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RESEARCH ARTICLE
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ANTI-ERYPTOTIC POTENTIAL OF SELENIUM AGAINST GENTAMICIN-INDUCED ERYPTOSIS

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Abstract
Gentamicin, an aminoglycoside, is used to treat serious infections such as blood infections (septicemia) and brain and spinal cord infections. Recent years have shown an increase in antibiotic use, which can be associated with side effects like eryptosis, a process mainly induced by oxidative stress, ceramide production, and energy depletion, which can lead to anemia. Eryptosis, quite similar to apoptosis, is defined by cell shrinkage, membrane blebbing, and membrane phospholipid scrambling with phosphatidylserine exposure at the cell surface. The core purpose of this research was to examine the eryptotic effect of gentamicin through the oxidative stress pathway and study the anti-eryptotic potential of selenium against gentamicin-induced eryptosis. In this study, erythrocytes were treated with therapeutical doses (9-18μM) of gentamicin and selenium for 48 hours according to experimental requirements. To assess the cytotoxicity of gentamicin hemolysis% was estimated, the oxidative potential of gentamicin and the anti-oxidative potential of selenium was confirmed by investigating the levels of glutathione peroxidase, superoxide dismutase and catalase, to confirm their eryptotic role mean cell volume(MCV) was measured, role of Ca^{+2} in eryptosis was confirmed by using a calcium-channel blocker, amlodipine. Results illustrate that an increase in gentamicin concentration directly affects hemolysis%, gentamicin treated cells showed low levels of enzyme activity while gentamicin+ selenium-treated cells showed better activity of antioxidants, less than normal MCV was observed in gentamicin-treated cells which is a direct indication that cell is going through eryptosis, calcium channel blocker showed the involvement of Ca^{2+} in eryptosis.

Introduction
Gentamicin an aminoglycoside is used to treat a broad range of aerobic infections in the body. Although it can be used against gram-positive as well as gram-negative bacterium, gentamicin is especially beneficial in the treatment of severe Gram-negative infections, such as those caused by *Pseudomonas Aeruginosa*. Whenever gentamicin is co-administered with beta-lactam antibiotics there is a huge synergistic advantage (Chaves & Tadi, 2020). An ototoxic impact (a toxic effect on the inner ear), renal toxicity, and neuromuscular blockade are the most common side effects of gentamicin. Gentamicin toxicity has no antidote. Gentamicin-induced toxicity may be prevented by medicines that protect the ear and kidney. Gentamicin users seem to benefit from N-protective

acetylcysteine properties which show protective effects (Sojo-Dorado & Rodríguez-Baño, 2017). In addition, gentamicin causes an oxidation stress-status in the testicles by raising the free-radical production and lipids peroxidation, and by declining the antioxidant levels. This leads to structural biochemical changes and cytotoxic fluctuations in the testis. Moreover, gentamicin also influences spermatozoa by disrupting their numbers, motility and morphology (Narayana, 2008).

Erythrocytes are the predominant cell type in the circulatory system and are nucleated in all vertebrates except mammals. The main function of these cells is the exchange of respiratory gases in the body (Pretorius et al., 2016). To help chemical processes of ATP generation. It must transfer oxygen to tissues in the periphery, from the lungs, and gather CO_2 from tissues to transfer it back to the lungs for excretion from the body in that short period (Berg et al., 2001). The oxygen-carrying ability of blood is disrupted when the number of erythrocytes decreases owing to any defect, disease, or injury. To limit the harmful effects of intravascular hemolysis, erythrocytes must be removed from circulation or the bloodstream before their physiological lifespan expires (Lang & Qadri, 2012).

Erythrocytes possess an organized and structured membrane, which comes across with different inflammatory molecules present in circulation. Although they lack nuclei or other double membrane-bound organelles they can carry programmed cell death called eryptosis, quite similar to apoptosis (Lang & Lang, 2015). Eryptosis, a form of programmed cell death specific to erythrocytes, is defined by cellular shrinkage, membrane blebbing, and the translocation of phosphatidylserine to the outer leaflet of the cell membrane. (Jilani & Lang, 2013). Oxidative stress triggers non-selective cation channels and allows calcium influx into the cell membrane, and the regular blebbing of the cellular membrane, causing constant exposure of phosphatidylserine on the cellular membrane and activating Calcium-dependent K^+ channels, leading to the removal of potassium, hyperpolarization, removal of chlorine and eventually shrinking of cell due to KCl and water loss due to osmosis (Bissinger et al., 2019).

Selenium is mediated as an essential micronutrient for the betterment of human health. The main property of selenium in organisms is associated with antioxidant functions and is known as a significant part of essential antioxidant enzymes. It plays a crucial role in managing the process of selenoproteins; selenoproteins are part of Se that have some active functions in the whole human body. Selenoproteins comprise iodothyronine deiodinases (IDD), thioredoxin reductase (TrxR), glutathione peroxidase (GPx), and function as antioxidant defense and halt oxidative damage. Therefore, selenium (Se) is a considerable element for optimum immune system activity and other cellular processes (Hasani et al., 2019). Selenoproteins are one of those proteins that contain the specific group that belongs to the selenocysteine residues. Almost twenty-five different selenoproteins have been classified in humans (Mehdi et al., 2013).

The research can demonstrate whether gentamicin has an eryptotic impact on erythrocytes or not, and with the treatment of antioxidant, selenium, whether this eryptotic effect can be minimized or not.

Material and Methods

The Cellular Research Laboratory at the University of Agriculture Faisalabad served as the primary research facility for cellular biology studies. Collection of screened human blood samples from laboratories or any blood banks was done and secure blood samples in heparin tubes.

The blood plasma was stored in a heparin tube, isolating about 2mL of blood and mixed with 2mL of ringer solution (to maintain an isotonic environment to erythrocytes) after this centrifugation was done for the isolation of red blood cells (Zbidah et al., 2012). Within two hours of blood washing with ringer solution, red blood cells were isolated from blood (Lupescu et al., 2014) by centrifuging at 1500 rpm for ten minutes. Different layers were formed, and the buffer and plasma layers had platelets, which were discarded carefully. The supernatant contained plasma that was discarded and erythrocytes were centrifuged again. The centrifugation of erythrocytes was performed meticulously because these cells are very fragile and sensitive. Erythrocytes separated by centrifugation were treated with the higher and lower doses of gentamicin along with 1 mL ringer solution and 4 µL of

blood for 48 hours at 37ºC, and a significant amount of selenium was added afterward. Control cells were left untreated. Gentamicin was solubilized in Dimethyl sulfoxide (DMSO).

Hemolysis measurement

When sample incubation was completed for 48 hours, the sample was centrifuged for three minutes at 500 rpm. Subsequently, the supernatant was collected and ELISA plates were prepared. The hemoglobin collected from the supernatant was measured at wavelength 405nm to check the hemolysis rate in treated cells. The absorbance of the supernatant, lysed in water, was considered to represent 100% hemolysis.

Measurement of oxidative stress

Several antioxidants are available for their action when needed. Different types of antioxidant enzymes, pharmacologic defense systems, trapping compounds and carrier proteins are found in the body (Kurutas, 2015). The defensive vitamins include selenium, vitamin E, A and beta-carotene while glutathione peroxidase, superoxide dismutase and catalase give complete defenses against free radicals (Singh et al., 2004). Thus, assays of these antioxidative enzymes were performed to determine the oxidative stress.

Superoxide dismutase (SOD)

The activity of superoxide dismutase was determined using the procedure outlined by (Rana et al., 2019) and (Giannopolitis & Ries, 1977). SOD was used to determine the radicals (superoxide) that were produced by hypoxanthine in the tetrazolium salt used. The spectrophotometer readings of chromophore 28 were taken at 525nm (Maier & Chan, 2002). Evaluation of SOD can lower the photo reduction of nitro blue tetrazolium.

The recipe to prepare superoxide dismutase was 0.222 gm methionine mixed in 15mL, NBT was taken 0.015 gm dissolved in 17.5mL, Triton-X 0.0375 mL dissolved in 17.5mL distilled water, and in the end, 0.0132 gm riboflavin was dissolved in 17.5mL and 0.2M buffer was prepared for getting readings by reaction solution.

Glutathione Peroxidase (GPx)

The reaction mixture of GPx consisted of 20 mM guaiacol, 50 mM phosphate buffer and 40 mM H_2O_2, as described by (Ullah et al., 2018). The absorbance reading was recorded at 470 nanometers.

Catalase

Catalase activity was measured according to the protocol outlined by (Ilyas et al., 2020). The catalase mixture consisted of H_2O_2 and phosphate buffer 50 mM, and absorbance readings were observed at 240 nm.

Measurement of cell size (MCV)

The change in mean cell volume of cells is a primary characteristic of eryptosis, for this, the mean cell volume of erythrocytes after treating them with gentamicin and selenium was examined. MCV was calculated with an automated hematology analyzer (d'Onofrio et al., 1995).

Calcium role confirmation

Amlodipine is a medication that blocks calcium channels in the body. Erythrocytes were exposed to gentamicin which resulted in eryptosis in the presence of calcium. Erythrocytes exposed to the 18 µM gentamicin were treated with a significant amount of Ca^{+2} channel blocker, amlodipine. Measurements taken by antioxidant enzyme activity confirmed the channel activity. Eryptosis inhibition was validated by determining the mean corpuscular volume. (Rana et al., 2019).

Statistical Analysis

Arithmetic means, with standard errors of the mean (SEM), were calculated to evaluate the overall data. Statistical analysis was conducted using ANOVA, with Tukey's test applied depending on the condition.

Results

Results are presented as mean values with standard errors of the mean (SEM), and statistical significance is denoted. As discussed earlier, oxidative stress is the key mechanism of eryptosis, so gentamicin-induced oxidative effects and radical scavenging by selenium in erythrocytes were analyzed through enzymes assays, by determining the antioxidative enzyme levels in treated and non-treated cells. To confirm their eryptotic role mean cell volume (MCV) was calculated, hemolysis percentage was to determine the cytotoxic effect of gentamicin, and Ca^{+2} role confirmation in eryptosis was done by using a calcium channel blocker (amlodipine). The stimulation of eryptosis is promoted with the opening of cationic channels by the high level of free radicals with the effect of the drug (Jilani & Lang, 2013). To examine the values of antioxidants, glutathione peroxidase, catalase, and superoxide dismutase enzyme assay were performed to examine the oxidative effect on erythrocytes (Gargouri et al., 2018). For the treatment of erythrocytes, gentamicin doses 0µM, 9 µM and 18 µM were used.

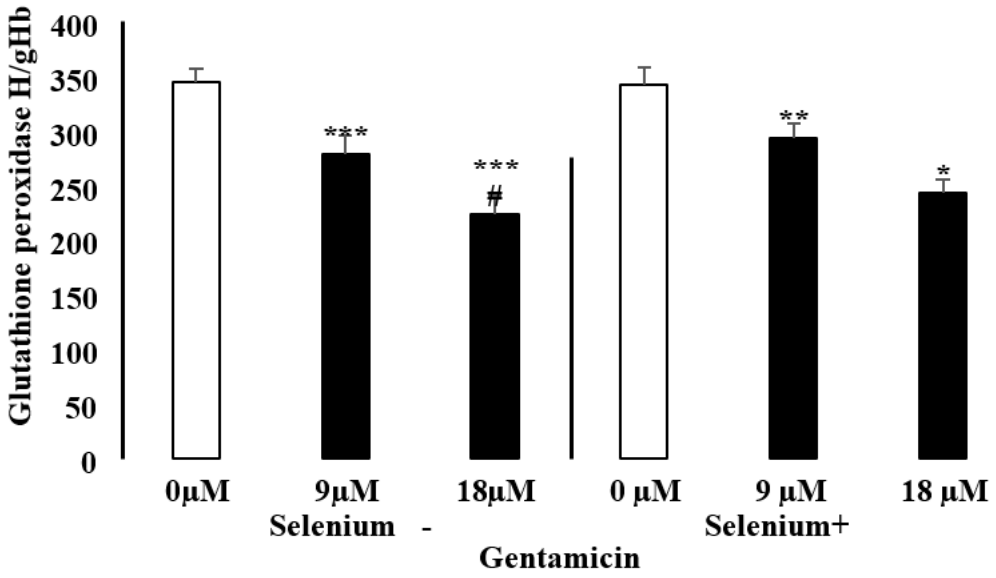

Figure 1 Variations in Glutathione peroxidase (U/g Hb) activities due to Gentamicin and selenium in red blood cells. Arithmetic mean ±SEM (n = 15) of erythrocytes incubated with ringer solution and without (white bars) or with (black bars) gentamicin and selenium for 48 hours was estimated. The Y-axis bar shows SEM *(p<0.01) and *** (p<0.001) mentioning that there is a remarkable difference in treated and non-treated cells (ANOVA).**

Figure 1 illustrates that the erythrocytes were exposed to gentamicin (9–18µM) and selenium for 48 hours, which demonstrates that in cells lacking selenium, the activity of glutathione peroxidase declined due to the oxidative stress caused by gentamicin. Meanwhile, in Selenium-positive cells, the glutathione peroxidase activity was increased because of the free radical scavenging induced by selenium. Similar results were mentioned about enzyme activity in (Neve, 1995) .

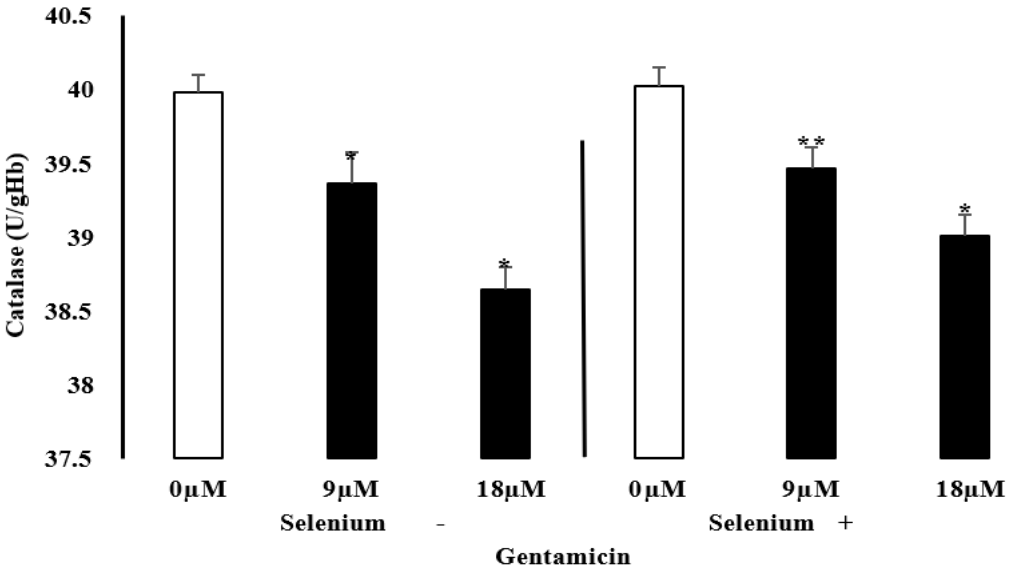

Figure 2 Variations in Catalase (U/g Hb) activities in human erythrocytes due to Gentamicin and selenium. Arithmetic mean ±SEM (n = 15) of erythrocytes treated and incubated with ringer solution and without (white bars) or with (black bars) gentamicin and selenium for 48 hours was calculated. The Y-axis bar shows SEM *(p<0.01) and *** (p<0.001) mentioning that there is a notable difference in treated and non-treated cells (ANOVA).**

Figure 2 depicts the activity of catalase enzyme after 48-hours gentamicin (9–18µM) and selenium exposure to human erythrocytes. Results demonstrated that in selenium-deficient cells, a substantial decline in the activity of enzyme was observed relative to the control, which may be attributed to the reactive oxygen species generation by gentamicin. But in selenium-rich cells, there was a significant rise in catalase activity which may be due to the antioxidative potency of selenium offering total protection against oxidative free radicals.

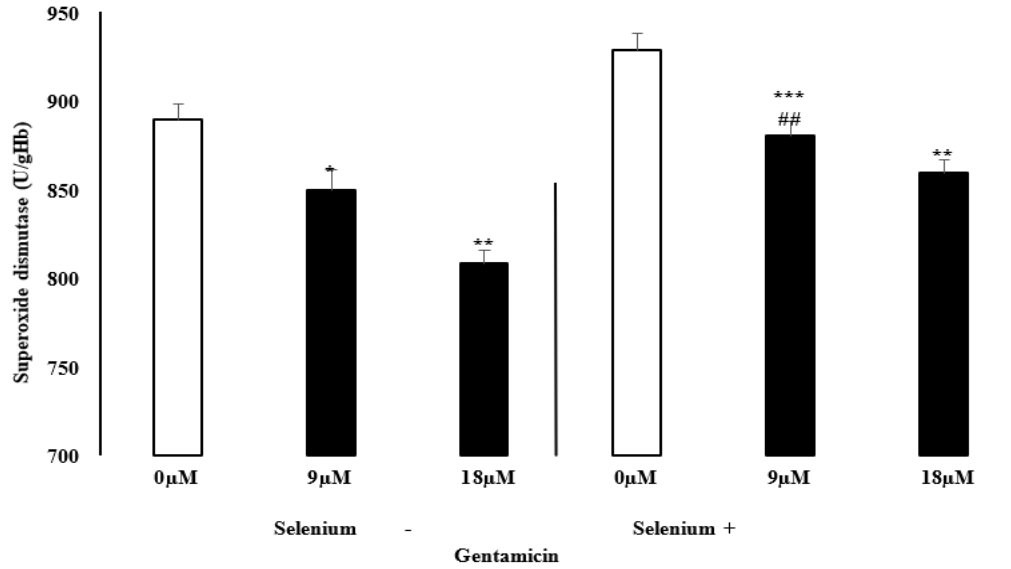

Figure 3 Variations in the superoxide dismutase (U/g Hb) activities in red blood cells due to gentamicin and selenium. Arithmetic mean ±SEM (n = 15) of erythrocytes exposed with ringer solution and without (white bars) or with (black bars) gentamicin and selenium for 48 hours was estimated. The Y-axis bar shows SEM *(p<0.01) and *** (p<0.001) mentioning that there is a contrast in reading witnessed in treated and non-treated cells (ANOVA).**

Figure 3 illustrates that the erythrocytes were exposed to gentamicin (9–18μM) and selenium for 48 hours which shows that in the absence of selenium, the activity of superoxide dismutase decreased which may be due to oxidative damage caused by gentamicin. In Selenium-containing cells, the activity of SOD is enhanced because of the antioxidative ability of selenium. Literature demonstrated the same results about enzymatic activity (Wang et al., 2010).

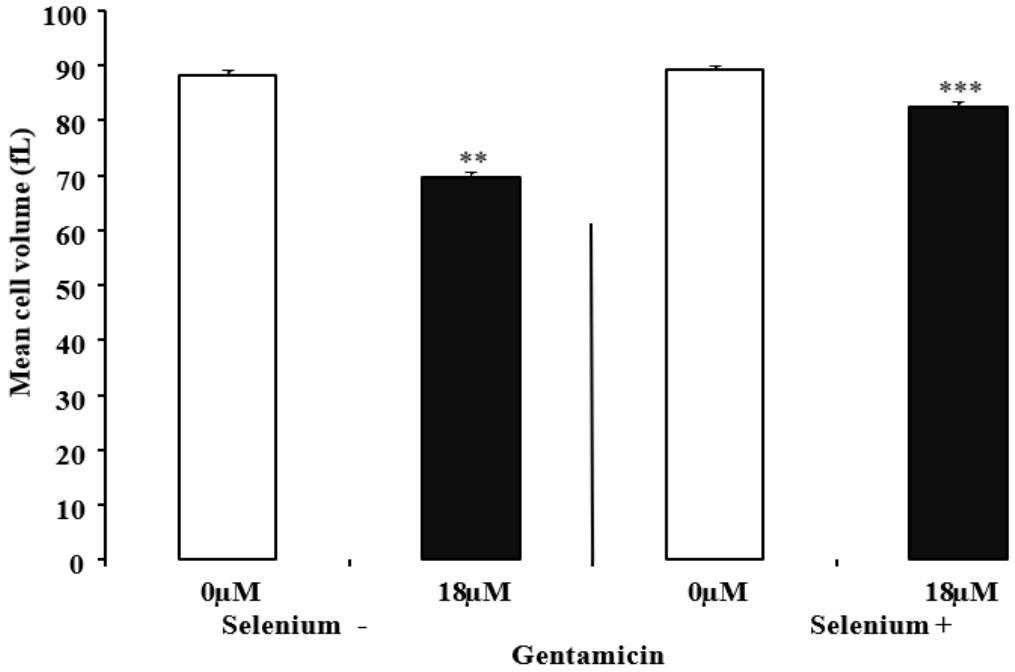

Figure 4 Variations in the mean cell volume (fL) of erythrocytes due to Gentamicin and selenium. Arithmetic means ±SEM (n = 12) of erythrocytes treated without (white bar) and with (black bar) gentamicin, selenium and ringer for 48 hours was measured. The Y-axis bar shows SEM *($p<0.01$) and *** ($p<0.001$) mentioning that there is a prominent difference in treated and non-treated cells (ANOVA).**

Figure 4 illustrates that human erythrocytes were exposed to gentamicin (0–18μM) and selenium for 48 hours. In selenium-negative cells, cell shrinkage was witnessed compared to control, which may be because of the damage exerted by reactive oxygen species produced by gentamicin, an indicator of eryptosis. Meanwhile, in selenium-positive cells, the mean cell volume of erythrocytes was apparently increased, and selenium's antioxidant activity may be responsible for this.

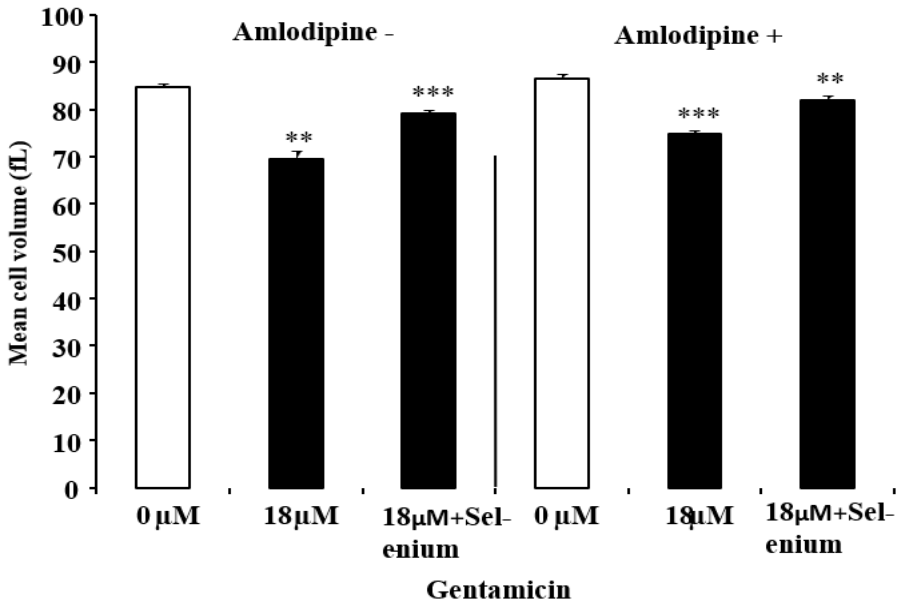

Figure 5 Variations in the mean cell volume (fL) of erythrocytes due to amlodipine (a calcium channel blocker). Arithmetic mean ±SEM (n = 12) of erythrocytes treated without (white bars) or with (black bars) gentamicin, selenium, amlodipine and ringer for 48 hours was measured. Y-axis bars show standard error means (SEM) ** (p<0.001) and * (p<0.01) declared that there is a slight difference in the volume of cells treated with amlodipine and amlodipine negative cells (Tukey's test).**

Figure 5 illustrates that human erythrocytes were exposed to gentamicin (18µM), selenium and amlodipine for 48 hours. In amlodipine-deficient cells, a substantial decrease in mean cell volume was observed compared to control, which may be due to oxidative stress exerted by gentamicin, an indicator of eryptosis. Meanwhile, there is a slight change in the mean cell volume of amlodipine-rich cells due to calcium channel blockage. Amlodipine nonselectively suppresses cation channels and prevents calcium entry into the cell (Hoth & Penner, 1992).

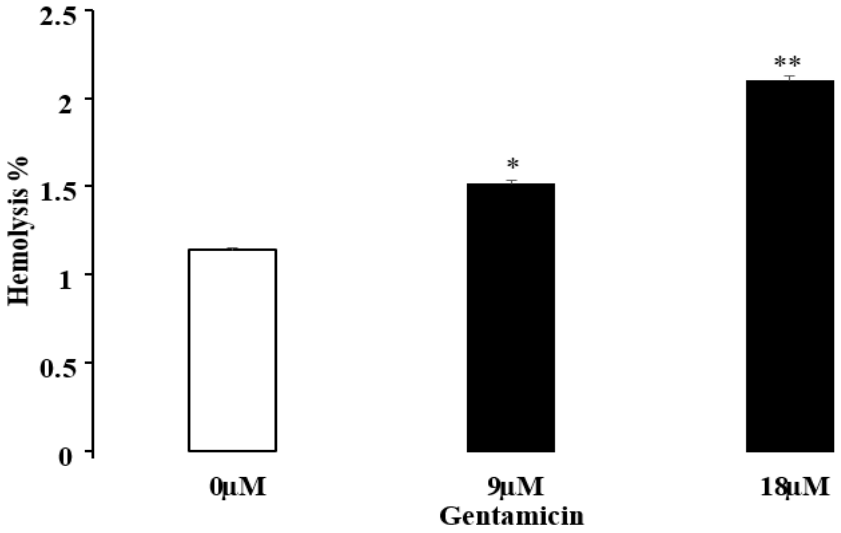

Figure 6 Variations in percentage of hemolysis due to Gentamicin in erythrocytes. Arithmetic mean ±SEM (n = 12) of erythrocytes exposed without (white bar) or with (black bars) gentamicin and ringer for 48 hours was measured. The Y-axis bar shows SEM *(p<0.01) and *** (p<0.001) mentioning that there is a prominent difference in treated and non-treated cells (ANOVA).**

Figure 6 demonstrates that when the erythrocytes were exposed with 9µM and 18µM doses of gentamicin for 48 hours, a substantial incline was noticed in the hemolytic activity.

Discussion

The primary goal of this research study was to evaluate the oxidative and eryptotic effects of gentamicin and the antioxidative role of selenium against gentamicin-induced eryptosis. To meet this objective, the activity of the antioxidative enzymes, mean cell volume of erythrocytes, their hemolytic activity, and the confirmation of Ca^{+2}'s role in the induction of eryptosis were determined. Oxidative stress results in eryptosis and the fluctuations in the activities of anti-oxidative enzymes are important indicators of oxidative stress (Shahid et al., 2016). Glutathione peroxidase(GPx) acts in the mitochondrial membrane, inhibits the assemblage of oxidized lipids, and disintegrates H_2O_2 into H_2O (Hassan et al., 2001). Oxidative stress declines its activity. Selenium works primitively as a cofactor of GPx, so participates in the reduction of peroxidases specifically hydrogen peroxidase. Superoxide dismutase is a vital antioxidant enzyme that neutralizes superoxide radicals, preventing oxidative stress. Accumulation of reactive oxygen species can result in declining SOD levels and hinders mitochondrial functioning (Jaleel et al., 2008). Selenium acts as a scavenger and reduces the O_2 free radicals and thus enhancing the activity of SOD. Similarly, catalase converts H_2O_2 into H_2O and O_2 (Vijayaraghavan & Paneerselvam, 2011). A decline in catalase activity leads to an increase in hydrogen peroxide levels. Selenium acts as an antioxidant that neutralizes harmful free radicals and boosts catalase activity. Previous studies depicted oxidation as the decline in the antioxidative enzymes activity due to the high accumulation of oxidants in the cell (Lucero et al., 2015), and, antioxidants increase the activity of these enzymes (Harris, 1992).

Cell shrinkage is a distinctive feature of eryptosis. This shrinkage is attributed to the activation of calpain, a calcium-dependent cysteine protease responsible for the degradation of the cytoskeleton in erythrocytes. The display of oxidative stress and cell shrinkage in the cells dosed with gentamicin evidences its eryptotic activity. Alternatively, the decline in oxidative stress and the shifting of the membrane to its normal size demonstrates the antioxidative potency of selenium. Intracellular calcium plays a critical role in triggering eryptosis in response to oxidative stress (Calabrò et al., 2015). Oxidative stress activates the non-selective cation channel. Amlodipine prevents the entry of calcium ions into cells by non-selectively inhibiting cation channels. Published studies regarding eryptosis showed the same effects (Duranton et al., 2002), oxidative stress causes eryptosis, and antioxidants scavenge harmful substances.

Excretion of old or damaged red blood cells from the bloodstream is the key physiological function of eryptosis (Lang et al., 2012). Hemolysis can lead to the passage of hemoglobin through kidney cells and its subsequent precipitation in the renal tubules (Malik et al., 2015). This hemoglobin can lead to severe clinical complications, such as decreased nitric oxide bioavailability, endothelial dysfunction, systemic vasoconstriction and vasomotor instability (Rapido, 2017)**.**

Conclusion

The research findings concluded that the therapeutic doses of gentamicin can cause erythrocyte death by increasing eryptosis and hemolysis. 18µM gentamicin may cause oxidative stress by declining the activities of antioxidative enzymes, enhancing the eryptosis rate, and, subsequently, these activities of antioxidative enzymes can possibly be increased by using selenium and hence lowering the rate of eryptosis.

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