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Effects of L-Arginine on the Reproductive System of Male Rabbits

Sukardi S[1], Yaakub H[2], Ganabadi S[3] & Cheng LH[1]

[1] *Department of Biomedical Sciences, Faculty of Medicine and Health Sciences;*
[2] *Department of Animal Sciences, Faculty of Agriculture;*
[3] *Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

L-arginine is an amino acid, which serves as the sole substrate for nitric oxide (NO) synthesis with the concomitant formation of L-citrulline in biologic system. NO has been demonstrated to be involved in smooth muscle relaxation and vasodilation, immune regulation and neurotransmission. It also has an important function as both intercellular and intracellular signals in many physiological systems, including the reproductive system where NO mediates penis erection. This study was undertaken to determine the effects of L-arginine on sperm motility, sperm count, and the nitric oxide level in the seminal plasma. Twelve sexually matured male rabbits (Oryctolagus cuniculus) were randomly divided into four groups with three rabbits each, which were control, low, medium, and high concentration groups. The treatment groups were force-fed with 100mg/kg, 200mg/kg, and 300mg/kg body weight of L-arginine for four weeks, whereas the control group was force-fed with water. Semen samples were collected every three days alternatively for a week before starting treatment and then after four weeks of treatment. Pre-treatment and post-treatment results were compared. Semen samples were collected using artificial vaginas from each group for sperm analysis such as sperm motility, sperm count and NO level in seminal plasma. Sperm motility and sperm count were analysed manually under microscope (twenty power objective), using a Makler counting chamber. NO levels in the seminal plasma were determined using Griess reaction. The results obtained from this study showed that oral consumption of L-arginine exerted a significant ($p<0.05$) effect in decreasing sperm motility in all treatment groups but showed a significant ($p<0.05$) increase in sperm count (25.5%) in group 3 (200 mg/kg of L-arginine). This experiment also showed that there was a significant ($p<0.05$) increase in NO concentration with L-arginine consumption. The levels of NO concentration were significantly correlated ($r = 0.624$) to L-arginine consumption. Besides, there was a significant ($p<0.05$) positive correlation ($r = 0.584$) between NO concentrations with sperm count. However, there was a significant negative correlation ($r = -0.775$) between NO concentrations with sperm motility. Thus, data suggests that oral consumption of L-arginine can increase NO level, which in turn increases sperm count but exerts a reduction in sperm motility.

Correspondence author: Assoc Prof Dr Sabrina Sukardi, email address: sabrina@medic.upm.edu.my

INTRODUCTION

L-arginine was identified in 1988 as the physiologic precursor of nitric oxide (NO) synthesis in mammalian cells. It is an amino acid, which serves as the sole substrate for NO synthesis with the concomitant formation of L-citrulline in biologic systems. NO is the major endothelium-derived relaxating factor, a mediator of immune responses, a neurotransmitter, a cytotoxic free radical, and a widespread signalling molecule in the body (Ignarro *et al.,* 1999).

It is now well established that NO plays a vital role in the regulation of male reproductive function (Forstermann *et al.,* 1995; McCann and Rettori, 1996). NO has been demonstrated to be involved in smooth muscle relaxation and vasodilation, immune regulation and neurotransmission. It also has an important function as both intercellular and intracellular signals in many physiological systems, including the reproductive system where NO mediates penis erection.

Objectives

The objectives are to study the effects of L-arginine on sperm count, sperm motility and NO levels in seminal plasma as well as the relationship between NO level with sperm count and motility.

MATERIALS AND METHODS

Animal

Twelve sexually matured (6-month-old) New Zealand White male rabbits (*Oryctolagus cuniculus*), weighing approximately 3.0 to 3.5 kg were obtained from the Department of Biomedical Sciences, UPM. Each rabbit was housed separately in a cage measuring 0.6 m x 0.45 m x 0.8 m. Rabbit pellets, which consist of the entire nutrient requirements needed, and water were given *ad libitum*.

L-Arginine preparation

The pure L-arginine was commercially available in powder form (Sigma Chemical). Approximately 100, 200 and 300 mg were dissolved in distilled water.

Treatment

The rabbits were randomly selected and assigned into four different treatment groups with three rabbits each. The first group was the control group and this was followed by the low, medium and high concentration treatment groups. The rabbits in group 2 (n = 3) were force-fed with 100 mg/kg of L-arginine, group 3 (n = 3) with 200 mg/kg of L-arginine, and rabbits in group 4 (n = 3) with 300 mg/kg of L-arginine for 4 consecutive weeks. However, group 1 (control group) (n = 3) were given water.

Semen collection

Semen samples were collected using artificial vaginas (AV) from each group for sperm analysis such as sperm count, sperm motility and NO level. Samples were collected every three days for a week before starting treatment and then after four weeks of treatment. In short, a week pre-treatment assessment would be compared with a week post-treatment assessment. Only one sample was collected from each animal at every collection. Semen samples were kept in tightly capped bottles and incubated at 37°C before analysis.

Sperm count analysis

Sperm count was calculated using Makler Counting Chamber under the light microscope. Spermatozoa were counted in 3 x 10 squares either horizontally or vertically (twenty power objective). This number represented the sperm concentration in millions per ml. The counts were

repeated 3 times to get the average of the total sperm count in a sample. Only sperm heads within those 30 square areas were counted.

$$\text{Sperm count} = \frac{\text{Total sperm count from 3 x 10 squares}}{3} \times \text{million/ml}$$

Sperm motility analysis

Sperm motility analysis was accomplished within 3-5 minutes after application of the sample to avoid errors due to tendency of sperm to migrate from the periphery. The spermatozoa were counted within 16 squares. All motile and non-motile sperm were counted within those 16 squares. The percent motility is the number of motile sperm divided by the total number of sperm (motile and non-motile) multiplied by 100.

$$\%\ \text{motile sperm} = \frac{\text{Number of motile spermatozoa}}{\text{Total number of spermatozoa (motile +non-motile)}} \times 100\%$$

Determination of Nitric Oxide (NO) concentration in the seminal plasma

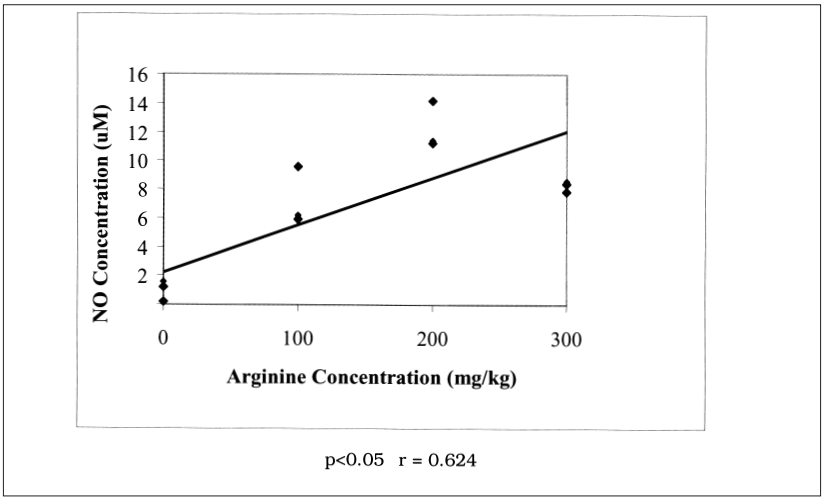
Semen was dispensed into eppendorf tubes and centrifuged at 3000g for 10 minutes in order to get the seminal plasma (supernatant). NO level in the seminal plasma was determined by using Griess reaction. Under physiological conditions, NO was readily oxidised to nitrite and nitrate, or it was trapped by thiols as an S-nitroso adduct. The Griess reagent provided a simple and well-characterised colorimetric assay for nitrites and nitrates that had been reduced to nitrites, with a detection limit of about 100 nM. Nitrites reacted with sulfanilic acid in acidic solution to form an intermediate diazonium salt that coupled to N-(1-naphthyl)ethylenediamene to yield a purple azo derivative (diagram 5) that could be monitored by absorbance at 548 nm. The readings obtained were estimated from the nitrite standard curve, which was prepared earlier to quantify the level of NO present in the seminal plasma.

Statistical analysis

The results were expressed as means ± SEM. The data obtained were analysed with SPSS (Statistical Package for Social Sciences) version 10. Two-way ANOVA and Duncan's Multiple Range Test were used to determine the degree of significance for the various mean variables obtained. Pearson correlation was used to study the association between dosage of L-arginine consumption and NO concentration, NO concentration with sperm motility and sperm count. P value < 0.05 was considered to indicate a significant difference.

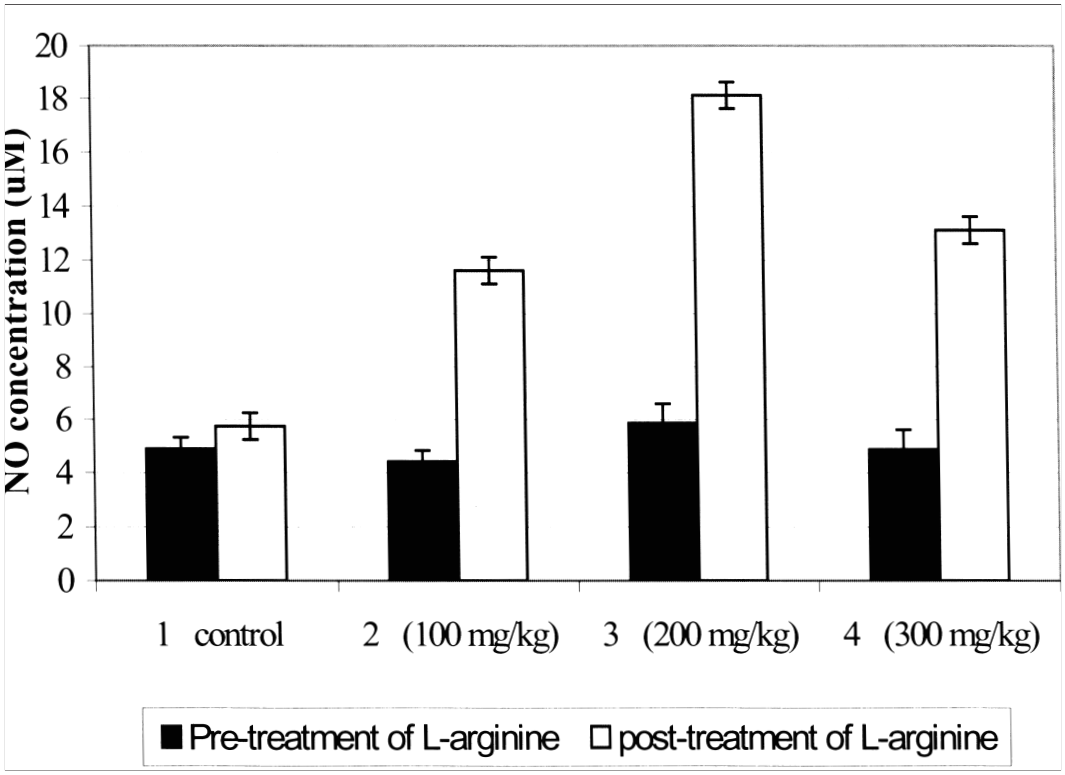
RESULTS AND DISCUSSION

This study demonstrated that repeated oral administration of L-arginine had a significant ($p<0.05$) increase in NO concentration in seminal plasma in rabbits; suggesting that the dosage and duration of L-arginine administration may produce a significant effect in elevating the NO concentration in this study. L-arginine is the sole substrate for NO synthesis with the presence of enzyme nitric oxide synthase (NOS), oxygen and nicotinamide adenine dinucleotide phosphate (NAPDH). Thus, higher oral consumption of L-arginine may increase NO concentration in the seminal plasma as shown in the Pearson correlation (Figure 1) where there was a significant ($p<0.05$) positive ($r = 0.624$) association between L-arginine consumption and NO concentration. However, the increment of NO concentration was



$p < 0.05$ $r = 0.624$

Figure 1. Scatter diagram depicting correlation between L-arginine consumption and NO concentration



Data are expressed as means ± SEM (n = 9) *$p < 0.05$

Figure 2. Effect of different dosages of L-arginine on NO concentration for pre-treatment and post-treatment

prominent in group 3 with 200 mg/kg of L-arginine treatment. This suggests that 200 mg/kg of L-arginine may be the optimum dosage to increase to the maximum level of NO concentration in the seminal plasma in rabbits. Further increase of oral administration of L-arginine treatment may not produce higher NO concentration in seminal plasma.

These findings support previous studies done, where L-arginine is converted to L-citrulline and NO endogenously *in vivo* by the catalytic action of the enzyme NOS (Forstermann *et al.,* 1995), which is widely distributed in the male reproductive tract (Burnett *et al.,* 1995; McCann and Rettori, 1996). The NADPH provides a source of electrons, and molecular dioxygen is incorporated into NO and citrulline, the coproduct of NO formation (Billar, 1995). NO produced in the body by L-arginine is called ADNO, or Arginine-Derived-Nitric-Oxide. NO is an extremely unstable and diffusible molecule that converts to stable form metabolites, nitrate and nitrite within ten seconds which can be detected in biological fluids (Ocho *et al.,* 1991). Recent studies also showed that certain concentrations of L-arginine used were capable of elevating the NO levels in the brain and the reproductive organs (Ratnasooriya and Dharmasiri, 2001).

The finding that free radicals can influence male fertility has received substantial scientific report (Gagnon *et al.,* 1991). The proposed mechanism for loss of sperm function upon oxidative stress has been shown to involve excessive generation of reactive oxygen species (ROS) (Aitken and Clarkson, 1987). The nitrogen-derived free radical nitric oxide (NO) and peroxynitrite anion ($ONOO^-$) also appear to play a significant role in reproduction and fertilisation.

Our study showed that there was a significant ($p<0.05$) drop in percentage of sperm motility in post-treatment semen samples in treatment groups as compared to the pre-treatment groups (Figures 2 - 4, Tables 1-3). In contrast, there was a significant increase in NO concentration in post-treatment seminal plasma with L-arginine treatment. Pearson correlation also demonstrated that the decline in sperm motility was significantly and strongly correlated ($r = -0.775$) to the NO concentration in the seminal plasma as shown in Figure 5. This indicated that sperm motility is inversely proportionate to the NO production. The results obtained here were closely related to the previous studies on human sperm, where NO supplied by a variety of donors inhibits human sperm motility *in vitro*, possibly by inhibiting cellular respiration (Weinberg *et al.,* 1995).

Studies carried out by Weinberg *et al.* (1995) also demonstrated that high concentration of NO (at milimolar to micromolar concentrations of sodium nitroprusside) inhibited sperm motility and viability. The results are consistent with other reports demonstrating that high concentration of NO have a cytotoxic effect on spermatozoa (Ratnasooriya and Dharmasiri, 2001). In contrast, lower concentrations (10^{-7} and 10^{-8} M) of NO had no effect. NO generated under physiological conditions, could be beneficial for sperm functions, especially in improving sperm motility and viability as well as aid in human sperm capacitation (Francavilla *et al.,* 2000). In addition, low concentrations of NO may protect against O^{2-} mediated reduction of sperm motility (Rosselli *et al.,* 1998). Other researchers also demonstrated that treatment of spermatozoa with micromolar sodium nitroprusside decreased sperm motility and viability in a dose-dependent manner (Rosselli *et al.,* 1995; Nobunaga *et al.,* 1996; Zhang and Zheng, 1996).

Sperm require high levels of adenosine triphosphate (ATP) to maintain their motility. ATP is generated by the glycolytic pathway and the mitochondrial electron transport system. Based on previous research, NO can reduce ATP levels in

Table 1. Effects of different dosages of L-Arginine on NO concentration in seminal plasma

Group	*Pre-treatment NO concentration (μM)*	*Post-treatment NO concentration (μM)*	*Increase in NO concentration (μM)*
Group 1 (control)	4.90 ± 0.44	5.75 ± 0.37	0.85[a]
Group 2 (100 g/kg)	4.42 ± 0.42	11.62 ± 1.00	7.20[b]
Group 3 (200 g/kg)	5.87 ± 0.74	18.13 ± 1.14	12.26[c]
Group 4 (300 g/kg)	4.88 ± 0.74	13.12 ± 1.02	8.23[b]

Values are means ± SEM with n = 9 for each group.
Means with same superscript are not significantly different at $p < 0.05$

Table 2. Effects of different dosages of L-arginine on sperm motility

Group	*Pre-treatment sperm motility (%)*	*Post-treatment sperm motility (%)*	*Changes in sperm motility (%)*
Group 1 (control)	64.52 ± 0.94	64.59 ± 0.65	0.07[a]
Group 2 (100 g/kg)	80.06 ± 2.34	68.79 ± 2.58	-11.27[bc]
Group 3 (200 g/kg)	78.79 ± 1.47	64.75 ± 2.86	-14.04[b]
Group 4 (300 g/kg)	78.33 ± 1.67	70.70 ± 2.23	-7.63[c]

Values are means ± SEM with n = 9 for each group.
Means with same superscript are not significantly different at $p < 0.05$

Table 3. Effects of different dosages of L-arginine on sperm count

Group	*Pre-treatment sperm count (million/ml)*	*Post-treatment sperm count (million/ml)*	*Changes in sperm count (million/ml)*
Group 1 (control)	277 ± 11	282 ± 16	5[a]
Group 2 (100 g/kg)	286 ± 20	326 ± 12	40[ab]
Group 3 (200 g/kg)	311 ± 19	390 ± 29	79[b]
Group 4 (300 g/kg)	248 ± 16	282 ± 11	34[ab]

Values are means ± SEM with n = 9 for each group
Means with same superscript are not significantly different at $p < 0.05$

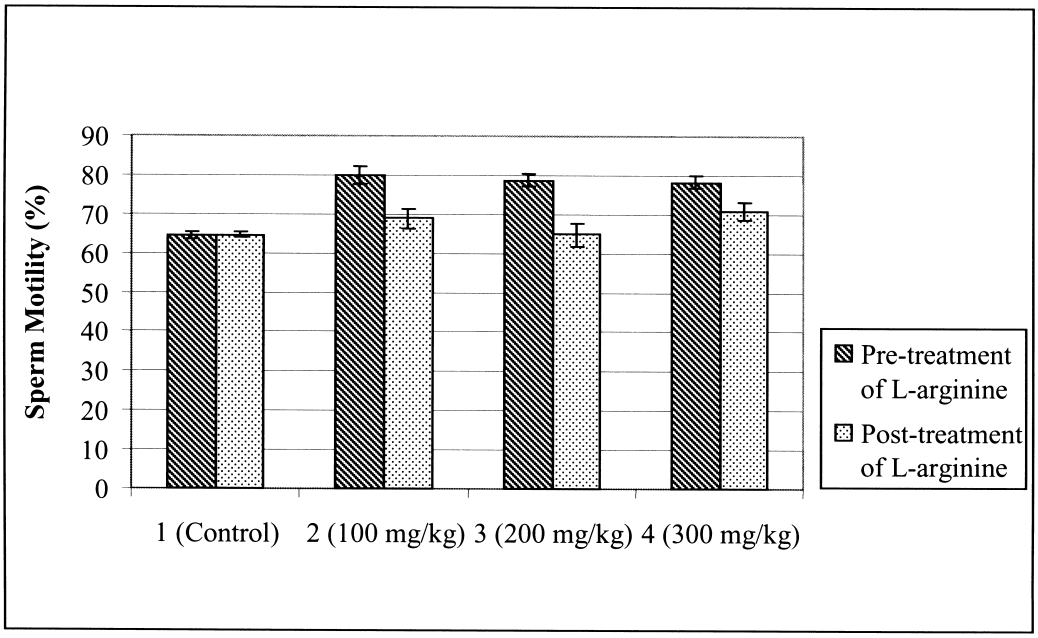
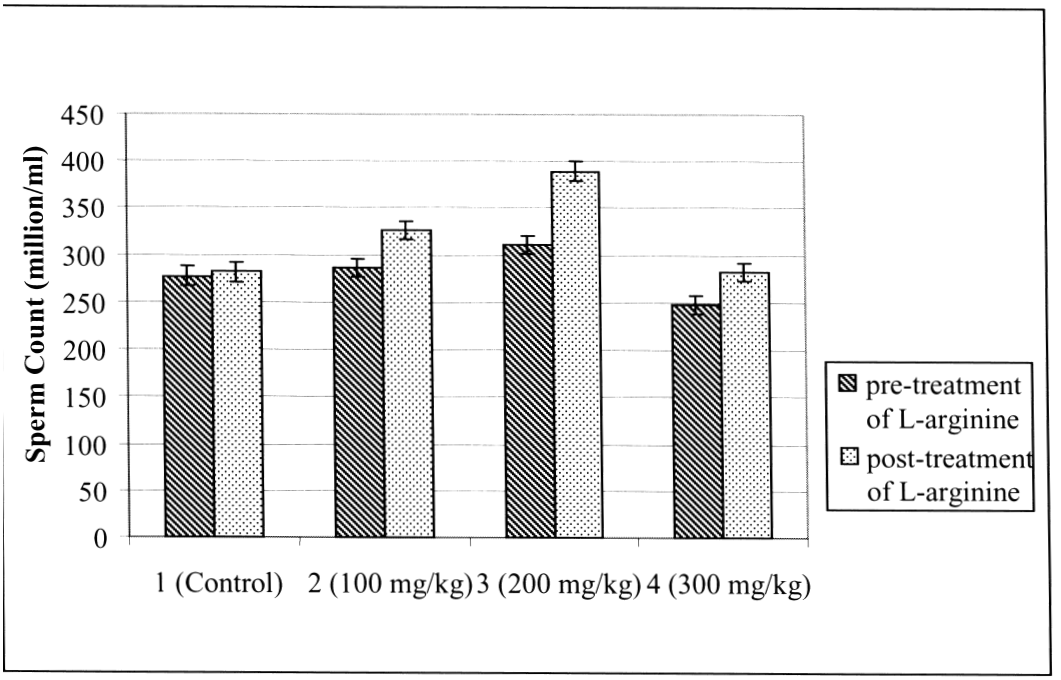


Figure 3. Effect of different dosages of L-arginine on sperm motility for pre-treatment and post-treatment

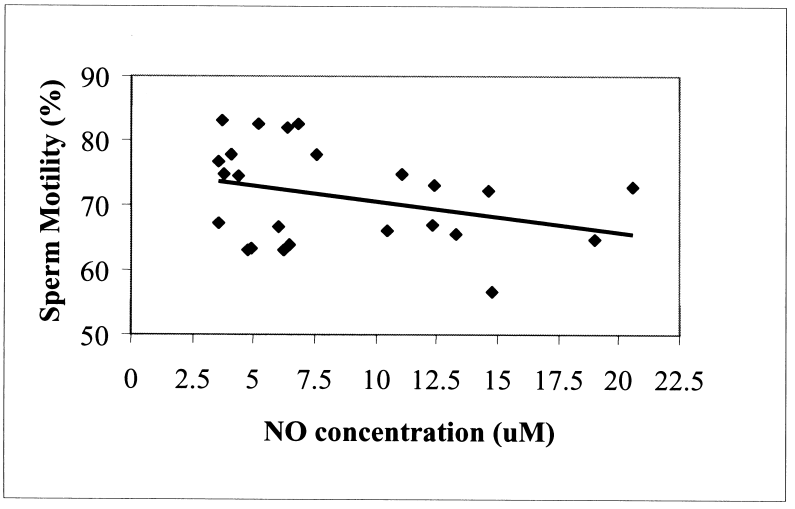
cells by inhibiting the ATP-generating ability of enzymes in these pathways (e.g., iron-containing proteins of the electron transport system and the tricarboxylic acid cycle and glyceraldehydes phosphate dehydrogenase of the glycolytic pathway) (Dimmeler *et al.,* 1992). NO may also inhibit heme containing the mitochondrial enzymes, aconitase, NAPH:ubiquinone, and NADH:succinate oxidoreductase, leading to the reduction in sperm motility by inhibition of ATP production. Thus, excessive amounts of NO may cause sperm dysfunction and toxicity (Weinberg *et al.,* 1995).

The results obtained from sperm count showed non significant results except in group 3, which showed a significant ($p<0.05$) increase in sperm count (25.5%). However, there was a significant positive correlation ($r = 0.584$) between NO concentrations with sperm count as shown in Figure 6. This indicated that elevation in sperm count was directly proportionate to the increase in NO concentration. The increase of the sperm count may be due to the fact that NO is an important regulator of gonadotrophin releasing hormone (GnRH), which is involved in the stimulation of luteinising hormone (LH) that regulates the function of the Leydig cells in testes to produce testosterone. Elevated testosterone may cause an increase in sperm count. This study also showed that 200 mg/kg of L-arginine could increase optimum NO concentrations, which in turn cause the maximum production of sperm count. Meanwhile, the lower concentration of NO may not produce a higher sperm count as seen in 100 mg/kg of L-arginine group. However, the further increase of the NO



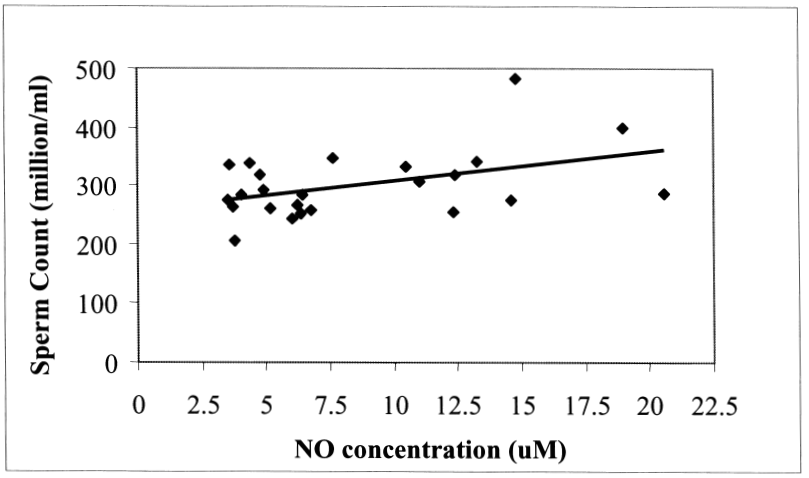
Data are expressed as means ± SEM (n = 9) *p < 0.05

Figure 4. Effect of different dosages of L-arginine on sperm count for pre-treatment and post-treatment



p < 0.05 r = -0.775

Figure 5. Scatter diagram depicting correlation between sperm motility and NO concentration



$p<0.05$ $r = 0.584$

Figure 6. Scatter diagram depicting correlation between sperm count and NO concentration

level did not exert a higher sperm count, which was probably due to the negative feed back mechanism of LH by testosterone.

These findings supported previous studies done where the anatomical localisation of NO neurons in close proximity to GnRH neurons in hypothalamus has been demonstrated and suggested that NO may be an important regulator of GnRH secretion (Bhat *et al.,* 1995). Studies carried out by Honaramooz *et al.* (1999) also reported that N-methyl-D-aspartate is an excitatory amino acid agonist, which stimulates LH release in part in prepubertal heifers.

In addition, this study also showed that L-arginine exerts its metabolite roles through the production of diverse metabolites including NO, ornithine, polyamines, proline, glutamate, glutamite, creatinine, agrnatine, and dimethylarginine, which play important roles in nutrition and physiology. Ornithine is an immediate precursor for the synthesis putrescine, which is converted into spermidine and spermine. These spermidine and spermine are thought to be involved in spermatogenesis (Morris, 2002). The dependence of normal spermatogenesis on the amino acid arginine was reported by Holt and Albanese (1944) where an arginine-free diet even a few days in duration will result in impaired spermatogenesis in previously normal individuals (Holt and Albanese, 1944). Schachter *et al.* (1973) also reported that arginine therapy increased sperm count and pregnancies occurred soon after the course of arginine.

CONCLUSION

In conclusion, the findings suggest that a dosage of 200 mg/kg L-arginine is the optimum dosage for maximum increase in NO concentration and sperm count. However, high NO concentration decreases sperm motility by inhibiting ATP production.

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