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What Happens to Erythrocytes When Ca^{2+} Accumulates in The Outside and Inside of The Cell?

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Abstract
The aim of our study is to examine the effect of extracellular and intracellular Ca ions (Ca^{2+}) accumulation on human erythrocyte morphology. Ca^{2+} balance is important for the normal progression of body functions. It is also important to be able to sustain it intracellular functions. As Ca^{2+} accumulation is sourced from various diseases, it can cause various diseases. Excessive accumulation of Ca^{2+} in blood plasma has adverse effects on erythrocytes. In our study, the effect of Ca^{2+} on the surface area and volume of erythrocytes was studied by calculating the circularity of erythrocytes. In this study, the erythrocytes isolated from normal human blood were used. Erythrocytes were incubated with $CaCl_2$ and sodium ortho-vanadate (SOV). SOV is a plasma membrane calcium ATPase (PMCA) blocker. Accumulation of Ca^{2+} in the cell was provided by SOV. Images of these erythrocytes were taken by scanning electron microscope. Morphological characteristics were examined using Image-J software. According to these datas, extracellular and intracellular Ca^{2+} accumulation decreased erythrocyte areas compared to the control group ($p < 0.001$). At the same time, according to erythrocyte circularity values Ca^{2+} accumulation inside and outside of the cell made erythrocytes spherical compared to the control group. In this study, the volume calculations were made according to the circularity values of erythrocytes, and the surface area to volume ratios of erythrocytes were approximately calculated. According to these calculations, the SA/V values of the erythrocytes accumulating Ca^{2+} inside and outside the cell decreased when compared to the control group ($p<0.05$, $p<0.01$, $p <0.001$).

Keywords: Erythrocytes Morphology, Ca^{2+}, Volume of Erythrocytes

1. Introduction

All healthy mammalian erythrocytes are disk shaped, have flexible membrane features and their surface areas to volume ratios are high under normal conditions. (Diez-Silva et al. 2010). One of the most important parameters determining the ability of mammalian erythrocytes to circulate within the body is the surface area to volume ratio. There are no intracellular structures in mature erythrocytes. Therefore, deformity of the erythrocyte membrane is not difficult. Since erythrocytes may be deformed, they pass easily through capillaries (Waugh et al. 1997). Its membrane structure is very important for deformation of the erythrocyte. The erythrocyte membrane consists of a phospholipid double layer and spectrin network distributions. Because of these features of the membrane, erythrocytes maintain their disk shapes under normal conditions (Tse and Lux 1999). Human erythrocytes are approximately 7.5-8.7 μm in diameter and 1.7-2.2 μm in thickness. The internal fluid volumes of erythrocytes are regulated by membranes. Average internal fluid volumes are 94 μm^3 at 300 mOSmol/kg (Diez-Silva et al. 2010). Defects in erythrocytes membranes also affect blood flow. One of the parameters affecting the viscosity of blood is the erythrocyte deformations. Deformation rates of erythrocytes are very high when passing

through very small capillaries. In addition to the surface areas and volumes of erythrocytes, circularity is an important parameter. The surface area to volume ratio decreases in the spheroidized erythrocytes. The volume of spheroidized erythrocytes increases and the surface area reduces. They move slower than normal erythrocytes. As the deformation rate decreases passing through very small diameter vessels would also be difficult. The life span of erythrocytes in the body must be preserved from chemical and physical effects for natural and healthy circulation. As a result of chemical changes, the enzymatic mechanism of the cell is affected and the cell resists these changes (Waugh et al. 2018).
Calcium is also important for cells as much as it is for the body. Due to various diseases, calcium ions (Ca^{2+}) increase or decrease in body fluids. In both cases, they have various effects in cells. Ca^{2+} must be stable in both the cell and body fluids.
Ca^{2+} enters the cell through channels on the cell membrane. The Ca^{2+} balance in the cell provides by Na/Ca exchanger and plasma membrane calcium pump (PMCA). PMCA works with ATP to pump excess calcium in the cell to out of the cell. If PMCA does not work, calcium accumulates in the cell and toxic effect for the cell occurs. In our study we used sodium ortho-vanadate (SOV) to block the PMCA pump.
The aim of this study is to examine the effect of Ca^{2+} accumulation on erythrocyte morphology both in the body fluid and in the cell. There may be complaints arising from morphological changes caused by Ca^{2+}, even though erythrocytes are found in the circulation for a short time. Therefore, by applying different concentrations of Ca^{2+} in vitro, the effect of extracellular Ca^{2+} increase on the erythrocyte morphology was examined. SOV was applied to prevent Ca ions in the cell from leaking out of the cell. Thus, how the morphology of erythrocytes changed with intracellular Ca accumulation was analyzed. The question we ask at the beginning of this study is how intracellular and extracellular Ca^{2+} accumulation affects erythrocytes morphology.

2. Material Methods

2.1. Isolation of Erythrocytes from Blood

The blood sample was taken from healthy donors into tube containing EDTA. For the separation of erythrocytes from white blood cells, the blood was centrifuged at 3000 rpm for 10 min. The erythrocytes were washed three times with an isotonic NaCl solution. After the erythrocyte suspension was adjusted to a hematocrit of 20%, it was incubated with $CaCl_2$ (1 nM, 10 nM, 25 nM), Sodium ortho-vanadate (1 nM, 10 nM, 25 nM) and $CaCl_2$ (1 nM, 10 nM, 25 nM) + 1 nM SOV at one hour. These erythrocyte suspensions were fixated with gluteraldehit for imaging on electron microscopy.

2.2. Imaging Erythrocytes on Scanning Electron Microscopy

The fixated erythrocytes were spread on glass slides in a thin layer. Then, slides were coated with gold to ensure conductivity. Scanning electron microscopy (SEM) (LEO 440 QEMSCAN SEM, Zeiss) was used for imaging the erythrocytes. Erythrocyte images were taken at 1.00 KX, 2.00 KX and 5.00 KX magnifications by SEM. Imaged erythrocytes were analyzed with Fiji-win 64 image-J software. For Image-J analysis, erythrocyte images with magnified by 2.00 KX were used (Table 3.1-3). The area, perimeter, major axis, minor axis and circularity value of erythrocytes images were measured by image-j software.

2.3. Measurement Circularity, Volume and Surface area of erythrocytes:

The circularity of erythrocytes was calculated with Image-J software. The circularity is known as a shape descriptor which can mathematically demonstrate the degree of similarity to a perfect circle. A value of 1.0 characterizes a perfect circle. As the circularity value approaches 0.0, the shape is getting less circular. The circularity was determined with the equation 2.1:

$$Circ. = 4\pi \frac{Area}{Perimeter^2} \quad (2.1)$$

When it is considered simply, the erythrocytes are almost disc-shaped (Fig.2.1a). So, the circularity value of normal erythrocytes approaches 0.0. When the circularity value of erythrocyte approaches 1.0, the erythrocytes are almost sphere-shaped (Fig. 2.1b).

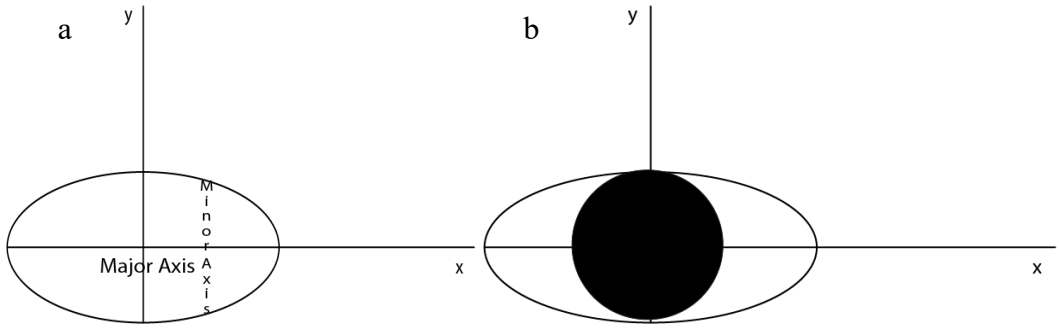


Fig.2.1. A normal and abnormal erythrocyte shapes. a. A normal erythrocyte with disc-shaped. There are major and minor axises. b. An abnormal erythrocyte (black) can be sphere- shaped.

The main problem with the erythrocyte shapes is that there are not the similar to each other. Therefore the calculations of volume and surface area are very complex and difficult. Their calculations need the diameter and the thickness values. Therefore, some approximated models (Fig. 2.2) can be used to calculate the volume and the surface area of erythrocytes. Udrio proposed some approximated models for erythrocytes and formulas for the volume and the surface area of these models (Udroiu 2014).

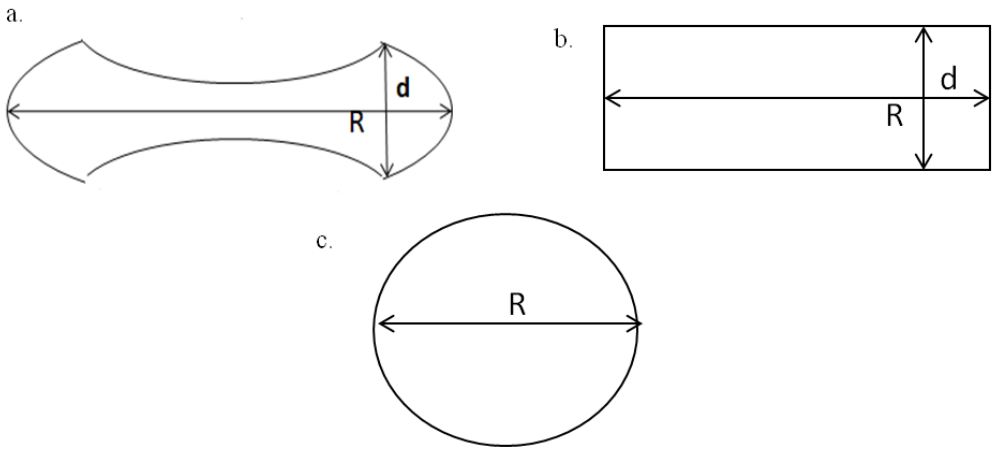


Fig. 2.2. Proposed models of erythrocytes. a. spheroid with concave caps, b. cylinder, c. sphere. (d=thickness, R= diameter).

For the sphere model, the formulas for the surface area (SA) and the volume (V) are:

$$SA_s = \pi R^2 \quad (2.2)$$

$$V_s = \frac{\pi}{6} R^3 \quad (2.3)$$

Where R is the diameter and d is the thickness.
For the cylinder model (almost as disc), the formulas are:

$$SA_c = \pi R \left(\frac{R}{2} + d\right) \quad (2.4)$$

$$V_c = \frac{\pi}{4} R^2 d \quad (2.5)$$

In our study, according to circularity values of erythrocytes, we determined of approximate erythrocytes shapes. According to these data, we calculated the volume and surface area to volume ratios of erythrocytes.

2.4. Statistical Analysis

All graphs were created by Prism 5 GraphPad software. Also, Prism 5 GraphPad was used for statistical analysis. One way ANOVA was used to compare differences between concentrations of chemicals.

3. Results

3.1. The measurement of area, circularity and perimeter of erythrocytes with $CaCl_2$.

To assess the effects of Ca^{2+} increase in body fluid on erythrocyte morphology, erythrocytes were incubated at different concentrations and imaged in SEM (Table 3.1).

According to control erythrocytes, the areas of erythrocytes with $CaCl_2$ were lower. There are significant differences between all concentration of $CaCl_2$, when we compare with control group ($p<0.001$). There is not any effect on area of erythrocytes between different concentrations of $CaCl_2$. Different concentrations of $CaCl_2$ does not affect on the areas of erythrocytes (Fig.3. 1a.).

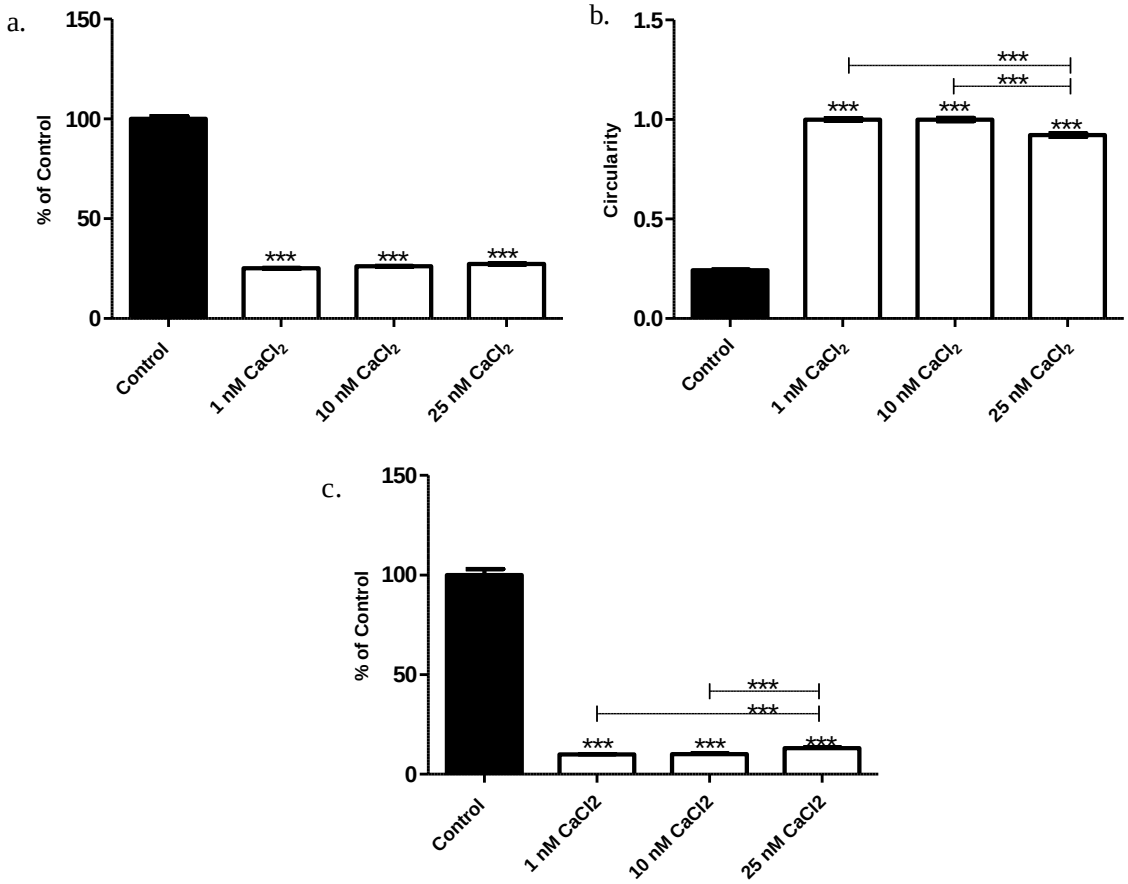


Fig.3.1. The graphs of morphology of erythrocytes with $CaCl_2$. a. The areas of erythrocytes with $CaCl_2$. b. The circularity of erythrocyte with $CaCl_2$. c. The perimeter of erythrocyte with $CaCl_2$.

Another parameter that needs to calculate for morphologic evaluation of erythrocytes is circularity. The circularity values of the $CaCl_2$ applied erythrocytes are shown in Fig. 3.1b.. According to the circularity values of the control group erythrocytes, the control group erythrocytes are approximately disc-shaped. The circularity values of erythrocyte with the $CaCl_2$ are approximately 1.0. These erythrocytes can be considered spherical. There is a significant difference between the control erythrocyte and erythrocytes with the $CaCl_2$ ($p<0.001$). Also, there is a significant difference between concentrations of the $CaCl_2$. Erythrocytes with 25 nM $CaCl_2$ are slightly closer to the control group erythrocytes than erythrocytes 1 nM and 10 nM $CaCl_2$.

When we evaluate the erythrocytes with the CaCl2, according to the perimeter measurements, there is a significant difference between the control erythrocytes and the erythrocytes with the $CaCl_2$ (Fig. 3.1c.). There is a significant difference between perimeter of erythrocytes with 1 nM and 10 nM $CaCl_2$ and perimeter of erythrocyte 25 nM $CaCl_2$.

3.2 The measurement of area, circularity and perimeter of erythrocyte with SOV.

To determine the effect of Ca^{2+} accumulation in the cell on erythrocyte morphology, erythrocytes were incubated with SOV at different concentrations. According to the areas of the control erythrocytes, the area of the erythrocytes treated with SOV showed a large decrease in area (Fig. 3.2a.). There is a significant difference between them ($p<0.001$). Accumulation of Ca^{2+} in the cell reduces the erythrocyte areas. There is no significant difference between SOVs at different concentrations of application.

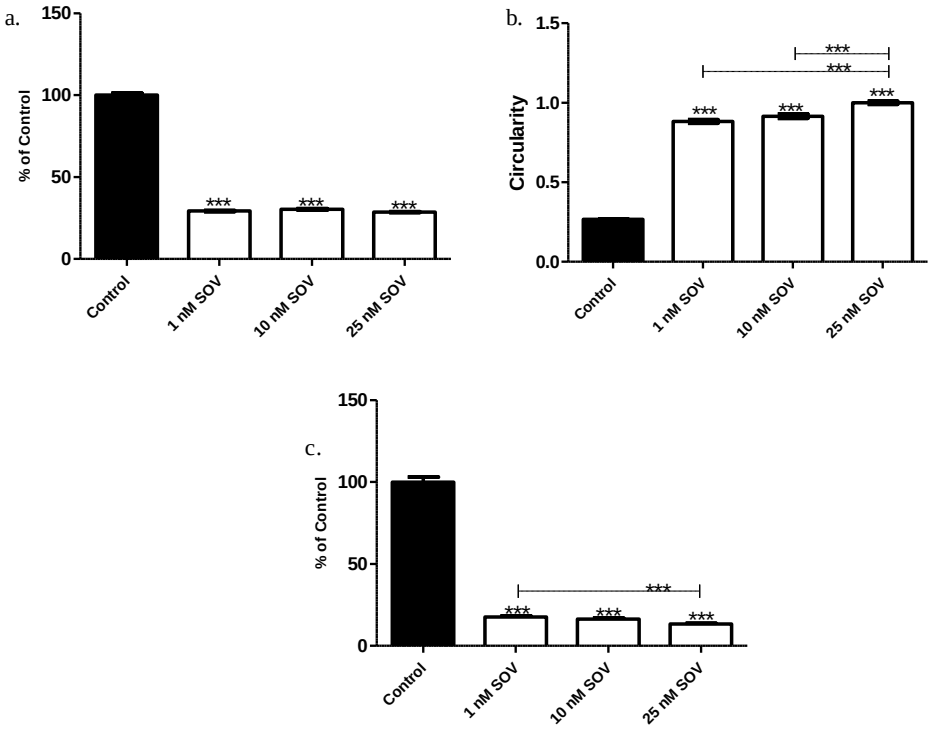


Fig. 3.2. The graphs of morphology of erythrocytes with SOV. a. the area of erythrocyte with SOV. b. the circularity of erythrocyte with SOV. c. the perimeter of erythrocyte with SOV.

When evaluated according to their circularity values, the SOV applied erythrocytes are spherical (Fig. 3.2b.). There is a significant difference when compared to the control group ($p<0.001$). At the same time, as the concentration of SOV increases, the spheroidized shape of erythrocytes also increases significantly ($p<0.001$).

When evaluated according to the perimeter of erythrocytes, the perimeter of erythrocytes with the SOV is significantly lower than the control erythrocytes (Fig. 3.2c.). There is a significant difference between the control group and applied the SOV groups ($p<0.001$). While there is no difference between 10 nM SOV and 25 nM SOV groups, there is a significant difference between 1 nM SOV and 25 nM SOV.

3.3. The measurement of area, circularity and perimeter of erythrocyte with $CaCl_2$+1 nM SOV.

In addition to the Ca^{2+} increase in the body fluid, the changes in erythrocyte morphology were examined when the pump which is responsible for pumping out Ca^{2+} in the cell was blocked (Table 3.3).

After adding $CaCl_2$ at the different concentrations, the area measurements of erythrocytes which have blocked Ca^{2+} exit were lower (Fig. 3.3a). There is a significant difference between the control erythrocytes and the erythrocytes with $CaCl_2$+1 nM SOV ($p<0.001$). There is no difference between changes in the Ca^{2+} concentrations on the area of erythrocytes.

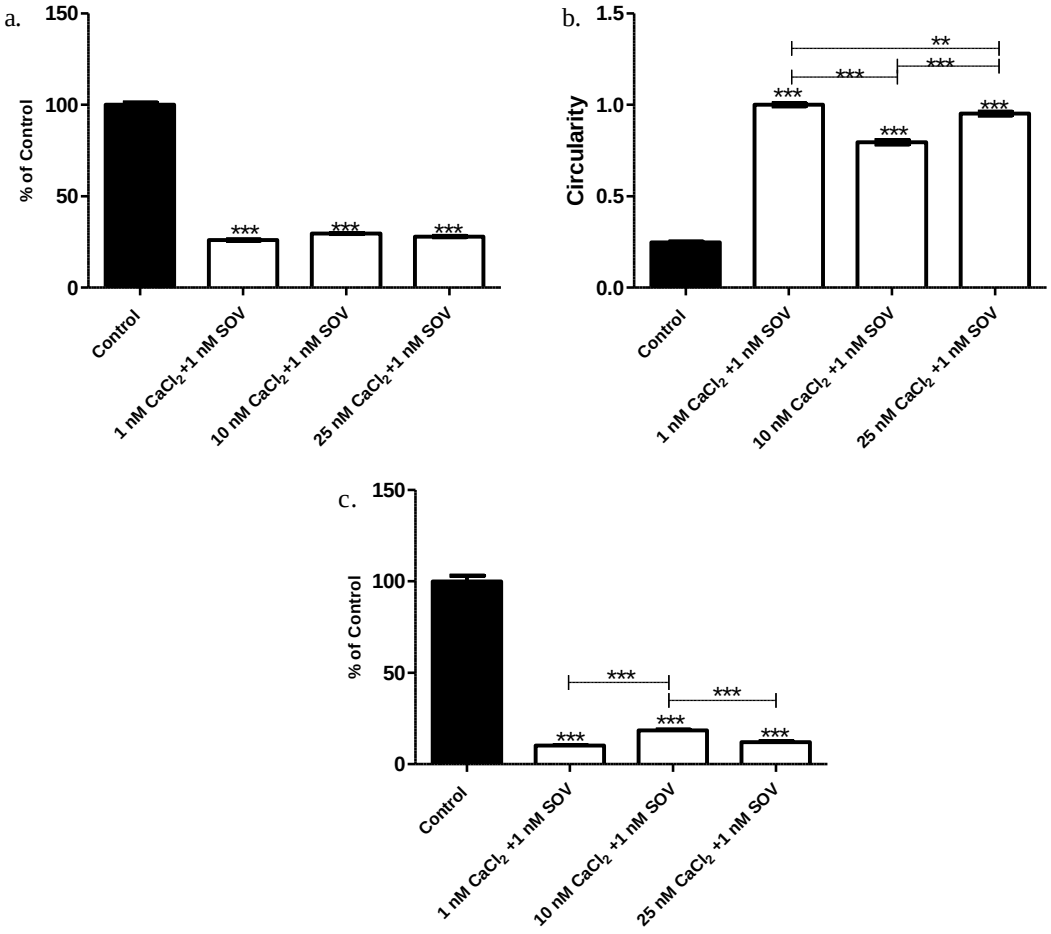


Fig.3.3. The graphs of morphology of erythrocytes with $CaCl_2$+1 nM SOV. a. the area of erythrocyte with $CaCl_2$+1 nM SOV. b. the circularity of erythrocyte with $CaCl_2$+1 nM SOV. c. the perimeter of erythrocyte with $CaCl_2$+1 nM SOV.

The circularity values of the erythrocytes combination with the Ca^{2+} and the SOV are significantly different from the control erythrocytes ($p<0.001$) (Fig. 3.3b.). Ca^{2+} concentration changes affected the circularity values of erythrocytes. Erythrocytes with 1 nM $CaCl_2$+1 nM SOV was more spherical than other concentrations ($p<0.001$). 10 nM $CaCl_2$+1 nM SOV combination did not spherize the erythrocytes, their shape was closer to the control.

There is a significant difference in the perimeter values between the erythrocytes with the $CaCl_2$+1nM SOV combination and the control erythrocytes ($p<0.001$) (Fig. 3.3c.). There is also a significant difference between $CaCl_2$+SOV concentrations in the perimeter values ($p<0.001$).

All chemicals and the concentrations applied to the erythrocytes were compared with each other according to the area, circularity and perimeter of erythrocytes (Fig. 3.10-11). When compared to the areas of erythrocytes (Fig 3.4a.), there is a meaningful difference between 1 nM $CaCl_2$ and 1 nM SOV ($p<0.001$), but no significant difference between 1 nM $CaCl_2$ and 1 nM $CaCl_2$+1 nM SOV. There is a significant difference between 1 nM SOV and 1 nM $CaCl_2$+1 nM SOV ($p<0.001$). There is a significant difference between 10 nM $CaCl_2$ and 10 nM SOV ($p<0.001$). There is a significant difference between 10 nM $CaCl_2$ and 10 nM $CaCl_2$+1 nM SOV ($p<0.001$). There is not any difference between 10 nM SOV and 10 nM $CaCl_2$+1 nM SOV. In the same way, there is not any significant difference between 25 nM $CaCl_2$ and 25 nM $CaCl_2$+1 nM SOV.

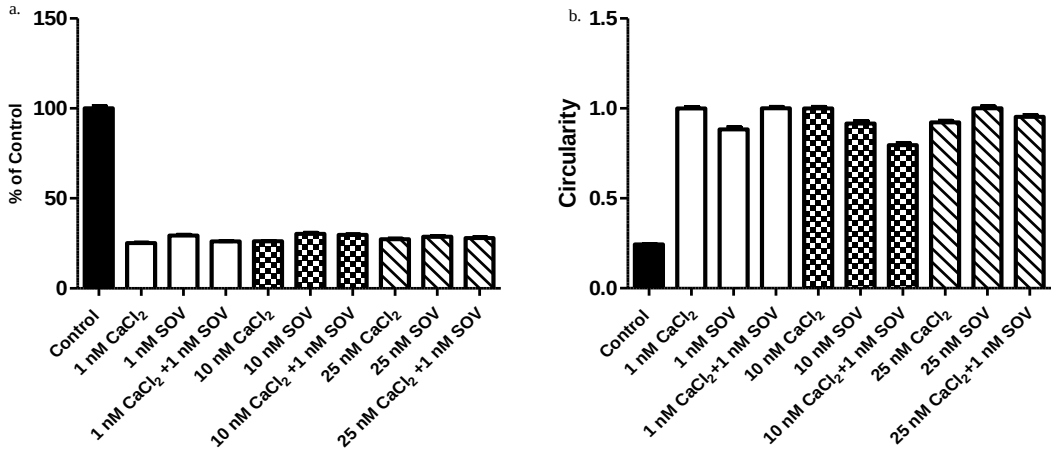
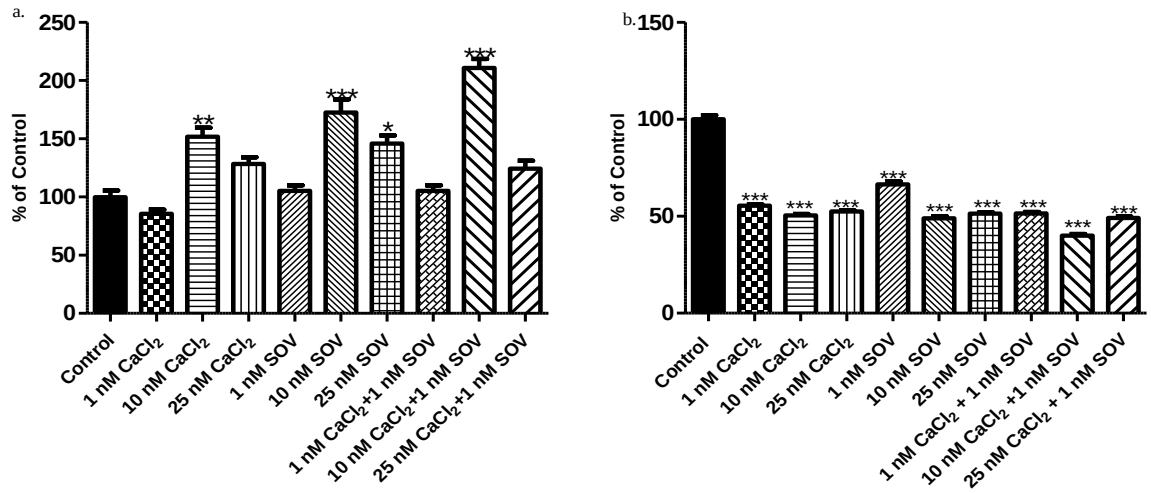


Fig. 3.4. a. The compared area of erythrocytes with all applied chemicals. b. The compared circularity of erythrocytes with all applied chemicals.

According to the circularity values (Fig. 3.4b.), there is no difference between 1 nM $CaCl_2$ and 1 nM $CaCl_2$+1 nM SOV. But there is a significant difference between 1 nM SOV and 1 nM $CaCl_2$+1 nM SOV ($p<0.001$). There is a significant difference between 10 nM $CaCl_2$ and 10 nM $CaCl_2$+10 nM SOV ($p<0.001$).

3.4. The measurement of volume and surface area/volume of erythrocytes with all applied chemicals.

The volume calculations were made using the approximate shapes of erythrocytes determined according to their circularity values (Fig. 3.5a.). The volumes of erythrocytes differed between the control erythrocytes and the erythrocytes applied chemicals ($p<0.001$, $p<0.01$, $p<0.05$). At high concentrations of chemicals, the erythrocytes volume increased. There is no significant difference between 1 nM $CaCl_2$+1 nM SOV, 10 nM $CaCl_2$, 10 nM $CaCl_2$+1 nM SOV, 25 nM $CaCl_2$ and 25 nM $CaCl_2$+1 nM SOV with 1 nM $CaCl_2$ when the concentrations are compared among themselves.



There was a significant difference between the control erythrocytes and erythrocyte applied chemicals ($p<0.001$), when the chemicals were evaluated according to the SA/V ratio (Fig. 3.5b.). When the chemical applied erythrocytes compared the control erythrocytes, their SA/V ratios were low. There is a significant difference between 1 nM $CaCl_2$ and 1 nM $CaCl_2$+1 nM SOV when the chemicals are compared among themselves ($p<0.05$). There is a significant difference between 10 nM $CaCl_2$ and 10 nM $CaCl_2$+1 nM SOV ($p<0.001$), while there is no significant difference between 25 nM $CaCl_2$ and 25 nM $CaCl_2$+1 nM SOV.

Table 3.1. The images of erythrocytes with $CaCl_2$ by SEM.

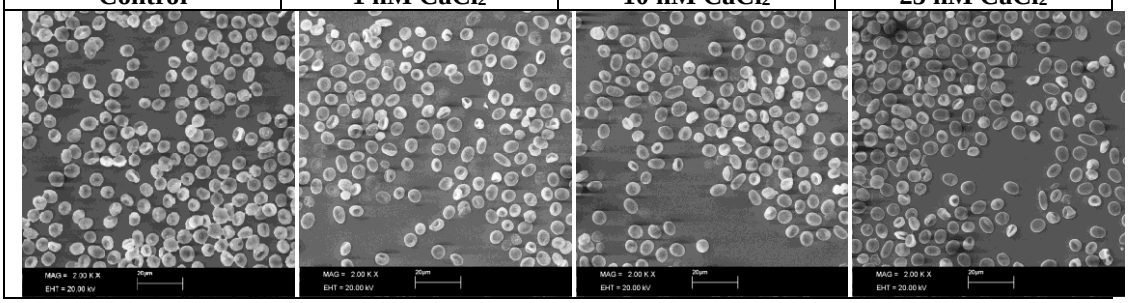
Control	1 nM $CaCl_2$	10 nM $CaCl_2$	25 nM $CaCl_2$
			

Table 3.2. The images of erythrocyte with SOV by SEM.

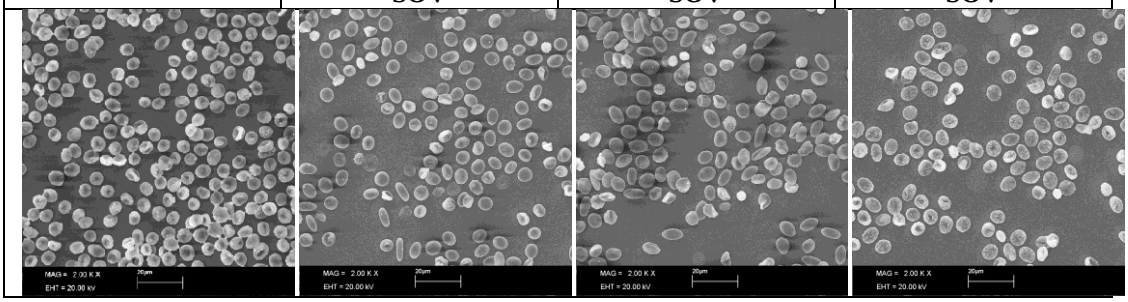
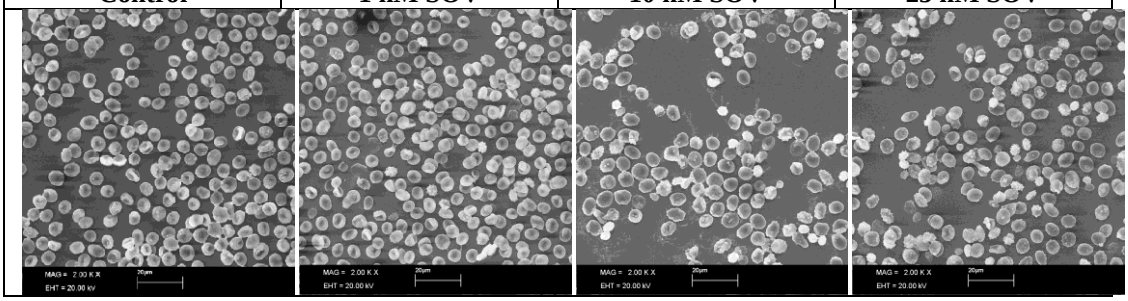
Control	1 nM $CaCl_2$ + 1 nM SOV	10 nM CaCl2 + 1 nM SOV	25 nM CaCl2 + 1 nM SOV
			

Table 3.3. The images of erythrocytes with $CaCl_2$ + 1 nM SOV

Control	1 nM SOV	10 nM SOV	25 nM SOV
			

4. Discussion

According to the data we have obtained, Ca^{2+} accumulation inside and outside the cell affects morphology of erythrocytes. Furthermore, when erythrocytes have a large surface area, it is easier for O_2 to enter the erythrocytes by diffusion. In this study, it was shown that the erythrocyte areas decreased due to the excess of Ca^{2+} in or out of the cell.

Decreased in the area of erythrocytes due to the presence of Ca^{2+} in and out of the cell provided these erythrocytes to get sphere-shape. Decrease in the area of erythrocyte means a decrease in the area of O_2 diffusing into the cell. Thus, increasing the sphere shape reduces the transport of O_2. The movement of O_2 to the tissues will also decrease. The decreased surface area of erythrocytes also affects the movement of erythrocytes in the lungs. While taking O_2 from the lungs, it is necessary to cling to the lung capillaries for a period of time. As the surface area decreases, the clinging of O_2 will decrease and consequently the contact with the O_2 will be reduced (Kiefmann et al. 2008).

The erythrocytes have changed their shapes due to Ca^{2+}. Therefore, measuring the volume of erythrocytes is very difficult because they have different shapes. In this study, we measured the circularity values of erythrocytes. If this value is close to 1, erythrocytes have approximately the spherical shape and if the value is close to 0, they are gradually elliptical. According to this information we calculated the erythrocyte volume (Zdilla et al. 2016).
The equations that Udroi defined for erythrocytes were used (Udroiu 2014). According to the obtained results, the erythrocytes shapes which applied Ca, SOV and Ca+SOV chemical combination were close to the spheres, so their volume was calculated as approximately the equation 2.3. Since the control erythrocytes are close to the elliptic shape, the equilibrium is calculated to be the equation 2.5.
The conversion from the ellipsoidal disc-shape to the spherical shape can be caused by the various reasons. The pH change is the best known of these reasons. According to our results, the accumulation of Ca^{2+} outside and inside the cell is also the reason for sphereizedation (Rudenko 2010).
Another parameter that is thought to influence the erythrocyte shape is the transmembrane potential (Rudenko 2010; Glaser 1979). However, the effect of the transmembrane potential is controversial because other authors have argued that there is no causal relationship between the membrane potential and the cell shape (Gedde and Huestis 1997). This topic remains unclear. Because the Ca^{2+} causes the transmembrane potential change we can attribute the resultant shape change to this in our results. Under the physiological conditions, the free Ca^{2+} concentration in the healthy human erythrocytes is estimated to be in the range of 30 to 60 nM (Tiffert, Bookchin, and Lew 2003a). There is a gradient of at least 40.000 fold between the cytosol and the blood plasma reaching the free Ca^{2+} concentration of 1.8 mM. This gradient is preserved by the low permeability of the membrane to Ca^{2+}~ 50 µmol/(1cells h). In this case, the effect of the PMCA is too high (Tiffert, Bookchin, and Lew 2003b). In our study we used SOV, a PMCA blocker to trap Ca^{2+} in the cell. By this way, it has been shown that the erythrocyte areas changes and the shapes are spherodized when the Ca^{2+} accumulates in the cell.
Prolonged increases in Ca^{2+} permeability cause severe impairment of multiple cellular functions. Intracellular Ca^{2+} activates a large number of Ca^{2+} dependent proteins. The gradual increase in the Ca^{2+} levels causes the physiological and the pathophysiological changes in the erythrocytes. In our study, there were morphological changes by the accumulation of Ca^{2+} in the cell by applying SOV (Bogdanova et al. 2013). The erythrocyte volume regulation is a complicated process because of the contribution of many molecules. An abnormal increase in the Ca^{2+} concentration in the erythrocytes results in the development of life-threatening systemic pathologies (Romero and Romero 1999; Clark 1988).
Attempts were made to describe the shape of erythrocytes in the blood for many years [6–8]. In the normal conditions, the disk-shaped erythrocytes change their form when exposed to chemical or physical stress [4]. It significantly affects the function of erythrocytes in varying forms. Although the morphological characteristics of erythrocytes can be defined as the diameter, the length, and the area, their volume and the surface area to volume ratio gives better information about the function of erythrocytes.
The surface area to volume ratio is the amount of surface area per unit the volume of an object. The SA/V is used in the chemistry to determine how quickly a chemical can be dissolved. The higher the rate, the chemical dissolves faster. In the biology, the SA/V ratio is related to the ease of movement of the material, the transport function, and the diffusivity of the cell membrane. The large surface area of erythrocytes increases movement functions and speeds of erythrocytes in the blood and allows them to move using less energy.

5. Conclusions

As a result, the Ca^{2+} accumulation both outside and inside the cell change the erythrocyte morphology. The erythrocytes are responsible for transporting O_2 to the tissues, so Ca^{2+} accumulation will also affect the O2 transportation of erythrocytes because the SA/V ratio decreases and erythrocytes get more spherical shape according to data we have obtained from our study. For sphere-shape erythrocytes passing through very small capillary vessels are difficult. As the SA/V ratio is low, the amount of O_2 to be transported will decrease. Although the erythrocytes stay briefly in the body cycle, the effects of morphologic disorders are long-lasting. Our study proved that Ca^{2+} imbalance affects the erythrocytes negatively.

Ethical number: 2010/70 This ethical approval is the ethics committee decision of Erciyes University.

REFERENCES

Bogdanova, Anna, Asya Makhro, Jue Wang, Peter Lipp, and Lars Kaestner. 2013. "Calcium in Red Blood Cells-a Perilous Balance." *International Journal of Molecular Sciences* 14 (5). Multidisciplinary Digital Publishing Institute (MDPI): 9848–72. doi:10.3390/ijms14059848.

Clark, M. R. 1988. "Senescence of Red Blood Cells: Progress and Problems." *Physiological Reviews* 68 (2): 503–54. doi:10.1152/physrev.1988.68.2.503.

Diez-Silva, Monica, Ming Dao, Jongyoon Han, Chwee-Teck Lim, and Subra Suresh. 2010. "Shape and Biomechanical Characteristics of Human Red Blood Cells in Health and Disease." *MRS Bulletin* 35 (5). NIH Public Access: 382–88. http://www.ncbi.nlm.nih.gov/pubmed/21151848.

Gedde, M.M., and W.H. Huestis. 1997. "Membrane Potential and Human Erythrocyte Shape." *Biophysical Journal* 72 (3): 1220–33. doi:10.1016/S0006-3495(97)78769-1.

Glaser, R. 1979. "The Shape of Red Blood Cells as a Function of Membrane Potential and Temperature." *The Journal of Membrane Biology* 51 (3–4): 217–28. http://www.ncbi.nlm.nih.gov/pubmed/43897.

Kiefmann, Rainer, Joseph M Rifkind, Enika Nagababu, and Jahar Bhattacharya. 2008. "Red Blood Cells Induce Hypoxic Lung Inflammation." *Blood* 111 (10). American Society of Hematology: 5205–14. doi:10.1182/blood-2007-09-113902.

Romero, Pedro J., and Eneida A. Romero. 1999. "The Role of Calcium Metabolism in Human Red Blood Cell Ageing: A Proposal." *Blood Cells, Molecules, and Diseases* 25 (1): 9–19. doi:10.1006/bcmd.1999.0222.

Rudenko, Sergey V. 2010. "Erythrocyte Morphological States, Phases, Transitions and Trajectories." *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1798 (9). Elsevier: 1767–78. doi:10.1016/J.BBAMEM.2010.05.010.

Tiffert, Teresa, Robert M. Bookchin, and Virgilio L. Lew. 2003a. "Calcium Homeostasis in Normal and Abnormal Human Red Cells." In *Red Cell Membrane Transport in Health and Disease*, 373–405. Berlin, Heidelberg: Springer Berlin Heidelberg. doi:10.1007/978-3-662-05181-8_15.

Tiffert, Teresa, Robert M. Bookchin, and Virgilio L. Lew. 2003b. "Calcium Homeostasis in Normal and Abnormal Human Red Cells." In *Red Cell Membrane Transport in Health and Disease*, 373–405. Berlin, Heidelberg: Springer Berlin Heidelberg. doi:10.1007/978-3-662-05181-8_15.

Tse, W T, and S E Lux. 1999. "Red Blood Cell Membrane Disorders." *British Journal of Haematology* 104 (1): 2–13. http://www.ncbi.nlm.nih.gov/pubmed/10027705.

Udroiu, Ion. 2014. "Estimation of Erythrocyte Surface Area in Mammals," March. http://arxiv.org/abs/1403.7660.

Waugh, Richard E., James B. McKenney, Richard G. Bauserman, Donna M. Brooks, C.Robert Valeri, and L.Michael Snyder. 1997. "Surface Area and Volume Changes during Maturation of Reticulocytes in the Circulation of the Baboon." *Journal of Laboratory and Clinical Medicine* 129 (5). Elsevier: 527–35. doi:10.1016/S0022-2143(97)90007-X.

Waugh, Richard E, Mohandas Narla, Carl W Jackson, Thomas J Mueller, Takashige Suzuki, and George L Dale. 2018. "Rheologic Properties of Senescent Erythrocytes: Loss of Surface Area and Volume With Red Blood Cell Age Isolation of Aged Erythrocytes." *Blood*. Vol. 79. Accessed August 1. www.bloodjournal.org.

Zdilla, Matthew J, Scott A Hatfield, Kennedy A McLean, Leah M Cyrus, Jillian M Laslo, and H Wayne Lambert. 2016. "Circularity, Solidity, Axes of a Best Fit Ellipse, Aspect Ratio, and Roundness of the Foramen Ovale: A Morphometric Analysis With Neurosurgical

Considerations." *The Journal of Craniofacial Surgery* 27 (1). NIH Public Access: 222–28. doi:10.1097/SCS.0000000000002285.