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Original Article

Methaemoglobin and Malondialdehyde Levels of Human Erythrocytes Incubated with Acetylsalicylic Acid

Marika Louise Graham[1], Paul Chidoka Chikezie[1,2,*]

[1]School of Sciences, Coventry University, Coventry, England, UNITED KINGDOM.
[2]Department of Biochemistry, Imo State University, Owerri, NIGERIA.

ABSTRACT

Background: MetHb and MDA are biomarkers for erythrocyte redox status and membrane lipid peroxidation, respectively. This present study aims to investigate the levels of human erythrocyte redox status and membrane lipid peroxidation indicators in the presence of ASA over time using *in vitro* models. **Materials and Methods:** The %MetHb was evaluated by measuring the absorbance of human erythrocyte suspension at λ_{max}=630 nm and 540 nm. Human erythrocytes were incubated in 0.125 mg/mL, 0.25 mg/mL and 0.5 mg/mL ASA over a 6 hr incubation period. The human erythrocyte MDA concentration was measured using the TBARS assay. **Results:** The %MetHb of human erythrocytes following 3 hr of incubation with 0.125 mg/mL ASA gave a peak value of 2.70±1.36%, indicating a relative significant (p<0.05) increase of 45.16% compared with the control samples. Furthermore, an increase in the incubation time resulted in a decrease in erythrocyte %MetHb, although not to the levels observed at the beginning (t=0 hr) of incubation with ASA. Conversely, MDA concentration of human erythrocytes was 4.45±0.89 µM after 3 hr of incubation with 0.125 mg/mL ASA, showing a significant (p<0.05) decrease of 27.88%. **Conclusion:** The present study showed a negative correlation between %MetHb and MDA levels in human erythrocytes incubated with ASA over time, suggesting that the erythrocytes redox status did not specifically induce membrane lipid peroxidation.

Keywords: Acetylsalicylic Acid, Erythrocyte, Malondialdehyde, Methaemoglobin.

Correspondence:
Dr. Paul Chidoka Chikezie
[1]School of Sciences, Coventry University, Coventry, England, UNITED KINGDOM.
[2]Department of Biochemistry, Imo State University, Owerri, NIGERIA.
Email: ae2389@coventry.ac.uk

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INTRODUCTION

Erythrocytes are readily exposed to endogenous and exogenous oxidants, which produce RONS.[1,2] RONS elicits a multistage process of oxidative stress that can readily cause damage to the erythrocyte membrane and cytoskeleton proteins,[1,3] as well as cause intracellular structural damage.[4] Examples of exogenous sources of oxidation are leukocytes, vascular endothelium and drugs.[3] RONS can particularly oxidise membrane PUFAs, leading to membrane lipid peroxidation as well as loss in the structure-and-function interplay of critical biomolecules that contribute to the functionality of the erythrocytes.[1,4,5]

Erythrocyte cytosolic proteins are 95-97% comprised of haemoglobin.[6] MetHb is a reversible product of haemoglobin oxidation via increased endogenous oxidant production.[2] This autoxidation produces a low yield of weak superoxide and MetHb,[7] which can propagate a chain reaction leading to membrane lipid peroxidation.[3] The oxidising reaction of haemoglobin is where the ferrous state iron (Fe^{2+}) prosthetic group is oxidised to the ferric state (Fe^{3+})-MetHb.[8] MetHb is incapable of binding to oxygen, unlike the stable Fe^{2+} due to its high oxygen affinity.[8] The functionality of oxygen transport relies on the reduced Fe^{2+}, with a higher percentage content of MetHb being directionally proportional to the reduction in oxygen transport in plasma.[9]

Erythrocytes main mechanisms are for the gaseous exchange and transport of oxygen, carbon dioxide and amino acids;[10] the lack of organelles increases the efficiency of transport.[2] Increased ROS generation can cause structural changes to the erythrocyte membrane, leading to the dysfunction of oxygen transport.[11] Furthermore, this oxidative damage to the membrane can propagate dysfunction in oxygen transport and decrease deformability, which is essential for gaseous transport.[12] According to Becatti *et al.*,[13] the status of erythrocyte deformability is directly proportional to erythrocyte membrane lipid peroxidation.

The ASA is a common therapeutic NSAID.[14] ASA exerts its therapeutic effects by inhibiting COX-1 and COX-2 activity, thereby suppressing prostaglandin and thromboxane; this mechanism accounts for its anti-inflammatory and antiplatelet properties.[15] Previous studies have stated that ASA increases ROS production in different cell types,[16-18] which contributes to the

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induction of oxidative stress and lipid peroxidation. However, there is not much evidence denoting this in erythrocytes.

MetHb and MDA are important biomarkers that indicate the redox status of erythrocytes and the extent of membrane lipid peroxidation, respectively.[1,19-21] The present study aims to explore the impact of ASA on the redox status and the levels of membrane lipid peroxidation within human erythrocytes in an *in vitro* setting. This study will give insights into the capacity of measured concentrations of ASA to influence oxidative stress and membrane integrity of the human erythrocytes over time.

MATERIALS AND METHODS

Collection and preparation of blood samples

Venous blood samples were collected from consenting participants between 15th January and 9th February 2024 using 10 mL tubes with EDTA-Na_2 anticoagulant using the venipuncture procedure. The erythrocytes were washed according to the methods of Chikezie,[22] with slight modifications. A volume of 5 mL of the collected blood was suspended in 5 mL of PBS (pH 7.0) and centrifuged at 3000×g for 5 min. The washing of the erythrocytes was repeated thrice and the supernatant was discarded each time. Finally, the erythrocyte suspension was adjusted to a haematocrit of 10% and was stored at 4°C.

Exclusion criteria

Participants excluded include individuals who smoke and/or are on routine medications, suffering from blood disorders, e.g., sickle cell anaemia, undergone blood transfusions and on medications 4 weeks before blood sampling.

Ethics

The collection of blood samples from participants adhered to the ethical principles outlined in the October 2008 Declaration of Helsinki. The present study was approved by the Coventry University Ethics Committee (CU Ethics). Ethics Approval Number: P163330. Written consent was obtained whereby all the participants filled out and signed an Informed Consent Form.

Experimental design

The experimental design was according to the methods reported by Chikezie.[22] A 0.8 mL ASA of increasing concentrations in the order of 0.125 mg/mL, 0.25 mg/mL and 0.5 mg/mL was added to corresponding test tubes containing 2.2 mL of erythrocyte suspension (10% haematocrit). The incubation mixture was allowed to stand at a regulated temperature of 37°C in a water bath. The incubated erythrocytes devoid of ASA represented the control sample. At regular time intervals of 3 hr for 6 hr, appropriate aliquots of the incubation mixtures were drawn and used for the determination of erythrocyte parameters, namely, MDA and MetHb levels. To eliminate variations in erythrocyte parameters among blood donors, which may arise because of vagaries of their physiologic status, the erythrocytes were sourced from the same blood sample and analyses were carried out in triplicate.

Erythrocyte methaemoglobin

The %MetHb was measured according to the methods of Tietz,[23] as previously reported.[24,25] In a test tube containing 5.0 mL of distilled water, 0.05 mL of erythrocytes suspended in PBS osmotically equivalent to 0.9 g/100 mL (pH 7.4) was added. The suspension was allowed to stand for 60 min at room temperature (24-27°C) and the absorbance was measured at two different λ_{max}=540 nm and 630 nm using a spectrophotometer (Digital Blood Analyser®; SPECTRONIC 20, Labtech). Erythrocyte %MetHb of total haemoglobin concentration was evaluated using Equation 1 as previously described.[24,25]

$$\%\text{MetHb} = \frac{A630^2}{A630^2 + A540^2} \times 100 \quad \text{..... Equation 1}$$

Erythrocyte malondialdehyde

Measurement of erythrocyte MDA concentration was according to the methods previously described by Tjahjani *et al.*,[26] with minor modifications.[22] A 2:1 ratio mixture of 20% TCA and 0.67% TBA (3.0 mL) was introduced into a test tube. A volume of 0.2 mL of erythrocyte hemolysate was added to the mixture and boiled for 10 min in a water bath. The mixture was centrifuged for 10 min at 3,000×g after cooling to 24°C. A spectrophotometer (Digital Blood Analyser®; SPECTRONIC 20, Labtech) was used to measure the absorbance of the supernatant at maximum wavelength (λ_{max})=532 nm. Using the MDA standards, the absorbance of the samples was converted to MDA concentrations in µM.

Statistical analysis

The data collected were expressed in mean±SD and analysed in one-way ANOVA and Least Significant Difference (LSD). The comparison was made between groups and significance was established by ANOVA at a 95% confidence level. A difference of $p<0.05$ was considered statistically significant. The correlation coefficient (R^2) was evaluated using Excel Software (Microsoft, 2010 version).

RESULTS

As shown in Figure 1, erythrocyte %MetHb of the control sample increased by 1.06 folds between 0 and 3 hr of incubation, whereas at 6 hr of incubation, the erythrocyte %MetHb decreased by 0.90 folds at 6th hr. The overall alterations in erythrocyte %MetHb at 3 and 6 hr showed no significant difference ($p>0.05$) compared to the value at 0 hr.

The erythrocyte %MetHb following 3 hr of incubation with 0.125 mg/mL ASA gave a peak value of 2.70±1.36%, indicating a significant ($p<0.05$) increase of 45.16% compared with the

control samples. Similarly, 0.25 mg/mL ASA caused an increase in erythrocyte %MetHb by 26.88% (p<0.05) compared with the control sample at 3 hr of incubation. However, 0.5 mg/mL did not significantly (p>0.05) alter erythrocyte %MetHb at 3 hr of incubation. Overall, the levels of erythrocyte %MetHb in the presence of ASA were significantly (p<0.05) higher compared to those at the commencement of the incubation time (t=0 hr) (Figure 1).

Figure 2A showed that there was a significant difference (p<0.05) between the erythrocyte %MetHb in the presence of 0.125 mg/mL ASA at 3 and 6 hr intervals, which corresponded to a relative decrease of 21.48%. Figure 2B showed that 0.25 mg/mL ASA did cause a non-significant difference (p>0.05) in erythrocyte %MetHb (0.92-fold). Likewise, incubation of the erythrocytes with 0.5 mg/mL ASA showed a non-significant difference (p>0.05) between erythrocyte %MetHb at time intervals of 3 and 6 hr (1.04-fold) (Figure 2C).

Figure 3 showed that the erythrocyte MDA concentration of the control sample decreased from 7.59±0.98 µM to 6.17±1.50 µM, representing an 18.71% reduction (p>0.05). Conversely, erythrocytes incubated with separate 0.125, 0.25 and 0.5 mg/mL ASA for 3 hr exhibited significant (p<0.05) decreases in MDA concentration, representing 27.88, 30.15 and 30.60%, respectively (Figure 3).

Although the erythrocyte MDA concentrations changed following the incubation with 0.125, 0.25 mg/mL and 0.05 mg/mL ASA, the erythrocyte MDA concentrations showed no significant difference (p>0.05) at time intervals of 3 hr for 6 hr (Figure 4 A-C).

The calculated correlation coefficient (R^2) between erythrocytes MDA concentration and %MetHb following the incubation with varying concentrations of ASA showed a strong negative correlation. Specifically, the correlation coefficient between erythrocyte %MetHb and MDA concentrations was -0.99 in the presence of 0.5 mg/mL ASA. Furthermore, an R^2=-0.81 was in the presence of 0.125 mg/mL ASA, whereas 0.25 mg/mL ASA gave an R^2 value of -0.95.

DISCUSSION

The redox status and structural integrity of the erythrocytes can be ascertained using cellular parameters such as MetHb and MDA levels, as well as other redox biomarkers.[11,27,28] Previous reports have identified a wide range of xenobiotics and biological agents capable of altering the redox status of erythrocytes.[29-32] By altering the redox status of the erythrocytes, these agents can disrupt the functional and structural relationship of haemoglobin molecules, ultimately affecting the overall functionality of erythrocytes under oxidative stress.[33]

The present findings appear to indicate that ASA impacted the redox status of human erythrocytes, as exemplified by the changes in the levels of erythrocyte %MetHb in the presence of ASA over time of incubation of the human erythrocytes. This alteration is influenced by both the concentration of ASA and the duration of incubation of the erythrocytes. Specifically, higher

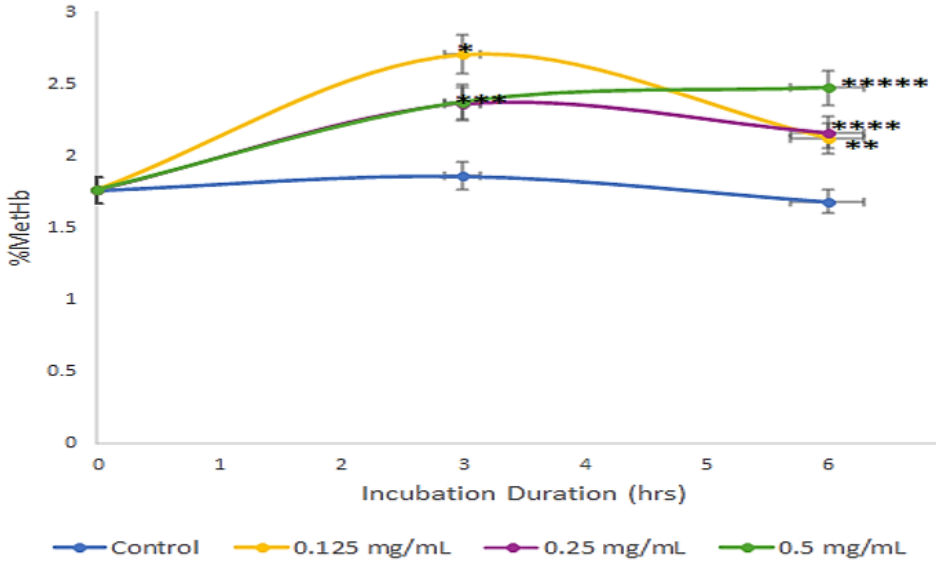

Figure 1: Human erythrocyte %MetHb in the presence of varying concentrations of ASA at time intervals of 3 hr for 6 hr. (The mean (X)±S.D of six (n=6) determinations.

*The mean erythrocyte %MetHb of erythrocytes incubated in 0.125 mg/mL ASA at t=3 hr showed significant difference (p<0.05) compared to the control sample. ** The mean erythrocyte %MetHb of erythrocytes incubated in 0.125 mg/mL ASA at t=6 hr showed significant difference (p<0.05) compared to the control sample. ***The mean erythrocyte %MetHb of erythrocytes incubated in 0.25 mg/mL ASA at t=3 hr showed significant difference (p<0.05) compared to the control sample. ****The mean erythrocyte %MetHb of erythrocytes incubated in 0.25 mg/mL ASA at t=6 hr showed significant difference (p<0.05) compared to the control sample. *****The mean erythrocyte %MetHb of erythrocytes incubated in 0.5 mg/mL ASA at t=6 hr showed significant difference (p<0.05) compared to the control sample.)

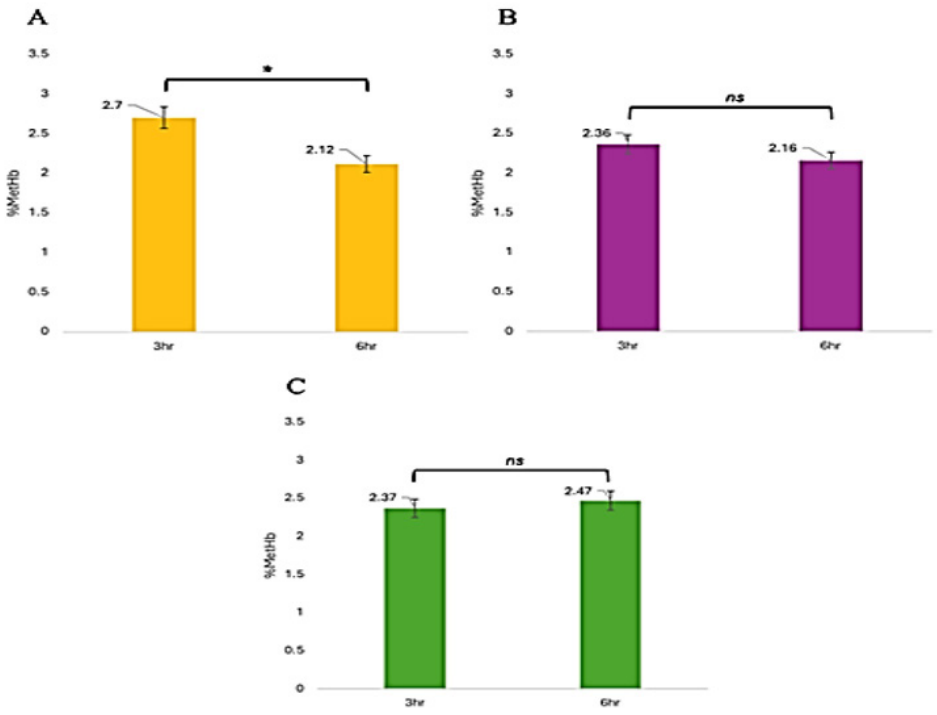

Figure 2: Human erythrocyte %MetHb in the presence of ASA at time intervals of 3 and 6 hr. (A: 0.125 mg/mL ASA. B: 0.25 mg/mL ASA. C: 0.5 mg/mL ASA. ns: no significance difference (p<0.05).

*The mean erythrocyte %MetHb of erythrocytes incubated with 0.125 mg/mL showed a significant difference (p<0.05).)

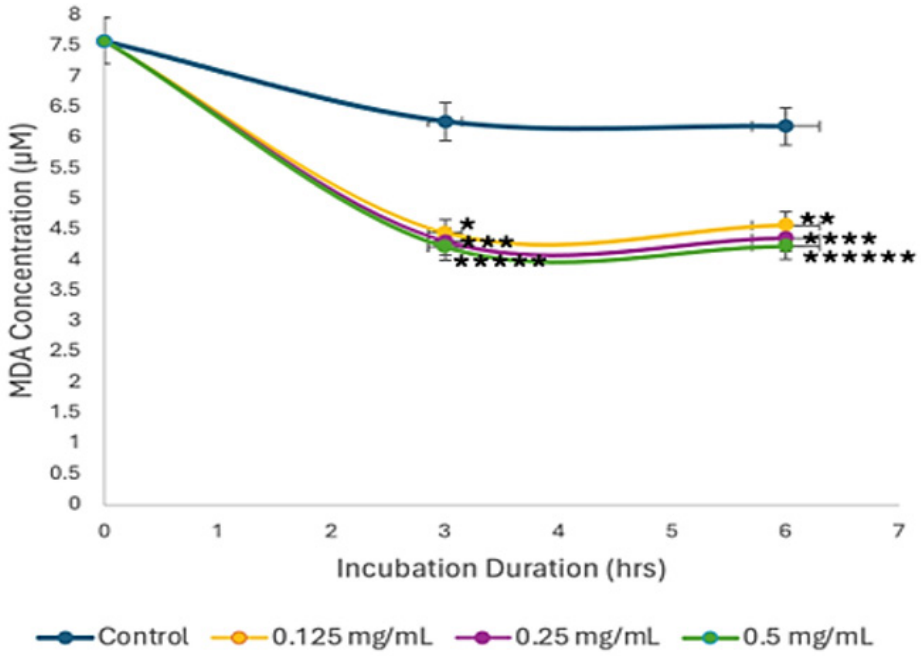

Figure 3: Human erythrocyte MDA concentration in the presence of varying concentrations of ASA at time intervals of 3 hr for 6 hr. (The mean (X)±S.D of six (n=6) determinations.

*The mean erythrocyte MDA concentration of erythrocytes incubated in 0.125 mg/mL ASA at t=3 hr showed significant difference (p<0.05) compared to the control sample. ** The mean erythrocyte MDA concentration of erythrocytes incubated in 0.125 mg/mL ASA at t=6 hr showed significant difference (p<0.05) compared to the control sample. ***The mean erythrocyte MDA concentration of erythrocytes incubated in 0.25 mg/mL ASA at t=3 hr showed significant difference (p<0.05) compared to the control sample. ****The mean erythrocyte MDA concentration of erythrocytes incubated in 0.25 mg/mL ASA at t=6 hr showed significant difference (p<0.05) compared to the control sample. ******The mean erythrocyte MDA concentration of erythrocytes incubated in 0.5 mg/mL ASA at t=3 hr showed significant difference (p<0.05) compared to the control sample. ******The mean erythrocyte MDA concentration of erythrocytes incubated in 0.5 mg/mL ASA at t=6 hr showed significant difference (p<0.05) compared to the control sample.)

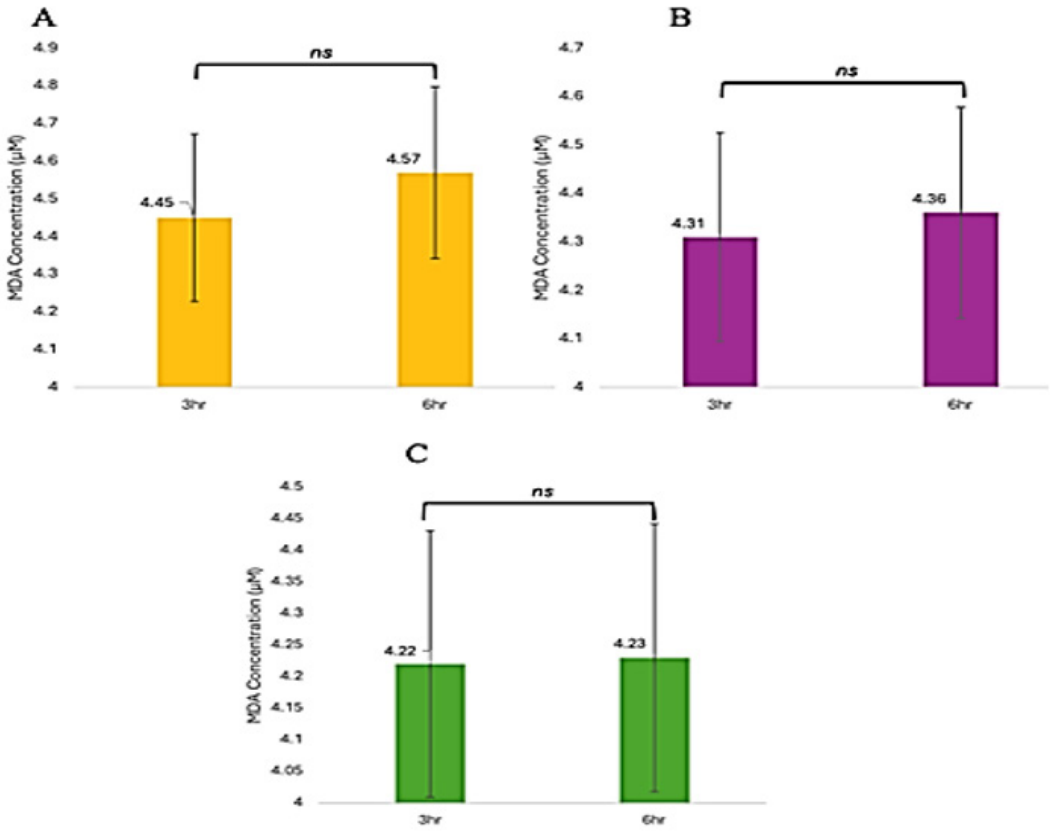

Figure 4: Human erythrocyte MDA concentration in the presence of ASA at time intervals of 3 and 6 hr. (A: 0.125 mg/mL ASA. B: 0.25 mg/mL ASA. C: 0.5 mg/mL ASA. ns: no significance difference (p<0.05).)

concentrations of ASA appear to exhibit a tendency to produce more pronounced changes in the redox status of the human erythrocytes. In the same manner, the duration the erythrocytes were exposed to ASA also played a crucial role in determining the extent of alterations of these biochemical indicators of redox status and ultimately oxidative stress. Therefore, the results of the present study suggest a concentration- and time-dependent relationship between ASA treatment and the levels of oxidative stability indicators of the human erythrocytes. These findings aligned with previous research outcomes, which demonstrated that chemical agents could alter the redox status of human erythrocytes and %MetHb was considered a reliable indicator.[27,34]

Previous studies have identified various mechanisms by which chemical agents can elicit elevated levels of MetHb under physiological and *in vitro* settings.[31] Generally, MetHb production can occur through 2 main processes: direct or indirect oxidation of haemoglobin. In this context, the chemical agent involved is either an oxidant or a pro-oxidant.[35] Additionally, MetHb production can occur when the pathways that reduces MetHb are disrupted, which involves NADH-cytochrome *b5* and NADPH-flavin reductase system, largely so-called NADH-methaemoglobin reductase activity as well as related redox enzymes and low molecular weight antioxidants.[32,36] In a related *in vitro* study, according to Raza *et al.*,[16] incubating isolated HepG2 cells with 5 and 10 µmol/mL ASA for between 24 and 48 hr caused a reduction in cellular levels of GSH with a concomitant increase in ROS generation.

On the contrary, previous reports have shown that ASA did not directly oxidise haemoglobin *in vitro*.[37] However, ASA can acetylate haemoglobin *in vitro* and *in vivo*, which is a different chemical process from oxidation.[37] The oxidation of haemoglobin in the presence of ASA only occurs when combined with an oxidising agent like H_2O_2.[38] In a similar manner, *in vitro* studies showed that primaquine oxidised haemoglobin in the presence of H_2O_2, $O_2\bullet^-$ and $^{\bullet}OH$ and metal ions[39] and H_2O_2 has been largely implicated in MetHb generation.[40] Specifically, ASA on its own did not significantly oxidise haemoglobin or GSH at a concentration of 10 mM,[38] which is much higher than the concentrations of ASA used in this study. By implication, the increased levels of MetHb observed in a pattern that depended on the concentration of ASA and the duration of exposure of human erythrocytes suggest that ASA did not directly oxidise haemoglobin. Rather, ASA may have disrupted the NADH-methaemoglobin reductase pathway necessary to reduce MetHb, thereby elevating the %MetHb in the present *in vitro* setting. From this standpoint Durak *et al.*[41] had previously reported that, in an *in vivo* setting, ASA impaired the antioxidant system of human erythrocytes, leading to alterations in their redox status.

Similarly, ASA, alongside the presence of H_2O_2 provoked membrane haemolysis through membrane lipid peroxidation

under physiological conditions.[38] Therefore; ASA can only induce haemolysis and oxidative changes in the presence of oxidative stress. The present study outcomes do not suggest that the erythrocytes incubated in the ASA were oxidatively stressed per se. This is because ASA alone did not lead to significant direct oxidation of haemoglobin since there was no H_2O_2 production in the presence of ASA in erythrocytes under *in vitro* settings as previously reported.[38] They also noted that ASA alone did not promote haemolysis *in vitro*, but the reverse was the case after the exposure of erythrocytes to 25 mg/100 ASA and 1.2% H_2O_2. In a related study, Kirkova *et al.*[42] had previously reported that relatively high ASA concentrations (>5.0 mM) inhibited the MDA formation, the membrane lipid peroxidation product, whereas the inhibitory action was promoted at relatively low ASA concentrations (<1.0 mM) activated by ASA-metal complexes in rat liver homogenate. They further noted that the biphasic dependence on the concentrations of ASA effects on MDA formation was engendered by ASA-metal complexes rather than ASA acting alone.

The result of the present study therefore implies that ASA alone appears not to have promoted membrane lipid peroxidation that could elicit haemolysis. Accordingly, the decreasing levels of MDA in the presence of ASA in a concentration and the time-dependent manner imply that ASA did not provoke membrane lipid peroxidation in human erythrocytes under *in vitro* settings. Consistent with the current findings, previous studies have shown that ASA inhibited membrane lipid peroxidation, as measured by TBA levels, in both *in vitro* and *in vivo* models.[43,44] Additionally, an overview of the current findings aligns with the reports of McMillan *et al.*[45] regarding drug-induced haemolytic anaemia. Their study previously confirmed that specific haemolytic agents elicited the generation of ROS within the erythrocytes but did not produce any evidence of membrane lipid peroxidation.

CONCLUSION

In conclusion, the present study showed a negative correlation between %MetHb and MDA levels in human erythrocytes incubated with ASA over time, suggesting that the erythrocytes redox status did not specifically induce membrane lipid peroxidation. Further studies will investigate the specific mechanisms underlying this relationship and its implications for erythrocyte function and oxidative membrane damage *in vivo*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

%MetHb: Percentage Methaemoglobin Concentration; **ASA:** Acetylsalicylic Acid; **COX:** Cyclooxygenase; **EDTA-Na_2:** Disodium ethylenediaminetetraacetic Acid; **MDA:** Malondialdehyde; **MetHb:** Methaemoglobin; **NSAID:** Non-Steroidal Anti-Inflammatory Drug; **PBS:** Phosphate Buffered Saline; **PUFAs:** Polyunsaturated Fatty Acids; **RONS:** Reactive Oxygen and Nitrogen Species; **TBA:** Thiobarbituric Acid; **TBARS:** Thiobarbituric Acid Reactive Substances; **TCA:** Trichloroacetic Acid.

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