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Roselle Calyx Extract as an Antioxidant Supplement on Tris-Base Extender for Goat Semen

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ABSTRACT

In this study, we used extract from Roselle (*Hisbiscus sabdariffa* L.) Calyx to determine its ability as an antioxidant supplement to improve motility, progressive score and abnormality of goat's sperms. Pooled semen from four male goats with average of live body weight 34.4 ± 0.6 kg and body condition score 2.5 ± 0.1 were aliquot into two groups: Control group; 0 mg/ml and Treatment groups; (Treatment 1:13 mg/ml, Treatment 2: 20 mg/ml, Treatment 3: 33 mg/ml, Treatment 4: 43 mg/ml of Roselle's extract. All the semen parameter was observed at 0 hour, 0.5 hour, 1.0 hour, 1.5 hours after semen collection. The supplementation of extender with 13 mg/ml, 20 mg/ml, 33 mg/ml, 43 mg/ml of extract from Roselle Calyx does not affect sperm motility, progressive score and morphology of goat ($p>0.05$). However, all sperm, regardless with supplementation and without supplementation of Roselle extract were maintained in good quality (60%) up to 1.5 hours. It is quite difficult to conclude that there are no effect of Roselle extract on sperm quality due to arise possibility of factors that may contribute to inefficiency effect of Roselle extract on sperm parameter. There were high possibility that the causes are due to inefficiencies of extraction method and/or degradation of anthocyanin pigment during extraction and storage process. Unsuitable extraction method could results in low yield and unstable of anthocyanin pigment. Moreover, temperature, pH and light causes the pigment to become unstable during processing and storage leading to degradation. Thus we suggest anthocyanin content monitoring in extract Roselle Calyx are required for future study.

1. Introduction

Application of artificial insemination is widely used in breeding program where the good quality of semen program depends on good semen quality which generally relies on viability, motility, progressive score and morphology of the sperm. To assure semen quality, numerous study on techniques maintaining and improving semen performance were conducted. As early as year 1988, antioxidant has been acknowledged as an effective supplement that has positive effect to sperm parameters. Since then, variety of antioxidant has been use to determine their potential ability in enhancing the semen quality. Abundant studies have proved that varieties of antioxidant and antioxidant rich content such as ascorbic acid [1], L-Arginine, glutathione [2], a-tocopherol (vitamin E) [3], Crocin (crocetin di-gentiobiose ester) [4], Nigella sativa Oil and Honey [5] and Coenzyme Q10 (CoQ10) [6] posse an ability to improve the semen performance by reducing the effects of reactive oxygen species (ROS).

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While small amounts of ROS are required for normal sperm functioning, unbalanced levels can negatively impact the quality of spermatozoa and impair their overall fertilizing capacity. Overwhelms of ROS against body's antioxidant defences trigger oxidative stress to sperm. Oxidative stress has been identified as one of the many intermediaries of male infertility in reducing the sperm viability, and increasing mid-piece sperm morphological defects, all of which contribute to decreased sperm motility [7].

Body commonly responds to oxidative stress by producing an antioxidants that act as defence system and suppress ROS activity from damaging the sperm cells [8]. However, when the cell itself cannot produce requirement amount of antioxidant to balance the ROS, supplementation of antioxidant is required to prevent membrane sperm from damage. Therefore we use extract from Roselle (*Hibiscus Sabdariffa L.)* Calyces as antioxidant supplementation to improve the fresh semen quality of goat.

The importantance of antioxidant as effective supplement to enhance the ability of semen parameter has been described before by [9]. High antioxidant properties of *Hibiscuss Sabdariffa* plant led to general hypothesis of extract Roselle Calyx improved semen performance in term of sperm motility, progressive score and abnormality. Ability of extract Roselle Calyx in reducing oxidative stress of sperm has been reported before in rat [10, 11]. Study by [11] on rat sperm proved that aqueous extract of Roselle Calyx able to decrease the concentration of Malondealdehyde (MDA) and reduced lipid peroxidation in diabetic induced rat sperm.

Recently, numerous research showed that *Hibiscus Sabdariffa L.* contains high antioxidant properties and this antioxidant activity highly contributed by anthocyanin, a phenolic content in Roselle [12]–[14]. *In vivo* studies in diabetics rat suggested that Roselle Calyx extract suppressed glucose elevation and alleviate oxidative damage in kidney and liver [15]. In particular, Roselle shown potential protective role against diabetes-induced sperm damage [16]. However, scarce evidence is available on the potentcy of extract Roselle Calyx as antioxidant supplement on goat semen. Therefore, the aim of this study is to investigate the effects of aqueous extract from Roselle Calyx on sperm function in fresh semen of goat by examining sperm motility, progressive score and abnormality.

2. Method

2.1 Extraction of Roselle Calyx

Fresh Roselle (Hibiscus Sabdariffa L.) fruits variety of Terengganu (UMKL-1) aged 20 to 30 days after bloom were harvested from Taman Pertanian Universiti, Universiti Putra Malaysia (UPM). Immediately after harvesting, the fruits were washed with tap water until all dirt were removed and soaked with distilled water for about 15 minutes. Seeds were removed and Roselle calyces were collected. Dried Roselle Calyces (50000 g) were cut to small and placed in distilled water (500 ml) to be heated until air bubbles appeared. Prepared stock solution from Roselle was stored in -20 ^{0}C until further diluted into four difference treatment concentrations; 13 mg/ml, 20 mg/ml, 33 mg/ml and 43 mg/ml.

2.2 Animal and Location

The semen from four sexually matured mixed-boer males aged of 2 to 3 years old with the average of live body weight 36.4 ± 0.6 kg and body condition score 2.5 ± 0.1 were used in this study. The study was conducted at Ladang Ternakan Haji Kassim, Kuantan Pahang. All bucks were fed with requirement maintenance of feeding (6:3 ratio of napier to pellet) and water was available ad libitum.

2.3 Semen collection and evaluation

Semen was sampled from all bucks around 0730 to 0800 and the semen was collected once for three consecutive weeks by using artificial vagina to mimic internal vagina of the female goat. Immediately after semen collection, the samples of semen were placed in water bath (37 ^{0}C) to maintain the semen quality. All semen collected from each individual bucks was analyzed to ensure the pooled semen was prepared from consistent quality semen (percentage of motilty >80%, progression score>3.5). All accepted ejaculations were pooled to eliminate individual buck differences. Pooled semen was divided into five aliquot and was diluted (1:9 ratio of semen to extender) with the base extender

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containing 13 mg/ml, 20 mg/ml, 33 mg/ml, 43 mg/ml extraction of Roselle calyces and one group as control (without extract Roselle calyces). A Tris-based extender containing 3.63 g tris, 0.50 g fructose, 14 ml egg yolk, 1.99 g citric acid, 1.0 streptomycin and 0.60 g penicillin [17] was used as the base extender. Each group of semen was then evaluated for their sperm motility, progressive score and morphology.

2.4 Assessment of sperm motility and progressive score

Percentage of motility were determine by counting the motile and non-motile sperm cells while progressive score was evaluate based on the forward movements of individual sperm cell using scoring of 0 = no movement to 5 = fast forward movement [17]. Five microliter (µl) of diluted semen from each groups were placed on pre-warm slide, covered with a cover slip and observed under compound light microscope. Four fields (about 200 spermatozoa) were observed to obtain a thorough reading of percentage motility and progressive score.

2.5 Assessment of sperm morphology

The percentage of normal and abnormal head, midpiece and tail were determined from sperm smears stained with eosin-nigrosin examined under compound light microscope at 40x magnification objective. Briefly, 7 µl of suspension semen was pipetted on centre of pre-warmed slide and slowly smeared with 7 µl eosin-nigrosin stain. 200 spermatozoa were observed in which an average of the four fields is the reading for that particular semen. The abnormal sperm were classified into several types of abnormality. Sperm abnormalities were classified into head, midpiece and tail. Tail were sub-classified into several types of tail defect (Figure 1).

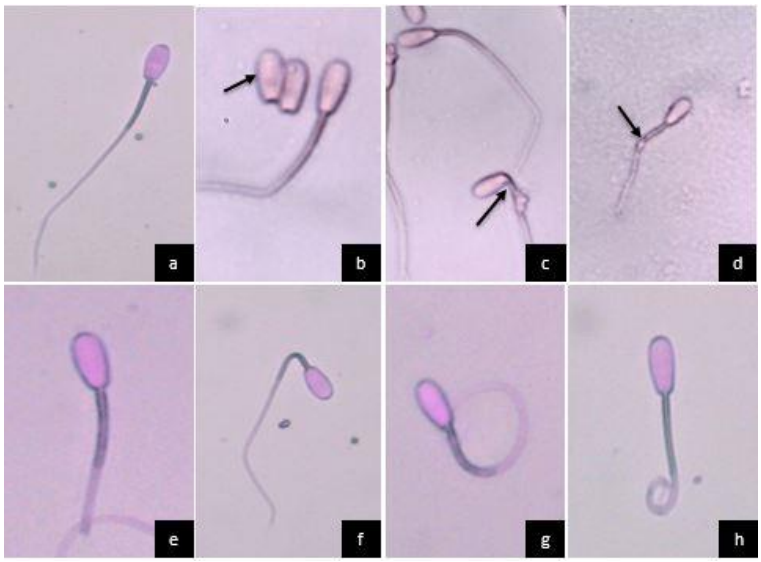

Figure 1. Normal sperm and sperm with different abnormality features: (a) Normal sperm; (b) Detached head; (c) Bent midpiece; (d) Proximal droplet; (e) Detached tail; (f) Bent tail; (g) Coiled spiral tail; (h) Bent coiled tail

2.6 Statistical analysis

The software of SPSS version 22 was used to carried out the statistical analysis. Multivariate Analysis of Variance (MANOVA) was performed in this study with $P<0.05$ considered significant. All of the values were expressed in mean ± standard error (SE). Normality of the data's were analysed using Kolmogorov-Smirnov test. Differences were examined by two-factor mixed (split-plot) factorial MANOVA with interaction including time and groups as main effects. Pairwise multiple comparisons were performed using the method of Tukey.

3. Results

3.1 Percentage of Sperm Motility and Progressive Score

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Table 1 summarises the results by showing the average percentage of sperm motility and progressive score of sperms with time interval between 0 to 1.5 hours. There were no significant differences between the Control and Roselle extract supplemented groups and within time interval (0-1.5 hours; $p > 0.05$). In general after 1.5 hours of sperm observation, the progressive score in all Treatment groups and Controls are above 70%, while the progressive score remains above 2.5.

Table 1. The Mean ± SE of sperm motility (%) in five different concentrations of calyx extract.

		Treatment (Roselle Calyx extract)			
Hour	**Control**	**13 mg/ml**	**20 mg/ml**	**33 mg/ml**	**43 mg/ml**
0	83 ± 2.58	81 ± 2.35	74 ± 3.68	81 ± 1.02	76 ± 3.41
0.5	82 ± 3.62	84 ± 2.03	83 ± 2.06	80 ± 1.78	79 ± 3.11
1.0	82 ± 2.40	78 ± 2.49	79 ± 2.88	81 ± 2.43	76 ± 4.02
1.5	78 ± 2.65	64 ± 9.40	78 ± 1.87	78 ± 2.77	73 ± 4.32
Average	**81 ± 2.81[a]**	**77 ± 4.07[a]**	**78 ± 2.62[a]**	**80 ± 2.00[a]**	**76 ± 3.71[a]**

[a] are not significantly difference. There were no significant differences between the Control and Treatment groups (P > 0.05).

3.2 Sperm Morphology

The results of sperm morphology are reported in percentage of normal and abnormal sperm (Table 2). The extender that supplemented with extract Roselle Calyces shows no significant effect on sperm shape abnormality and there were no interaction between group and time factor (Table 1). Generally, the result shows fluctuation mean data for sperm head, midpiece and tail abnormalities. However, the comparison between normal and abnormal sperm shows that for all groups mean sperm count for normal sperm are higher than abnormal (Table 2). Moreover, overall results of sperm morphology indicate that sperm tails are the most defected feature presence compare to other sperm structure.

Table 2. Mean ± SE of normal and abnormal sperm for head, midpiece and tail between Groups and time. There were no significant differences between the Group and time (P > 0.05).

Hour	**Group/mg/ml Calyx conc.**	**Head**		**Midpiece**		**Tail**	
		Normal	*Abnormal*	*Normal*	*Abnormal*	*Normal*	*Total abnormal*
0.0	0	168 ± 0.7	32 ± 0.4	162 ± 3.5	38 ± 1.8	110 ± 7.1	90 ± 7.1
	13	154 ± 0.7	46 ± 0.4	158 ± 12.7	42 ± 6.4	139 ± 2.1	61 ± 2.1
	20	157 ± 2.1	43 ± 1.1	166 ± 8.5	34 ± 4.2	113 ± 5.7	87 ± 5.7
	33	166 ± 0.7	34 ±0.4	166 ± 7.8	34 ± 3.9	109 ± 4.9	91 ± 4.9
	43	136 ± 2.8	64 ± 1.4	149 ± 4.9	51 ± 2.5	126 ± 4.2	74 ± 4.2
0.5	0	160 ± 0.4	41 ± 0.4	167 ± 7.1	33 ± 7.0	127 ± 3.5	73 ± 3.5
	13	154 ± 0.7	46 ± 0.7	175 ± 4.9	25 ± 4.0	114 ± 6.4	86 ± 6.4
	20	127 ± 2.5	76 ±0.7	166 ± 1.6	34 ± 4.9	118 ± 12.0	82 ± 12.0
	33	149 ±4.6	54 ± 3.2	167 ± 0.7	33 ± 0.7	112 ± 7.1	88 ± 7.1
	43	147 ± 2.5	55 ± 1.8	165 ± 4.9	35 ± 1.9	128 ± 4.2	72 ± 4.2
1.0	0	147 ± 1.8	59 ±2.1	160 ± 4.0	40 ± 4.9	110 ± 1.4	90 ± 1.0

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	13	136 ± 4.2	62 ± 2.8	158 ± 2.1	42 ± 2.1	122 ± 0.7	78 ± 1.7
	20	159 ± 6.0	43 ± 5.3	164 ± 0.7	36 ± 1.7	122 ± 7.1	78 ± 6.0
	33	148 ± 4.2	52 ± 4.2	157 ± 2.8	43 ± 2.0	110 ± 0.7	90 ± 0.7
	43	140 ± 2.1	61 ± 1.8	170 ± 1.8	30 ± 1.2	116 ± 3.5	84 ± 4.7
1.5	0	144 ± 1.1	58 ± 1.0	159 ± 2.1	41 ± 1.8	123 ± 2.1	77 ± 2.1
	13	161 ± 1.8	35 ± 2.8	156 ± 2.8	44 ± 2.8	113 ± 6.4	87 ± 6.4
	20	156 ± 6.1	34 ± 6.6	163 ± 2.1	37 ± .2.6	127 ± 0.7	73 ± 0.7
	33	149 ± 4.0	46 ± 3.9	157 ± 2.1	43 ± 1.0	118 ± 7.8	82 ± 7.8
	43	147 ± 3.5	58 ± 3.9	167 ± 0.7	33 ± 0.7	121 ± 0.7	79 ± 0.7

4. Discussion

Overall, these study unable to demonstrate the positive effect of aqueous extract of Roselle Calyx on sperm parameter. Although there is no significant difference between control and treatment group, however, good quality of sperm (60%) able to be maintain up to 1.5 hours. It is quite difficult to conclude that there are no effect of Roselle extract on sperm quality. Therefore, we would like to discuss the possible factors that contribute to inefficiency effect of Roselle extract on sperm parameter.

Extraction of Roselle using acidified solvent (methanol or ethanol) results in great effect in stabilizing anthocyanin and increasing extraction efficiency. Supported by [18], extraction of grape marc, by using acidified extraction media obtained high yield of anthocyanin pigment than using water based extraction which could only give minimum yield of anthocyanin in the extract. Acid is a good lysis buffer, it is more efficient when breaking down the plant cell membranes, thus dissolves the anthocyanins and stabilizes them [19]. In this study Roselle Calyx were extracted using distilled water as a solvent. This can be a possible factor of anthocyanin degradation thereby inefficiency effect of Roselle extract on sperm parameter.

Also reported, the dependency of antioxidant activity of Roselle extract on pH anthocyanin yield are high and most stable at low pH [19, 20] (pH 2 to 7). Acidification aids in maintaining a low pH thus providing a favourable medium for the formation of flavylim chloride salts. Stabilities of flavylium cation in highly acidic medium contribute to the efficiency of anthocyanins extractions [19]. Furthermore, anthocyanin is especially sensitive to light, pH and temperature. These factor cause the pigment to become unstable during processing and storage leading to degradation [12, 21].

Extraction of Roselle Calyx at 100°C may also cause degradation of anthocyanin pigment in this study. [22] has reported that the extraction temperature of Roselle Calyx at 70°C gave the highest yield, while at 90°C anthocyanin start to degrade. [23] suggested that Maillard reaction is the main explanation behind the degradation of anthocyanin by temperature. High temperature could provoke the interaction between anthocyanin pigment and sugar or reaction product and speed up the anthocyanin degradation.

From observation of sperm abnormalities several types of sperm tail abnormalities were determined in this study. Coiling of sperm tail is a sign of sperm abnormalities due to osmotic resistance of membrane on sperm tail [24]. The osmotic differences experiencing by the sperm, where the difference between internal and external solution causes biochemically-active sperm increase their volume in order to establish equilibrium [25]. The swelling process promotes an expansion of cell membrane covering the tail, that resulting the tail to coil. From the result, all groups have high number of tail abnormalities specifically on coiled tail. Therefore, it can be concluded that sperm tend to coil their tail as a response to high osmolality of extender. This evidence was supported by [26] which found that the high tail abnormalities of sperm are mainly cause by lower osmotic resistance of sperm in extender.

5. Conclusion

This study shows that extender supplemented with Roselle Calyx extract gives no significant effect on sperm parameter; sperm motility, progressive score and morphology. Contradictory results of this study with literature may be contributed by; 1) inefficiency of Roselle Calyx extraction method and

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2) degradation of pigment throughout the extraction and storage process. Therefore, further studies required monitoring of anthocyanin content in the extract Roselle Calyx to give clear effect to the semen parameters.

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