



## **Evaluation of low-grade *In Vitro* Oxidative Stress in Goat Erythrocytes Exposed to Hydrogen Peroxide and Dexamethasone**

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### **ABSTRACT**

Under conditions of oxidative stress, erythrocytes of goats could be predisposed to haemolysis. This study was aimed at evaluating the effect of oxidant exposure to goat erythrocytes using an *in vitro* model. Blood samples from 10 goats were incubated with 0.06 – 0.18 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) either singly or in combination with 0.02 µM dexamethasone for 60 min, and erythrocyte parameters such as packed cell volume (PCV), red blood cell count (RBC), mean corpuscular volume (MCV) and percentage haemolysis in hypotonic sucrose solution (250 mOsmol/L) were determined thereafter. No significant changes in the mean values of all parameters were observed. The *in vitro* model indicated that erythrocyte parameters remained stable under low-grade oxidant exposure in goats. Therefore, plasma H<sub>2</sub>O<sub>2</sub> concentration of ≤ 0.18 mM, whether in the presence or absence of 0.02 µM dexamethasone, may not induce apparent oxidative damage in goat erythrocytes that could be estimated by PCV, RBC, MCV and sucrose-based osmotic fragility at low hypotonicity.

**Keywords:** Erythrocyte, Dexamethasone, Goat, Hydrogen Peroxide, Haemolysis, Oxidative stress

### **INTRODUCTION**

Oxidative injury contributes to the induction of eryptosis and aging processes in erythrocytes with shrinking of erythrocyte (Hale *et al.*, 2011; Lang *et al.*, 2014; Mohanty *et al.*, 2014; Lang and Lang, 2015) or may cause oncosis of erythrocytes when injury is severe with swelling of erythrocytes (Schildknecht and Vidal, 2004, Igbokwe, 2016). *In vitro* treatment of erythrocytes with hydrogen peroxide causes oxidative stress which increases production of by-products of lipid peroxidation when antioxidant capacity is overwhelmed (Igbokwe *et al.*, 1994, 1996). Hydrogen peroxide initiates the generation of hydroxyl radicals and subsequently propagates free radical accumulation in the cell with associated injury to the cell membrane (Gradinsky-Vrbanac *et al.*, 2002; Hale *et al.*, 2011; Lang *et al.*, 2014; Morabilto *et al.*, 2016) and followed by haemolysis due to disruption of the erythrocyte membrane (Reddy *et al.*, 2007; Qasim and Mahmood, 2015).

Glucocorticoids, such as cortisol, hydrocortisone, prednisolone, methylprednisolone and dexamethasone, have anti-inflammatory actions (Schimmer and Parker, 1996) with the capacity to induce oxidative stress and cell death by apoptosis (Torres-Roca *et al.*, 2000; Eid *et al.*, 2003, 2006; Jaramillo *et al.*, 2009). Glucocorticoids have the capacity to induce oxidative stress in erythrocytes by enhancing the production of reactive oxygen species (Bjelakovic *et al.*, 2017). Increased osmotic fragility of goat erythrocytes was presumed to be mediated by cortisol produced due to stress

after loading and transportation of goats (Minka and Ayo, 2010). Repeated injection of hydrocortisone resulted in increased osmotic fragility of rat erythrocytes, whereas an *in vitro* treatment of erythrocytes with 10<sup>-6</sup>M hydrocortisone caused a contrary effect (Nezhentsev, 1981). *In vitro* treatment with methylprednisolone and hydrocortisone prevented the decrease in erythrocyte membrane deformability, a variable closely related to osmotic fragility, during incubation (Rand *et al.*, 1977).

We hypothesized that an experimental model of *in vitro* exposure of goat erythrocytes to hydrogen peroxide and/or dexamethasone would affect the erythrocyte size through erythrocyte shrinking or swelling after oxidative stress, with subsequent effect on packed cell volume and the erythrocyte osmotic fragility. Therefore, the study evaluated the outcome of oxidative stress to goat erythrocytes after incubation of the blood with aliquots of hydrogen peroxide and dexamethasone using estimation of erythrocyte parameters and sucrose-based osmotic fragility test in order to determine whether these exogenous substances influenced erythrocyte size and membrane osmotic stability.

### **MATERIALS AND METHODS**

#### **Blood Sample Collection from Goats**

Blood sample (10 ml) was collected with syringe and needle from the jugular vein of each of 10 apparently healthy male Sahel goats, aged 2-4 years and weighing 15-37 kg. The

blood was anti-coagulated in heparinised sample bottles and transported on ice pack to the laboratory.

### In Vitro Incubation of Whole Blood with Hydrogen Peroxide alone or In Combination with Dexamethasone

Each blood sample from 5 goats (No. 1-5), after proper mixing, was also measured out with pipette and inserted into a set of 5 test tubes (T1-T5) so that each tube contained 1 ml and 0, 0, 2, 4, 6  $\mu$ l of 30 mM hydrogen peroxide were added to each tube to obtain concentrations of 0, 0, 0.06, 0.12 and 0.18 mM, respectively. Each blood sample from another 5 goats (No. 6-10), after proper mixing, was also measured out with pipette and inserted into another set of 5 test tubes (T6-T10) to contain 1 ml each, to which aliquots of 30 mM hydrogen peroxide were added as follows: 0, 0, 2, 4, 6  $\mu$ l corresponding to blood mixture concentrations of 0, 0, 0.06, 0.12, 0.18 mM, meaning that T6 and T7 received no aliquot and served as negative controls for hydrogen peroxide treatment. Again, T6 received no aliquot of dexamethasone (negative control), but T7-T10 also received 2  $\mu$ l each of 10  $\mu$ M dexamethasone amounting to a blood mixture concentration of 0.02  $\mu$ M. The blood samples were incubated at room temperature ( $\approx$  35°C) for 60 minutes. After the incubation period, blood samples in T1-T10 were gently mixed and taken for determination of erythrocyte parameters and estimation of osmotic fragility.

### Determination of Erythrocyte Parameters

Packed cell volume (PCV) was determined by microhaematocrit method. The erythrocyte or red blood cell (RBC) count was estimated using a haemocytometer. The mean corpuscular volume (MCV) was calculated with standard formula (Schalm *et al.*, 1975).

### Estimation of Sucrose-based Osmotic Fragility

Blood sample (5 $\mu$ l) was inserted into each of 3 test tubes (FT1-FT3) containing 5ml of distilled water (FT1), 5ml of hypotonic sucrose solution (FT2; 250 mOsmol/L) or 5ml of isotonic sucrose solution (FT3; 308 mOsmol/L) and allowed to incubate for 30 minutes at room temperature ( $\approx$  35°C). The tubes were centrifuged at 3000 x g for 15 minutes, and the supernatant harvested into cuvettes. The optical densities (OD) of the supernatants were read at 540 nm using a spectrophotometer (ALL PRO, Shibe, Qingdao, China) with the supernatant of FT3 as blank and FT1 as complete (100%) haemolysis. The percentage haemolysis for FT2 was calculated as ratio of OD of FT2 to FT1 in percentage as previously reported (Igbokwe and Igbokwe, 2016).

### Statistical Analysis

Data were summarized as means  $\pm$  standard deviations and variations among means were assessed by one-way analysis of variance with Tukey post-test using computer software for statistics (GraphPad® InStat®, version 3.05, 1992–2000, GraphPad Software Incorporated, USA).

### Ethical Statement

This experiment was carried out in accordance with internationally accepted guidelines on care and use of animals in research.

### RESULTS AND DISCUSSION

The *in vitro* effects of hydrogen peroxide alone or in combination with dexamethasone treatments on the goat erythrocyte parameters and osmotic fragility are presented in Tables 1-4. The variations in the mean values of erythrocyte parameters and osmotic fragility estimates among treatment groups after incubation were not significant ( $p > 0.05$ ).

The blood samples used in the study had erythrocyte parameters and osmotic fragilities comparable to those reported for apparently healthy Sahel goats (Glaji *et al.*, 2014; Igbokwe and Igbokwe, 2016). In this study, no significant variations in the erythrocyte parameters (PCV, RBC, MCV) and osmotic fragility were observed following *in vitro* treatments of goat erythrocytes with hydrogen peroxide and dexamethasone. The PCV did not decrease because of lack of *in vitro* haemolysis induced by oxidative damage of erythrocytes and there was no increase in osmotic fragility from destabilized plasma membrane of the erythrocytes. Moderate injury, which could have caused swelling or shrinking of the erythrocytes with associated alteration of erythrocyte size estimated by MCV, was not observed. The finding suggests that low-grade oxidant exposure in goats might not have affected the erythrocytes adversely by overwhelming the normal physiological adaptive mechanisms in oxidative stress. Hydrogen peroxide causes haemolysis through oxidative stress and membrane damage (Gradinsky-Vrbanac *et al.*, 2002; Hale *et al.*, 2011; Lang *et al.*, 2014; Qasim and Mahmood, 2015; Morabilto *et al.*, 2016); and dexamethasone, a glucocorticoid, has the capacity to enhance oxidative stress in erythrocytes (Bjelakovic *et al.*, 2017). When the plasma is removed and washed erythrocytes are exposed to H<sub>2</sub>O<sub>2</sub> at concentration of 1.0 mM (Srouf *et al.*, 2000; Wlodek and Kusior, 2006), erythrocyte destabilization and increased osmotic fragility have been reported to occur. In this model, the erythrocytes were exposed to the oxidants in the whole blood, which was assumed to be close to the *in vivo* setting of the animal.

**Table 1:** Effect of incubation of goat blood with added hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on the erythrocyte parameters

Erythrocyte parameters*	<i>In vitro</i> treatment with H <sub>2</sub> O <sub>2</sub> (mM)			
	0.00	0.06	0.12	0.18
Packed cell volume (%)	28.4 $\pm$ 2.0	27.6 $\pm$ 1.5	28.0 $\pm$ 0.7	28.4 $\pm$ 1.5
RBC (x10 <sup>6</sup> / $\mu$ l)	13.6 $\pm$ 0.5	12.2 $\pm$ 1.1	12.6 $\pm$ 0.8	12.6 $\pm$ 0.8
MCV (fl)	20.7 $\pm$ 0.9	23.9 $\pm$ 3.4	22.2 $\pm$ 1.4	22.5 $\pm$ 1.8

\*No significant ( $p > 0.05$ ) variation in parameters (means  $\pm$  standard deviations)

**Table 2:** Osmotic fragility of goat erythrocytes in hypotonic sucrose solution (250 mOsmol/L) after incubation of the blood with added hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)

<i>In vitro</i> Treatment with H <sub>2</sub> O <sub>2</sub> (mM)	Haemolysis (%)*
0.00	4.8±5.9
0.06	3.0±3.6
0.12	6.0±9.7
0.18	5.1±4.3

\*No significant ( $p > 0.05$ ) variation in parameter (means  $\pm$  standard deviations)

**Table 3:** Effect of incubation of goat blood mixed with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and dexamethasone (Dexa) on the erythrocyte parameters

Erythrocyte parameters*	<i>In vitro</i> treatment				
	H <sub>2</sub> O <sub>2</sub> (mM)				
	0.00	0.00	0.06	0.12	0.18
	Dexa ( $\mu$ M)				
	0.00	0.02	0.02	0.02	0.02
PCV (%)	29.80±1.30	29.00±1.73	30.00±1.41	29.20±1.10	29.20±1.10
RBC ( $\times 10^6/\mu$ l)	13.72±0.49	12.66±0.72	13.60±0.34	12.92±0.58	13.01±0.78
MCV (fl)	21.72±0.72	22.99±2.31	22.07±1.14	22.63±1.14	22.40±1.57

\*No significant ( $p > 0.05$ ) variation in parameters (means  $\pm$  standard deviations)

**Table 4:** Osmotic fragility of goat erythrocytes in hypotonic sucrose solution (250 mOsmol/L) after incubation of the blood with added hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and dexamethasone (Dexa)

<i>In vitro</i> Treatment		
H <sub>2</sub> O <sub>2</sub> (mM)	Dexa ( $\mu$ M)	Haemolysis (%)*
0	0	2.51±2.03
0	0.02	2.57±0.93
0.06	0.02	1.60±1.51
0.12	0.02	2.54±2.90
0.18	0.02	2.34±1.51

\*No significant ( $p > 0.05$ ) variation in parameter (means  $\pm$  standard deviations)

The plasma contains an array of antioxidants like albumin (Wlodek and Kusior, 2006), vitamins (Lightbody *et al.*, 2001), and plant-derived phytochemicals (Ajila and Rao, 2008; Oyebanji *et al.*, 2015; An *et al.*, 2016); in addition, the erythrocytes contain antioxidants in the cytosol and plasma membrane (Wlodek and Kusior, 2006). Unless oxidant effects overwhelm these protective mechanisms, oxidative stress may not be evident (Igbokwe *et al.*, 1994, 1996; Cooper, 2002; Myers *et al.*, 2012) and erythrocyte parameters are not likely to be affected.

The exposure of the erythrocytes to oxidants did not alter the erythrocyte size and osmotic fragility because dynamic factors that stimulate mobilization of water from or into the erythrocyte cytoplasm might not have taken place due to counteraction of the oxidative stress in the whole blood. Oxidative stress, associated with glucocorticoid surge during transport and environmental stress, has been reported to induce variation in erythrocyte size with subsequent haemolysis indicated by increased erythrocyte osmotic fragility (Minka and Ayo, 2010), but a review of the effects of oxidant-related factors on erythrocyte osmotic fragility of mammals indicated that this parameter may be increased or decreased (Igbokwe, 2018). A decreased osmotic fragility is an indication of osmotic resistance and may not be detected at low hypotonicity (250 mOsmol/L) of sucrose (Igbokwe, 2016). Low-grade toxicant exposure of goat erythrocytes caused decreased osmotic fragility at high hypotonicity (100 mOsmol/L) of sucrose, and it was suggested that an eryptotic

injury causing shrinking of erythrocytes could have been the reason (Igbokwe, 2016). Low-grade oxidative stress may possibly induce eryptotic injury that our experimental model could not detect. However, it is important to note that eryptotic erythrocytes do not undergo *in situ* haemolysis, but may be eliminated by erythrophagocytosis into macrophages as a result of alterations of the plasma membrane surface (Repsold and Joubert, 2018; Bissinger *et al.*, 2019).

In conclusion, a plasma H<sub>2</sub>O<sub>2</sub> concentration of up to 0.18 mM, whether in the presence or absence of 0.02 $\mu$ M dexamethasone, may not induce oxidative damage in goat erythrocytes that could be measured by effects on PCV, RBC, MCV and osmotic fragility.

#### Author's Contribution

MBM and IOI conceived the research, designed the experiment, analysed the data and wrote the final draft of the manuscript. ESP and ICU performed the experiments and wrote the first drafts with the supervision of MBM and IOI.

#### Conflict of Interest

The authors have no conflict of interest to declare.

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