J. Afr. Ass. Physiol. Sci.9 (1): 26-31, July 2021

Journal of African Association of Physiological Sciences

Official Publication of the African Association of Physiological Sciences
http://www.jaaps.aapsnet.org

Research Article

Influence of nanosilver on osmotic fragility responses of erythrocyte membrane following Na^+/K^+-ATPase blockade.

***O.K. Uche and S. Odara**

Department of Physiology, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, P.M.B 1154, Benin City, 300-001, Nigeria.

Keywords:
Nanosilver-induced lysis in Na^+/K^+ATPase blockade

ABSTRACT

Background: The molecular mechanisms and overt effects of nanoparticle-induced changes in red blood cells (RBCs) structure and function across membrane cell lines remain unclear despite the increasing use and application in nanomedicine. The aim of this study was to assess the impact of nanosilver exposure on osmoregulation of red cell membrane fragility in digoxin-induced Na^+-K^+ATPase blockade in vitro. **Materials and Method:** Samples from 50 subjects were obtained from consenting asymptomatic adults: male and female HbAA haemoglobin genotype. After separation and washing of erythrocytes, the samples were divided into three sets with each sample treated in duplicate with graded percentage concentrations of phosphate buffer solutions (0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1). The second and third set of samples were incubated with 0.05ml of erythrocytes, 1 ml phosphate buffer saline and 1 ml nanosilver or digoxin of 25 mg/ml. Thereafter, the content of each test tube was incubated for 1 hour and 3 hours respectively. The absorbance was recorded after 30mins incubation for each set with standard spectrophotometer at 540 nm wavelength. Haemolysis in each tube was recorded and expressed as percentage of the absorbance in distilled water. The average values recorded were plotted against the different concentrations used. **Results:** Erythrocytes from the sample incubated with nanosilver had significantly increased osmotic lysis compared with the untreated cells in rate-depended manner ($P<0.05$). Similar pattern was observed with digoxin pre-incubated cells. The mean osmotic fragility (MOF) index of the untreated, nanosilver and digoxin pre-incubated cells was in the order: digoxin>nanosilver>untreated. **Conclusion:** exposure of RBCs to nanosilver and in Na^+/K^+ATPase blockade may result in increased hemolytic effects by multifactorial cell membrane-mediated processes

©Copyright 2021 *African Association of Physiological Sciences* -ISSN: 2315-9987. All rights reserved

INTRODUCTION

Nanotechnology is the control and restructuring of matter at the nanoscale to atomic and molecular size range of about 1-100nm, in order to create materials, devices, and systems with fundamentally new properties and functions (Yuan *et al.*, 2017, Sanam *et al.*, 2021). Nanoscience is broad based, and the application of nanotechnology integrates multidisciplinary fields including material engineering, information high-tech, biogenetics and biomedical sciences and innovative commercial consumer products.

Owing to their small size physicochemical unique and probiotic properties with proven wide range of biomedical health benefits and potentials, especially in treatment of cancer, HIV and other related viral and bacterial diseases, there has been a sudden upsurge recently in nanomaterial use and applications (Larisa *et al.*, 2020).

Among several known nanomaterials of diverse structures, size and morphology, nanosilver (NS) is most commonly used nanoparticles. Nanosilver are heterogeneous particles of silver between 1nm and 100nm in size (Ghaffari and Hamedi, 2015) and has been used in medicine for centuries on account of its high antimicrobial properties. (Adriana *et al.*, 2020). Because of their large surface area-to-volume ratios, nanosilver offers a large antimicrobial spectrum and greater efficacy against bacteria than common antibiotics and provides new opportunities for use in

*Address for correspondence:
Email: oge.uche@uniben.edu

both consumer and biomedical applications, making them most commercialised nanoparticles (NPs) (Mohanty *et al*., 2012 and Abirami *et al.,* 2017, Mousumi *et al.,* 2020). Nanosilver reach the blood stream through different routes of application and is distributed to organs including liver, kidney and brain (Daniel *et al.,* 2018). RBCs are also responsible for carriage of respiratory gases for cellular metabolism and energy production. In the clinical, NS coat cardiovascular and neurosurgical catheters, surgical instruments, wound and burn dressings and bone substitute biomaterials (Li *et al*., 2015).

Nanoparticles are reported to induce toxicity in biological cell organs due to adherence of particles to cell membrane. The release of silver ions (Ag^+) in the medium due to oxidation of the Ag NPs imposes cytotoxicity, genotoxicity, and apoptosis (Mousumi *et al.,* 2020). Erythrocytes transmembrane transport is essentially mediated by Na^+/K^+ATPase (NKA) which is an integral membrane protein that spans the red cell membrane. The main function of the NKA is to regulate osmotic pressure. The main factors related to osmotic pressure are H_2O the concentration of ions and bioorganic molecules. Significant alterations in the extracellular ionic concentrations activates perpetual ion pump activity to maintain the cytosolic concentrations, which are important for numerous cellular enzymatic functions and regulations. Inhibition of NKA enzyme activity in gills; alterations in concentrations of plasma biochemicals (electrolytes, proteins, lipids), oxidative stress/cell damage markers and histopathological changes are among other deleterious effects of exposure to different sublethal concentrations of Ag-NPs LC50 (96h) in fish (Vali *et al.,* 2020). The affinity of NKA for sodium and potassium seems to be modulated by tissue-specific factors, such as the lipid composition of the membrane, ionic composition and pH (Juliana *et al.,* 2020) and activities of cardiac glycosides like ouabain and digoxin are known to inhibit NKA accompanied by an increase in cell volume and osmotic fragility and a decrease in the cytosolic K^+/Na^+ ratio (Kowluru *et al.,* 1989). It has been postulated that nanoparticle toxicity is increased by the adherence of nanoparticles (NPS) to cell membrane. The biocompatibility and toxicity of NPS have been widely studied and data from different nanomaterials in different cell lines and animal experimental models present conflicting reports and the molecular mechanisms and factors influencing NPS toxicity are still enigma. Therefore, in view of the increase in application, the biosafety and the mechanism by which NPS act at different levels of biological systems and cellular organs accumulation as well as environmental exposures have attracted much attention and become subject of great concern recently in nanotechnology investigations.

Functionally, osmotic fragility is widely used to determine erythrocyte integrity and elucidate mechanisms of the influence of different factors on the osmotic properties of RBC membranes, including shear stress and mechanical haemolysis, temperature, ultrasound effects, drugs and irradiation (Laloy *et al.*,2014, Tomasz *et al.,2014).* Osmotic fragility is a useful indicator for evaluating the interactions of various substances with the red cell membrane in vitro. The aim of the present study was to examine the influence of RBC exposure to nanosilver and Na^+/K^+ATPase regulatory activity on erythrocyte membrane osmotic fragility responses in RBCs from HBAA subjects following digoxin inhibition.

MATERIALS AND METHODS

Subjects:

Blood sample was collected from 50 consenting individuals of HbAA haemoglobin genotype erythrocytes, Both male and female apparently healthy asymptomatic subjects within 18-25 years participated in the study after informed consent was obtained.

Phosphate buffer solution:

Phosphate buffer of the following composition (mL): NaCl/ 8.0g KCl/0.2g Na_2HPO_4/1.42g KH_2HPO_4/ 0.24g with a pH of 7.4 . From this phosphate buffer solution, different osmolarities (0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1) were made by serial dilution with distilled water.

Blood sampling

Five millilitres (5 ml) of blood were collected from each subject into a lithium heparin anticoagulant bottles and analyzed within 2 hrs of collection. Blood was centrifuged at 2500 rpm for 10 minutes in order to separate the erythrocytes from plasma.The erythrocytes were washed three times by methods as described by Tsakiris *et al*., 2005 with 5ml of 0.9 Phosphate buffer saline. Thereafter, erythrocytes were re-suspended in 3ml of phosphate buffer saline and the test carried out with these washed and intact erythrocytes.

Fragility experimental protocols

The blood samples were divided into three sets, with each sample set treated in triplicate with graded percentage of phosphate buffer solutions (0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1) as control to assess their resistivity to osmotic stress and thereafter, the second set of test tubes, 0.05ml of blood, 1 ml phosphate

buffer saline and 1 ml nanosilver. The third set of test tubes contained 0.05 ml of blood, 1 ml phosphate buffer saline and 1 ml digoxin of 25mg/ml. The content of each test tube was incubated for 1 hour and 3 hours respectively as described by Dacie and Lewis, 1995; at room temperature 37^{0}C after which it was centrifuged at 2500 revolution per minute (RPM) for 10 minutes. The supernatant of the spun mixture was extracted, and optical absorbance was recorded with a standard spectrophotometer at 540nm wavelength. Haemolysis in each tube was expressed as a percentage of the absorbance in distilled water. The average values recorded were plotted against different concentrations used.

Evaluation of fragility index and stabilization of erythrocytes:

Mean osmotic fragility (MOF) (concentration of the solution when 50% of the cells are haemolysed) was graphically determined. While the relative capacity to stabilize or destabilize erythrocyte membrane was evaluated as percentage of quotient of the difference between MOF values of the test and control samples to the control sample (Parpart *et al.*, 1947; Chikezie, 2011).

$$\text{Thus, Relative stability (\%)} = \frac{(MOF_{control} - MOF_{test} \times 100}{MOF_{control}}$$

Statistical Analysis

Data analyses were done with GraphPad prism 5.0 Statistical software, followed by one-way analysis of variance (ANOVA). Values are presented as means ± SEM. P-values less than 0.05 ($p<0.05$) were considered statistically significant, while n-values denote number of animals in each experimental group.

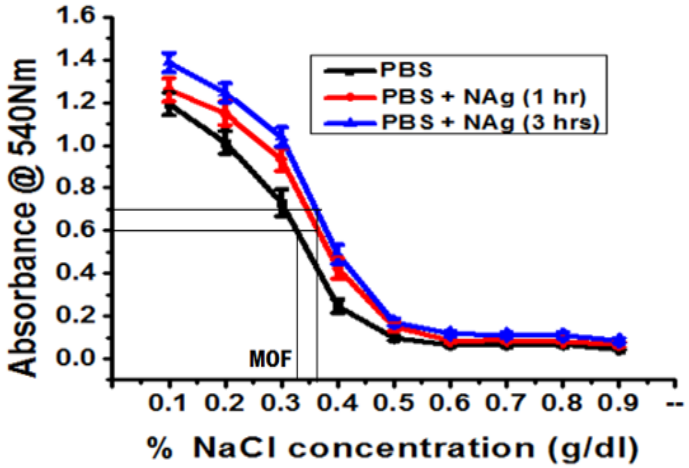

Fig.1: Nanosilver-induced haemolysis during 1 hr and 3 hrs incubation.

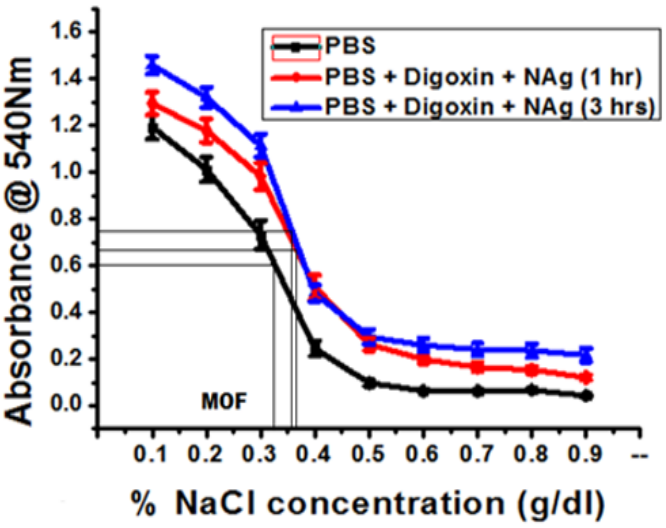

Fig. 2: Fragility response curves during 1 hr and 3 hrs nanosilver exposure in digoxin pre-incubated erythrocytes.

RESULTS

In the Osmotic fragility curves of erythrocytes following 1hour and 3 hours incubation in nanosilver in vitro (Fig. 1), there was a significant ($p<0.05$) increase in haemolysis by a right-ward shift of the curves with nanosilver treated cells compared with the untreated samples. This indicates decrease in osmotic burst resistance.

As shown in fig. 2 of hemolysate curves from cells pre-incubated with digoxin (Na^+/K^+ATPase) inhibitor and thereafter, following exposure to 10ppm nanosilver in vitro at 37^{0}C in 1hour and 3 hours incubation respectively. There was a right-ward shift of the curves with digoxin pre-treated cells compared with the untreated samples, suggesting a decrease in osmotic burst resistance.

In fig. 3 is the Comparative composite osmotic fragility curves of red cells treated with nanosilver and pre-incubated with digoxin for 1 hour and 3 hours respectively. The significant increase in red cell lysis by right-ward shift of the hemolysate curves with digoxin pre-incubated cells and nanosilver treated cells compared with the control indicate decrease in osmotic burst resistance. The degree of lysis in rate in the treated cells was in the order: 3hours>1 hour. Mean osmotic fragility (MOF) (concentration of the solution when 50% of the cells are haemolysed) (0.38, 0.36 and 0.32) g/dl was in the order: digoxin>nanosilver>untreated, suggesting an increase in osmotic fragility in treated cells.

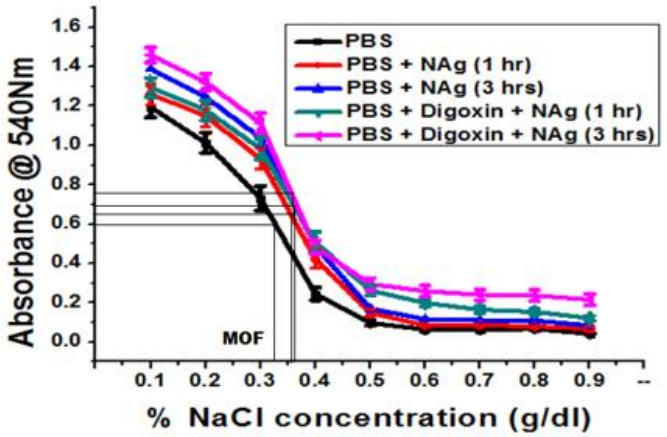

Fig. 3: Comparative effects of nanosilver-induced haemolysis during 1 hr and 3 hrs incubation in digoxin which inhibits Na^+/K^+ATPase.

The tenet was the same in relative capacity to stabilize or destabilize erythrocyte membrane evaluated as percentage of quotient of the difference between MOF values of the test and control samples.

DISCUSSION

The blood stream is the principal transport system for delivery of organic and non-organic substances including nutrients, biomolecules, respiratory gases, electrolytes and other biomaterials such as nanoparticles to target organ sites. Owing to the increasing importance and wide applications of nanomaterials in the advancement and improvement in healthcare service delivery in nanomedicine, biosystems, engineering and other industrial consumer products, focus on identification of cytotoxic effects associated with their use and applications has become imperative to guarantee biocompatibility within physiological systems. safe and sustainable development of nanotechnology for responsible and effective management of its potentials and safety concerns of patients and other end users. The haemolytic properties and interactions with red blood cells to ascertain the level of toxicity or undesirable effects are the main parameters for the hemocompatibility in nanotechnology.

The osmotic fragility evaluation is employed to measure the erythrocyte resistance to haemolysis following exposure to varying hypotonic concentrations of NaCl solution. Na^+/K^+ATPase plays an important role in the regulation of cellular Na^+ and K^+ concentrations and is one of the key enzymes responsible for maintaining the osmotic balance of cell and transmission of action potentials (Dao-Sheng *et al.*, 1990). The present study therefore examined the impact of 10ppm size nanosilver exposure on Osmoregulation of red cell membrane resistivity on fragility responses in digoxin-induced Na^+/K^+ATPase blockade in vitro. The data obtained from this study indicate an increase in osmotic lysis demonstrated by the rightward shifts of the fragility curves from the red cells pre-incubated with digoxin for one hour and three hours respectively and exposed to nanosilver compared with the untreated cell. The increase in fragility response was greater during the three hours exposure in both treated cells. This suggests rate-dependent nanosilver-induced decrease in erythrocyte burst resistance. The observation of nanosilver-induced hemolytic effect is in concordance with the work of Abirami *et al.,* 2017 who reported increase nanosilver haemolytic effects in fish and aquatic organisms. Furthermore, the mean osmotic fragility index (MOF) (concentration of the solution when 50% of the cells are haemolysed) obtained from the two different test samples compared with the control was in the order: digoxin>nanosilver>untreated, suggesting an enhancement in nanosilver-induced hemolytic action during Na^+/K^+ATPase blockade by digoxin. MOF is a measure of the osmotic fragility (OF) parameter (Dewey *et al.,* 1982, Krogmeier *et al.,* 1993). In clinical practice, osmotic fragility test is often done to aid with diagnosis of diseases associated with RBC membrane abnormalities. Some disease conditions linked to increased osmotic fragility include hereditary spherocytosis and hypernatremia, while some linked to decreased fragility include chronic liver disease, iron deficiency anaemia, thalassemia, polycythemia vera, and sickle cell anaemia after splenectomy (Ajayi and Igwilo, 2016). The mechanism of action associated with nanosilver-induced increase in red cell fragility and other cell line reported toxicity has not been fully characterised. From previous findings, the decrease in osmotic burst resistance following exposure to nanosilver could be associated with the unique small size and physiochemical properties of nanosilver particles which enhance rapid direct membrane increased permeability across cells (Andriana *et al.,* 2020). It has also been alluded that in vitro and in vivo animal models nanosilver particles non-covalently attached to red blood cells surfaces via electrostatic force attraction due to the presence of negatively charged proteins, lipids and polysaccharides on mammalian cells which offer a range of functional groups and surface properties that permit the attachment of cationic nanoparticles to their surface (Anselmo *et al*., 2013, Daniel *et al.,* 2018). Additionally, *in* vitro experiments have shown that cardiac glycosides including ouabain and digoxin inhibit the sodium pump of the red cell. The

physiological consequences of inhibiting Na^+/K^+ATPase may result in alteration in homeostasis of the red cell membrane in regulation of cell volume. The effect of digoxin-induced Na^+/K^+ATPase blockade would result in a reduction of the intracellular potassium and an increase in intracellular sodium content with concomitant increase in cell volume and ultimately increase in cell lysis; since sodium efflux is actively coupled to potassium influx at the cell membrane. In normal cell membrane function in biological systems, Na^+/K^+ATPase sodium-potassium sustained concentration gradient is crucial for physiological processes in many organs and has an ongoing role in stabilizing the resting membrane potential of the cell, regulation of the cell volume and in the cell signal transduction (Pirahanchi *et al.*, 2021). In conclusion, exposure of RBCs to nanosilver and Na^+/K^+ATPase blockade may result in increase hemolytic effects by multifactorial cell membrane-mediated processes.

REFERENCES

Abirami T, Alen GRJ, Bavani G, Karthikeyan J; (2017). Ecotoxicology of green synthesized silver nanoparticles on freshwater fish *mystus gulio*. Int J Pharm Sci; 9(11):192-198.

Adriana R.G, Amaya A, Ariane V, and Adela L. Genotoxicity of silver nanoparticles. Nanomaterials. 2020; 10; 251. doi: 10.3390/nano10020251

Ahamed M, Alsalhi MS and Siddiqui MK. (2010). Silver nanoparticles applications and human health. *Clin Chim Acta*. **411**: 1841-1848.

Ajayi, O.I., Igwilo, VO., (2016). Increased resistance to osmotic lysis of sickled erythrocytes induced by cocos nucufera (coconut) water. J. Afr.Ass.Physiol.Sci.4(2): 109-112

Anselmo AC, Gupta V, Zern BJ, Pan D, Zakrewsky M and Muzykantov V. (2013). Delivering Nanoparticles to Lungs while Avoiding Liver and Spleen through Adsorption on Red Blood Cells. *ACS Nano*. **7**:11129–11137.

Dacie J.V., Lewis S.M. (1995). *Practical Haematology*. Eight edition. Churchill Livingstone, New York. Pp 216-220.

Dewey, M.J., Mrown, J.L and Nallaseth, F.S., (1982). Genetic differences in red cell osmotic fragility: analysis in allophonic mice. *Blood*, **59**: 986-989.

Chikezie, P.C., (2011). Comparative in vitro osmotic fragility of three human erythrocyte genotypes in the presence of quinine and chloroquine phosphate. *Asian Journal of Biochemistry Research*, **6(1)**: 55-64.

Ghaffari T and Hamedi-Rad F. (2015). Effect of silver nanoparticles on tensile strength of acrylic resins. *J Dent Res Dent Clin Dent Prospects* .**9**:40-3.

Juliana SY, Heitor GS, Rosangela I, and Pietro C. (2020). Overview on solubilization and lipid reconstitution of Na, K-ATPase: enzyme kinetic and biophysical characterization. Biophys Rev. 12(1):49064.

Karthikeyan J, Abirami T.1, Alen Godfrey R. Jose1, Bavani Go, (2020). Ecotoxicology of green synthesized silver nanoparticles on freshwater fish mystus gulio. Int J Pharm Pharm Sci, 9 (11):192-198.

Krogmeier. D.E., Mao, I.L., Bergen, W.G. (1993). Genetic and non-genetic effects on erythrocyte osmotic fragility in lactating Holstein cow and its association with yield traits, *Dairy Sci*, **76:** 1994-2000.

Laloy J, Valentine M, Lutfiye A, Francois M, Sonja B, Olivier T, Stephen L and Jean-Michel D, (2014) Impact of Silver Nanoparticles on Haemolysis, Platelet Function and Coagulation Nanobiomedicine ; 1 (4) https://doi.org/10.5772/59346

Larisa Koleva , Elizaveta Bovt, Fazoil Ataullakhanov, and Elena Sinauridze , (2020). Erythrocytes as Carriers: From Drug Delivery to Biosensors. Pharmaceutics, 12, 27

Li QC, Li F, Jian L, Cheng ZD, Bin K and Cheng ZH,. Nanotoxicity of silver nanoparticles to red blood cells: Size dependent adsorption, uptake, and hemolytic activity. Chem. Res. Toxicol, 2015; 28: 501-509.

Mohanty S, Mishra S, Jena P, Jacob B, Sarkar B and Sonawane A. (2012). An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. *Nanomedicine*. **8**:916-924.

Mousumi M, Kaustav G, Rahul D, and Pradipta P; (2020). Development of Non-ionic surfactant and protein-coated ultrasmall silver nanoparticles: Increased viscoelasticity enables potency in biological applications. ACS Omega, 5: 8999-9006.

Parpart AK. Loren PB, Parpart ER, Gregg JR and Chase AM, (1947). The osmotic resistance (fragility) of human erythrocytes. J. Clin. Invest.,26: 636-640

Pirahanchi Y, Jessu R, and Aeddula NR. Physiology, sodium potassium pump. (Updated 2021), In: StatPearls (Internet). Treasure island (FL): StatPearls Publishing; 2021

Sanam N, Ali A, Leili A; (2021). Current applications and prospect of nanoparticles for antifungal drug delivery. *Excli J*; 20:562–584.

Tomasz W, LudmiBa CN, MaBgorzata K, and Wojciech W; (2014). Individual Osmotic Fragility Distribution: A New Parameter for Determination of

the Osmotic Properties of Human Red Blood Cells. BioMed Research International, Volume 2014, http://dx.doi.org/10.1155/2014/162102

Tsakiris S, Giannoulia-Karantana A, Simintzi I and Schulpis KH. (2005). The effect of aspartame metabolites on human erythrocyte membrane acetylcholinesterase activity. Pharmacol. Res. **53**:1-5

Vali S, Ghasen M, Kamran RT, Fatemeh M and Saeid SN, (2020). The effects of silver nanoparticles (Ag-NPs) sublethal concentrations on common carp (Cyprinus carpio): Bioaccumulation, hematology, serum biochemistry and immunology, antioxidant enzymes, and skin mucosal responses. Ecotoxicology and environmental safety, 194; (2020): 110353.

Yuan Y, Zhen Q, Wei Z, Ting Y, Yubin C, Chaorong M and Yu K; (2017). Toxicity assessment of nanoparticles in various systems and organs. Nanotechnol Rev. 6(3): 279-289.