IN VITRO PROTECTIVE ROLE OF MELATONIN AGAINST HEMOLYSIS INDUCED BY SODIUM FLUORIDE IN HUMAN RED BLOOD CELLS

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SUMMARY: This study was designed to evaluate the protective effect of melatonin against *in vitro* hemolysis induced by fluoride (F) in human red blood cells. Venous blood samples from twenty healthy, well-nourished male donors, age 20–25, residing in a nonfluorotic area of Ahmedabad, India, were collected for the preparation of red blood cell (RBC) suspensions in normal saline. When 2.0 mL of the RBC suspensions were treated for 4 hr at 37ºC with 0.05–0.5 mL of 4 mg NaF/mL in normal saline (50–500 µg NaF/mL in a final normal saline volume of 4.0 mL), they exhibited a significant dose-dependent increase in hemolysis. Addition of melatonin (5 µg/mL and 10 µg/mL) to the final volume caused a significant reduction in F-induced hemolysis with maximum amelioration occurring at 10 µg/mL. Melatonin therefore can exert a significant protective action against F-induced hemolysis *in vitro.*

Keywords: Amelioration of hemolysis; Fluoride-induced hemolyis; Human red blood cells (RBC) corpuscles; Melatonin and hemolysis.

INTRODUCTION

Fluoride (F) is a well-known soil, water, and air contaminant, and its toxicity in humans has been widely studied. Intake of excess F through drinking water, food, or inhalation causes a wide range of toxic effects known as 'fluorosis'. F can cross cell membranes and enter bone, soft tissues, and blood.[1,2,3] Chronic administration of F to animals under laboratory conditions induces various changes in all the organ systems including blood. These include abnormal behavior patterns,[4] altered neuronal and cerebrovascular integrity,[5] neurological manifestation,[6] and metabolic lesions.[7,8,9] Recent studies in our laboratory have demonstrated the ability of melatonin (N-acetyl-5-methoxytryptamine) to reduce F-induced hepato- and nephrotoxicity in mice.[10,11]

Melatonin is the major secretory product of the pineal gland in the brain and is well known for its functional versatility. In hundreds of investigations, melatonin has been documented as a direct free radical scavenger and an indirect antioxidant as well as an important immunomodulatory agent.[12]

The aim of present study was to evaluate the *in vitro* hemolysis by F in human red blood cells and its amelioration by melatonin.

MATERIALS AND METHODS

Samples of venous blood (2.5 mL) were obtained with voluntary consent from 20 well-nourished, healthy adult male donors, age 25–30 years, residing in nonfluorotic areas of Ahmedabad, India, and not having any sign of dental fluorosis. The samples were collected in 2.5-mL vials containing ethylenediaminetetraacetic acid (EDTA), diluted with normal saline (0.9% NaCl), and centrifuged at 300 g for 10 min. The RBC pellets were washed twice and finally suspended in normal saline to have a cell density of 2 x 10^4 cells/mL.

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For the experiments, solutions of 4 mg NaF/mL were prepared in normal saline. Melatonin was first dissolved in ethanol and further diluted with normal saline. The final concentration of ethanol in the solution was less than 1%.

To determine the effect of NaF on RBCs, two sets of spectrophotometric tubes were prepared, control and NaF treated tubes. Control tubes contained 2.0 mL RBC suspension and 2.0 mL normal saline. In the NaF-treated tubes different volumes of the NaF solution (0.05–0.5 mL) were mixed with 2.0 mL RBC suspension, and the final volume was made to 4.0 mL with saline. The concentrations of NaF in the 4-mL final volume therefore ranged from 50 to 500 µg/mL.

To determine the effect of melatonin on NaF-induced toxicity in RBCs, two sets of spectrophotometric tubes were prepared: tubes treated with melatonin alone and tubes treated with NaF plus melatonin. In the melatonin-treated tubes 0.1 mL melatonin was mixed with 2.0 mL RBC suspension. Final volume was made up to 4.0 mL with normal saline. In the NaF plus melatonin treated tubes 0.05-0.1 mL melatonin was mixed with 2.0 mL RBC suspension and the final volume was made up to 4.0 mL with normal saline. The concentrations of NaF and melatonin in the final volume were therefore 5–500 µg/mL and 5–10 µg/mL, respectively.

All tubes were incubated at 37ºC for 4 hr with intermittent shaking. Absorbance of the supernatants was obtained after centrifuging the incubated tubes at 300 g for 10 min. Absorbance was read spectrophotometrically at 540 nm, and the percent hemolysis was calculated by the formula:

$$\text{Percent hemolysis} = \frac{\text{Absorbance of individual tubes}}{\text{Absorbance with 100\% hemolysis}} \times 100$$

To achieve 100% hemolysis by hypotonic action, 2.0 mL distilled water was added to 2.0 mL RBC suspension. The percent hemolysis with reduction in hemolysis with melatonin was calculated using the following formula.[13,14]

$$\text{Percent reduction in hemolysis} = \frac{X-Y}{X} \times 100$$

X = sodium fluoride induced hemolysis,

Y = hemolysis caused by concurrent addition of melatonin.

Morphological alterations in RBC were also noted microscopically by staining RBC smears with Leishman's stain.

Statistical analysis of the data was performed using Student's t-test. Values of $p<0.001$ were considered statistically significant.

RESULTS

In control tubes the RBCs appeared as normal spheres or biconcave discs. The cells remained settled in the bottom of the tubes with clear supernatant indicating no hemolysis.

Addition of F (as NaF) to the RBC suspension showed a significant ($p<0.001$) increase in RBC hemolysis as compared to the control tubes. The cells remained settled at the bottom of the tube, but the saline developed a reddish colour, indicating hemolysis. As seen in the table, the results revealed a significant dose-dependent increase in hemolysis. Melatonin alone had essentially no effect and gave readings comparable to the control values. On the other hand, co-incubation of melatonin with NaF demonstrated a significant decline in F-induced hemolysis compared to the respective pro-oxidant group.

Table. Dose-dependent *in vitro* increase in hemolysis (%) of human RBCs by NaF and its reduction (%) by melatonin. (Mean ± SEM; n = 20)

Treatments		Hemolysis (%)	Reduction (%)
Concentration of NaF (µg/mL in final volume)	Concentration of melatonin (µg/mL in final volume)		
0	0	2.25 ± 0.052	-
0	5	2.38 ± 0.04	-
0	10	2.55 ± 0.010	-
50	0	4.35 ± 0.031[a *]	-
50	5	3.37 ± 0.09 [b *]	22.52
50	10	3.03 ± 0.09 [b *]	30.34
100	0	8.60 ± 0.080 [a *]	-
100	5	6.70 ± 0.25 [b *]	22.90
100	10	5.63 ± 0.32 [b *]	34.50
150	0	18.23 ± 1.71 [a *]	-
150	5	12.97 ± 0.39 [b *]	28.85
150	10	10.83 ± 0.20 [b *]	40.59
300	0	29.56 ± 0.063 [a *]	-
300	5	25.06 ± 2.022 [b *]	15.22
300	10	20.31 ± 2.58 [b *]	31.29
500	0	81.68 ± 0.051[a *]	-
500	5	52.95 ± 4.66 [b *]	35.17
500	10	45.73 ± 4.38 [b *]	44.01

[a]Compared with control; [b]compared with sodium fluoride control; *significant at the level $p< 0.001$.

DISCUSSION

The results demonstrate that NaF in the range of 50 to 500 µg/mL in the RBC suspensions affected membrane permeability leading to influx of water into the cells thereby causing hemolysis. It might also be due to an increase in lipid peroxidation and oxidative damage. Moreover, F can inhibit or activate various functions in blood cells. Neutrophils affected by F exhibit increased oxygen intake and production of superoxide anion along with decreased phagocytic ability.[15] F also affects erythrocyte membrane transport system, e.g inhibits K^+-Cl^- co-transport.[16] Alterations in cation pump activity caused by F occur as a direct inhibition of Na^+-K^+-ATPase.[17] This can be observed in erythrocyte shadows in people exposed to fluoride under both *in vitro* and *in vivo* conditions.[18] F ions *in vivo* have further been shown to decrease ATP concentration in erythrocytes.[19]

Under conditions of high oxidative stress *in vivo* and *in vitro* melatonin has proved superior to vitamins C and E in reducing oxidative damage.[20,21] As found here, addition of melatonin to RBC suspensions significantly reduced the rate of hemolysis compared to addition of NaF alone.

Melatonin exhibits scavenging action at both physiological and pharmacological levels. It protects membrane lipids, nuclear DNA, and protein from oxidative damage induced by a variety of free radical generating agents.[22-25] Reduction in hemolysis by addition of melatonin could be attributed to scavenging hydroxyl radical, stimulating antioxidative enzymes, inhibiting capacity of prooxidative enzymes. Studies also reveal that melatonin eliminates the decomposition products of peroxynitrite ($ONOO^-$), including $OH^\bullet$, $NO_2^\bullet$, and the carbonate ion radical ($CO_3^{\bullet-}$) in the presence of physiological carbon dioxide concentrations.[26,27] It also supports several intracellular enzymatic antioxidant enzymes under *in vivo* conditions.[28]

There are also indications that melatonin is efficient in reducing oxidative stress. Recently Bharti and Shrivastava have reported that melatonin is capable of improving the serum Na^+ level and therefore may prove beneficial in fluid electrolyte imbalance conditions[29]. Earlier research in our laboratory also demonstrated that melatonin supplementation provides protection against chromosomal anomalies induced by F under *in vitro* condition[30]. Gavella and Lipovac[31] found that the antioxidative property of melatonin suppresses experimentally induced lipid peroxidation in sperm membrane by protecting the integrity of the membrane and safeguarding against motor assembly deformities exerted by mercury ions.[33]

Possibly the efficiency of melatonin in reducing the oxidative stress may be due to its metabolites that are produced during its scavenging actions. These metabolites, i.e., cyclic 3-hydroxymelatonin (cyclic 3-OHM), N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), and N^1-acetyl-5-methoxykynuramine (AMK) also seem to be very efficient oxidative free radical scavengers.[34-35] Thus, second and third generation metabolites of melatonin might well contribute to the ability of the parent molecule to protect against oxidative burst. In this way, melatonin and its metabolites might be able to neutralize numerous adverse

reactions exerted by certain toxicants. As shown here, melatonin appears to be a potent antioxidant, since its use under *in vitro* conditions provides significant protection against hemolysis induced by NaF.

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