an index of general ATPase activity (Lehninger, 1988). At the sub-cellular level, magnesium regulates contractile proteins, modulates transmembrane transport of Ca^{2+}, Na^{+} and K^{+} and acts as an essential cofactor in the activation of ATPase, controls metabolic regulation of energy-dependent cytoplasmic and mitochondrial pathways and influences DNA and protein synthesis (Laurant and Touys, 2000; Hoenderop and Bindels,, 2005). The integrity of the cellular membrane, intracellular signaling and stabilization of tissue permeability depends upon the transepithelial regulation of Mg^{2+} ions (Parvez et al., 2006). Dissipation of the membrane potential releases mitochondrial Mg^{2+} into the cytoplasm (Kubota et al., 2005). Restraint stress increases the mitochondrial Mg^{2+}-ATPase levels. Extrusion of Mg^{2+} from mitochondria to cytosol may be the reason for increased ATPase activity in the mitochondrial membrane. As magnesium ion transporter, the Mg^{2+}-ATPase has a unique role in energy synthesis. Since a tight regulation of Mg transport exists across the membranes, the stress-induced metabolic alterations often result in the rapid production and mobilization of energy through the activation of mitochondrial electron transport chain and consequent production of ATP (Gardner, 2003; Warren et al., 2004). This might also elevate the mitochondrial Mg^{2+}-ATPase activity. Melatonin perfusion in stressed mice reduced the hyper- Mg^{2+}-ATPase levels. Romani and Maguire (2002) reported that the total cellular Mg^{2+} level changes upon addition of several hormones to cells. The altered glucocorticoid levels through melatonin action might work in the cells which may regulate the total Mg^{2+} levels thus suggesting a modulating effect of melatonin on transporter activity.

Exogenous *in situ* melatonin was found to abolish the ion transporter activity in all the tested tissues of restrained mice. A high-affinity binding site has been identified in the nucleus, where melatonin may regulate gene transcription or may prevent oxidative damage to DNA (Becker-Andre et al., 1994). Thus, the effects of melatonin may result from binding to high affinity receptors in the plasma membrane, cytosol or the nucleus, leading to changes in gene expression or altering second messenger levels (cAMP and Ca^{2+}). The effects of melatonin may also result from nonspecific interactions related to the antioxidant activity of melatonin or from direct binding to ion channels. The melatonin perfusion reduced the hyper-Na^{+}/K^{+}-ATPase activity due to the restraint stress. This suggests that pump activity can be altered in response to extracellular melatonin. Melatonin perfusion in stressed mice also regulates the hyper-H^{+}-ATPase, Ca^{2+}ATPase and Mg^{2+}-ATPase activites which suggest its protective role in maintaining cation homeostasis. Being a lipophilic hormone, melatonin has access to cytosolic, mitochondrial, and nuclear compartments and the reduction in the hyper-activity of ion transporters by melatonin perfusion can, therefore, be attributed to the protective effect including the antioxidant activity of melatonin or from direct binding to ion channels.

Overall, our results show that melatonin can exert a direct action on cation transport and can modify the ion transporter activity during restraint stress in mice.

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