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EXPERIMENTAL STUDY

Modulatory role of melatonin on superoxide release by spleen macrophages isolated from alloxan-induced diabetic rats

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Abstract: *The aim* of this study was to evaluate the modulatory effect of melatonin on superoxide release by spleen macrophages isolated from alloxan-induced diabetic insulin treated or non-treated rats.
Methods: Blood glucose, body weight, CuZn-superoxide dismutase concentration, and superoxide release by spleen macrophages were evaluated.
Results: The spontaneous superoxide release from the macrophages of the control group was lower when compared to diabetic rats without insulin treatment. Melatonin (MLT) significantly increased the superoxide release in the control group (11.5±1.5 with MLT x 6.8±1.0 without MLT). The macrophages from diabetic rats treated with insulin exhibited a decreased superoxide release (7.0±2.4), when compared to superoxide release of the macrophages from diabetic rats without insulin (14.7±3.7). CuZn-SOD concentrations were increased in both diabetic groups.
Conclusion: The pineal hormone melatonin in physiological concentration can stimulate natural immunity since it triggers the superoxide release from the macrophages, an important anti-infectious mechanism. On the other side, melatonin had antioxidant effects in the macrophages from insulin-treated alloxan-induced diabetic rats *(Tab. 1, Fig. 2, Ref. 51)*. Full Text (Free, PDF) *www.bmj.sk.*
Key words: melatonin, macrophage, superoxide, pro-oxidant, antioxidant, insulin.

Melatonin, a mammalian pineal gland neurohormone, has diverse functions such as ability to scavenge oxygen and nitrogen free radicals, stimulatory activity on the antioxidant enzyme cell systems (1–2). On the other hand, melatonin may be considered a potent immunomodulatory agent in both animals and man (3–5). Melatonin performs immunomodulatory effects partially due to its specific actions on the melatonin receptors in immunocompetent cells (6). For example, melatonin was found to enhance cytokines production in human lymphocytic cell line (7). In physiological concentrations, melatonin can also stimulate phagocytic activity of macrophages in animals (8).

It is well known that patients with diabetes mellitus are more susceptible to acquire infectious diseases. Disorders of phagocytosis and failure of the superoxide release are considered the main factors contributing to chronic and recurrent infections (9). Diabetic patients usually present with a delayed phagocytosis and microbicidal activity, decreased release of lysosomal and antioxidant defense enzymes as well as with a failure on oxidative-burst, an important microbe-killing pathway (9). It has been suggested that hyperglycemia impairs the leukocyte phagocytosis and microbicidal activity (10, 11).

In the alloxan- or streptozotocin-induced rat diabetes models, the following changes have been observed: 1) increased chemotaxis of neutrophils (12, 13); 2) decreased phagocytic activity (13); 3) decreased release of hydrogen peroxide (14); 4) increased oxidative stress (15); and 5) overproduction of the superoxide dismutase (16). These diabetic oxidative stress triggers the LDL peroxidation and compromise an antioxidant protection, the effects dependent on a hyperglycemic environment (17).

Nowadays it has been shown that melatonin exert beneficial effects in controlling diabetes complications (2), and scavenging reactive oxygen species (1) in a dose-dependent manner (18). Melatonin also improves diabetes and its associated metabolic disorders (18, 19). Melatonin stimulates antioxidant cell systems, which decisively influence insulin secretion and glucose homeostasis in hypercholesterolemic (20) and diabetic rats (21). On the other hand, diabetic patients have decreased melatonin levels, which may be involved in the impairment of insulin secretion, immune disorders, and other features of diabetes pathogenesis (22).

A failure of phagocytes functional activity may contribute to an increased susceptibility and severity of infections in diabetes (13). Melatonin has important immunomodulatory roles in disease control and release of free radicals. Then, this study evalu-

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ated the modulatory effect of melatonin on the superoxide release of spleen macrophages isolated from the alloxan-induced diabetic rats. The effects of hyperglycemia on superoxide dismutase were also studied.

Materials and methods

Animals and operation procedures

Adult (10–12 weeks old) male Wistar rats, weighting between 200 and 250 g, were kept in constant light conditions (photoperiod L:D 12 h:12 h), 25 °C temperature, with *ad libitum* access to water and food. The animals were separated in the three groups: control, alloxan-induced without insulin and alloxan-induced treated with insulin. For the experimental diabetes induction, animals were fasting 12 hours. After this time, they received a single intravenous injection of alloxan (42 mg/kg body weight) in the median region of the tail. Control animals were given an equivalent volume of saline (0.9 %).

Fifteen days after the alloxan administration, diabetes was confirmed by the measurement of the blood glucose level using the "Accu-Check Advantage" glucometer (Roche). The animals of each group were followed during next 21 days. Blood glucose levels were determined at the 1°, 7°, 14° and 21° day of the experiment. After confirming the diabetes, insulin therapy was started in the alloxan-induced diabetic group. The average blood glucose levels were calculated using all values obtained during experiments.

On the 21 day in the morning, animals were anesthetized with an ethyl ether and underwent the surgical procedure. Spleen was removed and placed in the phosphate buffered saline (PBS) solution. The blood was collected for blood glucose and superoxide dismutase (SOD) evaluation.

Obtaining plasma

Blood was collected together with an anticoagulant, and then centrifuged for 15 minutes at 160 G until plasma separated from the red blood cells. Plasma samples were stored as individual samples of 1 ml at 20 °C for the later evaluation of superoxide dismutase (SOD).

Isolation, Purification and identification of Macrophages

The spleens of animals were macerated in the PBS. Cells were fractioned by a centrifugation (160 x g, 30 min) through gradient density (density 1.077 g/l) using the Ficoll-Paque (Pharmacia, Uppsala, Sweden). Purified macrophages were re-suspended independently in the serum-free medium 199 (Gibco, MD, USA) and washed twice. Cell counts using the Newbauer hemocytometer and the macrophages suspensions were adjusted at the final concentration of $1x10^6$ cells/ml and used in the measurements of the superoxide release (23). Macrophages were identified under the light microscope and the viability was measured using the trypan blue exclusion test, before the procedures of the superoxide release.

CuZn-Superoxide Dismutase Determination (CuZn-SOD)

CuZn-SOD enzyme analysis was made from the plasma using the method of reducing the Nitro Blue Tetrazolium (NBT-

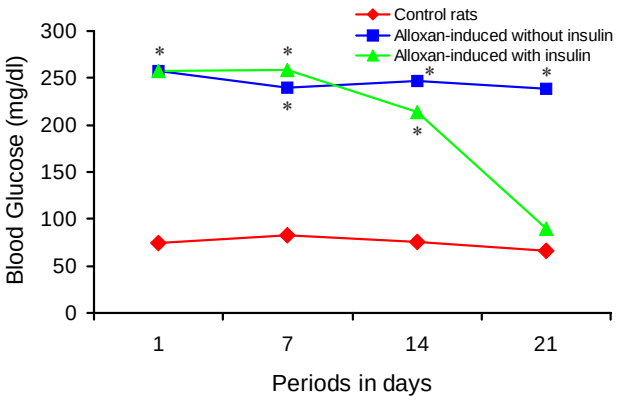

Fig. 1. Blood Glucose (mg/dl) in the alloxan-induced diabetic rats treated or not with insulin, during 21 days (n=10 for each treatment), * p<0.05 (ANOVA) comparing control rats to alloxan-induced diabetic rats.

Sigma) which used a reading on spectrophotometer at 560 nm (24). Individual samples were placed in glass tubes and another tube was set aside to write a standard solution. The volume of each sample was 0.5 ml and the standard tube consisted of 0.5 ml of hydro-alcoholic solution. Then 0.5 ml of chloroform-ethanol solution (1:1 ratio) and 0.5 ml of reactive mixture (NBT increased by EDTA) was added to all tubes. Then, in standard solution and in each sample, 2.0 ml of buffer carbonate, pH increased to 10.2 when hydroxylamine was added (25, 26).

All tubes remained at rest at room temperature without stirring for 15 min, and then each sample was read in extinction of 560 nm, hitting zero with the reactive mixture (total 3.5 ml). The analyzed superoxide dismutase was calculated by the following relationship:

SOD=(Ab standard - Ab sample/Ab standard) x 100 = % reduction of NBT/CuZn-SOD. The results were expressed in international units (IU) of CuZn-SOD.

Release of Superoxide anion

The release of superoxide was measured by determining the cytochrome C (Sigma) reduction as previously described (23, 27). The macrophages were centrifuged twice at 160 x g, for 10 min at 4 °C and 500 ml of phagocytes was re-suspended in PBS containing 2.6 mM $CaCl_2$ and 2.0 mM $MgCl_2$ and the cytochrome C (Sigma-2 mg/ml). The macrophages were stimulated with the 10 µL de Phorbol Myristate Acetate (PMA – Sigma, final concentration at 10^{-7} M) and 50 µL of melatonin (Sigma, final concentration at 10^{-7} M) (8). The suspensions (100 µl) were incubated for 60 min at 37 °C on culture plates. The reaction rates were measured by absorbance at 450 nm and the results were expressed as nmol/O_2^-. All the experiments were performed in duplicate or triplicate.

Statistical analysis

The quantitative data of blood glucose, weight, superoxide release, SOD concentration were analyzed using the variance analysis (ANOVA), the statistical F was calculated, and the mini-

Tab. 1. General Characteristics of alloxan-induced diabetic and control rats.

Parameter measured	Control rats (N=10)	Alloxan-diabetic rats without insulin (N=10)	Alloxan-diabetic rats with insulin (N=10)
Glucose level (mg/dl)	74.7±7.0	245.5±8.6*	205±79.4*
Body weight	318.6±16.6	288.5±14.1	293.8±24.9
Number of macrophages (x10^6 cells/ml)	4.6±0.6	3.2±0.5	3.8±0.4
Viability of macrophages (%)	89±4.7	88.9±5.7	85.8±5.9
Superoxide release (nmol) (unstimulated cells)	6.9±1.0	14.8±3.3*	9.49±4.3
CuZn-SOD	10.4±3.6	27.9±2.9*	26.3±10.6*

* $p<0.05$ (ANOVA) comparing control rats to the alloxan-induced diabetic rats

mal significant differences were calculated according to the Tukey's method (28). All results were expressed as mean±SD. The difference was considered significant when p-values were lower than 0.05 ($p<0.05$).

Results

Evaluation of Alloxan-induced hyperglycemia

Evaluation was performed in four different times and the alloxan-induced diabetic rats not treated by insulin presented significantly ($p<0.05$) increased blood glucose levels with no change over the study period (Fig. 1). However, in the alloxan-induced insulin-treated diabetic rats decreasing blood glucose levels were found over the time, reaching values similar to control group in the 21st day (Fig. 1).

The mean glucose level was significantly higher ($p<0.05$) in the aloxan-treated rats when compared to the untreated group (Tab. 1). There was no significant difference ($p>0.05$) in body weight of diabetic animals compared to the control group.

The effect of diabetes on the number and function of macrophages

Diabetes did not influence ($p>0.05$) neither the number nor the viability of spleen macrophages in rats (Tab. 1).

In contrast, the functional activity of macrophages from the alloxan-induced diabetic rats not treated by insulin significantly increased ($p<0.05$) (Tab. 1). Macrophages from the alloxan-induced diabetic rats treated with insulin presented no change of functional activity regarding a spontaneous superoxide release (Tab. 1).

The effects of diabetes on the CuZn-Superoxide Dismutase (CuZn-SOD)

Diabetes influenced the CuZn-SOD concentration in both diabetic groups. Compared to the control rats (10.4±3.6), a significant increase ($p<0.05$) of the CuZn-SOD concentration was observed in the alloxan-induced diabetic groups, regardless the insulin treatment (27.9±2.0 without insulin x 26.9±10.6 with insulin) (Tab. 1).

Melatonin and superoxide release in macrophages

The melatonin concentration used in this study (10^{-7} M) significantly stimulated ($p<0.05$) the superoxide release in macrophages from normal rats (11.5±1.5) compared to the superoxide release in non-stimulated macrophages (6.8±1.0) (Fig. 2). There was no significant difference ($p>0.05$) between the stimulation by melatonin compared to stimulation by the PMA (9.1±1.6) in the control group. Melatonin failed to stimulate the alloxan-induced diabetic rats without insulin treatment (14.7±3.7 with melatonin compared with 12.2±3.2 without melatonin). The superoxide release by the non-stimulated macrophages (12.2±3.2) present similar levels to those stimulated by the PMA (14.8±3.6) and melatonin (14.7±3.7). In the alloxan-induced diabetic rats treated with insulin, melatonin administration decreased ($p<0.05$) the superoxide release (7.0±2.4) when compared to the superoxide release in macrophages from the diabetic rats without insulin treatment and melatonin stimulation (14.7±3.7) (Fig. 2).

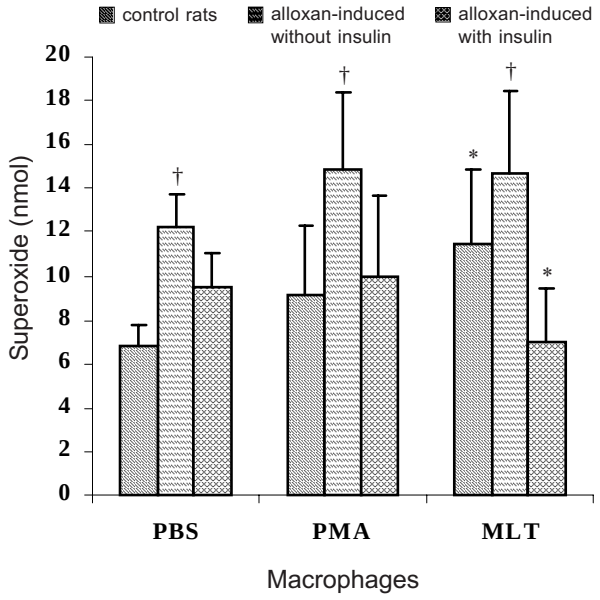

Fig. 2. Effects of melatonin (MTL – 10^7 M), PBS (control) and PMA (10^7 M – positive control) on the macrophage superoxide release (mean±SD) in control or alloxan-induced diabetic rats (N=10 for each treatment). * $p<0.05$ comparing the superoxide release in cells stimulated by the PMA or melatonin to PBS, considering the same experimental group. + $p<0.05$ comparing the control group to the alloxan-induced diabetic groups, considering the same cell treatment.

Discussion

The present study evaluated the modulatory effects of melatonin on the superoxide release in macrophages from the aloxan-induced diabetic rats.

Alloxan-induced diabetic rats showed hyperglycemia accompanied by a trend, not statistically significant, of a lower body weight (Tab. 1). Insulin therapy induced a reduction of blood glucose from the 14th day, restoring normal glucose levels on the 21st day, reaching levels similar to those in the control group. Insulin resistance *per se* is not a disease, but rather an abnormality that increases the risk of many biochemical disorders associated with glucose intolerance and its metabolic consequences (19, 29).

On the other hand, there was no change in the number and viability of macrophages present in the spleen in both diabetics and control group. Beyond, the superoxide release was higher in the alloxan-induced diabetic rats compared to the alloxan-induced diabetic rats treated with insulin and values observed in the control group. This data indicate that hyperglycemia did alter these cells in rats.

The alloxan induction of diabetes is not dose-dependent, due to low damaging effects on the β-cell membranes until the necrosis of pancreatic islet cells (30–32). The excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, which eventually culminate in triggering cell death pathways (33, 34). A great variety of mechanisms have been suggested in the formation of the reactive oxygen-free radicals. Glucose oxidation is believed to be the main source of free radicals in diabetes (35). Hyperglycemia has also been found to promote lipid peroxidation by a superoxide-dependent pathway resulting in free radicals' generation and release (36, 37). Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of advanced glycation-end products that contributes to the excessive mitochondrial free radicals-burst (33). In this study, the high blood glucose level in diabetic rats determined changes in the functional activity of macrophages. These cells were more active, as shown by an increased oxidative metabolism and the consequent enhanced superoxide anion production.

While hyperglycemia enhances the mitochondrial and cytosolic membrane production of free radicals, it also strongly impairs different endogenous antioxidant defense systems in diabetic patients (38). Antioxidant defense mechanisms involve enzymatic and non-enzymatic pathways. Among the enzymatic mechanisms, CuZn-SOD plays a relevant role. Changes in the SOD activity have been found in chemically-induced diabetic animals (33). In this study, an increase of the CuZn-SOD concentration was observed in diabetic groups when compared to the control group. It should be noted that the diabetic effects on the SOD synthesis is a matter of controversy. Paradoxical results have been observed considering the gender, animal species, onset of disease, and different target tissues. Some authors reported increased levels of SOD whereas others observed decreased or even normal SOD concentrations in diabetes (39–42).

Neuro-hormonal control is very important in modulating the immune effects (43). Melatonin has beneficial, free radical scavenging actions beyond its stimulatory effects on diverse cytosolic antioxidant enzymes' systems (1, 2, 5). Many studies reported that melatonin strongly stimulates the immune cells (8, 44, 45). This study showed that melatonin stimulated the cellular oxidative metabolism of control rats, proven by the increased superoxide release in macrophages reaching values similar to the PMA-induced macrophages (positive control). Moreover, the macrophages of diabetic rats not treated with insulin were not stimulated by melatonin (Fig. 2). This lack of stimulation of superoxide release in macrophages, e.g., a failure of pro-oxidant effect, is in accordance to the antioxidant effects of melatonin in the alloxan-induced diabetes models (46, 47). The diabetic group with insulin therapy presented a decreased superoxide release, an effect enhanced by the antioxidant action of melatonin. Similar results were found by Pawlak et al (8) who observed stimulating effects of melatonin on the phagocytosis in normal rats. In the same study, melatonin failed to stimulate phagocytosis in cells isolated from diabetic rats.

The generation of free radicals has been reported as an important protective mechanism during the infectious processes, mainly in intestinal infections (23, 48, 49). In this study, there was no failure of the superoxide anion release in diabetic animals (Fig. 2). Contrary to the cells from the control group, the superoxide release in macrophages from the diabetic rats failed to be modified by melatonin and PMA, indicating that hyperglycemia may affect macrophages' function. Most probably, those cells were activated by an uncontrolled glucose metabolism.

In conclusion, the pineal hormone melatonin in physiological concentration in control animals can stimulate natural immunity since it triggers the release superoxide from macrophages, which is an important anti-infectious mechanism (11, 23, 24). When animals were rendered diabetic, but not treated with insulin, an increase on spontaneous superoxide release was observed, with values equivalent to the cells of the control group stimulated by melatonin or PMA. On the other side, macrophages from diabetic rats treated with insulin in the presence of melatonin had potentially lower superoxide release. These results suggest the existence of a relationship between the glucose metabolism in diabetic animals and melatonin response in macrophages. In macrophages from diabetic animals without insulin therapy melatonin had no effect, while melatonin displays potent antioxidant effects on the superoxide release in macrophages from diabetic rats in the presence of insulin.

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