

Aging and destruction of blood erythrocytes in mice
(A Hypothesis)

Prof. Rajiv K. Saxena

School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067
South Asian University, New Delhi 110067

Introduction:

Erythrocytes constitute almost 99.9% of all blood cells (excluding platelets). Erythrocytes are terminally differentiated cells lacking nucleus and cannot undergo cell division. Since erythrocyte's main function is to remove carbon dioxide and supply oxygen to different organs, the cells shuttle between various organs and the lungs where carbon dioxide bound to hemoglobin in erythrocytes is replaced with oxygen. A reversal of the process occurs in peripheral organs where oxygen is released by the hemoglobin and carbon dioxide absorbed. These repetitive cycles of oxidation and reduction result in significant oxidative stress in erythrocytes. Erythrocytes are thus subject to significant oxidative

stress. In addition, erythrocytes traverse through skin where they may be exposed to damaging penetrating radiations or through areas with ongoing inflammatory responses where they may be exposed to damaging mediators. Since the cell repair machinery is highly deficient in erythrocytes, erythrocytes damaged due to oxidative or other forms of stress are unable to undertake self repair and are prone to be destroyed. For this reason, erythrocyte turnover rate is high and 1% of all circulating erythrocytes in human blood (2% in rodents) are destroyed each $^{-4}$. Mechanism of destruction of erythrocytes involves macrophages in the reticulo-endothelial system that recognize the erythrocytes that must be removed and eliminate them through the process of $^{-7}$. Evidence for an alternative mechanism of erythrocyte destruction through cell lysis has also been published ⁸⁻¹⁰.

Models of erythrocyte turnover:

Theoretically, two extreme models may be conceived for the destruction of circulating erythrocytes. These models can be termed (a) critical age model, and (b) random destruction model. First model may suppose that the destruction of erythrocytes depends purely upon their age. Thus erythrocytes may be destroyed when they reach a particular critical age and consequently have accumulated sufficient cellular damage. If this model is correct, all members of a cohort of erythrocytes that enters the blood at a given time, would survive till they reach a particular critical age and then be destroyed rapidly as shown in Figure 1A. In the random destruction model, erythrocytes in circulation may be killed at random irrespective of their age. In this case members of a cohort of erythrocytes freshly entering the blood would start dying from the very beginning and in

this case the expected survival kinetics of the erythrocyte cohort could be as depicted in Figure 1B. If both processes are partially responsible for the destruction of erythrocytes, the survival kinetics may lie some where in between the two curves shown in Figure 1. In any case, the destruction of erythrocytes in blood should be finely balanced with the entry of new erythrocytes in blood circulation so that at any given time the number of erythrocytes in circulation remains constant.

The problem we have been pursuing in our laboratory is to determine the actual survival kinetics of erythrocytes and correlate it with the two models discussed above. Understanding of these issues are important not only for gaining an insight into the mechanisms of normal turnover of circulating erythrocytes but also the changes that occur in the blood counts of erythrocytes in various forms of anemia and polycythemias.

There is a considerable amount of information in literature about the changes that take place in erythrocytes upon aging¹¹⁻¹³. However most of this data can be criticized since there are no good methods available for fractionating the circulating erythrocytes into populations of different age groups. Most popular method of physically separating the erythrocytes is base upon the buoyant density of erythrocytes. It is generally believed that the buoyant density of erythrocytes increase as they . There is however no clear proof of a direct relationship between the buoyant density and age of the circulating erythrocytes, and the assumption of such a relationship has been¹⁶. In an old study by Allison and , blood group-O bearing erythrocytes were infused in a host with blood group-A and at subsequent time points group-O bearing erythrocytes were isolated by removing the host

erythrocytes through agglutination by anti-group-A antibodies, in order to study changes in aging group-O erythrocytes. Unfortunately, this technique suffered from many technical problems. Ganzoni et al¹⁸ and later Mueller et al used another method involving sequential hyper-transfusing of blood cells in rats that allowed aging erythrocytes to be enriched. Besides being cumbersome and animal intensive, this technique also resulted only in relative enrichment of old erythrocytes and did not yield a pure preparation of old erythrocytes. Moreover, none of these techniques allowed a comparison of erythrocytes of different age groups within a given sample of erythrocytes.

A breakthrough in the technology for studying age related changes in blood erythrocytes came in 1987 when Suzuki and Dale demonstrated that erythrocytes could be labeled with biotin and the biotin label remains unchanged if the cells are transfused back in²¹. Hoffmann-Fezer et al.^{22,23} further improved the method by demonstrating that circulating erythrocytes could be biotinylated in vivo and the label could be used to assess the survival of erythrocytes and isolation of erythrocyte populations enriched in aged cells. In these techniques, biotinylated erythrocytes could be stained ex-vivo with avidin coupled to a fluorochrome and analyzed by flow cytometry. After labeling all circulating erythrocytes in vivo, if mice were bled after few days, the fresh erythrocytes released in circulation were biotin negative and very young erythrocytes could thus be distinguished from the rest by flow cytometry. Similarly, if such mice were bled after about 50 days of biotinylation step, only a small fraction of very old biotinylated erythrocytes survived in circulation that could either be analyzed flow cytometrically or even isolated by using avidin affinity. While this elegant technique could be used to study very young or very

old erythrocytes, it could not be used to isolate intermediate age groups of erythrocytes or for direct comparison of very young and very old erythrocytes from the same mouse. Recently, we have been able to further modify the single step *in vivo* biotinylation technique of Hoffmann-Fezer et al.^{22,23} by introducing a second *in vivo* biotinylation²⁵. Our modified technique allows for the first time a systematic study of changes in cohorts of erythrocytes from the time they are first released in circulation till they age and fade away from circulation. The technique can also be used to simultaneously study very young and very old erythrocytes from the same mouse²⁴⁻²⁷.

The double *in vivo* biotinylation technique for tracking age related changes in circulating erythrocytes:

The principle of double *in vivo* biotinylation (DIB) technique has been explained in Figure 2. In the first biotinylation step comprising three daily i.v. injections of BXN (Biotin-X-NHS Ester, 1 mg/mouse/day), all erythrocytes become labeled with biotin. After resting the mouse for 5 days, about 10% of the blood erythrocytes are biotin negative, these being the cells that were released in blood circulation *after* the completion of first biotinylation step. At this stage a single injection of 0.6 mg BXN labels the freshly released erythrocytes and render them as compared to older erythrocytes that were labeled in the first biotinylation step. Any time thereafter, blood erythrocytes isolated from a DIB labeled mouse may be categorized in three groups *viz* a (oldest age group), a biotinlow (intermediate age group) and a biotin-negative (youngest age group). All three groups of erythrocytes can be identified and enumerated by staining with streptavidin coupled to a fluorochrome like FITC or APC, followed by flow cytometric

analysis. It should be noted that instead of a 5 days gap, the second step of biotinylation could as well be done after a 2, 3 or 4 days gap. These altered gaps would result in a decrease in the proportion of the erythrocyte band (i.e. 4, 6 and 8% respectively instead of 10% for 5 days gap), without any effect on the actual pattern of age dependent changes on erythrocytes.

Results in Figure 3 show the flow cytometric patterns of biotin labeling on erythrocytes isolated after different steps in the DIB technique. By gating on any category of erythrocytes and using a second or a third staining antibody, it is possible to compare the expression of various markers of interest on the oldest, intermediate and the youngest erythrocyte populations. Furthermore, as the DIB labeled mouse ages, the cohort of erythrocytes gradually moves from very young to very old part of the spectrum. By analyzing test bleeds from DIB labeled mice taken at various time points, it is possible to track age related changes taking place in the cohort of erythrocytes in blood.

Kinetics of erythrocyte destruction in blood circulation in mice:

Survival kinetics of cohorts of blood erythrocytes expected from of two extreme models, of erythrocyte destruction has been discussed above. DIB technique allowed us to investigate the actual kinetics of loss of erythrocytes generated and released in blood during a short and defined window of time (an erythrocyte cohort). For this purpose, mice were DIB labeled and sacrificed at different time points when blood samples were taken and the percentage survival of the cohort of erythrocytes determined. Combined results of survival kinetics data obtained on 14 mice have been presented in Figure 4.

Scrutiny of whole survival data plotted against time indicated that up to 10 days of age, survival of erythrocyte cohorts remained around 100%. Thereafter, a decline in survival was observed. Mean rate of decline in survival was about 1.3% per day till 40 days of age and doubled thereafter to 2.8% per day. The three phases denoted in Figure 4 have essentially been demarcated based upon the best visual fit of the data points. Mean slopes of the survival curve in the three phases and their standard deviations are also shown in Figure 4. Correlation coefficients remained above 0.9 for phases 2 and three of the survival curve. These results indicate that a steady killing of erythrocyte cohort starts as early as 10 days after their release in the blood and the rate of killing becomes greater as the cohort after they are 40 days old. By day 60, almost all erythrocytes of the cohort vanish from the blood. This data agrees well with the accepted life span of erythrocytes in mouse ².

Age related changes in CD47, CD147 and phosphatidyl serine (PS) expression on erythrocytes:

We have tracked three important markers [CD47, CD147 and phosphatidyl serine (PS)] on erythrocytes that are important in regulation of cellular survival. CD47 is a marker on erythrocytes that protects erythrocytes from phagocytosis by ⁻³⁰. CD47 molecules on erythrocytes interact with their specific receptors on macrophages and send an inhibitory signal that suppresses the phagocytic response. CD147 expression on erythrocytes facilitates their recirculation from spleen to . Decreased CD147 marker results in trapping of erythrocytes in spleen. Results in Figure 5 show the kinetics of age related changes in the expression of CD47 and CD147 markers on erythrocytes cohort. These

results show that both markers decline significantly with age on circulating erythrocytes. Lower density of CD47 and CD147 markers would render the aging erythrocyte populations susceptible to phagocytosis and promote their entrapment in spleen. Both these mechanisms may be responsible for the gradual loss of erythrocytes from circulation.

PS externalization is an early marker for cells undergoing . While erythrocytes lack nucleus and may not generate a classical apoptotic response, a process like apoptosis termed eryptosis has been described for erythrocytes that also involves PS externalization^{33, 34}. It has been suggested that PS externalization may be responsible for the destruction of senescent⁻³⁶. Using DIB technique, we monitored changes in PS expression on erythrocytes as a function of age. Results in Figure 6 show that PS expression on unfractionated erythrocytes remains low (<1%). When cohort was monitored for PS expression, no significant age related increase was found in this parameter. Very young erythrocytes had a small percentage of PS positive cells (about 4%) but at later time points PS expression remained very low (Figure 6). We could not find an age related increase in PS expression on erythrocytes and our results therefore do not support a role of PS externalization in the destruction of old erythrocytes in circulation. It is possible that we did not see the enhanced PS expression because such cells were immediately removed from circulation by macrophages. To test this possibility, we depleted mice of macrophages (90% macrophage depletion) but did not find elevation of PS+ erythrocytes in macrophage depleted mice . Some other workers have demonstrated a significant increase in PS expression in old erythrocytes in mice by using annexin V-FITC reagent to

assess PS expression. We have recently found that old murine erythrocytes have elevated green autofluorescence with emission spectrum similar to that of . Annexin V-FITC may therefore not be an appropriate reagent for comparing PS expression in young and old erythrocytes since the autofluorescence in old erythrocytes would introduce artifacts resulting in erroneous results. We used Annexin V-APC in stead of annexin V-FITC to stain erythrocytes for PS expression. Emission spectrum of APC does not overlap with that of the green autofluorescence of old erythrocytes and we could avoid the artifact associated with using FITC labeled annexin V. Using annexin V-APC, we found no evidence for elevated PS expression on old erythrocytes (Figure 6).

Stress and erythrocyte turnover:

Exposure of mouse erythrocytes to a variety of stress inducing reagents results in high degree of PS externalization ³⁷⁻³⁹. We could confirm these results but found marked differences in the PS externalization in young and old erythrocytes, exposed to stress inducing reagents. In order to compare the PS expression on stress exposed youngest and the oldest populations of erythrocytes from the same mouse, we developed a fresh strategy of the two step biotinylation protocol that has been explained in Figure 7. By using this strategy of biotinylation, youngest and the oldest populations of blood erythrocytes could be identified and examined simultaneously for stress induced PS externalization. Results in Figure 7 indicate that exposure to deoxy glucose (DOG), and + calcium ionophore (reagents known to induce stress in erythrocytes), induced high levels of PS expression in young erythrocytes but relatively low levels on erythrocytes of intermediate ages. No increase in PS expression was seen on old erythrocytes exposed to

stress. Furthermore, when DIB labeled erythrocytes were exposed to stress *in vitro* and then infused back into mice, only young population of infused erythrocytes was rapidly and selectively eliminated from the blood (Figure 8). These results suggest that stress induced PS externalization is more prominent in young erythrocytes. We therefore hypothesize that young erythrocytes in blood circulation, exposed to stress may be eliminated through eryptosis or through phagocytosis by effector macrophages recognizing enhanced PS expression on erythrocytes.

Current Hypothesis about erythrocyte turnover in blood:

Due to lack of appropriate techniques, an objective assessment of erythrocyte turnover in mice was not available. A variety of techniques involving the infusion of tagged erythrocytes have been utilized to assess the life span of erythrocytes *in vivo*. However since the infused erythrocytes were unfractionated and represented a mixture of erythrocytes of all ages, precise determination of rate of erythrocyte destruction were not possible. Using the new DIB technique, we have been able to follow the survival kinetics of a cohort of erythrocytes of defined age, with a degree of precision not possible so far. The actual survival kinetics of an erythrocyte cohort determined experimentally by using the DIB technique appears to fall somewhere in between the patterns predicted by the two extreme models given in Figure 1. The fact that the numbers of erythrocytes within the age defined cohort started to fall as early as 10 days after the cells entered the circulation clearly indicates that the erythrocyte destruction is not solely determined by the age of the cohort and random killing must at least partially account for the loss of erythrocytes from circulation. Furthermore, the fact that the rate of decline in cell numbers is lower in

the beginning (10 -40 days) and increases in the later part of the life span of erythrocytes (>40 days) suggests that the cell destruction may also be partially determined by age related changes that occur on circulating erythrocytes. The factors that make circulating erythrocytes susceptible to random (age independent) killing are not known. However it is tempting to speculate that younger erythrocytes that get damaged due to oxidative or other forms of stress may extrude PS and be removed through phagocytosis. Since PS extrusion is more efficient in younger erythrocytes, it is likely that this mechanism of stress induced random killing may be relatively more prevalent in younger populations of erythrocytes.

We have demonstrated that the PS extrusion response to stress is significantly lower in senescent erythrocytes. Older damaged erythrocytes may therefore neither externalize PS nor be eliminated by the random killing route. We have also demonstrated a steady fall in CD47 and CD147 expression during the aging of erythrocyte cohorts. Factors like a fall in CD47 and CD147 expression (rather than PS extrusion) may render older erythrocytes susceptible to phagocytosis by macrophages. It is possible that the combined effects of these changes may result in a greater rate of destruction of erythrocytes above 40 days of age. That would explain an increase in the slope of the survival curve after 40 days time point.

Conclusion:

In conclusion, we have developed a new double in vivo biotinylation (DIB) technique that allows objective assessment of age related changes that occur in circulating blood

erythrocytes. Using this technique, we have been able to determine the precise survival of age defined cohorts of erythrocytes in mouse blood. Further, based upon our results of survival kinetics as well as changes in the expression of survival determining markers like CD47, CD147 and PS, we have suggested the factors that may determine the shape of the survival curve of erythrocytes in mouse blood.

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LEGENDS TO FIGURES:

Figure 1. Two extreme models of erythrocyte destruction. If age is the sole criterion for earmarking erythrocytes for killing, members of a cohort of erythrocytes generated at a given time point would survive *in toto* and keep circulating till a critical age is reached. Thereafter, accumulated damage to senescent cells may cross a critical mark for their recognition and destruction by macrophages (left panel). If erythrocytes are destroyed

randomly irrespective of their age, a given cohort of erythrocyte population would start dwindling as soon as it is released fresh in blood circulation (right panel).

Figure 2. Double *in vivo* biotinylation (DIB) technique for tracking age related changes on circulating erythrocytes. (A) Normal circulating erythrocytes are biotin negative. (B) After three daily injections of BXN (Biotin-X-NHS Ester 1 mg / mouse / day) all erythrocytes become biotin labeled. (C) After five days, about 10% erythrocytes that were produced fresh *after* the first biotinylation step are biotin negative. (D) A second *in vivo* biotinylation step with a single injection of 0.6 mg BXN results in relatively lower intensity of biotin labeling on freshly produced band of erythrocytes. (E) At all later time points, Biotin high population represents the oldest erythrocyte population in circulation, biotin-low population represents a cohort of erythrocytes produced during a limited time window (5 days in this example) between first and second biotinylation steps. Biotin negative population represents the youngest cells produced after the second biotinylation step. Erythrocytes can be stained with streptavidin-FITC / APC ex-vivo and analyzed on flow cytometer as shown in Figure 3. For details of methodology please see reference 24 and 25

Figure 3. Demarcation of three discrete erythrocyte populations in blood using a double *in vivo* biotinylation (DIB) procedure. C57Bl/6 mice were administered intravenously three daily doses of 1 mg biotin-X-NHS Ester (BXN) [first biotinylation step]. After a five day rest, a single additional dose of 0.6 mg BXN was administered [second biotinylation step]. Blood was collected at different time points and distribution of biotin

label on erythrocytes was examined by staining the cells with streptavidin-APC and flow cytometric analysis^{24,25}. Biotin label on circulating erythrocytes was examined 2 hours (panel A) and 5 days (panel B) after the first step of biotinylation, and 2 hours (panel C), 5 days (panel D), 25 days (panel E) and 50 days (panel F) after the second step of biotinylation. Erythrocyte populations in Boxes X, Y and Z represent , and biotin-negative populations of erythrocytes. Values in parentheses represent percentage of cells in different boxes.

Figure 4. Composite data on the decay of a defined erythrocyte cohort in circulation. C57Bl/6 mice were DIB labeled and sacrificed at different time points. At different time points, population (defined in Figure 2 and 3) were assessed as percentage of all circulating erythrocytes. Combined data of survival kinetics from 14 mice show the decay of the cohort of erythrocytes as a function of age. Correlation coefficients and slopes of the three phases of the decay curve have been calculated.

Figure 5. Kinetics of decline in CD147 and CD47 marker expression on erythrocyte cohort as a function of age. Mouse erythrocytes were labeled with DIB technique as described in Figure 2 and 3. CD147 (left panel) and CD47 (right panel) expression on cohort of erythrocytes was examined 5, 15, 25, 35, 45 and 50 days after the second biotin dose (panel B). At each time point erythrocytes were stained with anti-CD147 and anti-CD47 mabs and examined on flow cytometer. At each time point, mean channel of CD147 and CD47 expression was assessed for all erythrocyte as well as for cohort of cells. Mean channel values for cohort as percentage of mean channel for all

erythrocytes have been plotted. Each point on the graph represent mean \pm SD of observations on 5 mice. * p <0.005 for comparison of different time points with the first time point. Details of methodology are available elsewhere^{24,26}.

Figure 6. Kinetics of age dependent Phosphatidylserine (PS) externalization on circulating erythrocytes. Mice were labeled by DIB technique as described in Figure 3 and sacrificed at different time points. Erythrocytes samples were stained with streptavidin-FITC as well as Annexin V-APC and analyzed on a flow cytometer. PS expression was studied on (open circles) and whole erythrocyte (closed circles) populations at all time points. Each point in the graph represent mean \pm SD of data from 3-5 mice.

Figure 7. Double *in vivo* biotinylation (DIB) technique for simultaneous identification and study of youngest and oldest erythrocytes in blood circulation. (A) Normal circulating erythrocytes are biotin negative. (B) After three daily injections of BXN (Biotin-X-NHS Ester 1 mg / mouse / day) all erythrocytes become biotin labeled. (C) After 30 days, about 60% erythrocytes that were produced fresh *after* the first biotinylation step are biotin negative. (D) A second *in vivo* biotinylation step with a single injection of 0.6 mg BXN results in relatively lower intensity of biotin labeling on freshly produced band of erythrocytes. (E) After 5-6 days, biotin high population represents the oldest erythrocyte population in circulation, whereas the biotin negative population represents the youngest cells produced after the second biotinylation step. Biotin-low population represents a wide spectrum of erythrocytes of intermediate age group. Erythrocytes can be stained

with streptavidin-FITC / APC ex-vivo and analyzed on flow cytometer as shown in Figure 3. For details of methodology please see reference 24 and 25

Figure 8. Effect of various stresses on PS externalization on different age groups of erythrocytes. Mouse erythrocytes were biotin labeled by the DIB technique as described in Figure 7. This biotinylation strategy enabled identification of erythrocytes belonging to young (<10 days), intermediate (10-40 days) and old (>40 days) age groups. Labeled erythrocytes were collected and incubated for 12 h in HEPES buffered saline with (A) 5 mM 2-DOG, or (B) 1mM CaCl₂, or (C) 1 hrs with 1mM CaCl₂ and 0.5μM calcium ionophore. At different time points, panel A and B, percentage of PS⁺ erythrocytes was examined after staining with SAV-APC and annexin V-FITC. Each point represent mean ± SD of 3 observations. In panel A, PS expression on intermediate as well as old erythrocytes was significantly different from young group (p<0.05 at all time points except 0h). Intermediate and old groups were significantly different at 8 and 12h time points (p<0.05). In panel B also, PS expression on intermediate as well as old erythrocytes was significantly different from young group (p<0.05 at all time points except 0h). Intermediate and old groups were significantly different at 12h time points (p<0.05). In panel C, PS expression is significantly lower in old group (p<0.01) as compared to young and intermediate groups. Young and intermediate group values are not significantly different.

Figure 9. Clearance of different age group of PS⁺ erythrocytes from the circulation. Mouse erythrocytes were labeled using DIB technique as described in Figure 7.

Erythrocytes were collected and stained with a green fluorescent dye CFSE and incubated for 8 hrs in the HEPES buffer saline with 1mM (stress inducing agent). After *in vitro* treatment these erythrocytes were infused in fresh mice and blood samples were collected at different time points. Erythrocytes were stained with SAV-APC and annexin V-PE and analyzed flow cytometrically. Residual infused erythrocytes (CFSE⁺ population) were gated and proportions of different age group of infused erythrocytes and the numbers of residual PS⁺ erythrocytes in each age group was determined. Values of percentage of residual PS positive erythrocyte in different age group of erythrocyte population at different time point have been shown. Each point in the graph represents the mean \pm SD of values obtained in three separate experiments. As compared to first time point, the decline in the numbers of infused young PS⁺ erythrocytes in circulation was statistically significant (range $p < 0.05$ to 0.001).

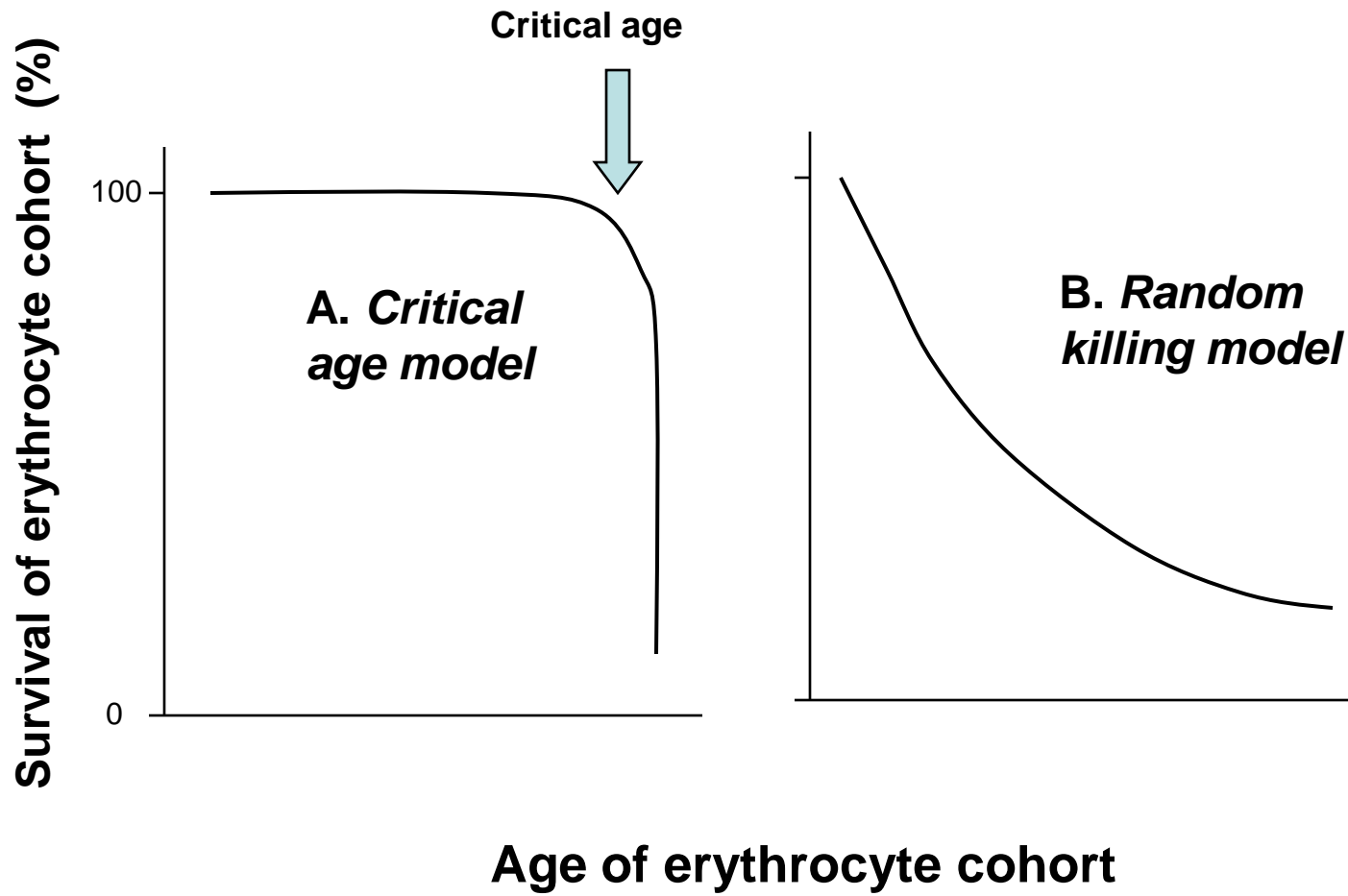


Figure 1

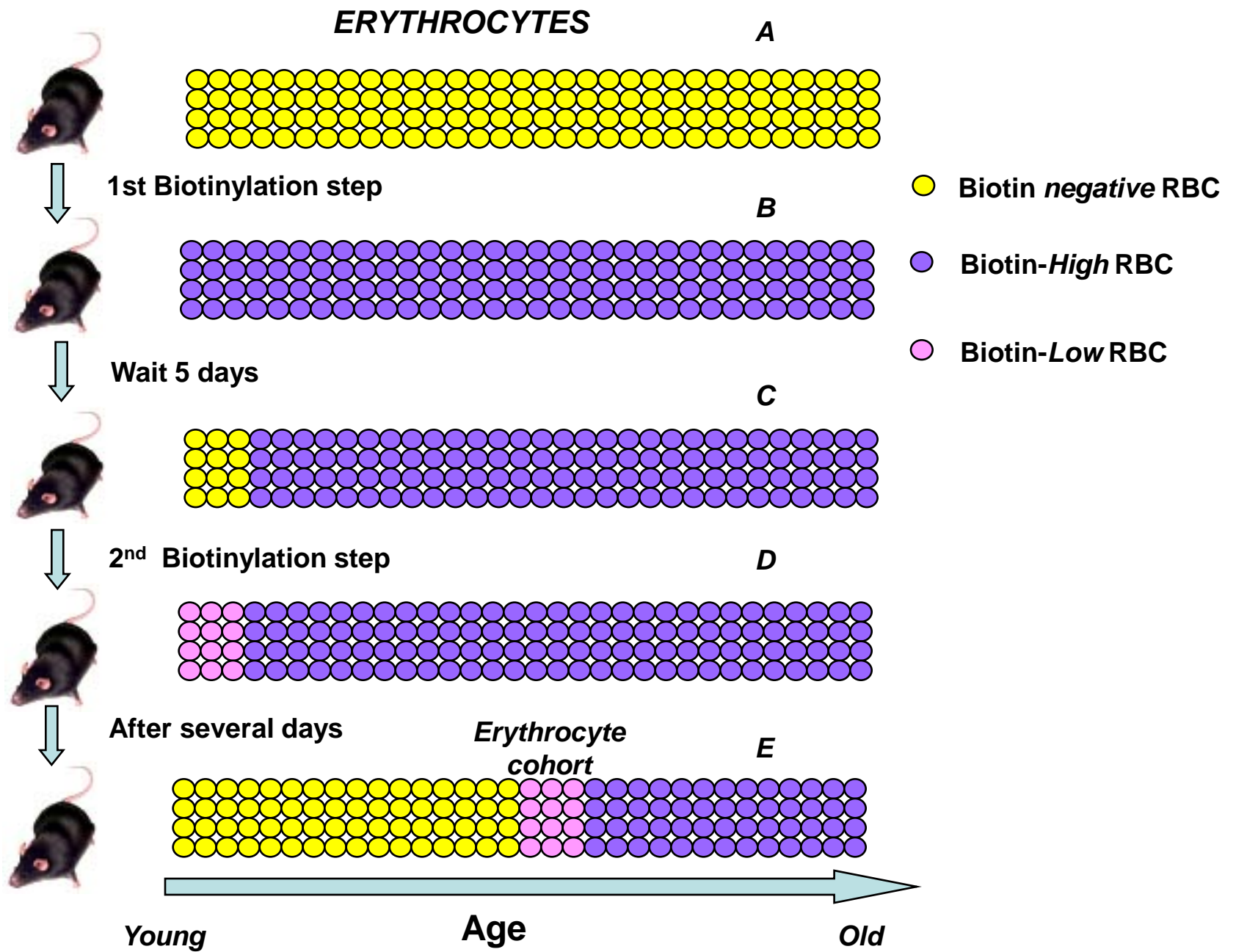


Figure 2

Relative Biotin Label

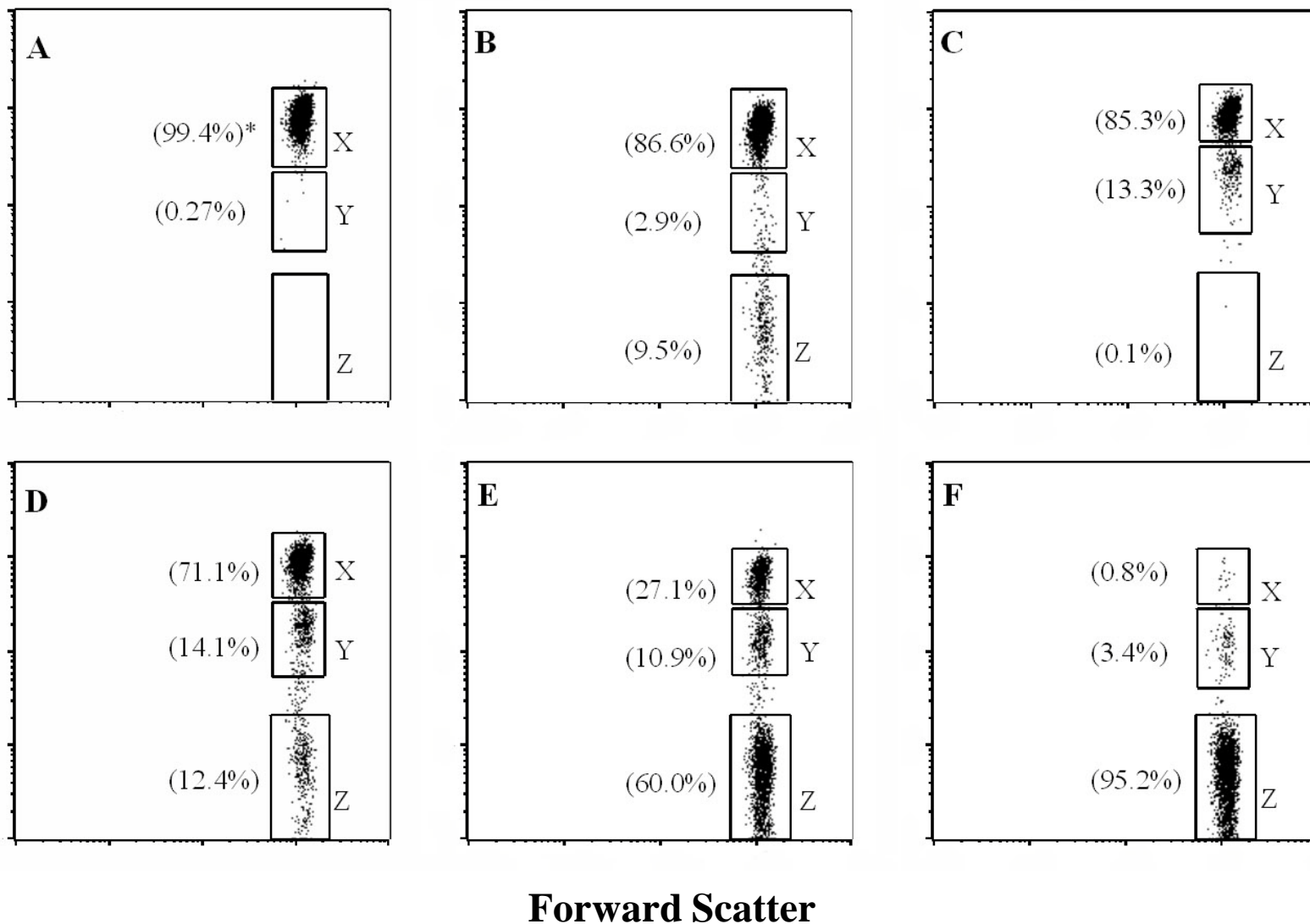


Figure 3

Fitting equation "y = mx + c" for the three phases of erythrocyte survival kinetics

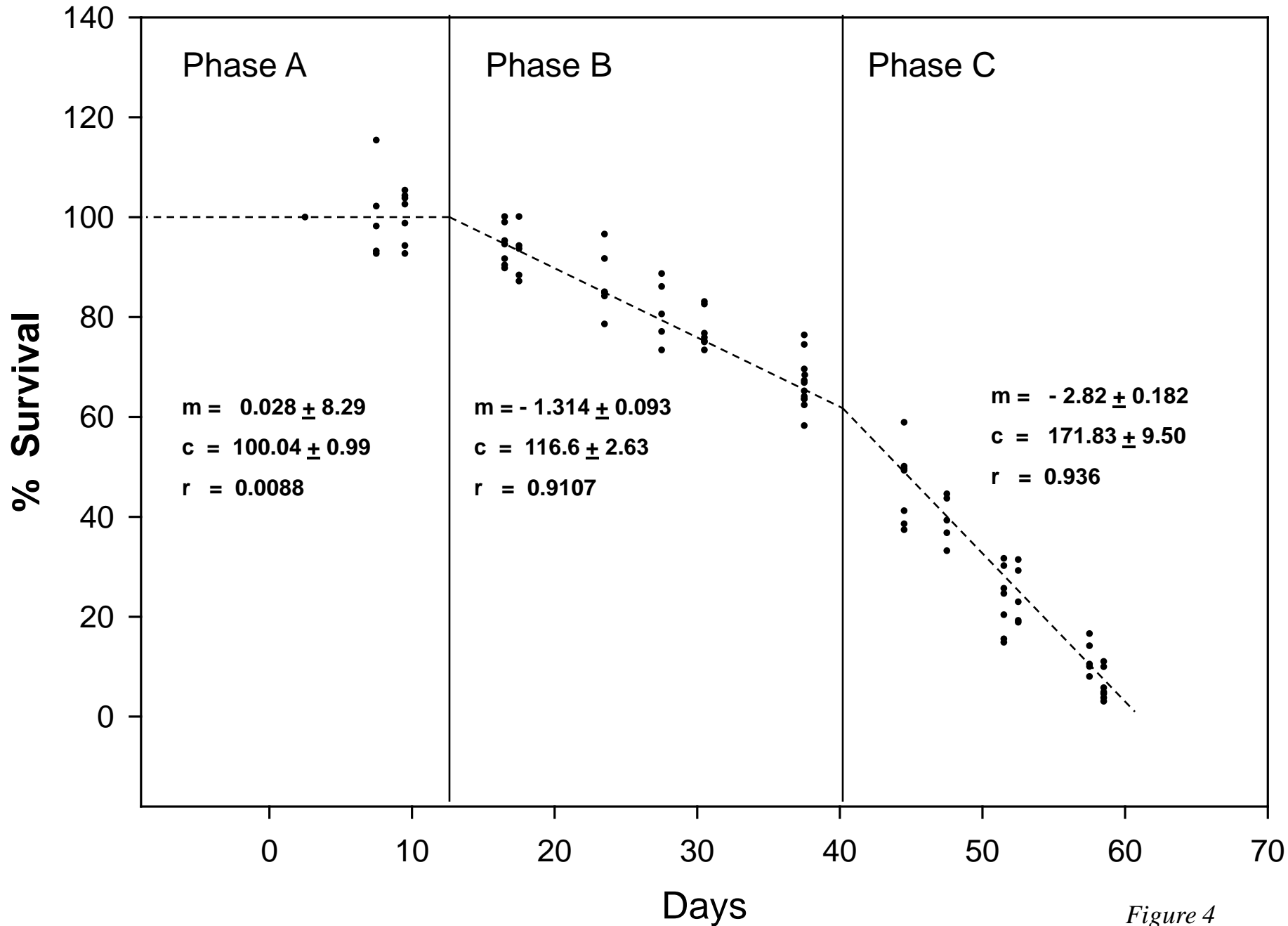


Figure 4

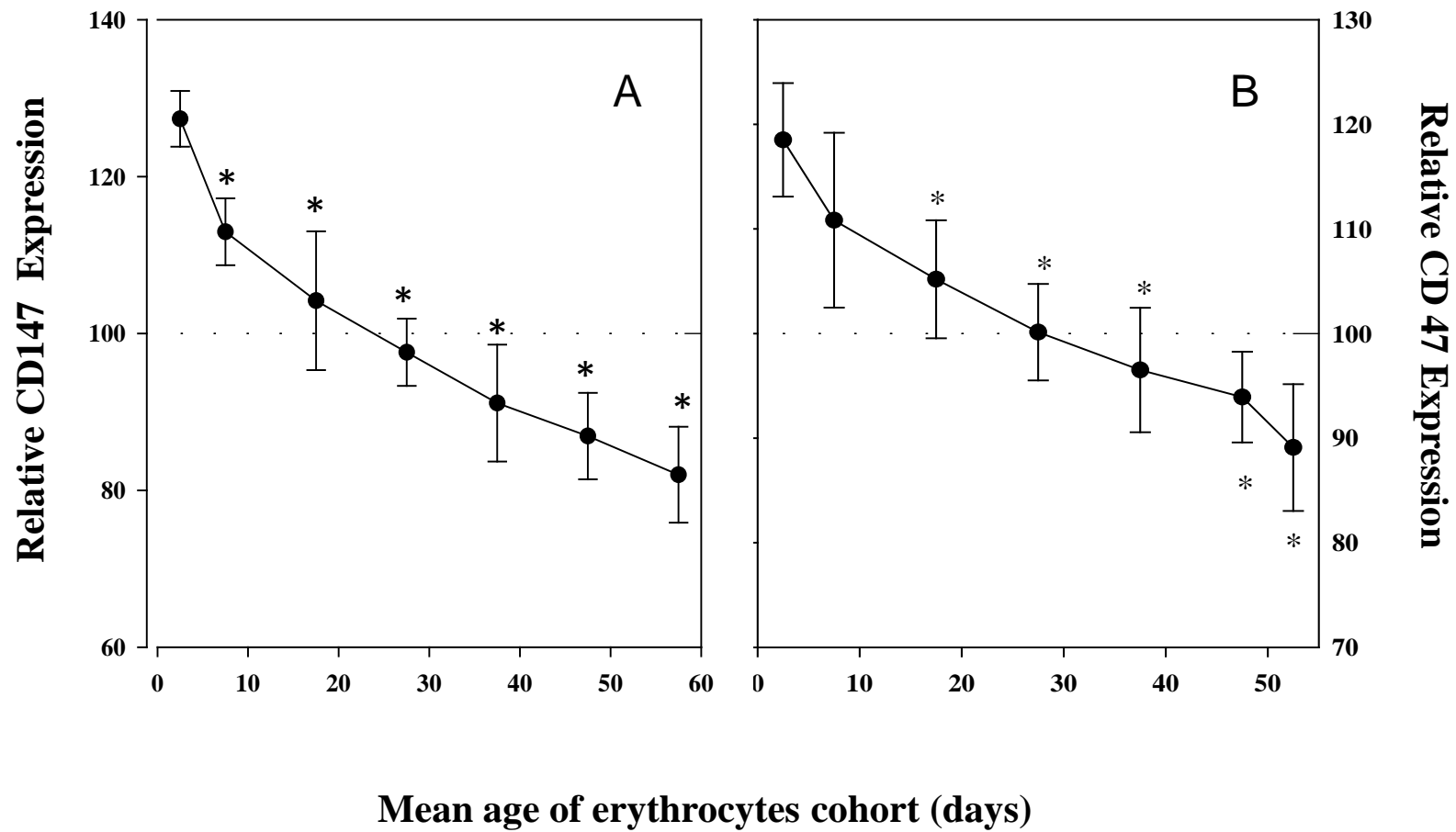


Figure 5

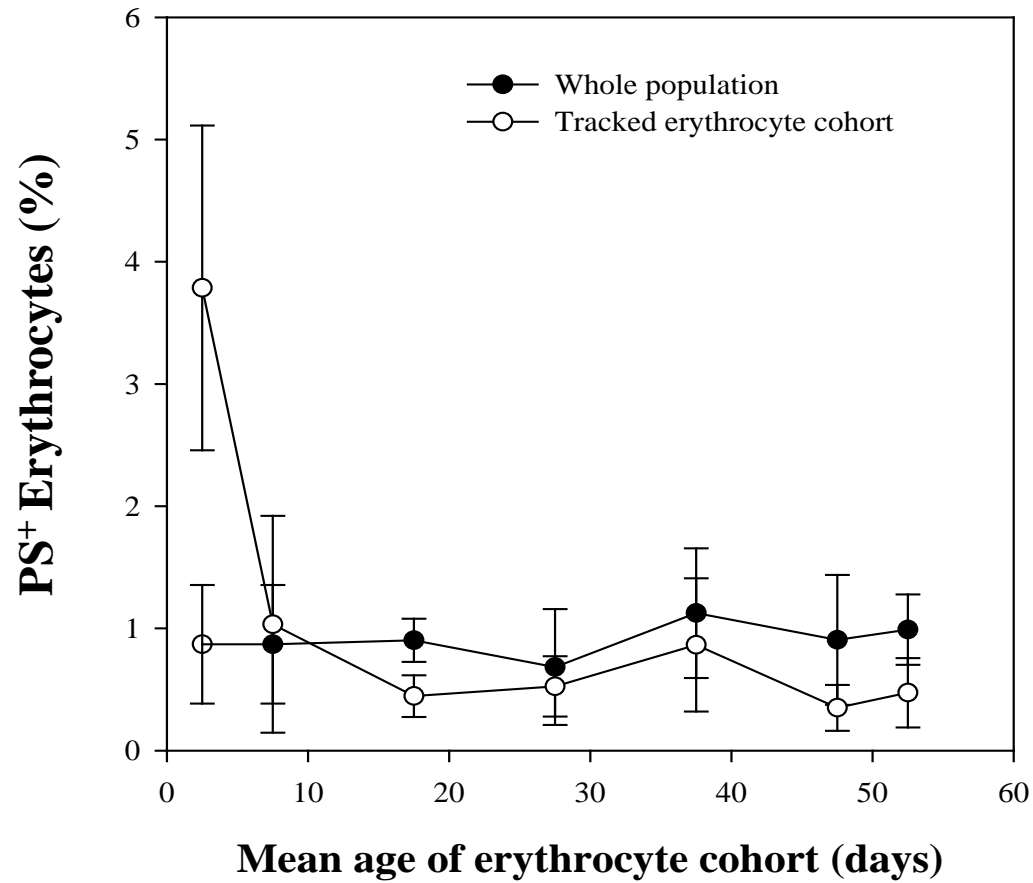
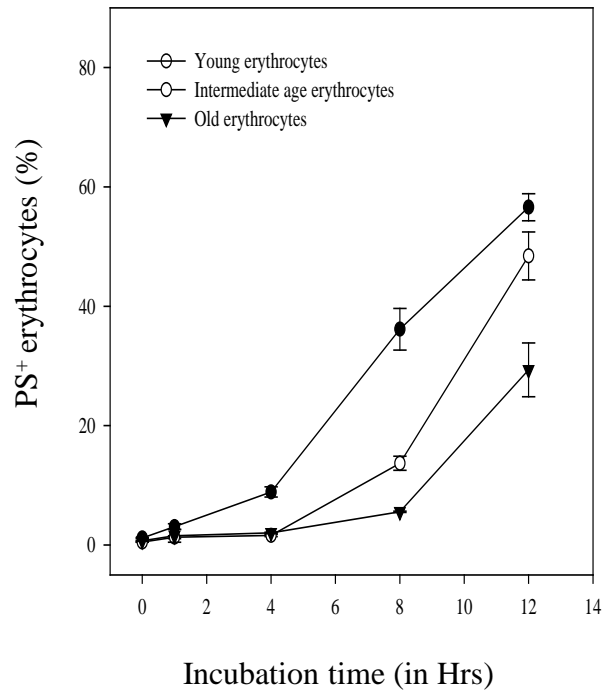
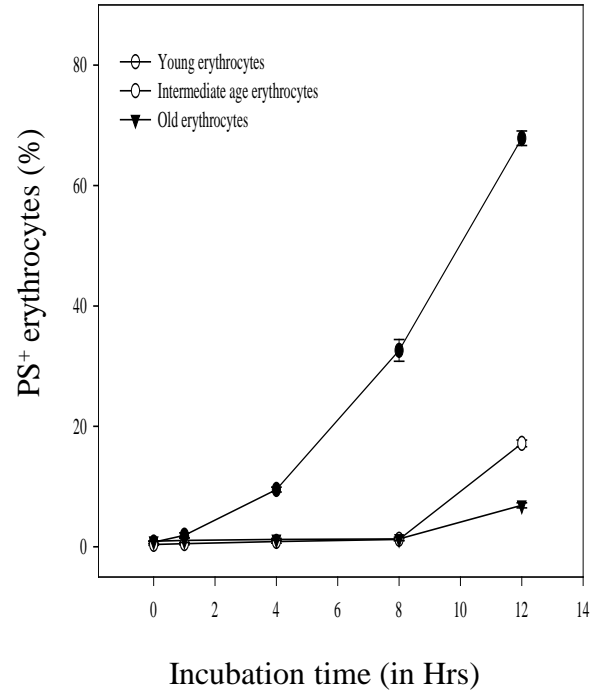


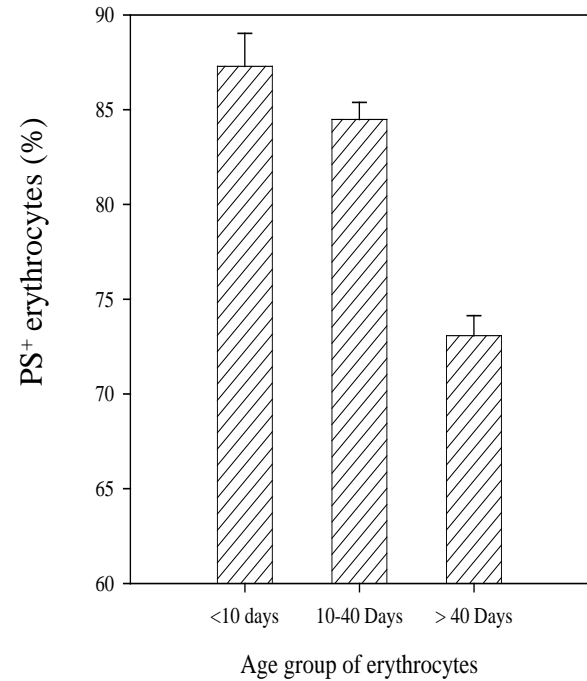
Figure 6



(A)



(B)



(C)

Figure 8

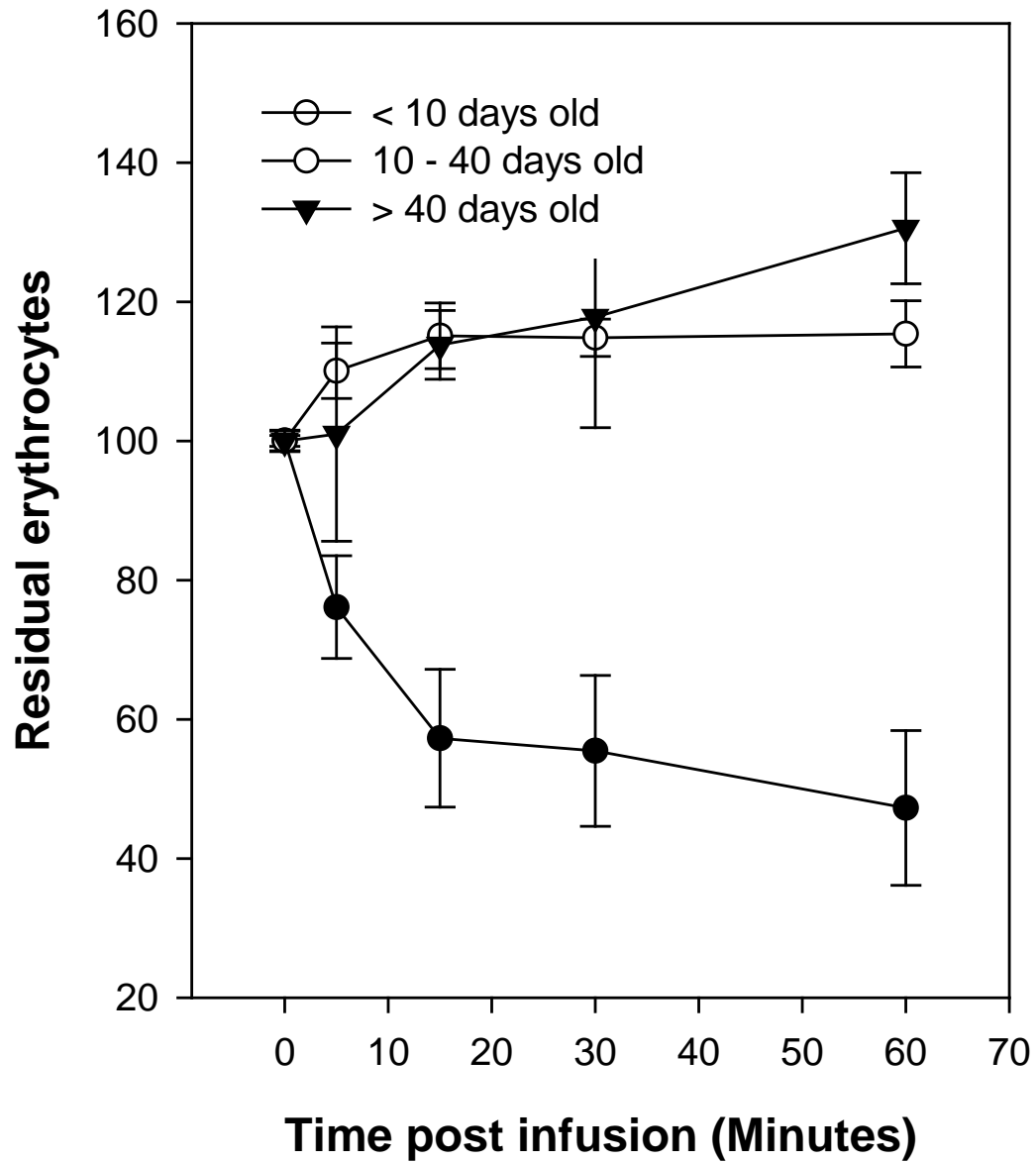


Figure 9