Age associated erythrocyte membrane damages in rats: potential role of chloroform extract of solanum trilobatum

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The current surge of attention to improve the quality of life of an organism is nowadays paying attention on natural therapies. Thus, this particular research is focused to study the age associated erythrocyte membrane damages in rats and the protective effect of the antioxidant rich Chloroform extract of \textit{Solanum trilobatum}. The erythrocyte membrane damages and the protective role of Chloroform extract of \textit{Solanum trilobatum} (150mg/kg body weight orally for 4 weeks) in aged rats was assessed by the levels of lipid per oxidation, lipid profiles, membrane fluidity, membrane bound enzymes and the antioxidant status as an incidence of oxidative stress on comparing with the young rats. A significant (p<0.05) increase in the lipid peroxidation levels with a significant alteration in lipid profiles, membrane fluidity and a decrease (p<0.05) in membrane bound enzyme activities in aged rat erythrocytes was restored (p<0.05) on treatment with Chloroform extract of \textit{Solanum trilobatum}. The results of this study showed that chloroform extract of \textit{Solanum trilobatum} on long-term administration in erythrocytes of rats showed immense membrane stabilizing effect with advancement of age for which the phenolic content of the studied plant material could be a good indicator of antioxidant capacity. With the development in techniques and recent researches, it has been proved that flavonoids which were earlier thought to be of no importance to human diet possess antioxidant properties.

\textbf{Keywords:} Antioxidant, Phenolic compounds, Chloroform extract of \textit{Solanum trilobatum}, Membrane fluidity, Membrane bound enzymes, Glutathione reducing enzymes, Lipid peroxidation.

INTRODUCTION

The imbalance between cellular production of Reactive Oxygen Species (ROS) and the ability of cells to defend themselves against the free radicals is referred to as Oxidative stress. Oxidative damage apparently increases with age and thus may overwhelm the natural repair system in the organism (Kowald, 1999) leading to the onset of age-related diseases with a progressive increase in the chance of morbidity and mortality. At the cellular level, the ROS inclination due to oxidative stress damages vital cell components like poly unsaturated fatty acids (PUFA), protein and nucleic acids (Bandyopadhyay et al., 1999). This further enhances the development of one or better-recognized age related modifications, such as alteration of intrinsic membrane properties (fluidity, ion transport, loss of enzyme activity, protein crosslinking), inhibition of protein synthesis and DNA damage (Stadtman et al., 1992), ultimately resulting in the cell death.

Erythrocyte is an ideal model for studies related to ageing as they are devoid of a nucleus and other intracellular organelles, thus cannot yield direct information on the DNA control of cellular ageing, and do allow the age related modifications of intracellular proteins and plasma membrane to continue without the interference of the repair processes and exchange of damaged macromolecules (Bartosz, 1998). Changes in
the macromolecules of membrane are one of the earliest
signs of erythrocyte membrane alterations during ageing.
ROS generated in the aqueous or lipid phase can attach
to the erythrocyte membrane and can induce oxidation of
lipids, proteins and carbohydrates, triggering disruption in
membrane (Grune et al., 2001). Oxidation of lipids or
Lipid Peroxidation is characterized as a free radical chain
reaction initiated by the abstraction of a hydrogen atom
within the cells due to high concentration of PUFA in the
RBC membrane (Bennett, 1990). Studies have shown
membrane lipid alterations, as an important factor in
decreasing lipid fluidity during ageing in erythrocytes
resulting in erythrocytes membrane rigidity (Borst et al.,
2000). The rigidity of membrane seems to be an
important factor in reducing cell deformability, the
phenomenon due to enhanced intracellular viscosity
(Borst et al., 2000). Alterations in membrane fluidity
during ageing thereby lead to disturbance in blood
rheological behaviour contributing to various
pathophysiological implications including cardiovascular
diseases, hypertension and stroke (Szapary et al.,
2004).

The free radical theory of aging thus serves and plays
a prominent role in invoking antioxidants as powerful
defense mechanisms protecting the cells and tissues
from oxidative stress as an anti-ageing movement.
However erythrocytes are prone to have shown
cumulative oxidant mediated damages due to
endogenously and exogenously produced radicals with
advancement of age. Antioxidant vitamins Vitamin E and
vitamin C may prevent or mitigate lipid peroxidation.
Vitamin E (alpha-tocopherol) is considered the
predominant lipid-soluble, chainbreaking micronutrient
antioxidant. In vitro studies suggest that vitamin C may
enhance the effects of vitamin E by reducing tocopheroxyl radicals (Meagher et al., 2001). In some
animal studies, a high intake of vitamin C increased
tissue vitamin E concentrations (Afanas, 2005).
Deficiency of vitamin E may cause neurological
dysfunction, myopathies and diminished erythrocyte life
span (Kawamoto et al., 2005). The molecular mechanism
of these functions is believed to be mediated by either the
antioxidant action of the vitamin or by its action as a
membrane stabilizer (Ward et al., 2005).

Epidemiological data suggest that dietary antioxidants
may have a beneficial effect on many age-related
diseases. The beneficial impact of antioxidants on
various age-related degenerative diseases may forecast
an improvement in life span and enhance quality of life.
The intake of plant derived natural antioxidants is recently
more in practice, as the usage of synthetic antioxidants
has long been questioned for its safety. Solanum trilobatum
is one such medicinal herb rich in antioxidant
supplement with wide beneficial biological property.
Solanum trilobatum extract consists of compounds
including solasodine, linoleic acid, palmitic acid, oleic
acid, stearic acid (Saratda Vasanth, 1990), sobatum, a-
sitosterol, disogenin and ß-solamarine responsible for
many biological properties (Mohanan and Devi, 1998).
Recently a strong antioxidant activity has been reported
in the chloroform extracts of the plant Solanum trilobatum
due to the presence of phytochemicals such as iso
flavonoids, phenols, phenolic acids, xanthones and
lignans (Sini and Devi, 2004; Amir and Kumar, 2005).
Solanum trilobatum possessed a broad spectrum of
antibacterial, antifungal and antitumoural activities of
cough (Govindan et al., 1999). The decoction of various
parts of the plant is used in chronic bronchitis. The
berries and flowers of Solanum trilobatum, are widely
used to treat respiratory diseases in Indian traditional
system of Medicine (Kirtikar and Basu, 1993). Recently
Mohanan and Rathinam (1997) has also proved that
Solanum trilobatum possess free radical scavenging
activity when exposed to the ultraviolet rays. Reports
have shown that active principle from Solanum trilobatum
possessed antitumor effect against chemically induced
tumors (Govindan et al., 1999; Shahjahan et al., 2005).
Thus this particular research was aimed to evaluate the
age-related oxidative stress in erythrocyte membrane of
young and aged rats and the protective effect of the
antioxidant rich Chloroform extract of Solanum trilobatum
(CST).

MATERIALS AND METHODS

Preparation of chloroform extraction of Solanum trilobatum

The leaves of Solanum trilobatum were collected from the local
market and samples of the plant were identified and authenticated
by Dr. Brindha, Botanist, Department of Pharmacognosy, and
Captain Srinivasa Murti Drug Research Institute for Ayurveda
(CCRAS, New Delhi), Arumbakkam, Chennai, and Tamil Nadu. The
fresh leaves (700g) were shade dried, powdered and extracted
successively with 1.2 L of chloroform, in a Soxhlet extractor for 18-
20 h. The extracts were concentrated to dryness under reduced
pressure and controlled temperature (40°-50°C). The chloroform,
exttract yielded a brown semisolid residue weighing 8.2 g (2.39%
w/w). The extract was then filtered through Whatman No.1 filter
paper and concentrated using concentrator. Then it was frozen and
subjected to lophilization.

Animals

Male albino wistar Young (3-4 months, 120-150 g) and aged (22-24
months, 380-410 g) rats were used for the experiments. The rats
were housed in polypropylene cages on a 12L: 12D cycle and fed
ad libitum on commercial laboratory food pellets and water. All
animal experiments were conducted as per the instructions of
Institutional Animal Ethics Committee

Experimental Design

The animals were divided into four major groups of six animals
namely, Group I: Control young rats were received sterile water
only, Group Ib: Young rats were administered with CST for 90 days,
Group II: Control aged rats were received sterile water only, Group
Preparation of erythrocytes and erythrocyte membrane

The erythrocyte membrane was isolated according to the method of Dodge et al. (1963) with slight modification. 4 ml of packed cells was washed thrice with Tris-HCl buffer (310 mM, pH 7.4). Hemolysis was then performed by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes that contained 5 mM sodium phosphate buffer, pH 8.0 and left for 1 hr. Erythrocyte ghosts were sedimented by centrifuging for 40 min at 30,000-x g. The supernatant (or hemolysate) was decanted carefully and saved, while the pellet was washed repeatedly. The colorless framework of erythrocyte remaining after hemolysis is referred to a ‘ghost’ or ‘stroma’ or ‘stroma’ or ‘post-hemolytic residues’. The membrane ghost was then immediately re-suspended in 0.1 M Tris-HCl buffer, pH 7.4 and frozen, stored by submerging the tubes with the membrane suspensions in liquid nitrogen at −80°C until further assay.

Determination of fluorescent anisotropy

Lipid fluidity of erythrocytes was measured by fluorescence anisotropy technique, using fluorescent lipid probe 1, 6 diphenylhexa 3, 4, 5 triene (DPH), according to the method of Shinitzky and Barenholz (1974). A small volume of DPH solution (2 mM) in tetrahydrofuran (THF) was injected with rapid suspension and was stirred for at least 2 hr after which no odour of THF was detected. The erythrocytes incubated in PBS were mixed with DPH suspension to give a final probe concentration of 1 n mol/ml and the mixture was incubated at 37°C for 15 min. Fluorescence polarization was measured at 25°C in Perkin Spectrofluorimeter equipped with polarizers using an excitation wavelength of 360 nm and emission wavelength of 420 nm with a period of exposure of sample to the excitation light less than 10 sec. The steady-state fluorescence anisotropy (rDPH) was calculated according to Van Blitterswijk (1981).

Biochemical estimation

Erythrocyte Membrane Bound Lipids

Extraction

Total lipids were extracted from the membrane by the method of Folch (1957) using Chlorform: Methanol (2:1 v/v) mixture. The membrane pellet obtained from 0.5 mL of packed cells was treated with 7.0 mL of methanol and homogenized in a Polter-Elvehjem homogenizer. The contents were filtered and the residue on the filter paper was carefully allowed to evaporate below 60°C. The lipid extract was redissolved in a known volume of chloroform - methanol mixture (2:1 v/v). To this was added 2.0 mL of 0.1 M potassium chloride. The contents were shaken well and centrifuged. The aqueous phase was washed again with potassium chloride: methanol: Chloroform taken in the ratio of 10: 10:1 (v/v), 3 times and each time the upper was discarded. Aliquots of the final lipid extract (chloroform layer) were used for lipid assay. Total cholesterol level in erythrocyte membrane and plasma were estimated by the method of Parekh and Jung (1970). Total phospholipids in erythrocyte membrane and plasma were estimated by the method of Rouser (1970).

Membrane bound assays

Lipid peroxidation in erythrocyte membrane was assayed according to the method of Beuge and Aust (1978) with slight modification. Na+ K+ ATPase was assayed according to the method of Bonting, (1970). Ca2+ ATPase was estimated according to the method of Hjerten and Pan (1983). Mg2+ ATPase was assayed by the method of Ohnishi et al, (1982). Inorganic phosphorus was estimated by the method of Fiske and Subbarow, (1925).

Assay of antioxidants

Total sulphhydryl group was estimated according to the method of Sedlack and Lindsay (1968). Ascorbic acid was estimated by the method of Omaye et al. (1979). The level of α-tocopherol was estimated by the method of Desai (1984).

Statistical Analysis

Values are mean ± SD for six rats in each group and significance of the differences between mean values were determined by one-way analysis of variance (ANOVA) followed by the Duncan test for multiple comparison.

RESULTS

Figure 1 illustrates the level of lipid peroxidation, blood ascorbic acid and α-tocopherol in control and CST treated young and aged rats. Levels of malonaldehyde (MDA) were profoundly increased by 1.7 fold in erythrocytes and 1.8 fold in plasma of aged rats when compared to young rats. Administration of CST significantly reduced the level of lipid peroxidation in erythrocyte membrane by 1.3 fold and in plasma by 1.5 fold respectively, thereby displaying the antioxidant capacity of CST in combating oxidative stress in aged animals. Significant decrease in the levels ascorbic acid by 35% and α-tocopherol by 30% was observed in aged rats when compared to young rats. CST supplementation to aged rats significantly increased ascorbic acid (32%) and α-tocopherol (30%) levels, demonstrating the non-enzymatic antioxidant elevating property of CST.

Table 1 shows the level of fluorescence anisotropy, the levels of cholesterol and phospholipids and the cholesterol / phospholipid (C/P) ratio in erythrocyte
Table 1. Total Cholesterol, Total Phospholipid Cholesterol: Phospholipid Ratio and Fluorescence Anisotropy in Erythrocyte Membrane of Control and CST Treated Young and Aged Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Young rats</th>
<th>Group Ib</th>
<th>Aged rats</th>
<th>Group Iib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Ia</td>
<td></td>
<td>Group Iia</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>120.52 ± 12.24</td>
<td>115.12 ± 11.28</td>
<td>181.32 ± 17.01</td>
<td>135.26 ± 14.23</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>241.59 ± 23.72</td>
<td>246.40 ± 21.02</td>
<td>173.16 ± 15.12</td>
<td>228.24 ± 20.71</td>
</tr>
<tr>
<td>C/P ratio</td>
<td>0.53 ± 0.05</td>
<td>0.51 ± 0.04</td>
<td>1.21 ± 0.09</td>
<td>0.59 ± 0.05</td>
</tr>
<tr>
<td>DPH</td>
<td>0.29 ± 0.02</td>
<td>0.27 ± 0.03</td>
<td>0.38 ± 0.04</td>
<td>0.33 ± 0.03</td>
</tr>
</tbody>
</table>

'a' - Group Ila compared with Group Ia, 'b' - Group IIb compared with Group Ila * represents p < 0.05

Units: cholesterol, phospholipid - µg/mg protein ;
Values are expressed as Mean ± SD for six rats

Figure 2 represents the activities of ATPase and the levels of total thiol in the erythrocyte membrane of control and CST treated young and aged rats. The levels of cholesterol were elevated by 50% and phospholipids were declined by 28% significantly in erythrocyte membrane of aged rats when compared with young rats. CST treatment to aged rats significantly decreased the levels cholesterol by 25% and increased phospholipids by 31% in erythrocyte membrane. Significant increase (p<0.05) in the C/P ratio was observed in aged rats (p<0.05) when compared to young control rats. CST supplementation to aged rats decreased the cholesterol levels and increased phospholipid levels thereby restoring the C/P ratio.

Figure 2 represents the activities of ATPase and the levels of total thiol in the erythrocyte membrane of control and CST treated young and aged rats. Significant decline in the activities of Na⁺K⁺ATPase by 36%, Ca²⁺ATPase by 28%.
Figure 2: Activities of Membrane-Bound Enzymes and Total Thiols in Control and CST Treated Aged and Young Rats

DISCUSSION

Several lines of evidence have been used to support the free radical theory including the claims that: enhanced expression of antioxidative enzymes in experimental animals can produce a significant increase in longevity and cellular levels of free radical damage increases with age (Fusco et al., 2007). Since the ageing process is characterized as time dependent, the age related modification of lipid structures should be considered as one of the major contributors for the age dependent diseases (Pepe et al., 1997). The high polyunsaturated fatty acid content of erythrocyte membrane and the continuous exposure to high concentration of oxygen and iron in hemoglobin are the factors that make erythrocytes very sensitive to the lipid peroxidative injury (Hulbert et al., 2007). The end products of LPO, namely malonaldehyde (MDA) are a highly reactive bifunctional molecule and have been shown to cross link erythrocyte phospholipids and proteins to impair variety of the membrane related functions and ultimately to diminished RBC survival (Kumar et al., 2009). Consequently such alterations in lipids have been suggested to be the cause of ageing and age-associated degenerations (Spiteller, 2003; Spiteller, 2002). Significant increase in the levels of MDA in erythrocyte membrane of aged rats indicated the possibility of increased radical production, and higher rate of LPO in the present study. Our results are in agreement with that of other authors who has reported the increase in concentrations of LPO products including MDA in erythrocytes of rats (Gurer et al., 1997), man (Marotta et al., 2007) and rabbits (Brzezi ska-Slebodzińska, 2007). Administration of CST brought down the oxidative stress substantially and decreased the MDA levels in the erythrocyte membranes of aged rats. Flavonoids (isoflavonoids) present in CST scavenge lipidperoxides by binding metal ions and by inhibition of enzymatic systems responsible for free radical generation (such as 5-lipoxygenase, cyclo-oxygenase, and mono-oxygenase or xanthine oxidase) thereby declining LPO levels in aged rats (Cotelle et al., 1996).

The age related membrane rigidity and impaired membrane-related functions diminished RBC survival which may probably be due to the increase in lipid peroxide levels formed upon oxidation of iron released by 56% and Mg\textsuperscript{2+}ATPases by 51% were observed in erythrocytes of aged rats. CST administration to aged rats improved the activities of these ion motive ATPase by 32% for Na\textsuperscript{+}K\textsuperscript{+}ATPase, 21% for Ca\textsuperscript{2+}ATPase and 58% for Mg\textsuperscript{2+}ATPases. This confirms the protective effect of CST in maintaining the ionic gradient in erythrocytes with advancement of animal age. Significant decrease in the levels of total thiols by 34% was noticed in erythrocytes of aged rats compared to young rats. CST supplementation to aged rats increased the total thiol levels in erythrocytes by 20%.
from hemoglobin that could in turn cause alteration in erythrocyte antioxidant defense systems (Yanagawa et al., 1999). Thus decrease in the antioxidants such as vitamin E and vitamin C in erythrocytes of aged rats, could enhance the lipid peroxidation process in the present study. Therapeutic supplementation of CST enhanced significantly the levels of ascorbic acid and α-tocopherol in aged rats to near normalcy. The increase in the level of vitamin C would have been possibly due to the increased absorption and stabilization of ascorbic acid, reduction of dehydroascorbate to ascorbate, and metabolic sparing effect of ascorbic acid by the flavonoids (Hughes and Wilson, 1977) in CST. As an antioxidant, α-tocopherol intercalates into phospholipid bilayers with the long axis of the molecule oriented parallel to the lipid hydrocarbon chains and prevents the propagation of free radical reactions, (Vannucchi et al., 2003; Ward et al., 2005). Elevation of vitamin E levels corresponds to the action of polyphenols that regenerate α-tocopherol from tocopherol radical through an H-transferring mechanism which thereby behaves as a sacrificial antioxidant (Carini et al., 2000). Moreover, the metal chelating property of CST increase vitamin E content by preventing the involvement of hemoglobin iron in lipid peroxidation processes (Maffei Facino et al., 1996).

Cell membrane fluidity at an optimum state is necessary for the right functioning of the membrane (Zicha et al., 1999). Various gerontological studies have illustrated altered membrane fluidity with advancement of animal age (Yu et al., 1992). In the present study, DPH probe was used to monitor the fluidity changes in erythrocyte membranes. Their fluorescence anisotropy values respond to lipid arrangement in various regions of membranes. Significant increase in fluorescence anisotropy therefore indicated the increased membrane rigidity in aged rat erythrocytes in the present study. A significant increase in lipid hydroperoxide content and a marked decrease in the fluidity of the erythrocyte membrane in old rats can be attributed to our study (Takasaki et al., 2002). Cholesterol plays a key role in erythrocyte membrane fluidity as it appears to be the main bilayer matrix in an intermediate fluid that regulates the mobility of phospholipid fatty acyl chains by condensing hydrophobic interaction leading to increased rigidity to membrane lipids. Reports by Marino et al., 2002 recognized an increase in cholesterol with the advancement of age may be the underlying cause for the membrane rigidity. In accordance our experimental results, demonstrated a significantly higher susceptibility to peroxidation, and a significant increase in the cholesterol and decrease in phospholipids contents with a concomitant increase in the ratio between cholesterol and phospholipids of the erythrocyte membranes of aged rats in comparison with the young rats. Parallel studies evidenced a significant decrease in erythrocyte phospholipids (Lopez-Revuelta et al., 2005) and augmented cholesterol levels during aging (Harman, 2003). Reduction in total amount of phospholipids in erythrocyte membrane, the composition of its subclasses and the asymmetrical distribution across the membrane bilayers may be owed to cause instability and dysfunction of the membrane (Schroeder, 1984).

Supplementation of CST to the aged rats increased the membrane fluidity to near normalcy indicating the protection afforded by the active components (saponins, tannins, anthocyanin, betacyanin, isoflavonoids, phenols, polyphenolic acids, xanthenes and lignins) present in the CST. Phytochemicals present in solanum trilobatum can potentially reduce oxidative modifications of membrane by restraining the access of oxidants to the bilayer and propagation of lipid oxidation in the hydrophobic membrane matrix thereby improving their fluidity (Haller and Bhaduri, 1998). The polyphenols effectively lowers the total cholesterol and LDL oxidability level by inhibiting cholesteryl ester hydroperoxides formation (Preuss et al., 2000; Vinson et al., 2002) thereby supporting the decreased levels of cholesterol levels in CST treated aged rats. Further the decrease in erythrocyte membrane rigidity on CST may possibly due to the hypolipidemic role and ROS scavenging effect of flavanoids (quercetin) present in CST (Sudheesh et al., 1992). CST was found to be effective in increasing the phospholipid levels, due to the property of flavanoids which are known to anchor the polar head of main phospholipids through hydrogen bonds forming reversible physiochemical complexes (Sendra et al., 2007; Roychowdhury et al., 2001).

Erythrocyte membrane bound ATPases play an important role in the maintenance of the ionic gradients between the intracellular and extra cellular compartment of the cell. Changes in the ionic concentration can bring about diverse ripple of cell injury and ultimately cell death (Trump et al., 1993). Significant decrease in the activities of Na+K+ ATPase, Ca2+ ATPases and Mg2+ATPases was observed in the erythrocytes of aged rats compared to young rats. It is evident that modification in the fatty acid composition of red cell phospholipids change the allosteric behavior of membrane bound enzymes (Yu et al., 1992). The most possible mechanism for the alterations in ATPases activity is through changes in lipid-protein interactions due to the amendment of the lipid-bilayer environment caused by free radicals and LPO during the transport of ions in ageing (Tsakiris et al., 2006). As thiol status also contributes in maintaining the structure and function of ATPases (Liu and Wei, 1999), the decrease in thiol levels in erythrocytes of aged rats as evidenced from the present study could also be a possible reason for the decrease in ATPases activity in aged rats erythrocyte. The phenolic compounds present in CST, (Zainol et al., 2003) with different functional properties such as scavenging of ROS, LPO chain breaking activity and thiol group replenishing properties (Laranjinha et al., 1995) accounts for the improvement of ATPases in aged rats. Further, membrane stabilizing
effects and the Ca\textsuperscript{2+} chelating property of phenolic compounds of CST (Pawlikowska-Pawlega et al., 2000) might also contribute for the enhanced activity of ATPases in aged rats.

Thus the identification of free radical reactions as promoters of the aging process implies that interventions aimed at limiting or inhibiting them should be able to reduce the rate of formation of aging changes with a consequent reduction of the aging rate and disease pathogenesis. In recent times the search for natural antioxidants and other preparation of plant origin to slow, stop or reverse ageing has been intensified to promote beneficial effects in aged animals. Plant-derived polyphenols are significant constituent of the human diets and their free radical scavenging capabilities protect erythrocytes against oxidative insult during ageing. The antioxidant and membrane protective role of Solanum trilobatum provide an efficient way to alleviate the macromolecular damages and protect from premature cell death with advancement of age. In expense of these studies, Solanum trilobatum is suggested as a novel life prolonging paradigm in laboratory animals. Therapy of flavonoid rich extract may not necessarily increase mean life span but they may extend the quality of life. Ultimately the study will help in designing elderly care which will lead to increase mean life expectancy.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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