Effects of Aqueous Extract of Hibiscus sabdariffa L. (Roselle) Calyx on Bovine Sperm Membrane

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ABSTRACT
Sperm cryopreservation is a crucial in preserving sperm quality and studying male fertility defects. However, the process will cause several sperm abnormalities as it will produce excessive ROS that damage the sperm. As consequence, membrane cell disrupt, DNA of the sperm will damage and sperm motility diminished as wells as its viability deteriorated. Therefore, this study was conducted to determine the ability of aqueous extract of roselle calyx in protecting sperm freezing-thawing condition. UKMRN2 type has been chosen because it contains high antioxidants compare to other variety of roselle. All the control and treated group are incubated at 37°C for six hours. Extraction of sperm membrane is done to get pure sperm membrane. Sperm membrane that was treated with aqueous extract of roselle calyx of 15 mg/ml, 25 mg/ml and 50 mg/ml showed a significant increased compared with control. Concentration of malondealdehyde (MDA) in sperm membrane treated with aqueous extract of roselle calyx concentration of 15 mg/ml, 25 mg/ml and 50 mg/ml are decreased compared to control. It showed less lipid peroxidation occurred in the membrane that was treated with aqueous extract of roselle calyx. The increasing and decreasing of protein and MDA were depending on the dose of the roselle concentration. There were no significant changes in the sperm morphology of the three treatment groups of the aqueous extract of roselle compared with control group. In conclusion, this study found that the aqueous extract of roselle calyx could reduce ROS production in sperm freezing-thawing process.

Keywords: Roselle, UKMR-2, bovine sperm, lipid peroxidation, freezing-thawing process
INTRODUCTION

Cryopreservation involves freezing process that produce excessive reactive oxygen species (ROS) and cause destroy the sperm (Anger et al. 2003). According to Zhiling et al. (2009) ROS level after the thawing process will increased and have related with sperm quality. Defect during the freezing that have been reported are membrane defect, decreased in mortality rate viability (O’ Connell et al. 2002) and DNA defect (Donnelly et al. 2001, Chohan et al. 2004). Study from Yoshimoto et al. (2008) suggested that sperm cryopreserve with variety of extender supplemented with antioxidant to prevent the defect. Antioxidant supplementation showed that increased in quality of cryopreserve sperm (Grossfeld et al. 2008).

Roselle is in the family Malvaceae, which is under the same family with Hibiscus. Calyx extract can be a healthy drink because it has high antocyanin and vitamin C. The other products that produced from roselle calyx were jam and jelly (Intan 2008). In traditional medicine, roselle was use as antihypertension (Odigie et al. 2003) and antioxidant (Forambi & Fakoya 2005). Study that has been done before proved that roselle can prevent testis toxicity induce by cisplatin to the male rats (Amr & AlaaEldin 2006). This antioxidant activity was known contributed by phenolic content in roselle or also known as anthocyanin.

MATERIALS AND METHOD

**Sampling**

Sperm was obtained from Institut Biodiversiti Veterinar Kebangsaan (IBVK), Jerantut, Pahang Malaysia through artificial vagina. The bull type which used was Banting bred. Roselle aqueous extract was took from Roselle Research Laboratory, Science and Technology Faculty, Universiti Kebangsaan Malaysia, Bangi, Malaysia. Roselle that been used was UKMR-2.

**Study Design and Sample Preparation**

Cryopreserve sperm that was received was thawed at 37°C before used. The sperm then incubate for 6 hours at 37°C with different concentration of roselle calyx extract; 15 mg/ml, 25 mg/ml and 50 mg/ml meanwhile positive control of the group was incubated with vitamin C and control group sperm without treatment. Next, sperm was processed for observation under the scanning electron microscope and sperm left were undergoing membrane extraction process (Hinsch et al. 1992). Pellets were diluted with PBS to produce 5% sperm membrane suspension. The suspension was aliquots before stored in -40°C freezer until use.

**Biochemical Parameter**

Protein was measured using Lowry technique (1951). MDA was measured according to Stocks & Dormandy (1971).

**Sperm Morphology Observation**

Treated sperm was processed and observed under scanning electron microscope without membrane extraction process.

STATISTICAL ANALYSIS
Analysis of Variance (ANOVA) was performed in this study using SPSS (version 19.0) with p<0.05 considered significant. All of the values were expressed in mean ± standard error (SEM).

RESULTS

Protein Concentration
Protein concentration increased significantly (p<0.05) in bull sperm membrane treated with different concentration of roselle calyx aqueous extract than control sample (0.08 ± 0.01 µg/ml). Control positive sample which is sperm membrane treated with vitamin C protein concentration is 0.54 ± 0.04 µg/ml. Sperm membrane treated with roselle calyx aqueous extract concentration of 15 mg/ml protein concentration is lower (0.16 ± 0.01 µg/ml) than bull sperm membrane treated with 25 mg/ml roselle extract. 0.19 ± 0.01 µg/ml and 50 mg/ml roselle extract which is 0.28 ± 0.02 µg/ml. Protein concentration increased in bull sperm membrane treated with vitamin C for 554.2% followed by sperm membrane that treated with roselle calyx aqueous extract concentration 15 mg/ml, 25 mg/ml dan 50 mg/ml of 88.0%, 127.7% and 236.1% respectively.

MDA Level
The study showed MDA decreased significantly (p<0.05) in bull sperm membrane treated with different concentration of roselle calyx aqueous extract than the control sample (4.30 ± 0.81 nmol/g protein). Positive control the MDA concentration is 0.14 ± 0.04 nmol/g protein. Sperm membrane treated with roselle calyx aqueous extract concentration of 15 mg/ml MDA concentration is higher (1.10 ± 0.34 nmol/g protein) than sperm membrane treated with roselle calyx crude extract concentration of 25 mg/ml (0.97 ± 0.35 nmol/g protein) and 50 mg/ml which is 0.34 ± 0.25 nmol/g protein. MDA concentration decreased in membrane sperm treated with vitamin C for 96.74% followed by membrane treated with 15 mg/ml, 25 mg/ml dan 50 mg/ml which is 74.7%,77.4% dan 92.1% respectively. Table 1 showed comparison protein mean level and MDA for control and treated group.

Sperm Morphology Observation
Figure 1 showed comparison sperm morphology between control and treated group. A few cracked in control sperm which was sperm without treated with antioxidant. After sperm membrane treated with roselle calyx extract at 3 different concentration 15 mg/ml, 25 mg/ml and 50 mg/ml, no abnormality and significant changes were seen.

TABLE 1: Comparison between protein and MDA level for control and treated group of sperm.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Vitamin C (Positive control)</th>
<th>Roselle aqueous extract 15 mg/ml</th>
<th>Roselle aqueous extract 25 mg/ml</th>
<th>Roselle aqueous extract 50 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration</td>
<td>0.08 ± 0.01</td>
<td>0.54 ± 0.04</td>
<td>0.16 ± 0.01*</td>
<td>0.19 ± 0.01*</td>
<td>0.28 ± 0.02*</td>
</tr>
<tr>
<td>MDA concentration (nmol/g protein)</td>
<td>4.30 ± 0.81</td>
<td>0.14 ± 0.04</td>
<td>0.10 ± 0.34*</td>
<td>0.97 ± 0.35*</td>
<td>0.34 ± 0.25*</td>
</tr>
</tbody>
</table>

Value in mean ± S.E.M.
* : different with significant (p< 0.05)

**DISCUSSION**

Sperm membrane have polyunsaturated fatty acid (PUFA), clupanodonic and docosahexananoic higher than somatic cell, thus ROS tend to attack sperm cell (Apel-Paz et al. 2003). Therefore, the effect of roselle extract was analyzed in three parameters which is protein concentration, MDA and sperm morphology.

In this study roselle aqueous extract showed the protective effect towards sperm cell by increasing protein concentration significantly. Lipid peroxidation was decreased because MDA decreased significantly in sperm membrane treated with roselle extract. MDA was an indicator for lipid peroxidation. This evidence was supported by Wang et al. (2000) where MDA level is decreased in hepatotoxicity rats induce by tert-butyl hydroperoxide (t-BHP) after treated with roselle extract at 0.1 mg/ml and 0.2 mg/ml. MDA result was parallel with research done by Ologundudu et al. (2009) that shown roselle extract decreased MDA in

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**Figure 1:** Control bull sperm morphology (A) had cracked head. Sperm treated with vitamin C (positive control) (B), sperm treated with roselle calyx crude extract 15 mg/ml (C), 25mg/ml (D) dan 50 mg/ml (E).

K: head; MP: mid-piece; R: crack
rabbit tissue induced by 2, 4-dinitrophenylhydrazine. Lipid oxidation caused dilayer and cell integration damage. However, this phenomenon was prevented by roselle extract.

Total protein in sperm membrane treated with roselle calyx aqueous extract increased significantly with roselle concentration. Balamurugan & Muthusamy (2008) shown increased in protein concentration in rat’s hepar treated with *Trianthema decandra* Linn. After induced with carbon tetrachloride. Other than that protein in sperm membrane is not oxidized by ROS. Research by Martinez-Cayuela (1995), showed free radicals attacked lipid, protein, nucleic acids and carbohydrate and damage them causing cell metabolism imbalance. Reaction protein with ROS would produced oxidized protein (Soszyński et al. 1996) which was also the product of MDA and protein reaction (Traverso et al. 2004). This oxidized protein could act as secondary free radicals (Davies et al. 1995). However, MDA effects would be different in protein sperm membrane because protein content of sperm was low compared to other cells (Flesch & Gadella 2000).

This research also found that the effect of roselle extract was dose dependent. This was supported by Hirunpanich et al. (2005) which found that the effect of antioxidant in dried calyx of roselle aqueous extract against hypocholesterolemic rats was depending on dose of roselle (roselle concentration 0.001, 0.01, 0.25, 5 mg/ml). The higher dose of roselle extract could reduce more MDA. At 5 mg/ml roselle, it showed the complete inhibition of MDA production induced by copper sulphate (CuSO₄). This research also suggested that roselle aquoues extract have the antioxidant activity and protect lower density lipoprotein from oxidized. Therefore, higher roselle concentration was better because it had higher antioxidant content. This antioxidant acts as hydrogen donor to unstable free radicals and inhibit free radicals cascade. Furthermore, antioxidant would not change to free radicals because of the molecular stability.

Previous study proved that cryopreservation produced excess ROS that cause sperm damage (Anger et al. 2003). Freezing-thawing process increased the ROS production in human sperm (Alvarez & Storey 1992) and bull (Chatterjee & Gagnon 2001). As a result, extender treated with antioxidant before cryopreservation process would give protective effect rather than antioxidant treated after freezing-thawing process. This is because antioxidant treated after freezing-thawing process cannot against the ROS attack during cryopreservation process but against during freezing-thawing process only (Yoshimoto et al. 2008).

From sperm morphology observation, there was a slightly crack head in control group and no changes in morphology of treated sperm. Crack head sperm was the result of the action of ROS in the sperm membrane. From the study done by Girotti et al. (1991), MDA will damage the amino group structure, phospholipids bilayer and other structure and cause the production of small pore in the sperm membrane of the head. The result of treated sperm is same with the previous study which is no changes in sperm morphology in the head, mid-piece and tail after treated with vitamin C.

**CONCLUSION**

The result of this study show that sperm that treat with roselle calyx crude extract can reduce the oxidative stress caused by freezing-thawing process. Therefore roselle extract has a
potential to be an additive as antioxidant in extender medium of sperm cryopreservation instead of vitamin C.

REFERENCES


