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Research Article

REVERSAL OF LIPOPOLYSACCHARIDE INDUCED OXIDATIVE STRESS BY SELECTED POLYPHENOLS

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ABSTRACT

Lipopolysaccharide (LPS) can induce oxidative damage in tissue/organ through increased production of reactive oxygen species. Flavonoids, polyphenolic compounds present in foods of plant origin have been shown to protect biological membranes against free radical-induced oxidative damage. Hence, an *in vitro* study was carried out to screen the ameliorative potential of rutin, morin and ascorbic acid on LPS induced oxidative stress biomarkers in erythrocytes. RBC cell suspension (10%) was prepared from sheep blood and was incubated with rutin (0.5 mM), morin (1.46 mM) and ascorbic acid (2.37 mM) for half an hour followed by LPS (250 µg/ml) incubation for one and half hour. After the incubation in LPS, RBC cell suspension was subjected for estimation of various oxidative stress biomarkers like % hemolysis, TBARS, superoxide dismutase (SOD), reduced glutathione (GSH). LPS has caused a significant increase in the % hemolysis, TBARS, SOD and intracellular GSH level. Rutin, morin and ascorbic acid produced significant protection against the LPS induced oxidative damage as evidenced by the decrease in % hemolysis, TBARS, SOD, GSH and increase in total protein level in RBC.

Keywords: Antioxidants, Flavonoids, Lipopolysaccharide, Oxidative stress

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Oxidative stress plays an important role in many physiological and pathological conditions. Erythrocytes have been used as a model to investigate oxidative damage in bio-membranes because of their high vulnerability to peroxidation due to presence of high polyunsaturated fatty acid content in their membranes and high cellular concentration of oxygen and hemoglobin, a powerful promoter of the oxidative process (Neelam *et al.*, 2017).

In recent times, there is an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing oxidative injury by free radicals (Jimoh *et al.*, 2009). Flavonoids, polyphenolic compounds present in foods of plant origin have been shown to protect biological membranes against free radical-induced oxidative damage. Flavonoids like rutin (quercetin-3-rhamnosyl glucoside) and morin (3,5,7,29,49-pentahydroxyflavone) are used in traditional medicine and reported to have antioxidant properties (Umarani *et al.*, 2015). Ascorbic acid, the reduced form of vitamin C, is a potent antioxidant quenching ROS produced by normal metabolism to prevent damage (Heaney *et al.*, 2008).

Keeping the above in view, the present study was planned to study the amelioration of *in vitro* oxidative stress induced by LPS (lipopolysaccharide) in erythrocytes by selected polyphenols and ascorbic acid.

MATERIALS AND METHODS

Chemicals

LPS, rutin trihydrate, morin and L-ascorbic acid were purchased from M/s Sigma Aldrich, USA. All other chemicals used were of analytical grade and purchased from Merck, India, Sigma Aldrich, USA, SRL Pvt. Ltd. and S.D fine chemicals.

Collection of blood and preparation of erythrocytes:

Blood was collected and pooled from sheep in a heparinized vial from a nearby slaughter house and was immediately brought to the laboratory. Plasma was separated after centrifugation at 3000 rpm for 10 min at 4 °C in a refrigerated centrifuge and the plasma and buffy coat were discarded. The erythrocytes (RBC) were collected and washed thrice by centrifugation at 3000 rpm at 4 °C for 10 min in cold phosphate buffered saline (PBS, pH-7.4) and supernatants were discarded after each centrifugation. After the final wash, the packed erythrocytes were re-suspended in phosphate buffer and 10% RBC cell suspension (v/v) was prepared and kept at normal refrigeration condition until analyses.

In vitro treatment of erythrocytes for oxidative stress induction

Sheep RBC cell suspension 10% (v/v) were divided as described below in various groups and pre-incubated with rutin (0.5 mM), morin (1.46 mM) and ascorbic acid (2.37 mM) for half an hour followed by LPS (250 µg/ml) incubation for one and half hour on shaking incubator. EC50 values of ascorbic acid, rutin and morin were calculated based on phosphomolybdate assay for screening of antioxidant activity. After the incubation

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period, RBC cell suspension was subjected for estimation of various oxidative stress biomarkers.

Groups of RBC cell suspension for evaluating the ameliorative potential of rutin, morin and ascorbic acid

Group	Treatment
I	RBC Cell suspension (10%, v/v)
II	RBC Cell suspension + LPS (250 µg/ml)
III	RBC Cell suspension + LPS (250 µg/ml) + Ascorbic acid (2.37 mM)
IV	RBC Cell suspension + LPS (250 µg/ml) + Rutin (0.5 mM)
V	RBC Cell suspension + LPS (250 µg/ml) + Morin (1.46 mM)

Oxidative stress indices

% Hemolysis

The hemolysis as percentage in the reaction mixtures were measured as described (Baumann *et al.*, 2000). Briefly, the reaction mixtures were centrifuged at 1,000 rpm at 4 °C for 10 min. The supernatants were measured at 540 nm and the percentage hemolysis was calculated.

$$\%\,\text{Hemolysis} = \frac{\text{Abs 540 nm in the reaction mixtures}}{\text{Abs 540nm in100\% hemolysis control}} \times 100$$

Erythrocyte lipid peroxidation

Lipid peroxidation was assayed as described (Stock and Dormandy, 1971), using formula and a molar extinction coefficient of $1.56\times10^{5}\,M^{-1}cm^{-1}$.

$$\mu\text{ moles of MDA} = \frac{2 \times \text{volume of sample taken} \times \text{absorbance of sample}}{1.56 \times 105 \text{ X ml}} \times 10^{6}$$

Protein determination

Total protein of the RBC cell suspension was quantified by using bovine serum albumin as standard (Bradford, 1976). The results are expressed as mg/ml as calculated from the standard calibration curve.

The LPO was expressed as µM MDA/mg of protein

Erythrocyte superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined as mentioned by Madesh and Balasubramanian (1998). The enzyme activity is expressed as SOD units (One unit of SOD is the amount in mg of protein required to inhibit the MTT reduction by 50%).

$$Y\,\% = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

$$\text{SOD (Units/mg of protein)} = \frac{\text{mg of protein in 0.01 ml of hemolysate}}{Y\%} \times 50 \times 10$$

Reduced glutathione

Reduced glutathione was assayed by Ellman method (1959). The GSH levels were expressed as mmol/ml of blood as calculated from GSH standard calibration curve.

Statistical analysis

Data were expressed as mean± standard error means (SEM). 'P' (<0.05) value was considered statistically significant. Comparisons of means between groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical analysis was conducted using GraphPad prism version 9.1.1 (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Oxidative stress is disruption of the balance between pro-oxidant - antioxidant activity in favor of the former, leading to potential damage (Rahal *et al.*, 2014). Erythrocytes are a convenient model to understand the membrane oxidative damage induced by various xenobiotic pro-oxidants because of high polyunsaturated fatty acid content of their membranes and high cellular concentrations of oxygen and haemoglobin (Mohanty *et al.*, 2014).

In vitro exposure of erythrocytes to oxygen radical generating systems (such as H_2O_2, ascorbate/Fe_3+, cumene hydroperoxide, tert-butyl hydroperoxide, etc.) was shown to induce lipid peroxidation, protein degradation, loss of deformability, an increase in osmotic fragility, membrane lipid bilayer perturbation, inhibition of enzymes and hemolysis (Srour *et al.*, 2000). The effect of LPS on erythrocyte lipid peroxidation and antioxidative systems and its amelioration by selected polyphenols in present study are presented in Table 1.

In the present study, there was significant increase in erythrocyte TBARS level in LPS treatment as compared to non LPS exposure. Selected polyphenols of rutin, morin and ascorbic acid treatment has ameliorative effect on LPS induced oxidative stress in erythrocytes and the TBARS were significantly restored back to the normal. The finding is in agreement with the findings of Hou *et al.* (2014).

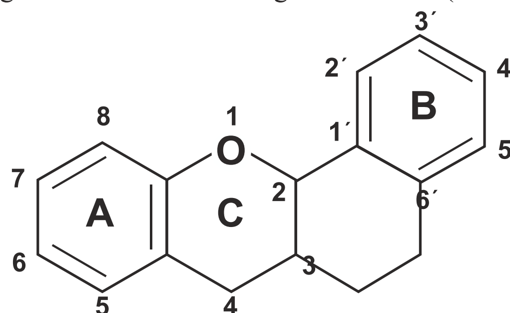

Fig. 1. Chemical structure of flavonoids

Table 1

Effect of lipid peroxidation and antioxidative systems in erythrocytes of sheep exposed to lipopolysaccharide and its amelioration by selected polyphenols

Groups	TBARS	GSH	SOD	Total protein	% Hemolysis
RBC Cell suspension	1.301±0.037[a]	0.102±0.001[a]	37.034±1.184[a]	9.745±0.103[a]	16.561±1.548[a]
RBC Cell suspension + LPS (250 μg/ml)	1.557±0.088[b]	0.148±0.008[b]	24.082±0.260[b]	9.441±0.068[ab]	68.605±12.22[b]
RBC Cell suspension + LPS (250 μg/ml)+ rutin (0.5 mM)	1.010±0.032[c]	0.133±0.004[bc]	30.604±0.250[c]	10.070±0.125[ac]	10.534±4.365[a]
RBC Cell suspension + LPS (250 μg/ml)+ morin (1.46 mM)	1.026±0.028[c]	0.133±0.002[bc]	32.475±0.638[c]	9.587±0.098[ab]	19.339±7.413[a]
RBC Cell suspension + LPS (250 μg/ml) + ascorbic acid (2.37 mM)	1.016±0.014[c]	0.113±0.001[ad]	32.445±0.549[c]	9.425±0.095[ab]	34.643±7.023[a]

Note: TBARS: Thiobarbituric acid (μM MDA formed/mg of protein); GSH: reduced glutathione (mM/mg of protein); SOD: superoxide dismutase (Units/mg protein); Total protein: mg/ml of blood. Values (mean ± SEM, n =6) bearing no superscript common in the same column vary significantly ($p \leq 0.05$)

Structure of flavonoids is shown in Fig. 1 for better understanding of action. The antioxidant activity is related to the acid moiety and the number and relative positions of hydroxyl groups on the aromatic ring structure. Significant inhibition of MDA production and lipid peroxidation in LPS treated erythrocytes by rutin is because of ortho 3', 4'-dihydroxy substitution in ring B. Further, the scavenging activity of flavonoids for hydroxyl radicals increases with the number of hydroxyl groups substituted in ring B (Pietta, 2000). The oxidation of meta-2', 4'- dihdroxy groups by free radicals could be responsible for the anti-lipid-per oxidant activity of morin observed in this study (Yousif *et al.*, 2012).

SOD is family of metalloproteins that catalyzes dismutation of superoxide radicals to the less reactive H2O2. It provides the first line of defense against free radical damage (Marklund, 1984). In LPS treated group, the activities of SOD were significantly decreased. Treatment with selected polyphenols has ameliorative effect on LPS induced oxidative stress in erythrocytes and the SOD was significantly restored back to the normal by pre-treatment with rutin, morin and ascorbic acid. These findings obtained for SOD agree with findings of Katan *et al.*, (1998).

Reduced glutathione is a major non-enzymatic antioxidant. Under oxidative stress, thiol groups protect cellular structures against free radicals by undergoing oxidization and forming disulfide bonds (Rangkadilok *et al.*, 2007). This may be the reason for increase in the erythrocyte GSH level in LPS treated group as compared to the control group which was restored back to the normal in erythrocytes that are pre-treated with rutin, morin and ascorbic acid.

In LPS treated group there was significant increase in % hemolysis when compared to RBC control. This might be due to increased membrane permeability leading to influx of water into the cells there by causing hemolysis. It might also be due to an increase in lipid peroxidation and oxidative damage. Reduction in hemolysis by addition of rutin could be attributed to its property of scavenging hydroxyl radical, stimulating antioxidative enzymes, inhibiting capacity of prooxidative enzymes. Previous study established that rutin is an effective antioxidant in protecting human red blood cells hemolysis from free radical induced oxidative damage (Dai *et al.*, 2006).

Flavonoids due to their phenolic ring and hydroxyl substituents that have ability to quench free electrons can function as effective antioxidants and also have the ability to incorporate into the hydrophobic core of the bilayer membranes thus decreasing the oxidative process throughout the membrane (Asgary *et al.*, 2005). On the other hand, ascorbic acid is a most powerful antioxidant molecule under physiological conditions. It exists in the reduced form which directly scavenges the superoxide radicals, hydroxyl radicals and single oxygen (Scribbans *et al.*, 2014).

CONCLUSION

The present work revealed that rutin, morin and ascorbic acid exerted a protective effect by reversal of oxidative stress induced by LPS in erythrocytes. There is need to work in future to validate the effects of these phytochemicals in *in vivo* models.

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