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RESEARCH ARTICLE

ASSAY OF *IN VITRO* EFFECT OF MORIN-5'-SULFONIC ACID SODIUM SALT (NAMSA) ON ANTIOXIDANTS IN ERYTHROCYTES

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Abstract

Flavonoids has proved its action against allergy and against metal chelation. Hence A preliminary study was designed to assess the *In vitro* effect of Morin-5'-Sulfonic Acid Sodium Salt in goat erythrocytes. In order to determine the optimum dose for morin-5'-sulfonic acid sodium salt 3 different concentrations of 25 µM, 50 µM and 100 µM morin-5'-sulfonic acid sodium salt used and from that 50 µm was found to be the optimum dose. Themorin-5'-sulfonic acid sodium salt25 µM, 50 µM administration maintained the CAT, SOD, vitamin E and levels slightly higher than control group and vitamin C remains similar to control group. Whereas administration of 100 µM NaQMSA, the CAT, SOD, vitamin Evalues were maintained similar to 25 µM, 50 µM group at the same time vitamin C levels was slightly decreased compared with the control group. NaMSA higher concentration may inhibits the activity of vitamin C in erythrocytes in a dose-dependent manner. From the obtained results it was concluded that, Addition of morin-5'-sulfonic acid sodium salt up to 50 µM did not generates free radical and enhances CAT, SOD, vitamin C and vitamin E activity in erythrocytes. On the other hand addition of 100 µM of NaMSA to the RBC suppresses vitamin C activity and does not enhance CAT, SOD and vitamin E activities. Hence, 50 µM of NaMSA was found to be the optimum dose for *in vitro* assay of RBC (5ml) activity.

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Keywords: NaMSA-Morin-5'- sulfonic acid sodium salt, CAT-Catalase, SOD-Super oxide dismutase,

Introduction

Flavonoids

Flavonoids have recently attracted a great interest as potential therapeutic agents against a large variety of diseases, such as anti-viral, anti-allergic, anti-platelet and anti-inflammatory, and possibly protective effects against chronic diseases (Chantal et al., 1996: Hollman *et al*., 1999).

Flavonoids are effective scavengers of free radicals in the test tube. However, even with very high flavonoid intakes, plasma and intracellular flavonoid concentrations in humans are likely to be 100-1,000 times lower than concentrations of other antioxidants, such as ascorbate (vitamin C), uric acid, or glutathione. Moreover, most circulating flavonoids are actually flavonoid metabolites, some of which have lower antioxidant activity than the parent flavonoid. For these reasons, the relative contribution of dietary flavonoids to plasma and tissue antioxidant function in vivo is likely to be very small or negligible. (Victoria and Drake, 2008) The metal-chelating activities of flavonoids may be beneficial in pathological conditions of iron or

copper excess; it is not known whether flavonoids or their metabolites function as effective metal chelators *in vivo* .

Morin other names are aurantia, calico yellow, toxylonpomiferum. It is aubiquitous phenolic secondary metabolite foun din orange, almonds, mill, fig, onion, guava and apple. Morin is an integral part of the human diet.

Morin hydrate is a flavonoid with antioxidant properties shown to protect cells against oxygen radical damage in vitro (Zeng*,* 1997). Morin not only scavenges oxy radicals, but also moderately inhibits xanthine oxidase, a free-radical generating enzyme. Chelating gents are primarily sulfhydril-containing compounds such as mono- or dithiol molecules. At the molecular level, the chelation process appears as an inevitable tug of war between the chelating agents and the competing biological ligands (Andersen and Molecular, 2002). Hence, it is necessary to convert Morin into morin-5-sulfonic acid sodium salt (NaMSA).

NaMSA is easily soluble in water and keep properties of the parent compounds. The aqueous solubility of NaMSA under the same conditions was 2.7.10-2 mol/dm3. Sulfonic morin derivative can be considered to be multiprotonic acids, which dissociate in aqueous solutions yielding respective anions and NaMSA was used as antioxidant. NaMSA is characterized by low toxicity to laboratory animals (mice and rats) (Kopacz., 2002 : Szelag., 2003). Flavonoids are mutagenic only under aerobic conditions (Nagao *et al.,* 1981) strongly suggests a role for active oxygen.

Evaluation of herbal toxicity

Herbal toxicity can be evaluated by (1) observing human or animal populations exposed to the plant material, (2) administering the plant medicine to animals under controlled conditions and observing the effects (*in vivo*) and (3) exposing cells, sub-cellular fractions or single-celled organisms to the plant material (*in vitro*) (Timbrell, 2002).A large number of plants contain appreciable levels of biosynthetically produced chemical substances and many of these have either been reported to be toxic to humans or are predictably toxic based on extensive animal or in vitro studies (Tomlinson and Akerele, 1998).Intravenous administration of queretin at doses of 945 mg/m^2 or more was associated with renal (kidney) toxicity in that trial (Roderick and Dashwood, 2008) .

Antioxidants

An antioxidant is defined as any substance present at low concentration compared to those of an oxidisable substrate significantly delays or prevents oxidation of those substrates(Diplock, 1991; Halliwell and Gutteridge, 1999). The intracellular antioxidants include low molecular weight scavengers of oxidizing species, and enzymes, which degrade various radicals especially O2•- .

Enzymatic antioxidants

Antioxidant enzymes such as superoxide dismutase, catalase, thio redoxin reductase, glutathione peroxidase, glutathione-S-transferase, aldo-keto reductase and aldehyde dehydrogenase exist in the cells convert ROS into less noxious compounds. These enzymes collectively provide a defence against various radicals and oxidants (Arner and Holmgren, 2000; Kuhn and Borchert, 2002).

Superoxide dismutase (SOD) is a primary antioxidant enzyme, which catalyses the dismutation of O2•− to the less-reactive species H_2O_2 and O_2. The cellular SOD is represented by a group of metalloenzymes with various prosthetic groups. The three forms are cytosolic Cu-Zn-SOD, mitochondrial Mn-SOD and extracellular Cu-SOD. Catalase (CAT) is a tetrameric hemeprotein, which is located in peroxisome and very efficiently promotes the conversion of H2O2 to water and molecular oxygen. (Halliwell and Gutteridge, 1999; Valko et al., 2007).

Non enzymatic antioxidants

The non-enzymatic antioxidants are low molecular weight substances, which includes vitamin C, vitamin E, carotenoids, thiol antioxidants (glutathione, total sulphydryl groups (TSH), thioredoxin (TRX) and lipoic acid (Valko et al., 2007).

Materials and Methods

Chemicals used

The fine chemicals Alanine, used for the present study purchased from Morin, from Sigma chemicals, USA procured from its Bangalore supplier. Nitroblue tetrazolium salt

(NBT),reduced nicotinamide adenine dinucleotide (NADH),reduced nicotinamide adenine dinucleotide phosphate (NADPH), phenazine methosulphate (PMS) and thiobarbituric acid (TBA) were purchased from Merck Company (AR Grade) dealer, Chennai.

Methods

Standard methods were used for the estimation of factors. SOD by the method of Kakkar *et a*l. (1984), catalase by the method of Sinha (1972).Vitamin E was estimated by the method of Desai (1984) and the level of ascorbic acid was estimated by the method of Omaye *et al*., (1979).

Preparation of Morin-5'-Sulfonic Acid Sodium Salt

Morin was purchased from sigma chemicals, USA is not soluble in water but soluble in alcohol 50 mg/ml.so, it is necessary to convert insoluble form of morin to water soluble Morin-5'-Sulfonic Acid Sodium Salt .Hence, sulphonation reaction was carried out and morin was converted into Morin-5'-sulfonic acid sodium salt (Kopacz., 2003) and used for the present experiment.

Erythrocyte preparation

120 millilitres of fresh goat blood was collected in dry tubes from a healthy goat through cervical decapitation and using heparin anticoagulant. Erythrocytes were separated from blood plasma by centrifugation (1600 rpm at 40^0C for 5 min), then washed three times with ice cold isotonic saline solution (0.9% NaCl). The supernatant and the buffy coat were carefully removed after each wash. After separation, packed erythrocytes were suspended in phosphate buffer (170 ml of Na2PO4H (1.41 g/l) solution+77 ml of NaPO4H2 (1.19 g/l) solution + NaCl (8.8 g/l)], at pH 7.40 to obtain a 50% cellular suspension.

The mixtures were thawed, the erythrocytes were destroyed by osmotic pressure and then subjected to centrifugation, Supernatants were isolated and the activities of SOD, CAT,Vitamin C and Vitamin E were measured by spectrophotometer (Shimadzu UV-1700, Japan).

Experimental design

Group A :(Control) 5 ml of erythrocytes incubated for1hour at 37^0C with 0.9% saline.
Group B: 5 ml of erythrocytes incubated for 1 hour at 37^0c with 25µM NaMSA
Group C: 5 ml of erythrocytes incubated for 1 hour at 37^0c with 50 µM NaMSA
Group D: 5 ml of erythrocytes incubated for 1 hour at 37^0c with 100 µM NaMSA

Statistical Analysis

The values were expressed as mean value (n=6) of + S.E.M, The *in vitro* experimental data were analysed using one way analysis of variance by the Duncan's Multiple Comparison Test to determine the level of significance and $p<0.05$was considered as statistically significant.

Results

Table -1: *In Vitro* Assay of Effect of NaMSA on Catalase, SOD, vitamin C and vitamin E level of Erythrocytes

In the present work, administration of morin-5'-sulfonic acid sodium salt 25 µM, 50 µM maintained the CAT, SOD and vitamin E levels slightly higher than control group and vitamin C remains similar to control group. Whereas,administration of 100 µM NaMSA, the CAT, SOD, vitamin E values were maintained similar to 25 µM, 50 µMadministered group at the same time vitamin C levels was slightly decreased compared with the control and other groups.

Table 1: Effect of NaMSA on Catalase, SOD, vitamin C and vitamin E level in Erythrocytes.

Parameters	Control(RBC+0.9% Saline)	RBC+25 µM NaMSA	RBC+ 50 µM NaMSA	RBC+100 µM NaMSA
Catalase (nmol/mg Hb)	42.51± 4.90 [a]	46.22± 5.21[b]	51.21 ± 5.11 [c]	52.33±7.31 [c]
SOD (nmol/mg Hb)	20.11± 1.95 [a]	21.66 ± 1.47 [b]	23.32 ± 2.12 [b]	24.13± 2.89 [b]
Vitamin C (µM**/** mg Hb)	5.99 ± 0.41[a]	6.57 ± 0.05b	6.63 ± 0.31[b]	5.09 ± 0.21[c]
Vitamin E (µM/ mg Hb)	4.21 ± 0.09[a]	4.97 ± 0.12b	5.43 ± 0.18[c]	5.32 ± 0.13[c]

NaMSA – Morin **-**5'**-**sulfonic acid sodium salt
Values are means ± S.D for six tubes.
Values not sharing a common superscript and differ significantly at $p< 0.05$ (DMRT)

Discussion

Influence of NAMSA induced changes in the activities of SOD, CAT, vitamin C and vitamin E in erythrocytes

Table-1: Explains the NAMSA induced changes in the activities of SOD, CAT, vitamin C and vitamin E in erythrocytes.Normal erythrocyte function is wholly dependent on an intact erythrocyte membrane. The toxic effect of many environmental chemicals and pesticides is largely due in large part to their effect on erythrocyte membranes (Schara *et al*., 2001; Brandao *et al*., 2005).

As per the results recorded in the experiment, The CAT,SOD,vitamin C and vitamin E values increasedwith respect to control group significantly($p<0.05$) upon the addition of 25 µM ,50 µM of NaMSA to the RBC. Whereas, administration of 100 µM NaMSA to the RBC, the CAT, SOD, vitamin E values were maintained similar to 25 µM, 50 µM administered groups at the same time vitamin C levels was slightly decreased compared with the control group and other groups.

Morin is a constituent of many fruits and used as a food additive because of its antioxidant activity (Hanasaki et al., 1994; Ramanathan et al., 1994). In addition, it possesses anti-inflammatory potential (Baumann *et al*., 1980; Galvez *et al*., 2001; Nakadate *et al*., 1984).

The sulfonic derivative of morin is potent in its cytostatic and cytotoxic activities. Its solubility in water was greater than that of the original agents and higher culture medium concentrations of NaMSA was obtained (Krol *et al*., 2002).

In vivo studies, morin hydrate has also been found to prevent necrosis of liver (Wu., 1993,; Wu., 1994). Morin (2', 3, 4', 5, 7-pentahydroxyflavone), a member of the flavonoid family, demonstrated cytoprotective properties against oxidative stress via antioxidant effects. Although previous papers reported that morin exhibits antioxidant effects on free radical, in addition to protecting cells such as myocytes, hepatocytes, and neuron cells against oxidative stress (Wu.,1995,:kok.,2000: Gottlieb, 2006) .

Early studies of flavonoids compounds investigated their mutagenic and genotoxic activity in a number of *in vitro* assays and caution should be exercised in ingesting them at levels above that which would be obtained from a typical vegetarian diet. The unborn fetus may be especially at risk, since flavonoids readily cross the placenta (Skibola and Smith,2008).

Studies in cell culture indicate that a number of flavonoids inhibit the transport of vitamin C into cells, and supplementation of rats with quercetin and vitamin C decreased the intestinal absorption of vitamin C. More research is needed to determine the significance of these findings in humans (JaneHigdon, 2008).Morin hydrate is an effective protector for human erythrocytes against lysis by peroxyl radical (Wu, 1994).

Thus, in high doses, the adverse effects of flavonoids may outweigh their beneficial ones, and caution should be exercised in ingesting them at levels above that which would be obtained from a typical vegetarian diet. The unborn fetus may be especially at risk, since flavonoids readily cross the placenta (Skibola and Smith, 2008**).**

The present investigation results derived that 25 µM, 50 µM of NaMSA was capable of enhancing the catalase, super oxide dismutase, vitamin C and vitamin E levels. On the other hand 100 µM of NaMSA decreased vitamin C activity and CAT, SOD, vitamin E levels were remained similar to 25 µM, and 50 µM of NaMSA administered groups. Hence, 50 µM of NaMSA was found to be safer than 100 µM of NaMSA for the *in vitro* erythrocytes and also determined that 50 µM of NaMSA is found to be the optimum dose for *in vitro* erythrocytes assay(5ml) using morin-5'-sulfonic acid sodium salt.

Conclusion

The present study results and earlier evidences concluded that morin-5'-sulfonic acid sodium salt 50 µM of NaMSA highly enhances enzymatic and non-enzymatic antioxidant activities level in RBC, which will be very helpful to act against free radicals and many other toxicants. At the same time caution should be taken while determining the dose of the Morin and other flavonoids prior to ingestion.

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