UNIVERSITI PUTRA MALAYSIA

THE EFFECT OF SELENIUM SUPPLEMENT ON ANTI-OXIDANT STATUS AND AST CONCENTRATION IN BEEF CATTLE

ZHARIF ATIQ BIN HASHIM

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THE EFFECT OF SELENIUM SUPPLEMENT ON ANTI-OXIDANT STATUS AND AST CONCENTRATION IN BEEF CATTLE

ZHARIF ATIQ BIN HASHIM

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

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It is hereby certified that we have read this project paper entitled "The Effect of Selenium Supplement on Anti-Oxidant Status and AST Concentration in Beef Cattle", by Zharif Atiq bin Hashim and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 – Final Year Project.

PROF. DR. NOORDIN MOHAMED MUSTAPHA

DVM (UPM), MSc (UPM), PhD (Murdoch)

Lecturer

Department of Veterinary Pathology and Microbiology

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

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DEDICATION

To,

My dearest family and friends.

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LIST OF ABBREVIATIONS

dH_2O	distilled water
DIW	deionized water
DTNB	5, 5-dithiobis- 2- nitrobenzoic acid
EDTA	ethylenediaminetetraacetic acid
GSH- Px	glutathione peroxidase
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

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OH^-	hydroxyl
RBC	red blood cell
ROS	reactive oxygen species
Se	selenium
STDE	standard deviation
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
TEP	tetraethoxypropane

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti PerubatanVeterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek.

KESAN SUPLEMEN SELENIUM TERHADAP TAHAP ANTIOKSIDAN DAN KEUTUHAN OTOT PADA LEMBU PEDAGING

Oleh

Zharif Atiq bin Hashim

2016

Penyelia: Prof. Dr. Noordin Mohamed Mustapha

Selenium (Se) merupakan mikronutrien penting yang diperlukan untuk pertumbuhan normal dan pertahanan antioksidan. Objektif kajian ini adalah untuk menentukan kesan pemberian Se terhadap tekanan oksidatif (malondialdehid, MDA dan glutation peroksidase, GSH-Px) dan integriti otot pada lembu pedaging. Sepuluh ekor lembu betina berusia setahun telah dipilih secara rawak dan dibahagikan kepada dua kumpulan iaitu kawalan dan rawatan. Kumpulan kawalan telah disuntik secara intra-otot dengan 10 ml normal salin, manakala kumpulan rawatan telah disuntik secara intraotot dengan 0.1mg/kg sebanyak dua kali, iaitu Se pada permulaan dan

dua minggu ujikaji. Darah diambil secara mingguan sehingga minggu keempat. Plasma, sel darah merah dan serum kemudian diproses untuk mendapatkan kepekatan MDA, GSH-Px dan AST. Hasil kajian menunjukkan bahawa tidak terdapat perbezaan yang keertian pada kepekatan MDA dan AST. Walau bagaimanapun, kepekatan GSH-Px menunjukkan perbezaan keertian ($p<0.05$) kenaikan hanya pada minggu 1 dan 2 ujikaji. Kesimpulannya, rejim tambahan Se yang digunakan mungkin tidak begitu berkesan dalam menyekat tekanan oksidatif dan meningkatkan keutuhan otot pada lembu pedaging.

Kata Kunci: *Selenium, tekanan oksidatif, malondialdehyde, glutathione peroxidase, AST*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD4999 - Project.

THE EFFECT OF SELENIUM SUPPLEMENT ON ANTI-OXIDANT STATUS AND MUSCLE INTEGRITY IN BEEF CATTLE

By

Zharif Atiq bin Hashim

2016

Supervisor: Prof. Dr. Noordin Mohamed Mustapha

Selenium (Se) is an essential micronutrient required for normal growth development and antioxidant defense. The objective of this study is to determine the effect of Se supplementation on the oxidative stress (malondialdehyde, MDA and glutathione peroxidase, GSH-Px) and muscle integrity (aspartate transaminase, AST) in beef cattle. Ten 1-year-old cows were selected randomly and divided into a control and treatment group. The control group was injected intramuscularly with 10ml normal saline, while the treatment group received two intramuscular injections of 0.1mg/kg of Se, viz at the start and two weeks post-experimentation. Blood was

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collected weekly until the fourth week of the experimental period. The plasma, red blood cell and serum were then subjected to MDA, GSH-Px and AST assays, respectively. The results showed that there was no significant difference in the concentration of MDA and AST between both groups. However, the concentration of GSH-Px showed a significant ($p<0.05$) increment only on the weeks 1 and 2 post-treatment. It is concluded that supplementation regime of Se used may not be effective in abating the oxidative stress and improving muscle integrity in beef cattle.

Keywords: *Selenium, oxidative stress, malondialdehyde, glutathione peroxidase, AST*

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1. INTRODUCTION

Selenium (Se) was first recognized as an essential micronutrient in 1957 (Schwarz and Foltz, 1957). Selenium and vitamin E both have complementary but independent roles as antioxidants in the protection of cells against damaging effects of free radicals produced during normal metabolism (Villar *et al*., 2002). On the other hand, Se and vitamin E deficiency can results in nutritional muscular dystrophy, infertility, stillbirths or retained placenta (Bill, 2010).

The acidic soil of Malaysia with a range of pH of 3 to 5 (Shamshuddin *et al*., 2011) may precipitate the formation of selenium complexes with iron hydroxide leading to low uptake by pastures (Lyons *et al*., 2007) and thus being less bioavailable to animals. Such scenario is likely to be manifested in grazing ruminant as a form of ill-thrift (Noordin, 1995). Selenium fertilization of pasture and supplementation programmes in animals via feed concentrate, mixture or boluses would be an effective way to increase selenium content in the animal diets (Lyons *et al*., 2007).

Owing to the likely scenario mentioned above, an investigation into the role of Se in grazing ruminants in Malaysia is warranted. Therefore, the objectives of this study are to:

i. determine the status of oxidative stress in beef cattle with and without selenium supplementation
ii. assess the muscle integrity via AST concentration in beef cattle with and without selenium supplementation.

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2. LITERATURE REVIEW

Selenium (Se)

Selenium is an essential micronutrient required in small quantities in the diet of animals (Bill, 2010). Selenium acts as antioxidant responsible in the protection of cells against the damaging effects of lipid peroxides and free radicals produced during normal metabolism (Villar *et al*., 2002). Selenium is acquired usually from the pastures and supplementation of the diets in animals and the content of selenium in food depend on the selenium content of the soil where the plants are grown or animals are raised (Stephen, 2006). Selenium deficiency can lead to nutritional muscular dystrophy, infertility, abortions, stillbirth or retained placenta (Bill, 2010).

Antioxidant

All cells in the body are equipped with a defense mechanism in the form of antioxidants to combat the damaging effects of free radicals which could lead to potential oxidative stress (Hardwick *et al*., 1990). Antioxidants act by reacting with free radicals and other reactive oxygen species (ROS) to protect other chemicals in the body from the damaging oxidation reactions (Aktar, 2008).

Free Radicals and Reactive Oxygen Species (ROS)

A free radical is defined as any chemical species that contains one or more unpaired electrons that in consequence will cause alteration in the chemical reactivity of an atom or molecule by making it more reactive. This happens because molecules with an unpaired

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electron will act like an electron acceptors and therefore will 'steal' electrons from another susceptible molecules. Oxidation generally occurs when a molecule loses an electron. Due to the fact that free radicals tend to make other molecules donate their electrons, they are hence referred to as 'oxidizing agents' (Kooter, 2004).

Free radicals are regarded as very dangerous as they can react indiscriminately with important cellular components such as the cell membrane or DNA and thus causes the cells to die as their function is impaired (Aktar, 2008; Kelly, 2003).

Reactive oxygen species (ROS) are basically free radicals that contain oxygen. Well known ROS include superoxide anion radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), which is not a free radical but still considered as one (Halliwell, 1999).

The ROS are constantly produced and eliminated in the body. This is because of the existence of endogenous defenses against oxidants. Oxidative stress occurs when there is an imbalance of ROS whereby production of ROS exceeds the rate of elimination either due to overproduction during detoxification process or trauma or as a result of damage to the natural antioxidant system. This imbalance will further lead to cellular damage to proteins, lipids, DNA and eventually death to cells (Kooter, 2004).

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Oxidative stress

Oxidative stress as illustrated in Figure 1 can be defined as a disruption in the balance between the pro-oxidants and the antioxidants either due to overwhelming production of oxidants or free radicals in the body or the insufficient level of the protective antioxidants leading to damages and injury to proteins, lipids and DNA (Kelly, 2003).

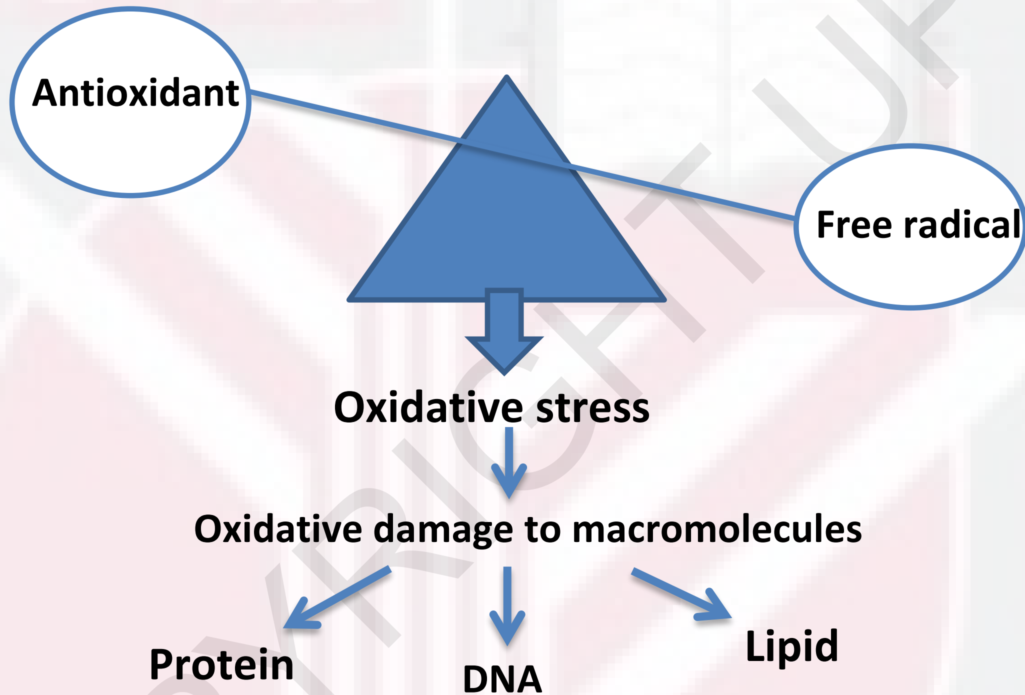

Figure 1: An illustration of oxidative stress and its implications. (Modified from Kelly, 2003)

Malondiadehyde (MDA)

This organic compound is one of the end products of lipid peroxidation and is often used as a good measure of oxidative stress which is responsible in the mechanism of cell damage leading to necrosis and apoptosis (Chlubek, 2003). Malondialdehyde is a known mutagen that has the potential to react with proteins and amino acids (Kooter, 2004).

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Glutathione peroxidase (GSH-Px)

This enzyme contains in selenium and generally found in the cytosol and mitochondria and it functionally seeks out to eliminate H_2O_2. The GSH-Px also plays a crucial role in detoxification of lipid peroxides in tissues to protect cellular membrane from oxidative damages. Therefore making GSH-Px is a very important antioxidant enzyme as it can be said to have dual function (Sagai and Ichinose, 1987).

This antioxidant enzyme acts by reducing H_2O_2 to H_2O by oxidizing GSH and produces reduced glutathione (GSSG) besides water and diatomic oxygen (Aktar, 2008; Kooter, 2004).

Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST) is present in many tissues and is useful in evaluating muscle and liver damage in small and large animals. Asparate aminotransferase is present in high concentration in the cytoplasm and mitochondria of cells and will elevate in states of altered membrane permeability. Under such circumstances, the elevation is used to indicate a breach of muscle fiber integrity (Martin *et al*., 2015).

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3. METHOD & MATERIALS

Animals, Management and Experimental Design

Ten healthy, Kedah-Kelantan cows aged around 1 year old were selected from Ladang 16, Taman Pertanian Universiti, Universiti Putra Malaysia. These animals were divided equally into two groups; control and treatment group.

About 3 ml of blood was collected into heparinized tube and another 3 ml were collected into plain tube via jugular venipuncture from both groups of animals. The cows in the treatment group were then injected with 0.1 mg/kg of selenium intramuscularly while the control group was given 10 ml of normal saline. Blood collection was repeated for another four weeks and the intramuscular injection of selenium is repeated after two weeks. The collected blood samples were subjected to MDA, GSH-Px and AST concentration analyses.

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General sample processing

The blood samples were processed according to the protocols outlined in Figure 2.

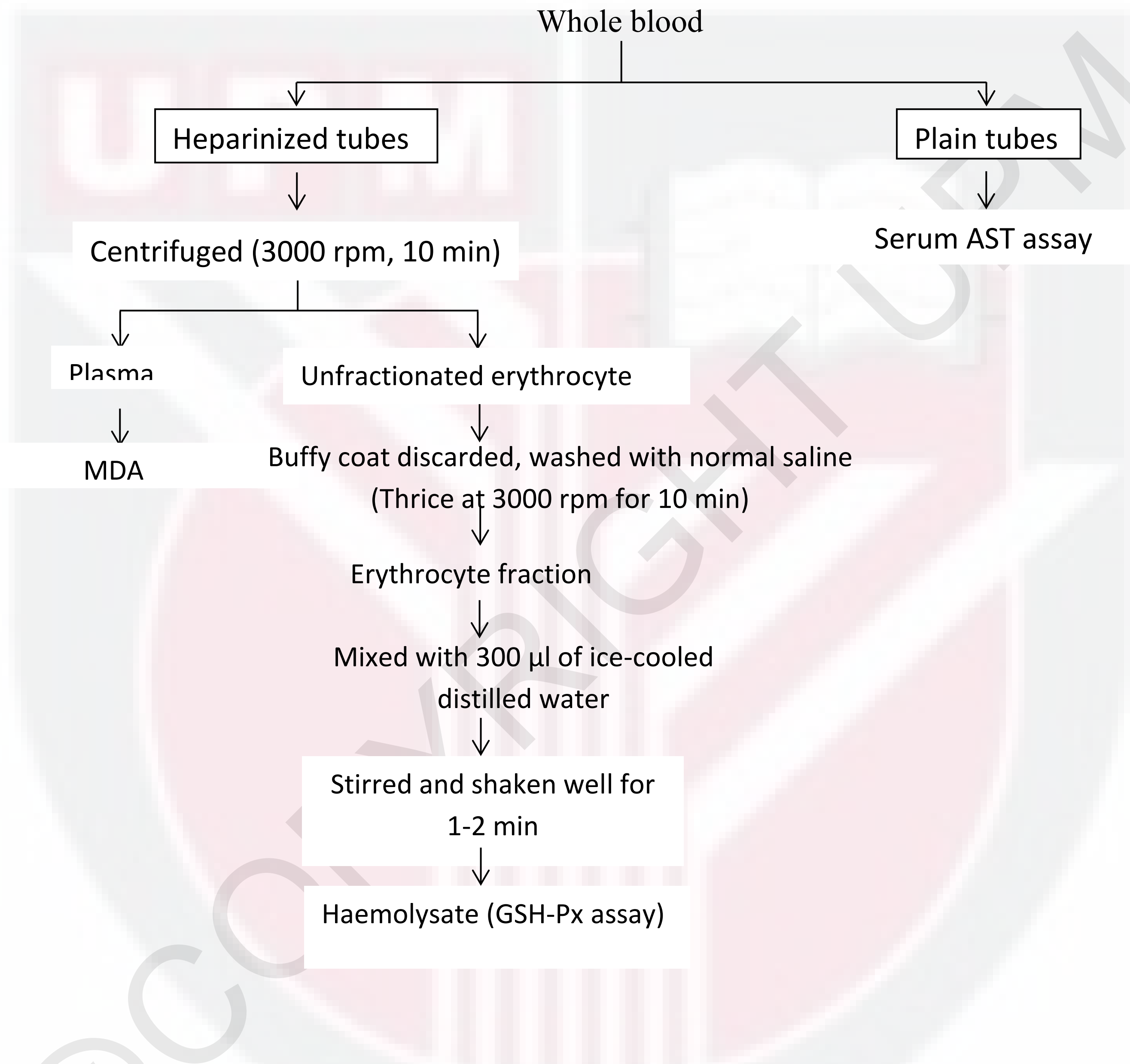

Figure 2: Schematic diagram depicting blood sample processing and analyses.

MDA concentration

The measurement of MDA concentration in plasma was done following the thiobarbituric acid reactive substances (TBARS) assay as described by Zhang (2000) with a slight modification of method by Ohkawa *et al*. (1979). Firstly, a mixture was made by adding 0.3 ml of plasma with 2.4 ml of 1/12 H_2SO_4 and 3 ml of 10% of sodium tungstate (Na_2WO_4). This mixture was then kept at room temperature for 10 minutes and later centrifuged at 3500 rpm for 10 minutes. Next, the supernatant was thoroughly removed by tapping the tubes upside-down on a few layers of absorbent paper towels and leaving the pellet behind.

The reactive mixture was formed by adding the pellet with 3 ml of 0.05M HCl, 1 ml of 1% thiobarbituric acid (TBA) and approximately 0.5ml of distilled water (dH_2O) so that the final volume is 5 ml.

The well-mixed mixture was then left in water bath for 1 hour at 95°C in centrifuged tubes covered with glass marble. Then, the centrifuge tubes containing the reactive mixtures were cooled down under running water and later centrifuged at 3500 rpm for 10 minutes. The supernatant layer was collected and its absorbency was measured using a spectrophotometer at 532 nm.

Standard control was prepared by adding 0.3 ml of 10 nmol/ of 1,1,3,3-tetraethoxypropane (TEP) with 3 ml of 0.05M HCl, 1 ml of 1% TBA and approximately 0.3 ml distilled water to make final mixture volume to 5 ml. the mixture treated the same as the sample by heating

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and centrifugation as described earlier. Finally, pink colored supernatant was retrieved and its absorbency measured immediately using spectrophotometer at 532 nm.

The concentration of MDA was expressed as nmol/ml plasma and was calculated by the equation: **C (nmol/ml) = (As / Ab) / (Vs / Vm)**, where;

As	=	Absorbance of sample		
Ab	=	Absorbance of standard control		
Vs	=	Volume of plasma sample	=	0.3 ml
Vm	=	Volume of total reactive mixture	=	5 ml

Glutathione Peroxidase (GSH-Px) in RBCs

The activity of GSH-Px in RBCs was measured by the DTNB direct method as mentioned by Sazuka *et al*. (1989) with slight modifications as described by Zhang (1997).

First, 100 µl of haemolysate (previously obtained by adding 100 µl of packed RBCs with 300 µl of ice cold DIW making it 1 part of RBCs to 3 parts of water dilution) was added to 0.4 ml of 1 nmol/L of glutathione (GSH). The solution was mixed well and then let prewarmed in water bath for 5 minutes at 37°C. Next, to initiate the reaction, 0.2 ml of 1.3-1.5 nmol/L of hydrogen peroxide (H_2O_2) was added and left for 3 minutes to react accurately in water bath at 37°C. The reaction was terminated immediately by adding 4 ml of 1.67% meta-phosphoric acid (HPO_3) solution. Then, the well-mixed solution was centrifuged at 3000 rpm for 10 minutes. Later, 2 ml of supernatant was taken and added to

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2.5 ml of 0.32 mol/L disodium phosphate (Na_2HPO_4) solution. Finally, the absorbency of the final solution was measured within 5 minutes at 422 nm after adding 0.5 ml of DTNB solution.

Blank control which served as the baseline without GSH and has the lowest absorbance value was prepared by replacing the supernatant with 0.4 ml of DIW and 1.6 ml of 1.67% HPO_3 mixture and processed the same way as the sample.

Standard control was prepared by substituting the haemolysate with 0.4 ml of DIW and treated the same manner as samples. This represented the non-enzymatic GSH oxidation and has the highest absorbance value.

The enzyme activity unit in RBCs was defined as units per gram of Hb and calculated using the following formula:

U/g Hb = log (Ac - AB) – log (As – AB) / 0.012, where;

Ac = absorbance of standard control

AB = absorbance of blank control

As = absorbance of sample

0.012 is the product of time (3 minutes) with the dilution factor (0.004).

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Statistical Analysis

Data were expressed as mean± standard deviation (SD) and subjected to independent t-test and repeated measures analysis of variance (SPSS version 22.0). Only p values of less than 0.05 are considered significant.

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4. RESULTS

The concentration of the MDA is presented in the Table 1. The concentration of MDA in both groups revealed a decrement overtime. However, that of the treatment group showed a much more obvious decreasing pattern. Although there was no significant difference between the groups, the treatment group has a slightly lower concentration of MDA at the end of the experiment despite starting with a much higher concentration.

Table 1: The blood MDA concentration (nmol/mL) of cows during the experimental period (Mean±STDE)

Group \ Week	0	1	2	3	4
Control	0.3±0.04	0.2±0.12	0.2±0.04	0.2±0.02	0.3±0.05
Treatment	0.4±0.25	0.2±0.05	0.2±0.03	0.2±0.05	0.2±0.04

The blood concentration of GSH-Px is as shown in Table 2. There exists no pattern fluctuation of GSH-Px in both groups. However, at Weeks 1 and 2, those of the treated cows has significantly higher ($p<0.05$) concentrations than that of control group.

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Table 2: The blood GSH-Px concentration (U/gHb) of cows during the experimental period (Mean±STDE)

Group \ Week	0	1	2	3	4
Control	3.2±1.39	2.5±0.23*	5.2±0.41*	3.2±0.83	3.2±0.52
Treatment	3.5±0.52	4.1±0.42*	6.7±0.70*	4.1±0.67	3.9±0.59

The blood concentration of AST is shown in the Table 3. Although the cows in the control showed an increasing trend in the AST concentration overtime, that in the treated group depicted a reversed response. However, these discrepancy in the AST concentration did not yield any significance between the groups.

Table 3: The concentration of AST (U/L) of cows during the experimental period (Mean±STDE)

Group \ Week	0	1	2	3	4
Control	83.8±24.92	88.1±13.89	86.6±14.14	107.6±32.02	105.8±30.22
Treatment	103.9±23.04	78.0±11.03	73.2±10.52	81.5±13.18	88.9±27.15

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5. DISCUSSION

Although a factor on stress may exist in this experiment, it ripples through both groups. This, spurious result stemming from stress is thus accounted in both groups and significant changes are statistically devoid of that. Throughout the experiment, the concentration MDA in the treated group shows a decreasing pattern denoting that Se supplementation did exert a protective effect against oxidative stress. Mathematically, with an increase in MDA, more GSH-Px would be generated and thus utilized to combat or prevent a worsening oxidative stress status. This in turn has led to an increase in GSH-Px production during the first two weeks of experimentation. However, the reduction of GSH-Px concentration after the second dose of supplementation is explained by its utilization in scavenging the readily available and newly formed MDA.

The plausible reasons for the lack of efficacy of the Se used in this study could be attributed to several reasons. One of the reason could be due to the existence of the Se used in this experiment is a concoction containing vitamin B12, adenosine triphosphate tetrasodium, potassium aspartate semihydrate and magnesium aspartate tetrahydrate. These compounds may have a negative or antagonizing effect with selenium or a much higher dose of selenium is required when this preparation is used (Young and Lowe, 2001). Vitamin B12 has been found to increase Se methylation and excretion which leads to lower Selenium concentration and activity (Chen *et al*., 1993), which could have also happen in this study.

Usage of vitamin E is also important to enhance the effect of antioxidant. Vitamin E and Se have complementary but independent roles as antioxidants in the protection of cells

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against the damaging effects of lipid peroxides and free radicals produced during normal metabolism (Villar *et al*., 2002). These two work synergistically and have sparing effect, which Se destroys peroxidase before it can destroy cell membrane, while vitamin E act within the membrane to prevent the formation of fatty acid hydroperoxides.

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6. CONCLUSION

In conclusion, selenium does play a role in reducing oxidative stress and aids in maintaining muscle integrity. However, supplementation of selenium alone is not significantly effective in reducing oxidative stress.

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7. RECOMMENDATIONS

In future study, it is recommended to supplement a combination of selenium and vitamin E to enhance the antioxidant effect. The duration of the study can be prolonged and bigger sample size can be used to have better result on the antioxidant status.

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APPENDICES

Appendix 1 (MDA assay)

1. 1/12 H_2SO_4

   8.33 ml of H_2SO_4 topped up to 100 ml with dH_2O

2. 10 % Na_2WO_4

   5 g $Na2WO_4$ dissolved to 50 ml of dH_2O

3. 0.05M HCl

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

4. 1% TBA

   1 g TBA dissolved to 100 ml of dH_2O

5. 10 nmol/L TEP (standard control)

   22.7 µl 97% TEP topped up to 1000 ml dH_2O

Appendix 2 (GSH-Px assay)

1. 1 mM GSH (MW=307.33)

   0.307 g GSH topped up to 100 ml with DIW

   (Prepared fresh and kept cold until used)

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

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

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ii) 1.3 – 1.5 mmol/L H_2O_2 stock solution

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

3. 1.67% HPO_3 solution

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

4. 0.32 mol/L Na_2HPO_4

   22.72 g Na_2HPO_4 added to 500 ml of dH_2O

5. DTNB colour generating solution

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

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