UNIVERSITI PUTRA MALAYSIA

EFFECT OF SELENIUM ON OXIDATIVE STRESS IN GOATS

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EFFECT OF SELENIUM ON OXIDATIVE STRESS IN GOATS

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It is hereby certified that we have read this project paper entitled “Effect of Selenium on Oxidative Stress in Goats”, by Agnes Ng Wei San and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD4999 – Final Year Project.

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DEDICATION

To,

my dearest family and friends.

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CONTENTS

Page No.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
dH_2O	distilled water
DIW	deionized water
DTNB	Ellman's solution/5,5-dithiobis-2-nitrobenzoic acid
EDTA	ethylenediaminetetraacetic acid
GSH	glutathione
GSH-Px	glutathione peroxidase
GSH-Rx	glutathione reductase
GSSG	reduced glutathione
Hb	haemoglobin
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
HPO_3	meta-phosphoric acid
H_2SO_4	sulphuric acid
MDA	malondialdehyde

NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	disodium phosphate
Na_2WO_4	sodium tungstate
O_2^-	superoxide anion
OH^-	hydroxyl
RBC	red blood cell
ROS	reactive oxygen species
Se	selenium
SE	standard error
SH	sulfhydryl
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
TEP	tetraethoxypropane
Tris-HCl	tris-hydrochloric acid

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901- Project.

KESAN SELENIUM PADA TEKANAN OKSIDATIF DALAM KAMBING

Oleh

AGNES NG WEI SAN

2015

Penyelia: Prof. Dr. Noordin Mohamed Mustapha

Tujuan kajian ini adalah untuk menentukan kesan daripada pemberian selenium (Se) terhadap tegasan oksidatif (malondialdehid, superoksid dismutase dan glutation peroksidase) dan prestasi pertumbuhan kambing. Dua belas anak kambing selepas cerai susu dibahagikan kepada kumpulan kawalan dan rawatan. Kumpulan kawalan disuntik secara subkutis 2ml normal salin manakala kumpulan rawatan menerima suntikan 100mg natrium selenit. Pengumpulan darah dilakukan sebelum rawatan dan diulangi selama empat kali dalam tempoh 3-4 hari. Tiada perbezaan keertian pada purata pertambahan berat badan harian, aras malondialdehid, superoksid dismutase dan glutation peroksidase antara kumpulan rawatan dan kumpulan kawalan. Kesimpulannya, adalah Se bentuk ramuan campuran tidak berkesan sebagai suplemen Se dalam mengurangkan tegasan oksidatif dan pengalak pertumbuhan kambing.

Kata kunci: Selenium, tekanan oksidatif, antioksidan

ABSTRACT

Abstract of project paper presented to Faculty of Veterinary Medicine in partial requirement for the course VPD4999- Project.

EFFECT OF SELENIUM ON OXIDATIVE STRESS IN GOATS

By

AGNES NG WEI SAN

2015

Supervisor: Prof. Dr. Noordin Mohamed Mustapha

The aim of this study is to determine the effect of selenium (Se) supplementation on oxidative stress status (malondialdehyde, superoxide dismutase and glutathione peroxidase) and growth performance in goats. Twelve post-weaning kids were divided into a control and treatment group. While the control group was injected with 2ml of normal saline, the treatment group was subcutaneously injected with 100mg sodium selenite. Blood collection was done before injection and these were repeated four times within a 3-4 day interval. There was no significant difference on the average daily gain, blood malondialdehyde, superoxide dismutase and glutathione peroxidase levels between the treatment and control group. It is concluded that supplementation of Se in a

mixture concoction is not effective in reducing oxidative stress and promoting growth in kids.

Keywords: Selenium, oxidative stress, antioxidant

1. INTRODUCTION

The acidic soil of Malaysia with a range of pH from 3 to 5 (Shamshuddin *et al*., 2011) can form insoluble selenium (Se) complexes with iron hydroxide fixing it strongly in the soil leading to low uptake by pastures (Lyons *et al.,* 2007). This will decrease the bioavailability of Se to animals. In contrast, Se in soils with high pH will be present mainly as selenite which is soluble and easily available in plants.

Plants are a major source of Se in animals where other factors affecting Se availability to plants include rate of artificial fertilization and rainfall (Lyons *et al*., 2007). Although Se deficiency can occur in all animal species, ruminants appear to be more susceptible (Hefnawy & Tórtora-Pérez, 2010). Absorption of Se is much lower in ruminants than in non-ruminants. Low absorption of Se in ruminants is believed to result from modification in the strong reducing rumen environment, in which dietary Se is converted to insoluble forms such as selenides (Ahmad & Dakshinkar, 2012).

Therefore, the objective of this study is to determine performance of Se supplemented and non-supplemented goats under a small holder system, with emphasis on their:

i. status of antioxidant enzyme (glutathione peroxidase, superoxide dismutase), peroxidation (malondialdehyde) concentrations
ii. growth performance via average daily body weight gain

2. LITERATURE REVIEW

Selenium (Se)

Selenium is an essential trace element vital for the normal growth and health of animals (Pechova *et al*., 2012) acting centrally as anti-oxidants and growth promoter. Biological functions of Se are mediated through its presence in selenoprotein, for example glutathione peroxidase (GSH-Px). In a study by Kumar *et al*. (2009), Se supplementation resulted in improved performance antioxidant status, growth rate and humoral immune response in lambs. Domestic animals require between 0.1 to 0.3 ppm of Se in their diet. Selenium deficiency has been linked to many health problems in animals such as increased neonatal mortality, higher occurrence of infectious diseases, reproductive disorders and white muscle disease (Hefnawy & Tórtora-Pérez, 2010; Ramirez-Bribiesca *et al*., 2004). The immunostimulant effect of Se and its role in the metabolism of thyroid hormones are mechanism through which Se might influence livestock health and production (Hefnawy & Tórtora-Pérez, 2010).

Free Radicals and Reactive Oxygen Species (ROS)

Free radicals are molecules that contain one or more unpaired electron in the outer orbit (Halliwell and Gutteridge, 2007). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Free radicals with unpaired electron act like electron acceptors and 'steal' electrons from other molecules. Free radicals are therefore

called oxidising agents or oxidants as they tend to make other molecules donate their electrons (Kooter, 2004).

Reactive oxygen species (ROS) are free radicals derived from molecular oxygen. Well known ROS include superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) which are formed during the intermediate steps of oxygen reduction by reduction of one, two, three electrons respectively (Guerin, 2001). Low levels of ROS can facilitate several physiological processes, however when produced in excess, ROS can damage cell functionality.

Exogenous sources of ROS that can contribute directly or indirectly to the total oxidant load include effects of ionising and non-ionising radiation, air pollution and natural toxic gases, such as ozone, as well as chemicals and toxins (Lykkesfeldt and Svendsen, 2007). The most abundant endogenous source of oxidants is the respiratory chain in the mitochondria that converts molecular oxygen to water where a few percent of oxygen molecules continuously leaks from the electron transport chain as ROS intermediates (Lykkesfeldt & Svendsen, 2007). Immune reactions may also contribute considerably to the generation of oxidants. During respiratory burst, activated neutrophils release ROS produced enzymatically by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. These ROS released intended to target foreign pathogens, but the lack of specificity in these reactions also results in tissue damage to the host (Kooter, 2004; Lykkesfeldt & Svendsen, 2007).

Oxidative Stress

The disturbed balance in which the generation of ROS in a system exceeds the system ability to eliminate them is known as oxidative stress (Stanczy *et al.*, 2005). It is also commonly defined as an imbalance between oxidants and reductants (antioxidants) at the cellular or individual level. This can cause damage to cellular lipids, proteins or DNA and subsequent death.

Lipid peroxidation

Lipids, in particular those that are polyunsaturated are prone to oxidation. Lipid peroxides causes phospholipid degradation, membrane injury which can cause rupture of the cell. Lipid oxidation is self-propagating in cellular membranes. In this process, polyunsaturated fatty acids are attacked by ROS causing a subtraction of hydrogen leading to formation of a lipid radical. This radical then quickly reacts with molecular oxygen to produce lipid peroxyl radical capable of oxidising a neighbouring lipid and thus propagating the oxidative damage (Lykkesfeldt & Svendsen, 2007).

Malondialdehyde (MDA)

Biomarkers of lipid peroxidation are easily measured and considered the best indicators of oxidative stress (Georgieva, 2005). The MDA is one of the end products of lipid peroxidation which readily reacts with thiobarbituric acid producing a red pigment that can be easily measured by spectrophotometry in the form of thiobarbituric acid reactive substances (TBARS) (Janero, 1990).

Rezaei and Dalir-Naghadeh (2009) has shown the association between MDA and cardiovascular diseases induced by acute Se deficiency in lambs. Their study revealed that MDA level was higher in affected lambs compared to healthy lambs due to higher oxidative stress. Similar finding was found whereby sheep with higher oxidative stress as a result of *Dicrocoelium dendriticum* infestation have higher MDA level than control group (Simsek *et al.*, 2006).

Antioxidant

Antioxidants can be defined as any substance that delays, prevents or removes oxidative damage to target molecules (Halliwell & Gutteridege, 2007). Antioxidants donate electrons to oxidants to stop their reactivity making them harmless to cellular macromolecules. The antioxidants thereby become radicals themselves, but these are far more stable and thus not capable of inducing cellular damage.

Endogenous antioxidants can be divided into three major groups, ie. enzymatic antioxidants, non-enzymatic protein antioxidants and non-enzymatic low-molecular weight antioxidants (Celi, 2010). Enzymatic antioxidants are the main form of intracellular antioxidant defense and often possess specialized functions. They include superoxide dismutase (SOD), glutathione-peroxidase (GSH-Px) (Celi, 2010). Non-enzymatic protein antioxidants are primarily found in plasma and are mainly represented by sulfhydryl (SH) groups of albumin. They are considered a significant element of the extracellular antioxidant defense system (Ueland *et al*., 1996). Non-enzymatic low molecular weight antioxidants which include vitamins C and vitamin E and glutathione

(GSH) are found mainly in plasma but also in other extracellular and intracellular fluids (Kooter, 2004).

Superoxide Dismutase

This enzyme is considered as the first defense against attack of free radicals (Pincemail *et al*., 2009). There is two different forms of SOD, ie. copper-zinc superoxide dismutase which located in cytosol and manganese superoxide dismutase which located in mitochondria (Guerin *et al*., 2001). The SOD catalyses the dismutation of two molecules of O_2 into one molecule of dioxygen and one of H_2O_2 (Kooter, 2004; Lykkesfeldt & Svendsen, 2007). Since SOD activity increases H_2O_2 production, protection from reactive oxygen would only be given by a simultaneous increase in catalase, GSH-Px activities and availability of glutathione.

$2O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$

Study by Rezaei and Dalir-Naghadeh (2009) showed that lambs with nutritional muscular deficiency due to Se deficiency had lower SOD activity compared to healthy lambs.

Glutathione peroxidase

A selenoenzyme found in the cytosol and mitochondria. In fact, determination of cellular or plasma GSH-Px activity is often used as a diagnostic tool when assessing Se status of animals (Sordillo & Aitken, 2009). Parenteral injection of Se increased the GSH-Px level in Se deficient goats (Ramirez-Bribiesca *et al*., 2004). Alternatively,

Rezaei and Dalir-Naghadeh (2009) also showed decreased in GSH-Px level in Se deficient lambs. As a part of the glutathione (GSH) oxidation-reduction cycle, GSH-Px acts by reducing H_2O_2 to water and produces reduced glutathione (GSSG) and water (Kooter, 2004).

$2GSH + H_2O_2 \rightarrow H_2O + GSSG$

The GSSG is then reduced by glutathione reductase (GSH-Rx) with NADPH as the electron donor.

$GSSG + NADPH \rightarrow 2GSH + NADP^+$

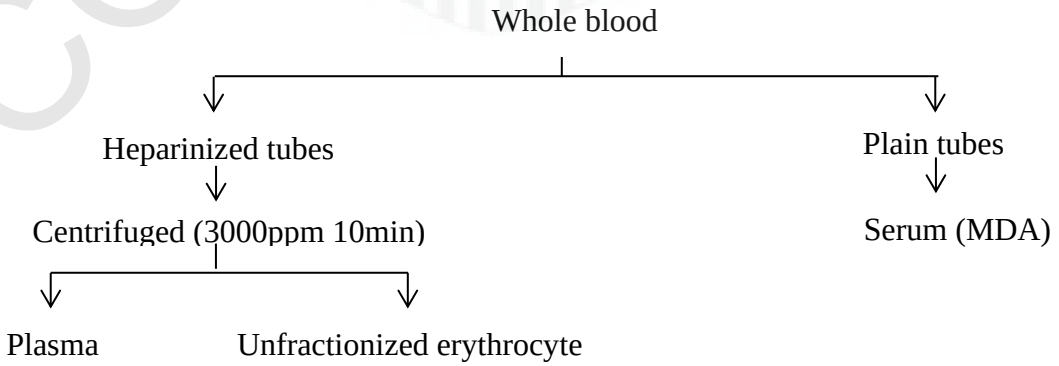
3. MATERIALS AND METHODS

Animals, Management and Experimental Design

Twelve male post-weaning goats aged between 4-5 months were selected from a goat farm. These animals were weighed and divided into two groups, ie control group and treatment group with six goats each. About 3ml of blood was collected into heparinized tube and another 3ml into plain tube by jugular venipuncture from both groups of animals. The treatment group was then injected with 2ml of 100mg selenite subcutaneously while the control group was given 2ml of normal saline subcutaeneously. Weighing, subcutaeneuos injection and blood collection were repeated for another four times at 3-4 days interval. The blood collected was subjected to SOD, GSH-Px and MDA concentration analysis.

General sample processing

The blood samples were processed according to the protocols outlined in Figure 3.1.



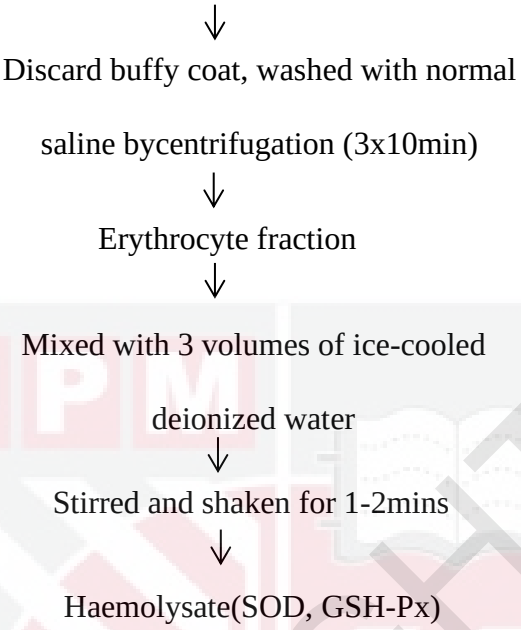


Figure 3.1 Schematic diagram depicting blood sample processing and analyses.

MDA concentration

The measurement of MDA concentration was done following the thiobarbituric acid reactive substance (TBARS) assay as described by Zhang (2000). A mixture was obtained firstly by adding 2.4ml of 1/12 H_2SO_4 and 0.3ml of 10% sodium tungstate (Na_2WO_4) to 0.3ml of serum and centrifuged (3500rpm, 10mins) after being kept at room temperature for 10 minutes. Then the supernatant was thoroughly removed by tapping tubes upside-down on several layers of absorbent paper. The reactive mixture was formed by adding 0.5ml of distilled water, 3.0ml of 0.05N HCl and 1.0ml of 15 thiobarbituric acid (TBA), and made up to 5ml with distilled water. The well-mixed mixture was then kept in water bath at 95°C for 60 minutes within centrifuge tube

covered with glass marble. After cooling, the mixture was again centrifuged and the supernatant layer was retrieved and its absorbancy was measured at 532nm.

For the standard control, 0.5ml of distilled water, 3.0ml of 0.05N HCl and 1.0ml of 1% TBA were added to 0.3ml of 10mmol/L tetraethoxypropane (TEP). The absorbancy was measured following heating as described earlier. The concentration of MDA was expressed as mmol/ml of serum.

Nmol/ml= (AS/BS) / (VS/VT)
AS= absorbance of sample
AB= absorbance of the standard control
VS= volume of plasma or serum
VT= total volume in cuvette(ml)

SOD activity

The SOD activity was measured by pyrogallol oxidation inhibition assay as described by Zhang (2000) which consisted of 3 major steps:

a) A chloroform-ethanol extract was prepared by adding known volumes of hemolysate to 1.5ml of ice-cold distilled water, 0.5ml of absolute ethanol and 0.3ml of chloroform. The solution was mixed well during each addition and shaken for 1 minute after the last addition. The clear top layer as extract was collected for further analysis after centrifugation.

b) A pre-active mixture was formed by adding 75µl chloroform-ethanol extract to 2.25ml tris-HCl buffer, followed by 2.0ml deionized water, mixed well and left in 25°C water bath for 10mins. The oxidation reaction was initiated by adding a pre-warmed 0.15ml of 3mmol/l pyrogallol solution.

c) The rate of spontaneous oxidation of the mixture was measured immediately with UV/visible spectrophotometer. The measurement was done at 330nm at temperature of 25°C for 1 minute after a delay for 30 seconds. The rate of spontaneous oxidation was expresses as the absorbancy difference per minute (ΔA/min). A solution of 20% ethanol was used to act as blank control and measured as described earlier.

One unit of SOD activity was stipulated as the amount of enzyme that can make a 50% spontaneous oxidation inhibition per minute per milligram of Hb. The enzyme activity was expressed as units per milligram of Hb (U/mg Hb) to erythrocyte-SOD (E-SOD).

GSH-Px activity

The activity of GSH-Px in RBCs was measured by the DTNB direct method as described by Zhang (2000). The activity of GSH-Px in erythrocytes was measured by the DTNB direct methods. Firstly, 100µl of hemolysate was added to 0.4ml of 1.0mmol/L glutathione. The mixture was mixed well and pre-warmed at 37°C in a water bath for 5 minutes. The final mixture was kept at 37°C in a water bath and allowed to react accurately for 3 minutes. The reaction was initiated by adding 0.2ml of 1.3-1.3mmol/L pre-warmed H_2O_2 to the mixture, and stopped sharply by adding 4ml of 1.67%

meta-phosphoric acid solution. Secondly, the well-mixed solution was centrifuged at 3000rpm for 10mins, then 2.0ml of the supernatant was taken and added to 2.5ml of 0.32ml/L disodium phosphate solution. Finaly, the absorbancy of the final solution was measured within 5 minutes at 422nm after adding 0.5ml of DTNB solution.

Standard controls were measured following the same procedure but the sample was replaced with deionized water. The absorbancy of the mixture of 0.4ml deionized water and 1.6ml of 1.67% meta-phosphoric acid solution was measured and used as blank control.

The enzyme activity unit was defined as the decline in amounts of Log(GSH) per minute per gram of Hb after subtracting the decline amounts of Log(GSH) of standard control, and expressed as units per gram of haemoglobin (U/g Hb).

$$\text{Unit} = \frac{\text{Log (AS-AB)} - \text{Log(AT-AB)}}{3 \times 0.004}$$

Statistical Analysis

Data were expressed as mean± standard error and subjected to with independent t-test and repeated measures analysis of variance (SPSS version 16.0).

4. RESULTS

The concentration of the MDA is presented in Table 4.1 There exists fluctuation of MDA in both the control and treatment groups throughout the experiment. The MDA concentration increased from the commencement of the study until Day 7 which then decreased at Day 10 and remained constant since thereafter. However, no statistical significance ($p>0.05$) was found between and within group throughout the experimental period.

Table 4.1: Mean ± SE of MDA level (nmol/mL) in peripheral blood of control and treated goats

Day / Group	Day 0	Day 3	Day 7	Day10	Day 14
Control	1.4±0.21*,a	1.8±0.36*,a	2.4±0.35*,a	1.3±0.21*,a	1.3±0.23*,a
Treatment	2.2±0.67*,a	1.9±0.20*,a	2.6±0.80*,a	1.0±0.32*,a	1.9±0.26*,a

Means with different superscripts symbol between rows differ significantly ($p<0.05$).
Means with different superscripts letters between columns differ significantly ($p<0.05$).

The concentrations of blood SOD are shown as in Table 4.2. Similarly as seen in the MDA concentrations, fluctuations of SOD also occurred during the experimental period. Both control and treated goats showed a decrease in SOD concentration at the beginning, which later remained constant at mid-way and increase towards the end of the study.

These fluctuations did not yield any significant differences ($p>0.05$) between and within groups throughout the experimental period.

Table 4.2: Mean ± SE of SOD level (U/mgHb) in peripheral blood of control and treated goats.

Day / Group	Day 0	Day 3	Day7	Day 10	Day 14
Control	2.3±1.33*,a	2.1±0.84*,a	1.4±0.42*,a	1.6±0.69*,a	3.0±0.45*,a
Treatment	3.00±1.52*,a	1.1±0.42*,a	1.1±0.29*,a	2.0±0.47*,a	1.9±0.70*,a

Means with different superscripts symbol between rows differ significantly ($p<0.05$).
Means with different superscripts letters between columns differ significantly ($p<0.05$).

The blood concentration of GSH-Px is as shown in Table 4.3 Fluctuations in concentrations of GSH-Px resembled those seen in MDA and SOD. Both control and treatment groups had decreasing GSH-Px concentration from commencement, followed by an increase during the mid-stage and a decrease at the end of the study. Although there was comparable GSH-Px concentration between group at all intervals, that of the within the treated group showed significant differences.

The highest concentration of GSH-Px was seen at Day 10 which is significantly higher than those at Day 3, 7 and 14.

The statistical analysis of average daily gain is presented in Table 4.4. There was no significant difference in the average daily gain between control and treated goats ($p>0.05$). Correlation test was done for average daily gain and blood GSH-Px levels and

statistical results showed no significant correlation (r=0.205) between average daily gain and GSH-Px concentrations (p>0.05).

Table 4.3: Mean ± SE of GSH-Px level (U/gHb) in peripheral blood of control and treated goats.

Day / Group	Day 0	Day 3	Day7	Day 10	Day 14
Control	40.8±8.19*,a	23.6±6.78*,a	40.0±5.77*,a	53.3±7.66*,a	30.9±10.12*,a
Treatment	57.2±10.60*,a	17.7±5.88*,b	26.6±15.24*a,b	56.6±5.22*a,c	19.3±8.33*,a,b

Means with different symbols between rows differ significantly (p<0.05).
Means with different superscripts letters between columns differ significantly (p<0.05).

Table 4.4: Mean ± SE of average daily gain of control and treated goats.

Group	**Mean ± SE**
Control	0.111±0.024*
Treatment	0.107 ±0.020*

Means with different superscripts symbol between rows differ significantly (p<0.05).

5. DISCUSSION

The fluctuation of MDA concentration is probably due to handling. The stress may have earlier resulted in an increase level, while following acclimatisation later, the MDA level has begun to drop. A similar scenario is also seen with SOD, which decreased from Day 0 to Day 7 and then increased after Day 7. It is likely that handling stress causes an early drop in SOD levels and getting use to the procedure, has led to a much higher level of SOD levels. Alternatively, with an increase in MDA, much more SOD would have been utilised to combat or prevent a worsened oxidative stress status leading to its drop in the initial stages. A similar trend in GSH-Px concentrations is also seen offering an almost similar explanation as seen with SOD especially in reducing build-up of lipid peroxides and to reduce H_2O_2.

It appears in this study has failed to yield expected effect of Se supplementation especially with regards to the GSH-Px concentration and body weight gain. This can be due to the Se used which is a concoction containing vitamin B12, adenosine triphosphate tetrasodium dehydrate salt, potassium aspartate semihydrate and magnesium aspartate tetrahydrate. These compounds may have a negative or antagonizing effect with Se or a much higher dose of Se is required when this preparation is used (Young & Lowe, 2001). Study by Chen *et al.* (1993) found that vitamin B12 increases Se methylation and excretion resulting in lower selenium concentration and activity. Besides that, the animals were not absolutely selenium deficient which make the effect of selenium not significant. Additionally, the absence of

vitamin E in this preparation may have also contributed to lack of effectiveness of Se supplementation. These two elements interact synergistically and have sparing effect, in which Se destroys peroxidase before they attack cell membranes while vitamin E act within the membrane to prevent the formation of fatty acid hydroperoxides. A study by Hamam & Hala (2007) showed better effect of vitamin E and Se on natural antioxidant and immunity compared to Se alone. This explained why Se supplementation alone in our study showed no significant effects.

Other possible factors include the early allotment of goats into the respective groups. A different set of results may appear if the goats we grouped according to bodyweight and GSH-Px status. Such allocation or group would have minimize the experimental error leading to a much lower standard error and thus a much more well distributed means between and within groups.

Since there is no correlation between the average daily gain and GSH-Px, we can again say that the effect of Se alone in concoction preparation is not enough to alleviate oxidative stress to improve the growth performance. However, Se in this preparation is somehow affecting the GSH-Px causing significant difference in GSH-Px level within the treatment group. This is due to Se is an integral part of GSH-Px, mainly in the form of selenocysteine (Hefnawy & Tórtora-Pérez, 2010). This finding is also supported by Koller *et al*. (1984) who found out a strong relationship between erythrocyte GSH-Px activity and blood Se concentration.

6. CONCLUSION

In short, antioxidant SOD and GSH-Px function simultaneously to remove free radicals to mop excessive MDA concentrations. Selenium, as part of antioxidant enzyme plays a role in reducing oxidative stress. However, supplementation of Se in a mixture concoction is not effective in reducing oxidative stress and promoting growth in kids.

7. RECOMMENDATIONS

In future study, it is recommended to supplement pure Se rather than a mixture preparation to get a better and obvious effect. Two different types of antioxidant can be used such as Vitamin E and Se to enhance the antioxidant function. The duration of the study can be prolonged in order to get a better result on the antioxidant status with a much bigger sample size and assignment to groups based on bodyweight and GSH-Px prior to commencement of the study.

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APPENDICES

Appendix I (MDA assay)

1. 1/12 H_2SO_4

   8.33ml of H_2SO_4 topped up to 100ml with dH_2O

2. 10% Na_2WO_4

   5g $N_{a2}WO_4$ dissolved to 50ml of dH_2O

3. 0.05M HCl

   1.03ml 37% HCl topped up to 250ml with dH_2O

4. 1% TBA

   1g TBA issolved to 100ml of dH_2O

5. 10nmol/L TEP (standard control)

   22.7 µl 97% TEP topped up to 1000ml dH_2O

Appendix II (SOD assay)

1. 0.5M Tris-HCl + 1mM EDTA buffer pH8.0

   i) 78.8g Tris-HCl added to 450ml DIW and pH adjusted to8.0

   ii) 0.292g EDTA added to 450ml DIW

   (i)and (ii) were mixed and topped up to 1000ml and was adjusted to pH8.0 at room temperature

2. 10mM HCl

   82 µl of 37% HCl topped up to 100ml with DIW

3. 3mmol/L pyrogallol

   0.0378g dissolved in 10nM HCl to 100ml

4. 20% v/v ethanol (blank)

   20ml absolute ethanol diluted with DIW to 100ml

Appendix III (GSH-Px assay)

1. 1mM GSH (MW=307.33)

   0.307g GSH topped up to 100ml withDIW

   (prepared fresh and kept cold until used)

2. i) 0.13-0.15mmol/L H_2O_2 stock solution

   1.7ml of 30% H_2O_2 was added with dH_2O to 100ml

   ii) 1.3-1.5mmol/L H_2O_2 stock solution

   1ml of H_2O_2 stock solution was added with dH_2O to 100ml (prepared fresh)

3. 1.67% HPO_3 solution

   1.67g HPO_3 + 0.2g EDTA + 30g NaCl and all were dissolved with DIW to 100ml (chilled before use)

4. 0.32 mol/L Na_2HPO_4

   22.72g Na_2HPO_4 added to 500ml of dH_2O

5. DTNB colour generating solution

   0.04g DTNB + 1g sodium citrate were dissolved to 100ml of DIW (prepared fresh and stored at 0-5^0c, wrapped in aluminium foil)