Research Article

Erythrocyte Senescence in a Model of Rat Displaying Hutchinson-Gilford Progeria Syndrome

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Background. Increased oxidative stress is a major cause of aging and age-related diseases. Erythrocytes serve as good model for aging studies. Dihydrotachysterol is known to induce premature aging feature in rats mimicking Hutchinson-Gilford progeria syndrome. Aim. In the present study, attempts have been made to explore the differential response of young and senescent erythrocytes separated by density gradient centrifugation from accelerated senescence model of rats mimicking Hutchinson-Gilford progeria syndrome and naturally aged rats. Methods. The erythrocytes of naturally aged and progeroid rats were separated into distinct, young and old cells on the basis of their differential densities. The parameters of oxidative stress and membrane transport systems were studied. Discussion and Conclusion. Our study provides evidence that organismal aging negatively affects oxidative stress markers and membrane transport systems in both young and old erythrocytes. This study further substantiates that the changes in progeria model of rats resemble natural aging in terms of erythrocyte senescence.

1. Introduction

Hutchinson-Gilford progeria syndrome (HGPS) is a sporadic disorder characterized by premature aging. The primary etiology of the disease is a genetic mutation wherein cytosine to thymine substitution at position 1824 of Lamin A (LMNA) gene located in exon 11 erroneously produces a mutant prelamin A with the deletion of 50 amino acids including proteolytic cleavage site required for its post translational maturation. This mutant prelamin A protein is called progerin which retains farnesyl group through which it remains anchored tightly to the nuclear envelope leading to their accumulation and consequent nucleo-skeleton defect resulting into features of aging [1, 2]. Progerin accumulation is not only confined to HGPS but also its progressive accumulation has been studied in relevance to normal aging [3, 4]. Importantly, progerin accumulation has been reported to promote oxidative stress [5].

It has been reported that chronic intoxication with dihydrotachysterol (DHT) to young rats induces premature aging mimicking HGPS-like syndrome [6]. This progeroid rat model has been reported to mimic natural aging in terms of impaired redox status [7]. Erythrocytes exhibit numerous senescence markers including microvesiculation, decreased cellular volume, increased cellular density, and modulation in membrane transport system, which appear progressively during their lifespan before they exit the circulation at the end of their life span [8]. Although the erythrocytes are enucleated cells without any biosynthetic machinery, they maintain their viability till the end of their life span and are widely considered as viable model to study the aging process [9, 10].

The erythrocyte membrane, enriched with polyunsaturated fatty acids, is a soft target of reactive oxygen species (ROS) which makes them susceptible towards oxidative damage and consequently early removal from circulation [11]. Recently, we have reported an altered redox balance in DHT-induced HGPS mimicking premature aging in rats [7]. In view of above, the present study was undertaken to correlate organismal aging with cellular senescence and its differential effect on young as well as old erythrocytes from naturally aged and HGPS mimicking accelerated aging model of rat. To meet the above objectives, the biomarkers...
of oxidative stress were evaluated in young, old, and unfracti-
nated erythrocytes isolated from normal young, naturally aged and DHT-induced aged rats through density gradient
centrifugation.

2. Materials and Methods

2.1. Reagents and Chemicals. Dihydrotachysterol (DHT), corn oil, DIDS (4,4-disothiocyanatostilbene-2,20-disulphonic acid), amiloride hydrochloride, ouabain, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), reduced glutathione (GSH), 4,7-diphenyl-1,10-phenanthroline disulfonic acid sodium salt (DPI), 5,5-dithiobis nitro benzoic acid (DTNB), and percoll were obtained from Sigma Aldrich, St. Louis, USA. The rest of the chemicals used were purchased from SRL and HIMEDIA Lab, India.

2.2. Animal Model and Experimental Protocol. A total of 24 female rats of different ages were used in the study. Female rats were preferentially used because a higher DHT dose is required to induce progeria-like syndrome in corresponding aged male rats [12]. The rats were subjected to a week-long acclimation prior to start of dosing and were retained in polypropylene cages maintained at 25 ± 5 °C (4 rats/cage) temperature and 12/12 h light/dark periodic cycle. They were fed with standard laboratory pellet diet obtained from Paramount Techno Chem., Varanasi, India, with free access to drinking water. The total duration of the study was 30 days with the following treatment schedule.

(i) Group I: young control (3 months) group (n = 8) with body weight of 120 ± 20 g, receiving no treatment

(ii) Group II: DHT treatment (3 months) group (n = 8) with body weight of 120 ± 20 g, receiving 50 μg DHT dissolved in 0.5 mL corn oil, once daily through oral gavage route [6]

(iii) Group III: old control (24 months) group (n = 8) with body weight of 350 ± 20 g, receiving no treatment

The protocols and guidelines laid by animal ethical committee of University of Allahabad were followed during the study.

2.3. Blood Collection Procedure and Separation of Red Blood Cells from Plasma. After completion of the treatment schedule, blood samples were drawn by cardiac puncture under light anesthesia and collected using heparin as anticoagulant. The blood samples were centrifuged at 800 g for 15 min at 4 °C, and plasma was separated from packed red blood cells (PRBCs). After removal of the upper 15% buffy layer, PRBCs were washed thrice with cold phosphate-buffered saline (PBS) and finally suspended in glucose-containing PBS (GPBS) for use in biochemical analyses.

2.4. Density-Based Separation of Young and Old Erythrocytes and Isolation of Membrane Fraction. Erythrocytes of blood samples of different groups were separated into young and old populations on percoll-based density gradient centrifugation as described previously [13]. Briefly, the PRBCs were washed with RPMI 1640 medium twice followed by resuspending in the same medium maintained at 25% hematocrit. The suspension was then overlaid on percoll/sorbitol (4%, wt/vol) gradient and was subjected to centrifugation for 20 min at 10,752 × g. The different bands of young, old, and unfractonated erythrocytes thus obtained were isolated and used separately for the biochemical investigations. Membrane isolated as per our previously described protocol [14], and protein estimation in the membrane fraction was done by Lowry’s method [15].

3. Biochemical Investigations

3.1. Enzymatic Assay for Erythrocyte Acetylcholinesterase (AChE) Activity. The activity of membrane-bound acetylchol

3.2. Measurement of Lipid Peroxidation (LPO) Product. Malondialdehyde (MDA), the byproduct of lipid peroxidation (LPO), was measured in erythrocytes as per the protocol described by Esterbauer and Cheeseman [19]. In brief, the reaction mixture constituted by 1 mL of 10% trichloroacetic acid (TCA), 2 mL of 0.67% thiobarbituric acid (TBA), and 0.2 mL of PRBCs was incubated at 90–100 °C for 20 minutes. The mixture was then centrifuged at 1000 × g for 15 minutes after bringing to room temperature, and optical density of the supernatant was read at 532 nm. The MDA concentration was calculated using extinction coefficient (1.56 × 10^5 M^−1 cm^−1), and the values were expressed as nmol/mL of PRBCs.

3.3. Determination of Na+/K+ ATPase (NKA) Activity in Erythrocyte Membrane. NKA activity in erythrocyte mem

liberated inorganic phosphate was then calculated, and the activity of NKA pump is expressed as \( \mu \text{mol Pi released/mg protein/h} \) at 37°C.

3.4. Determination of Plasma Membrane Ca\(^{2+}\) ATPase (PMCA) Activity. The PMCA assay was performed following previously standardized protocol [20]. In brief, reaction mixture was prepared constituting 200 \( \mu \text{l} \) of erythrocytes membrane in solution of \( \text{MgCl}_2 \) (3 mM), NaCl (80 mM), KCl (15 mM), EGTA (0.1 mM), Tris-HCl (50 mM; pH 7.4), and 0.5 mM ouabain. The final concentration of 40 units/mL of calmodulin was added to the reaction mixture in the presence or absence of 0.2 mM CaCl\(_2\). The reaction was then initiated by adding 6 mM ATP to each tube and incubating at 37°C for 30 minutes. The reaction was then ended by adding 1.4 mL of solution containing 0.5 M H\(_2\)SO\(_4\), 0.5% ammonium molybdate, and 2% SDS. After 10 minutes, 0.04 mL of a solution containing 1.2% sodium metabisulphite, 1.2% sodium sulphite, and 0.2% 1-amino-2-naphthol-4-sulphonic acid (ANS-A) was added to each tube and incubated for 30 minutes. After centrifugation at 800 \( \times \) g for 5 min, absorbance of the supernatant of each tube was measured at 37°C for 30 minutes. The PMRS activity was calculated using extinction coefficient of 13,600 M\(^{-1}\) cm\(^{-1}\). The GSH content of erythrocyte was calculated and expressed as mg/mL PRBCs.

3.5. Measurement of Na\(^+\)/H\(^+\) Exchanger (NHE) Activity. The established protocol of Matteucci et al. [21] was followed to measure the NHE activity in intact erythrocyte. The PRBC suspension was prepared in a medium composed of 150 mM NaCl, 1 mM KCl, 1 mM MgCl\(_2\), and 10 mM glucose at 37°C for 5 min under continuous magnetic stirring. To the resulting suspension, 0.2 M HCl solution prepared in sodium phosphate-EDTA buffer was added slowly so as to bring the pH of suspension to 6.35–6.45 within 10 minutes. Finally, 0.2 mM DIDS was then added and the pH of the medium was again brought to 7.95–8.00 by adding 0.05 M NaOH solution in 150 mM NaCl. In a parallel set of experiment, amiloride along with DIDS was added. Soon thereafter, proton efflux was monitored and recorded. The activity of NHE was calculated subsequently and expressed as proton efflux in mmol/L RBC/hour at 37°C.

3.6. Measurement of Membrane-Associated Redox System Activity of Erythrocyte. Plasma membrane redox system (PMRS) activity was assayed following the method of Witko-Sarsat et al. [22] as described earlier [23]. In brief, PRBC (0.2 mL) suspension in glucose containing PBS (5 mM) was prepared, to which freshly prepared potassium ferricyanide (1 mM) was added followed by 30-minute incubation at 37°C. After centrifugation at 1800 \( \times \) g at 4°C, the resulting supernatant was used to measure ferricyanide content using 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt (DPD) which forms colored complex. The absorbance of the colored solution was measured at 535 nm. The PMRS activity was calculated using extinction coefficient of 20,500 M\(^{-1}\) cm\(^{-1}\), and results were expressed in \( \mu \text{mol ferricyanide/mL PRBCs/30 min} \).

3.7. Intracellular Erythrocyte Reduced Glutathione (GSH) Estimation. Erythrocyte GSH content was measured as per experimental method of Beutler [18]. The method is based on properties of glutathione -SH group to reduce 5,5-dithiobis, 2-nitrobenzoic acid (DTNB) that forms pale yellow anionic complex, the absorbance of which was measured spectrophotometrically at 412 nm. The GSH content of erythrocyte was calculated and expressed as mg/mL PRBCs.

3.8. L-Cysteine Influx Measurement in Erythrocytes. L-cysteine influx in erythrocyte was determined following previously reported protocol [24]. In brief, PRBCs (0.25 mL) were added to the reaction mixture composed of 1 mM PBS containing 8 mM glucose and 10 mM L-cysteine. After 1-hour incubation at 37°C, the mixture was centrifuged for isolation of erythrocytes. Free thiol (-SH) content in erythrocytes was estimated as per Sedlak and Lindsay [25]. Subsequently, erythrocytes were lysed in 100 \( \mu \text{l} \) of TCA (10%) prepared in sodium phosphate-EDTA buffer (0.01 M sodium phosphate/0.005 M EDTA) and centrifuged at 12000 \( \times \) g for 5 min. Finally, 100 \( \mu \text{l} \) of the supernatant was mixed with 1.9 mL of Tris-EDTA buffer composed of 0.6 \( \mu \text{M/mL} \) 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 262 mM Tris base, and 13 mM EDTA [pH 8.9]. The reaction mixture was incubated for five minutes at room temperature, and optical density was measured at 412 nm. The concentration of free -SH was calculated using extinction coefficient of 13,600 M\(^{-1}\) cm\(^{-1}\). The rate of L-cysteine influx was calculated by subtracting the value of control which was prepared in parallel in which erythrocytes were incubated for 1 h at 37°C in PBS glucose without L-cysteine.

3.9. Statistical Analysis. The data analysis was performed using GraphPad Prism 5, version 5.01 software. The values are represented as mean ± SD of eight different experiments of each group, and assessment of differences among the groups was determined by two-way ANOVA. The Bonferroni’s post hoc test was used for intergroup comparisons. Intragroup comparison was made using student’s t-test, and the results with probability (p) value less than 0.05 were assumed to be significant.

4. Results

4.1. Erythrocyte AChE Activity during Senescence in Naturally Aged and Progeroid Rats. Erythrocyte AChE activity in progeroid rats and its age-dependent variation are presented in Figure 1. A significant decline in erythrocyte AChE activity was noted with aging as represented by its activity in unfractionated erythrocyte of old control (17.12 ± 2.34) rats when compared to the young control (37.36 ± 2.12) rats. The progeroid rats showed diminished activity of erythrocyte AChE (24.22 ± 2.42) similar to the aged rats when compared to the young control rats. However, the value of AChE activity in progeroid rats was significantly higher compared to the old control group (17.12 ± 2.34). The induction of progeria-like phenotype in young rats also affected the AChE activity in both young erythrocytes (33.99 ± 3.06) and old erythrocytes (15.27 ± 2.46) which declined significantly in comparison to the young (45.93 ± 2.95) and old (30.41 ± 2.56) erythrocytes of the young control but remained significantly
higher than the young (22.77 ± 2.76) and old (11.11 ± 1.94) erythrocytes of the old control rats.

4.2. Erythrocyte Malondialdehyde (MDA) Level during Natural Aging and in Induced Progeria. The measurement of MDA as a marker of lipid peroxidation in erythrocyte of various groups is shown in Figure 2. Erythrocyte MDA level, as function of age, increased significantly in the old control group (129.43 ± 3.98) as well as induced progeroid groups of rats (125.40 ± 4.42) as compared to the young control group (114.51 ± 3.91) which is denoted by MDA level of unfraccionated erythrocytes of each group. However, the MDA level in young erythrocytes (122.90 ± 6.95) and old erythrocytes (128.64 ± 5.89) of the progeroid rat group was significantly lower when compared to corresponding young (125.97 ± 7.29) and old erythrocytes (132.16 ± 6.66) of the control group.

4.3. Change in Membrane NKA Activity in Response to Induced Progeroid Phenotype. The erythrocyte membrane-bound NKA activity was measured in naturally aged and induced progeroid rats (Figure 3(a)). A significant decline in membrane NKA activity of unfraccionated erythrocytes was noted in old control rats (225.02 ± 8.16) as compared to that of young control rats (260.38 ± 12.44). Induction of progeria with DHT proceeded with significant downregulation NKA activity in unfraccionated erythrocyte (239.55 ± 7.04) when compared to young control rats. Moreover, the NKA activity of young erythrocytes (296.55 ± 10.30) of progeroid rats approached the value near to the old control group (166.96 ± 9.25) significantly higher than that of the old control group (166.96 ± 9.25).

4.4. Variation in PMCA Activity with Age and in Response to Induced Progeria. The PMCA activity in erythrocyte membrane of different experimental groups is shown in Figure 3(b). The organismal age was associated with decreased activity of PMCA as demonstrated by its value in membrane of unfraccionated erythrocytes of old control rats (0.23 ± 0.015) compared to young control rats (0.47 ± 0.032). The young progeroid rats showed an overall decrease in their erythrocyte PMCA activity (0.34 ± 0.015) when compared with the young control group (0.47 ± 0.032); however, the value was significantly higher than that of the old control group (0.23 ± 0.015). An induction of progeroid features with DHT was found to negatively affect the PMCA activity of both the young (0.39 ± 0.02) and old (0.28 ± 0.026) erythrocytes as compared to respective young (0.55 ± 0.015) and old (0.43 ± 0.02) cells of the young control rats.
4.5. Erythrocyte NHE Activity in Induced Progeria and Naturally Aged Rats. The results of NHE activity are presented in Figure 3(c). The results of erythrocyte NHE activity demonstrated a significant upregulation in age-dependent manner as shown by the value of unfractionated erythrocytes of old control rats (23.47 ± 2.41) in comparison to the young control group (14.08 ± 1.35), and the value approached to the value of old control rats (23.47 ± 2.41). The NHE activity of old erythrocyte (17.90 ± 1.27) of progeroid rats was significantly higher than the old erythrocyte of young control rats (12.37 ± 2.08); however, a nonsignificant change in the activity of young erythrocytes (24.26 ± 2.16) of progeroid rats was observed as compared to that of young control rats (22.05 ± 1.62).

4.6. Increase of Erythrocyte PMRS Activity with Aging and Induced Progeria. Erythrocyte PMRS activity measured as a function of age in various groups is depicted in Figure 4. DHT-induced progeroid rats showed significantly increased PMRS activity (0.88 ± 0.04) in unfractionated erythrocytes...
rats (0.03 significantly higher than that from the old control group. More-
corresponding young erythrocytes of the old control group
decreased erythrocyte GSH content, the level was signi-
fi
each group. Although the induction of progeria signi-
evident from the values of unfractionated erythrocytes of
various groups is shown in Figure 5(a). The overall GSH con-
centration in different erythrocyte fractions of the naturally
aged old control group was more pronounced than that of the
corresponding progeroid group.

4.7. Impact of Induced Progeroid Feature on Erythrocyte GSH
Content. The variation in erythrocyte GSH content among
various groups is shown in Figure 5(a). The overall GSH con-
tent in erythrocytes of both the old control (0.02 ± 0.0040)
and progeroid rats (0.03 ± 0.0039) was significantly decreased
as compared to the young control (0.06 ± 0.0018) rats as
evident from the values of unfractionated erythrocytes of
each group. Although the induction of progeria significantly
decreased erythrocyte GSH content, the level was signifi-
cantly higher than that from the old control group. More-
over, the GSH content of young erythrocytes of progeroid
rats (0.03 ± 0.0043) was significantly higher than that of the
corresponding young erythrocytes of the old control group
(0.028 ± 0.0046). In contrary, a nonsignificant difference in
the value of GSH content was observed in old erythrocytes
of the progeroid group (0.02 ± 0.0029) and the young control
group (0.01 ± 0.0021).

4.8. Erythrocyte L-Cysteine Influx in Progeroid Rats and
Natural Aging. The results of L-cysteine influx as a marker
of aging are depicted in Figure 5(b). The overall L-cysteine
influx rate in the erythrocytes of old control rats declined
(2.59 ± 0.15) significantly as compared to the young control
rats (3.89 ± 0.15) as well as progeroid rats (3.13 ± 0.10). An
induction of progeroid features showed profound effect both
in the young (3.95 ± 0.12) and old (2.33 ± 0.04) erythrocytes
with significant decline in the value as compared to the cor-
responding young (4.83 ± 0.26) and old (2.88 ± 0.21) eryth-
rocytes fractions of the young control rats. However, the
value was higher than the corresponding erythrocytes of the
old rats.

5. Discussion
A plethora of evidence is available linking oxidative stress to
aging and age-related diseases [26]. Numerous studies sug-
mit that the molecular mechanisms involved in accelerated
aging phenomena of progeroid human subjects also occur
in healthy cells of older individuals. The experimental reports
from in vitro studies in human fibroblasts suggest that pro-
geroid subjects experience higher oxidative stress compared
to age-matched controls [5, 27]. It has been proposed that
progerin sequesters NRF2 at nuclear periphery and prevents
binding to ARE motifs. NRF2 is a major stress response fac-
tor which activates antioxidant and cytoprotective genes
through binding to ARE motifs. Therefore, the reduced avail-
ability of NRF2 for transcriprional activation of antioxidant
genes results in elevated oxidative damage and consequential
HGPS defects [28]. To the best of our knowledge, this is the
first report on oxidative damage caused by erythrocytes in
HGPS rat model. Since erythrocytes interact with multiple
organ systems in the circulation, they are exposed to signifi-
cant oxidative challenges from several sources during their
normal lifespan. Although erythrocytes have evolved robust
antioxidant systems to mitigate these challenges, the
increased oxidative stress incurred due to aging predisposes
them towards significant oxidative damage and premature
removal from the circulation [11].

In the present study, we separated young and senescent
erthrocytes from young control, old control, and DHT-
induced progeroid rats to analyze the capacity of young and
senescent erythrocytes to resist oxidative challenges during
the aging process. The successful separation was confirmed
by assessment of acetylcholinesterase (AChE) activity both in
the young and senescent erythrocytes. The AChE activity
of erythrocytes is a sensitive indicator of cellular aging [29].
It is noteworthy that the reticulocytes show about thrice the
value of AChE activity in comparison to mature erythrocytes
with the progressive depletion in its activity with advancing
of age [30, 31]. In our study, a marked decline in erythrocyte
AChE activity was noted in old erythrocyte fractions as com-
pared to the young erythrocyte of the same group. As a func-
tion of age, AChE activity of old rats was significantly lower
than the corresponding young control, which corroborated
our previous findings [32]. Induced progeria in young
rats following chronic DHT administration significantly
decreased the AChE activity in young and old erythrocytes.
of DHT-treated rats similar to naturally aged rats in comparison to corresponding young control rats. Normally, the functional integrity of erythrocytes is ensured by maintenance of its membrane fluidity which largely depends upon the degree of unsaturation of its lipid component and ratio of cholesterol to phospholipids [33]. Aging has been reported to increase the peroxidation of its lipid component, which leads to decreased fluidity, loss of membrane integrity, and increased hemolysis [34, 35]. Several studies suggest a linear correlation of increased erythrocyte MDA level with the advancing of age [13, 36]. In our study, the erythrocyte MDA level in young as well as senescent erythrocyte populations increased significantly in progeroid rats as compared to that of age-matched young control rats. Apart from this, our study also demonstrates noticeable changes in the activity of membrane-associated transport systems including NKA, PMCA, and NHE. It is noteworthy that the proper functioning of different ion transport systems is critical physiological events required to preserve the ionic balance across the plasma membrane of the cells to ensure their viability [37]. In the present study, NKA and PMCA activity was found to decrease in the erythrocyte membrane of progeria-induced rats similar to those noted for natural aging.

NKA conserves the ionic gradient by catalyzing the transport of $3\text{Na}^+$ outside and $2\text{K}^+$ inside of the cells which is crucial in determining their resting membrane potential. Furthermore, its activity has been widely reported to be influenced by various factors including membrane lipid peroxidation, alterations in lipid composition, fluidity, and permeability of the plasma membrane [38, 39]. The observed reduction in NKA activity in the present study thus could be justified from increased lipid peroxidation and consequent decrease in membrane fluidity as a consequence of oxidative stress. The reduced NKA activity has been linked to Ca$^{2+}$ overload which is known to trigger excitotoxicity leading to necrotic cell death [40].

Intracellular calcium plays crucial role in the regulation of several properties of erythrocytes, including cell volume and rheological properties, metabolic activity, redox state, and cell clearance [41]. However, the functional well-being of erythrocyte requires it to maintain a steep calcium gradient across the cell membrane with optimum functioning of PMCA which extrudes calcium out of the cell at the expense of ATP hydrolysis. There are several studies reporting intracellular calcium overload related to diminished PMCA activity with outcome of cellular rigidity, hemolysis, senescence, and apoptosis [42, 43]. Besides this, calcium overload in response to decreased PMCA activity has also been reported as a hallmark of aging [44]. Thus, decreased activity of PMCA in progeroid rats provides the evidence for age-

**Figure 5:** Erythrocyte GSH content and L-cysteine influx values represented as mean ± SD of eight independent experiments. (a) Effect of DHT treatment on GSH content of young, old, and unfractionated erythrocytes and its comparison with values of young and old. The represented values are mean ± SD of eight experiments performed independently. Overall erythrocyte GSH content in old erythrocytes of the young control group and the old control group was significantly ($p < 0.05$) lower as compared to the young and unfractionated erythrocytes of the same group. The progeria induction significantly ($p < 0.05$) decreased the overall GSH content in young, old, and unfractionated erythrocytes as compared to the corresponding erythrocytes of the young control group, but the level was higher than that of the naturally aged old control group. (b) L-cysteine influx of erythrocyte measured as marker of aging and membrane damage. The values represent the mean ± SD of eight independently performed experiments. L-cysteine influx was found to decrease significantly ($p < 0.05$) as function of age with lower values in different erythrocyte fractions of naturally aged old rats as compared to the corresponding young control group. The progeroid rats were associated with significant ($p < 0.05$) decline in L-cysteine influx compared the age-matched young control group, but the level was higher than that of the old control group.
related changes and significantly higher PMCA activity of young erythrocyte compared to old erythrocytes also corroborates with other similar studies [45, 46].

In response to increase in age, the overall activity of NHE has been observed to increase in our study as evident from the values of unfractionated erythrocytes and was found to follow the trends of other reports [14, 47]. Induction of progeroid features with dihydrotachysterol significantly enhanced the activity of NHE similar to natural aging. However, the old erythrocytes demonstrated significantly lower NHE activity as compared to the erythrocytes of the same group. It is worth noting that the plasma membrane NHE plays a crucial role in maintenance of intracellular pH of cells by catalytic expulsion of proton (H\(^+\)) in exchange for sodium (Na\(^+\)) ion being transported down their concentration gradient [48]. In addition, the optimal activity of NHE is also known to regulate growth and differentiation, cellular volume, and sodium absorption [49]. The hyperactivity of NHE is known to increase the intracellular Na\(^+\) which in turn increases intracellular calcium [Ca\(^{2+}\)], overload as secondary response to Na-Ca exchanger and is reported to mediate necrotic and apoptotic cell death [44]. However, report suggests that an elevated [Ca\(^{2+}\)], could be responsible for the increased NHE activity through phosphorylation-dependent or phosphorylation-independent pathways [50]. The decreased NHE activity has been shown to lower intracellular pH in old erythrocytes [11].

Normal red blood cells exhibit a degree of resistance towards oxidative damage primarily through their highly efficient antioxidant system [35]. In particular, the reduced glutathione is a component of GSH/GSSG redox couple and is present in high concentration in erythrocyte [51]. This provides the erythrocyte a reducing potential approximately 250 times more than its oxidizing capacity [52]. GSH is known to have effective free radical scavenging action on a wide range of reactive oxygen species including hydroxyl radicals, lipid peroxyl radicals, peroxynitrites, and H\(_2\)O\(_2\), either directly or indirectly acting as substrates for glutathione peroxidase (GSHPx) and glutathione-S-transferase (GST) enzymatic reactions [53]. Since erythrocytes experience a significant oxidative challenge from several sources including the auto oxidation of hemoglobin as well as those contributed by leukocytes, neutrophils, and other phagocytic cells in the circulation [54], reduced glutathione plays a vital role in mitigating the consequent damages. The erythrocyte GSH content is the net result of the rate of GSH synthesis and its intracellular utilization and efflux. Since free radicals readily oxidize GSH to its disulfide (GSSG) form, their high permeability leads to efflux from cells contributing to a net loss of intracellular GSH [24, 55]. The depletion of GSH is widely reported in reference to aging [56, 57]. In the present study, substantial decrease in intracellular GSH content was observed in erythrocytes of progeroid rats as compared to the age-matched control. Due to the lack of protein synthesis machinery, erythrocytes solely depend on GSH synthesis for defending the damage inflicted by oxidative stress encountered during its normal cycle. However, the rate of its synthesis depends largely upon the catalytic activity of the enzyme γ-glutamylcysteine ligase and adequate supply of cysteine [58]. The transport of cysteine in human RBC involves both the specific sodium-dependent (ASC system) cysteine transport as well as nonspecific but high capacity sodium-independent system, the L-transporter [59]. Our result of cysteine influx in rat erythrocytes, with reference to young control and old control, is in agreement with our previous report showing dependence of L-cysteine influx rate on human age [24]. The induced progeria model showed significant decrease in cysteine influx in both the young and old erythrocytes with reference to age-matched young control rats, thus providing evidence for aging like changes in comparison to the old control. The impaired membrane integrity and oxidation of transport proteins contribute to decreased L-cysteine influx in DHT-treated rat erythrocytes. The decreased L-cysteine influx might partially explain the decreased erythrocyte GSH content during aging.

As an adaptive response towards minimizing oxidative stress, the PMRS activity has been reported to get elevated with increase in oxidative stress during aging [57]. PMRS is constituted by several NAD(P)H reductases which participate in transmembrane electron transfer process [60] and acts to restore the redox homeostasis by modulating the oxidative status of various enzymes with antioxidant activity including ascorbate, coenzyme Q, and α-tocopherol [61]. The present study demonstrated an age-associated increase in erythrocyte PMRS activity in old control rats in comparison to young control. Similar to the changes observed for aging, induction of progeria in young rats activated erythrocyte PMRS activity. This study provides evidence that both the organismal age and cellular senescence positively affect PMRS activity.

6. Conclusion
Our study concludes that organismal aging negatively affects the oxidative stress markers and membrane transport systems in both the young and old erythrocytes. The DHT-induced progeria-like premature aging in rats resembles natural aging process in terms of erythrocyte senescence. This study further substantiates our findings of the previous study on DHT-induced premature aging rat model proving its suitability to study age-related changes.

Data Availability
The original data are available on request.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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References

[1] M. Eriksson, W. T. Brown, L. B. Gordon et al., “Recurrent de novo point mutations in lamin A cause Hutchinson–Gilford progeria syndrome,” Nature, vol. 423, no. 6937, pp. 293–298, 2003.

[2] R. D. Goldman, D. K. Shumaker, M. R. Erdos et al., “Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome,” Proceedings of the National Academy of Sciences of the United States of America, vol. 101, no. 24, pp. 8963–8968, 2004.

[3] P. Scaffidi and T. Misteli, “Lamin A-dependent nuclear defects in human aging,” Science, vol. 312, no. 5776, pp. 1059–1063, 2006.

[4] M. A. Singer, Human Ageing: A Unique Experience: Implications for the Disease Concept, World Scientific, New Jersey, 2015.

[5] G. Viteri, Y. W. Chung, and E. R. Stadtman, “Effect of peroxidation on the accumulation of oxidized proteins in fibroblasts from Hutchinson Gilford progeria patients,” Mechanisms of Ageing and Development, vol. 131, no. 1, pp. 2–8, 2010.

[6] B. Tuchweber, G. Gabbiani, and H. Selye, “Effect of vitamin e and methyltestosterone upon the progeria-like syndrome produced by dihydrotachysterol,” The American Journal of Clinical Nutrition, vol. 13, no. 4, pp. 238–242, 1963.

[7] M. K. Chaudhary, S. Singh, and S. I. Rizvi, “Redox imbalance in a model of rat mimicking Hutchinson-Gilford progeria syndrome,” Biochemical and Biophysical Research Communications, vol. 491, no. 2, pp. 361–367, 2017.

[8] S. Piomelli and C. Seaman, “Mechanism of red blood cell aging: relationship of cell density and cell age,” American Journal of Hematology, vol. 42, no. 1, pp. 46–52, 1993.

[9] L. Kaestner and G. Minetti, “The potential of erythrocytes as cellular aging models,” Cell Death & Differentiation, vol. 24, no. 9, pp. 1475–1477, 2017.

[10] A. Stier, S. Reichert, F. Criscuolo, and P. Bize, “Red blood cells open promising avenues for longitudinal studies of ageing in laboratory, non-model and wild animals,” Experimental Gerontology, vol. 71, pp. 118–134, 2015.

[11] S. Asha Devi, C. S. Shiva Shankar Reddy, and M. V. V. Subramanyam, “Oxidative stress and intracellular pH in the young and old erythrocytes of rat,” Biogerontology, vol. 10, no. 6, pp. 659–669, 2009.

[12] J. A. Schriefer and G. R. Spratto, “Examination of dihydrotachysterol-induced progeria as a model for aging changes in carbohydrate metabolism,” Journal of Pharmacological Methods, vol. 3, no. 4, pp. 297–304, 1980.

[13] D. Kumar and S. I. Rizvi, “Markers of oxidative stress in senescent erythrocytes obtained from young and old age rats,” Rejuvenation Research, vol. 17, no. 5, pp. 446–452, 2014.

[14] A. K. Singh, S. Singh, G. Garg, and S. I. Rizvi, “Rapamycin mitigates erythrocyte membrane transport functions and oxidative stress during aging in rats,” Archives of Physiology and Biochemistry, vol. 124, no. 1, pp. 45–53, 2017.

[15] O. H. Lowry, N. Rosebrough, A. Farr, and R. Randall, “Protein measurement with the folin phenol reagent,” The Journal of Biological Chemistry, vol. 193, no. 1, pp. 265–275, 1951.

[16] G. L. Ellman, K. D. Courtney, V. Andres jr., and R. M. Featherstone, “A new and rapid colorimetric determination of acetylcholinesterase activity,” Biochemical Pharmacology, vol. 7, no. 2, pp. 88–95, 1961.

[17] M. Suhail and S. I. Rizvi, “Erythrocyte membrane acetylcholinesterase in type 1 (insulin-dependent) diabetes mellitus,” Biochemical Journal, vol. 259, no. 3, pp. 897–899, 1989.

[18] E. Beutler, Red Cell Metabolism: A Manual of Biochemical Methods, Grune & Stratton, Orlando, FL, USA, 3rd edition, 1984.

[19] H. Esterbauer and K. H. Cheeseman, “[42] Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal,” in Methods in Enzymology, pp. 407–421, Elsevier, 1990.

[20] K. B. Pandey and S. I. Rizvi, “Role of resveratrol in regulation of membrane transporters and integrity of human erythrocytes,” Biochemical and Biophysical Research Communications, vol. 453, no. 3, pp. 521–526, 2014.

[21] E. Matteucci, S. I. Rizvi, and O. Giampietro, “Erythrocyte sodium/hydrogen exchange inhibition by (−) epicatechin,” Cell Biology International, vol. 25, no. 8, pp. 771–776, 2001.

[22] V. Witko-Sarsat, M. Friedlander, C. Capellere-Blandin et al., “Advanced oxidation protein products as a novel marker of oxidative stress in uremia,” Kidney International, vol. 49, no. 5, pp. 1304–1313, 1996.

[23] S. I. Rizvi, R. Jha, and K. B. Pandey, “Activation of erythrocyte plasma membrane redox system provides a useful method to evaluate antioxidant potential of plant polyphenols,” in Advanced Protocols in Oxidative Stress II, D. Armstrong, Ed., pp. 341–348, Humana Press, Totowa, NJ, USA, 2010.

[24] S. I. Rizvi and P. K. Maurya, “L-cysteine influx in erythrocytes as a function of human age,” Rejuvenation Research, vol. 11, no. 3, pp. 661–665, 2008.

[25] J. Sedlak and R. H. Lindsay, “Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman’s reagent,” Analytical Biochemistry, vol. 25, no. 1, pp. 192–205, 1968.

[26] D. P. Jones, “Redox theory of aging,” Redox Biology, vol. 5, pp. 71–79, 2015.

[27] L. Trigueros-Motos, “Hutchinson-Gilford progeria syndrome, cardiovascular disease and oxidative stress,” Frontiers in Bioscience, vol. S3, no. 1, pp. 1285, 2011.

[28] N. Kuppen, W. Zhang, L. Wang et al., “Repression of the antioxidant NRF2 pathway in premature aging,” Cell, vol. 165, no. 6, pp. 1361–1374, 2016.

[29] Y. G. Prall, K. K. Gambhir, and F. R. Ampy, “Acetylcholinesterase: an enzymatic marker of human red blood cell aging,” Life Sciences, vol. 63, no. 3, pp. 177–184, 1998.

[30] F. Herz and E. Kaplan, “A review: human erythrocyte acetylcholinesterase,” Pediatric Research, vol. 7, no. 4, pp. 204–214, 1973.

[31] A. A. Lawson and R. D. Barr, “Acetylcholinesterase in red blood cells,” American Journal of Hematology, vol. 26, no. 1, pp. 101–112, 1987.

[32] D. Kumar and S. I. Rizvi, “A critical period in lifespan of male rats coincides with increased oxidative stress,” Archives of Gerontology and Geriatrics, vol. 58, no. 3, pp. 427–433, 2014.

[33] J. E. Smith, “Erythrocyte membrane: structure, function, and pathophysiology,” Veterinary Pathology, vol. 24, no. 6, pp. 471–476, 1987.

[34] M. Y. B. Çimen, “Free radical metabolism in human erythrocytes,” Clinica Chimica Acta, vol. 390, no. 1-2, pp. 1–11, 2008.

[35] K. B. Pandey and S. I. Rizvi, “Markers of oxidative stress in erythrocytes and plasma during aging in humans,” Oxidative Medicine and Cellular Longevity, vol. 3, no. 1, 12 pages, 2010.
[36] T. Cebe, P. Atukeren, K. Yanar et al., “Oxidation scrutiny in persuaded aging and chronological aging at systemic redox homeostasis level,” Experimental Gerontology, vol. 57, pp. 132–140, 2014.

[37] G. R. Dubyak, “Ion homeostasis, channels, and transporters: an update on cellular mechanisms,” Advances in Physiology Education, vol. 28, no. 4, pp. 143–154, 2004.

[38] H. de Lima Santos, C. Fortes Rigos, and P. Ciancaglini, “Kinetics behaviors of Na,K-ATPase: comparison of solubilized and DPPC:DPPE-liposome reconstituted enzyme,” Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, vol. 142, no. 3–4, pp. 309–316, 2006.

[39] H. de Lima Santos, M. L. Lopes, B. Maggio, and P. Ciancaglini, “Na,K-ATPase reconstituted in liposomes: effects of lipid composition on hydrolytic activity and enzyme orientation,” Colloids and Surfaces. B, Biointerfaces, vol. 41, no. 4, pp. 239–248, 2005.

[40] S. P. Yu, “Na’s, K+-ATPase: the new face of an old player in pathogenesis and apoptotic/hybrid cell death,” Biochemical Pharmacology, vol. 66, no. 8, pp. 1601–1609, 2003.

[41] A. Bogdanova, A. Makrho, J. Wang, P. Lipp, and L. Kaestner, “Calcium in red blood cells—a perilous balance,” International Journal of Molecular Sciences, vol. 14, no. 5, pp. 9848–9872, 2013.

[42] F. Liu, H. Mizukami, S. Sarinaik, and A. Ostafin, “Calcium-dependent human erythrocyte cytoskeleton stability analysis through atomic force microscopy,” Journal of Structural Biology, vol. 150, no. 2, pp. 200–210, 2005.

[43] N. Stafford, C. Wilson, D. Oceandy, L. Neyses, and E. J. Cartwright, “The plasma membrane calcium ATPases and their role as major players in human disease,” Physiological Reviews, vol. 97, no. 3, pp. 1089–1125, 2017.

[44] S. M. Qadri, R. Bissinger, Z. Solh, and P.-A. Oldenborg, “Eryptosis in health and disease: a paradigm shift towards understanding the (patho)physiological implications of programmed cell death of erythrocytes,” Blood Reviews, vol. 31, no. 6, pp. 349–361, 2017.

[45] P. J. Romero and E. A. Romero, “The role of calcium metabolism in human red blood cell ageing: a proposal,” Blood Cells, Molecules & Diseases, vol. 25, no. 1, pp. 9–19, 1999.

[46] P. J. Romero and E. A. Romero, “Differences in Ca2+ pumping activity between sub-populations of human red cells,” Cell Calcium, vol. 21, no. 5, pp. 353–358, 1997.

[47] P. Kumar, S. Chand, and P. K. Maurya, “Quercetin-modulated erythrocyte membrane sodium-hydrogen exchanger during human aging: correlation with ATPase’s,” Archives of Physiology and Biochemistry, vol. 122, no. 3, pp. 141–147, 2016.

[48] J. Orlowski and S. Grinstein, “Na+/H+ Exchangers,” in Comprehensive Physiology, R. Terjung, Ed., John Wiley & Sons, Inc., Hoboken, NJ, USA, 2011.

[49] H. Cingolani, “Na+/H+ exchange hyperactivity and myocardial hypertrophy are they linked phenomena?,” Cardiovascular Research, vol. 44, no. 3, pp. 462–467, 1999.

[50] S. Wakabayashi, M. Shigekawa, and J. Pouyssegur, “Molecular physiology of vertebrate Na+/H+ exchangers,” Physiological Reviews, vol. 77, no. 1, pp. 51–74, 1997.

[51] M. D. Scott, L. Zuo, B. H. Lubin, and D. T. Chiu, “NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes,” Blood, vol. 77, no. 9, pp. 2059–2064, 1991.

[52] M. D. Scott, J. W. Eaton, F. A. Kuypers, D. T. Chiu, and B. H. Lubin, “Enhancement of erythrocyte superoxide dismutase activity: effects on cellular oxidant defense,” Blood, vol. 74, no. 7, pp. 2542–2549, 1989.

[53] Y.-Z. Fang, S. Yang, and G. Wu, “Free radicals, antioxidants, and nutrition,” Nutrition, vol. 18, no. 10, pp. 872–879, 2002.

[54] K. Gwozdzinski, A. Pieniazek, S. Tabaczar, A. Jegier, and J. Brzeszczyńska, “Investigation of oxidative stress parameters in different lifespan erythrocyte fractions in young untrained men after acute exercise,” Experimental Physiology, vol. 102, no. 2, pp. 190–201, 2017.

[55] O. W. Griffith, “Biologic and pharmacologic regulation of mammalian glutathione synthesis,” Free Radical Biology & Medicine, vol. 27, no. 9–10, pp. 922–935, 1999.

[56] L. Gil, W. Siems, B. Mazurek et al., “Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes,” Free Radical Research, vol. 40, no. 5, pp. 495–505, 2006.

[57] S. I. Rizvi and P. K. Maurya, “Markers of oxidative stress in erythrocytes during aging in humans,” Annals of the New York Academy of Sciences, vol. 1100, no. 1, pp. 373–382, 2007.

[58] R. Masella, R. di Benedetto, R. Vari, C. Filesi, and C. Giovannini, “Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes,” The Journal of Nutritional Biochemistry, vol. 16, no. 10, pp. 577–586, 2005.

[59] I. Bernhardt and J. C. Ellory, Red Cell Membrane Transport in Health and Disease, Springer Berlin Heidelberg, Berlin, Heidelberg, 2003.

[60] D.-H. Hyun, J. O. Hernandez, M. P. Mattson, and R. de Cabo, “The plasma membrane redox system in aging,” Ageing Research Reviews, vol. 5, no. 2, pp. 209–220, 2006.

[61] S. F. Leiser and R. A. Miller, “Nrf2 signaling, a mechanism for cellular stress resistance in long-lived mice,” Molecular and Cellular Biology, vol. 30, no. 3, pp. 871–884, 2010.