PENGARUH EKSTRAK DELIMA MERAH(Punicagranatum L.) TERHADAP PENINGKATAN PERSENTASE KAPASITASI DAN REAKSI AKROSOM SPERMATOZOA TIKUS PUTIH (Rattusnorvegicus)

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INFLUENCE OF POMEGRANATE EXTRACT (*Punicagranatum L.*) ON IMPROVEMENT OF PERCENTAGE OF SPERMATOZOA CAPACITATION AND ACROSOME REACTION OF WHITE RAT (*Rattusnorvegicus*)

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ABSTRACT

The aim of this research is to know the effect of pomegranate (*Punicagranatum L.*) on the improvement of spermatozoa quality that is increase of percentage of capacitation and acrosome reaction in white rat (*Rattusnorvegicus*). This research used 20 healthy white rat (*Rattusnorvegicus*) with average age 2-3 months old, 200-300 g. Research design using completely randomized design (CRD) with four treatments and 5 replications. Rats were randomly divided into 4 treatment groups. Rat in the negative control group not treated just kept alone, solvent control group were given CMC Na 0,5% and is exposed to heat, the treatment groups 1, 2, and 3 induced pomegranate extract standardized to 40% ellagic acid at a dose of 75mg / kg, 150mg / kg and 300mg / kg perorally with doses 1 ml/rat/day gastric for along 14 days and exposed to heat. Data were analyzed using One Way Analysis of Variant (ANOVA) followed by Duncan ($p<0,05$). The result showed that there was significant effect from therapy on the quality of rats spermatozoa especially to capacitation and acrosome reaction. The treatment from treatment 2 group showed the best result in maintaining the quality of rats spermatozoa with 27.6± 3.04 values for capacitation and 6,80 ± 1,30 for acrosome reactions. Extract of pomegranate (*Punicagranatum L.*) standardized to 40% ellagic acid has a significant change in improve the quality of spermatozoa, namely an increase in the percentage of capacitation and acrosome reactions in white rats (*Rattusnorvegicus*) exposed to heat.
Keywords : Pomegranate extract, Capacitation, Acrosome Reaction

Research Background

The quality of reproduction is in-fluenced by the ability of spermatozoa to fertilize. Fertilization plays a role in the process of capacitation, where spermatozoa cells can penetrate the ovum has an im-portant role in improving reproductive qua-lity (Lestari, 2014). Fertilization involves a process of capacitation and acrosome reac-tions. The process of capacitation is a series of changes that occur to prepare the sperm to meet and interact with the ovum at the ti-me of fertilization. The process of capaci-tation includes the ability to spermatozoa for motile or movement, the ability to fer-tilize and the removal of cytoplasmic dro-plet. Spermatozoa cannot afford to fertilize without a capacitation process (Bearden and Fuquay, 2000).

Acrosome reaction is the reaction of re-lease of enzymes from the acrosome to pe-netrate the oocyte layers by induction by zone proteins. Acoromic reactions occur only in spermatozoa that have intact mem-branes. In addition, the acrosome reaction takes place before the spermatozoa pene-trate into the zona pelucida / ZP (Grudzinskas and Yovich, 1995 in Anwar 2014).

Spermatozoa require reactive oxygen species (ROS) at low concentrations to in-duce the process of capacitation and acro-some reactions (Sikka, 2004) and bind to the zona pellucida (Sanocka and Kupisz, 2004) so that fertilization proceeds well. Physiologically free radicals are present in sperm (Zavos *et al*. 1998) and the onset of free radicals in the body is offset by en-

dogenous defense mechanisms, by producing substances that have an anti-free radical effect called antioxidants. However, as the ROS level rises above the body's antioxidant defense system, oxidative stress occurs (Saleh *et al*., 2003).To reduce ROS needs to be provided additional antioxidants (Makker *et al*., 2009).

Pomegranate (*Punica granatum L*) are one source of antioxidants from plants with high polyphenol content (Aviram *et al*., 2014).Anthocyanin is one of the pomegranate compound that have a powerful antioxidants that can prevent various damages caused by oxidative stress so as to protect cells from free radicals (Yanjun *et al*, 2009).

Research on the effect of antioxidants in pomegranate to the process of sperma-tozoa capacitation has never been done. Therefore this research was conducted find out the effect of giving pomegranate to spermatozoa by using experimental animal White rat (*Rattus norvegicus*)

Materials and Methods

This research was carried out for 4 weeks in May to June 2017. The research was conducted at three locations. LaboratoriumFarmakologiKedokteranHewan,DepartemenBiokimiaFakultasKedokteran, and Institute Tropical Disease (ITD). This research used20 healthy white rats (*Rattusnorvegicus*) with average age 2-3 months old, 200-300 g body weight (BW) and bought from Pusat Veterinaria Farma (PUSVETMA) Surabaya.

Research material used in this study is white rat (*Rattus norvegicus*), white mouse semen, pomegranate (*Punica granatum L*) extract powder that contains 40% ellagic acid produced by Xi'an Biof Bio-Technology Co., Ltd. (Room 1-1111, High-tech Venture Park, No. 69 Jinye Road, GaoxinDistric of Xian, People Republic of China), *FlourescentIsotiocianant* (FITC) Staining, PBS Aquabides, NaCl physiological, For-maldehyde, Ethanol 70 % and Glycerol.

Research tools used in this study is a scaled-semen container tube, epifluorecent microscope, object glass, glass cover, test tube, tube rack, petri dish, bath, 1 ml and 3 ml syringe pipette, thermometer, aluminum foil, tissue paper and camera digital.

White Rat aged 2-3 months weighing 200-300 grams were adapted for 7 days. Experimental animals consisted of 1 control group, and 3 treatment groups. Each group consisted of 5 white rats and randomly selected.Before giving pomegranate extract, experimental animals exposed to the sun at 09.45 WIB for 15 minutes to get free radical directly. The control group (K) was given only CMC Na 5% oral solvent, T1 group, T2, T3 white rat were treated with excretion of pomegranate orally with multilevel dose, i.e. dose 75 mg / kgBW, 150 mg / kgBW, and 300mg / kgBW once daily For 14 days.

At the end of the treatment all of the rat were terminated with ether, then the abdominal surgery was performed and a collection of testicular organ was performed (Aulanni'am *et al*., 2007). Spermatozoa taken from the epididymis, exactly 1 cm below chorda spermatica. In the place is clamped and cut. The cut part is removed by sperm by squeezing on the epididymis then given physiological NaCl and stirred to become homogeneous.

Testing of capacitation status was performed by *FlourescentIsotiocianant* (FITC) Staining. Briefly the FITC preparation process is as follows: (1) 100 µl of treated semen is fed into aluminum foil enclosed eppendorf tube and added with 100 µl dye FITC, vortex for 1 min, and add 8 µl FITC fixative vortex solution for 1 min, (2) Taken 10 µl (mixture 1 and 2) and placed on top of the glass object, added with 10 µl of DABCO solution then mixed with a micropipette tip, then covered with a glass cover and carefully pressed with thickly coated tissue palms. The side of the glass cover is adhesive with cutex.

(3) The observations were performed with an epifluourescence microscope using an ultra violet light source. FITC staining on spermatozoa exhibits 2 different forms of fluorescent flux, ie (1) The same fluorescent distribution in the spermatozoa (non-capacitated) head, and (2) Fluorescent is concentrated in the acrosomal region indicating the spermatozoa is capacitated. Observation of capacitation status is done after the next 24 hours (Utomo, 2011).

Examination of acrosome reaction were done by fixated semen with 4% formal dehyde, then washed by adding 3 ml PBS and

centrifuged 1500 rpm for 10 min, supernatant was removed and added with 0.3 ml of FITC con A (Sigma) with 10 µg / ml concentration in PBS dulbeccos. Staining is done for 25 minutes at room temperature, then washed 2 times with 1500 rpm centrifugation for 10 minutes. The supernatant was removed and the precipitate was scratched on the slide labs of the specimen, dripping with 90% glycerol. Further specimens were observed in the epifluorescent microscope with excitation B (excitation 490 rpm with 525 nm emission) to determine fluorescence in FITC spermatozoa. Observations show: (a) spermatozoa with intact acrosome, and (b) spermatozoa without acrosom (Utomo, 2011).
The data obtained were arranged in one table, then the white mouse capacitated results were analyzed using One Way Analysis of Variant (ANOVA) then proceed to Duncan to know the real difference between the treatments (Kusriningrum, 2008).

Result and Discussion

Experimental result of capacitation and acrosome reaction of rat (*Rattus norvegicus*) which was given pomegranate extract (*Punicagranatum L.*) after stained with FITC (Fluorescent Isotiocianat) and was observed with epifluorescent microscope 400x magnification as shown in Tabel 1.

Table 1. Experimental result of capacitation and acrosome reaction of rat (*Rattus norvegicus*) spermatozoa

Treatment	**Spermatozoa Capatitation (X ± SD)**	**Spermatozoa Reaction Acrosome (X ± SD)**
K	15,40^{a} ± 1,81	2,20^{a} ± 0,83
T1	17,60^{a} ± 1,81	3,00^{a} ± 0,70
T2	27,60^{b} ± 3,04	6,80^{c} ± 1,30
T3	25,00^{b} ± 1,58	5,20^{b} ± 0,83

Different superscripts in the same columnshowed significant differences (p <0.05). K= Control treatment, Rats were given CMC solvent Na 0.5%, T1 = Treatment 1, White rats given pomegranate extract dose 75 mg / kg BW, T2 = Treatment 2, White rats given pomegranate extract dose 150 mg / kg BW, T3 = Treatment 3, White rats given pomegranate extract dose 300 mg / kg BW

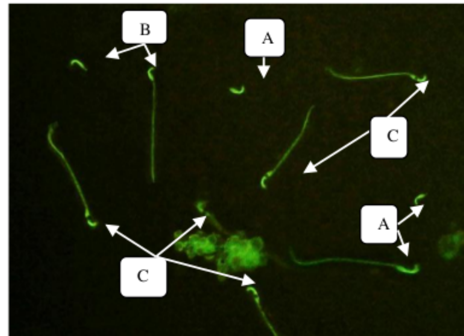

Figure 1 Results of observation of capacitation and white rat spermatozoa acrosome reaction of white rat with FITC staining using epiflouroecent microscope (A: Non-Capacitation, B: Capacitation, C: Acrocome Reaction).

Based on the results of the Analysis of Varian (ANOVA) test followed by Duncan test on the average of spermatozoa capacitation and acrosome reaction of white rats examination showed that there was a noticeable difference (P <0.05). The results of examination of percentage of spermatozoa capacitation on K(15,40) and T1 (17,60) have no significant differences, T3 (25,00) and T2 (27,60) also have no significant difference, but K and T1 differ significantly with T2 and T3.In the Acrosome Reaction results, K (2,20) and T1 (3,00) had no significant differences, T2 (6,80) differed significantly with T3 (5,20), K and T1 had significant differences with T2 and T3.
Normally, free radical levels are maintained in physiological levels by antioxidants. Endogenous antioxidants that play a role in maintaining free radical levels are glutathione (GSH), superoxide dismu-tase (SOD), and catalase. GSH plays an important role in coordinating antioxidants in the body to counteract free radicals. However, if the production of excessive free radicals, there remains a part of the remaining ROS. To absorb the remaining ROS needs to be provided additional antioxidants (Makker, 2009).
Pomegranate is a potent source of exogenous antioxidants. All parts of pomegranate plants contain polyphenols and have antioxidant activity. The main group of phytochemicals in pomegranates is

the polyphenols found in the fruit. Polyphenols comprise flavonoids, hydrolysable tannins and condensed tannins (Yuniarti, 2012).Hydrolyzable tannins (HTs) as a major part of polyphenols in pomegranate and punicalaginis the most dominant part of HTs responsible for nearly half of the pomegranate juice antioxidant activity. Punicalagin is one of ellagitannins compounds found in many fruit and pomegranate stalks. Punicalagin contained in pomegranate has antioxidant activity up to 89%(Gil *et al*., 2000).

In this study it was proved that the results of treatment using pomegranate ex-tracts had a significant effect on increasing the amount of capacitation and spermatozoa acrosome reaction. With the results of the average research conducted can show the maximum level of use of the pomegranate extension. The average result of capacitation in this study showed that the percentage of spermatozoa control treatment capacitation showed a yield of 15.40 ± 1.81. In the dosing group 75mg / kg BW showed a result of 17.60 ± 1.81 it can be concluded that the giving of pomegranate extract with a dose of 75 mg / kg BW can maintain spermatozoa capacitation of white rats. In the treatment with the dose of 150 mg / kg BW showed 27.60 ± 3.04 can be seen that with the provision of pomegranate extract 150 mg / kg BW proved abler to maintain spermatozoa capacitation to reach the ovum, because the success of fertility is determined by the spermatozoa capacitation status, membrane quality and penetration power against zona pellucida (Ba'a, 2009). In the examination of the percentage of capacitation with a dose of 300 mg / kg BW resulted in a 25,00 ± 1.58 percentage, the percentage result at the treatment of 300 mg / kg BW dose was decreased from a better dose of 150 mg / kg BW. This is thought to be due to the content in flavonoids and tannins contained in the pomegranate given has exceeded the optimal limit in dosing. One of the antioxidant content of tannin that can cause sperm clot, alkaloid suppress the secretion of reproductive hormone that is testosterone so that spermatogenesis process is disturbed (Winarno, 1997).

The results of percentage of acrosome reaction in this study showed that treatment with dosage of 150 mg / kg BW with a value of 6.80 ± 1.30 shows the highest rate of acrosome reaction results with different doses. At dosing 75 mg / kg BW increased compared with control treatment that was not given pomegranate extract. But the ANOVA statistic test followed by the Duncan test showed insignificant results. This suggests that administration of pomegranate extract at doses of 75 mg / kg BW may increase the acrosome reaction but the increase is not statistically significant. This increase shows that pomegranate extract acts as an exogenous antioxidant capable of neutralizing free radicals (Reynertson, 2007). This means that the polyphenols contained in the pomegranate are influential in maintaining the integrity of the spermatozoa membrane and by donating hydrogen atoms to free radicals and converting them into non-reactive forms (Bravo, 1998).

From the results it is clear that by gi-ving the dose of pomegranate extract 150 mg / kg BW per oral give the biggest result that is 6,80 ± 1,30 which is different with treatment without giving extract of pomegranate. In the treatment of doses of 300 mg / kg BW showed a decrease in yield compared with a dose of 150 mg / kg BW. The decrease can be caused by the head of the spermatozoa cannot be protected by cytoplasmic membranes that have been damaged by imbalance doses and free radicals so it will be easy to suck the color so it can be concluded that the percentage of sperm that included death (Nugraheni, 2003).

Conclusion

The conclusion that can be drawn from this research is the effect of giving pomegranate extract (*Punica granatum L.*) standardized 40% ellagic acid with doses of 75 mg / kg BW, 150 mg / kg BW and 300 mg / kg BW can improve the quality of spermatozoa, namely an increase in the percentage of capacitation and acrosome reactions in white rats (*Rattusnorvegicus*). The optimum dosage generated from this study was by administering a dose of 150 mg / kg BW.

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