**RESEARCH ARTICLE**

**The Effect of Continuous Multistage Countercurrent Extraction (CMCE) Propolis Extract Administration on Leydig, and Sertoli Cells Counts, and Sperm Quality Induced With Monosodium Glutamate (MSG) (An experimental study on male Wistar rat induced by monosodium glutamate)**

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**ABSTRACT**

**INTRODUCTION:** ROS is the most common cause of male infertility. Monosodium glutamate (MSG) is a compound which produces ROS and decreases FSH and LH levels. Continuous Multistage Countercurrent Extraction (CMCE) propolis extract as an antioxidant is evidently capable of reducing ROS concentration. **OBJECTIVE:** this research aims at evaluating the effect of CMCE propolis extract administration on Leydig cells and Sertoli cells counts and sperm quality of MSG-induced male wistar rats.

**METHODS:** employing the Post Test Only Control Group Design, 18 rats were divided into 3 groups: control group, treated only with MSG (MS-G), and treatment groups, respectively treated with CMCE propolis extract at doses 8.3mg/day (CM8-G) and 10.8mg/day (CM10-G). CMCE propolis extract was given orally for 21 days. At the end of treatment, sperm and testis were taken to evaluate the Leydig cells and Sertoli cells counts and sperm quality. The sperm obtained from epididymis was analyzed using the WHO standards, while Leydig cells and Sertoli cells were counted from preparation using the HE stain method.

**RESULTS:** the Post Hoc analysis result shows that the Leydig and Sertoli cells counts and sperm quality of CM8-G and CM10-G are significantly higher than those of MS-G, p<0.05. The Leydig cells and Sertoli cells counts and sperm quality of CM10-G are significantly higher than those of CM8-G, p<0.05.

**CONCLUSION:** CMCE propolis extract administration evidently increases the Leydig cells and Sertoli cells counts and sperm quality from the perspective of the count, motility and morphology of spermatozoa of MSG-induced male wistar rats.

**Keywords:** MSG, CMCE Propolis Extract, Leydig Cells Count, Sertoli Cells and Sperm Quality

**INTRODUCTION** Infertility is a problem in the reproduction system faced by men throughout the world. (Zegers-Hochschild et al., 2009). Twenty four to forty two percent of male infertility is caused by reduced sperm quality related to increasing ROS level (Singh, 2013; Martin-hidalgo et al., 2009).
al., 2019). Excessive ROS level in the body may cause oxidative stress and cause male infertility (Singh, 2013). Chronic use of Monosodium Glutamate (MSG) may trigger ROS production and reduce follicle stimulating hormone (FSH) and Luteinizing Hormone (LH) levels, followed with reduced level of testosterone (World Health Organization (WHO), 2010). Luteinizing Hormone (LH) produced by anterior hypophysis serves to stimulate Leydig cells in testis to produce testosterone. Meanwhile, FSH, which is also produced by anterior hypophysis, influences Sertoli cells to secrete growth factor and form androgen binding protein (ABP) serving to bind intratesticular testosterone produced by leydig cells and triggering spermatogenesis (Surikno, 2014). Therefore, decreased LH and FSH levels resulting from exposure to MSG may cause reduced sperm quality with mediation of reduced spermatogenesis.

Monosodium Glutamate is a compound often used to improve food deliciousness in Asia (Sharma, 2015). However, excessive use of MSG may endanger the body because of formation of free radicals and oxidative stress (Sharma, 2015). Free radicals and oxidative stress may occur to testis and various cells in the body including cells in hypothalamic, pituitary and gonadal axis. Interference in the axis causes reduced level of LH, FSH and testosterone hormones. Reduced LH level causes Leydig cells unable to produce testosterone, while reduced FSH level inhibits spermatogenesis (Martin-hidalgo et al., 2019). In addition, ROS may directly damage Leydig cells and Sertoli cells (Mahidan, Maulana and Susiyadi, 2018). Therefore, it is necessary to administer an antioxidant to reduce or even prevent excessive ROS production resulting from exposure to MSG. One of the important antioxidants derived from the nature is propolis, one of bee’s products.

Propolis is a compound containing Caffeic acid phenethyl ester (CAPE), constituting flavonoids which are able to donate its electron to neutralize free radicals, thus various cell damages resulting from oxidative stress may be prevented. Various studies show that propolis may reduce MDA level and improve sperm quality because of free radicals from exposure to smoke (Hoesada, Nasihun and Isradji, 2016). Another study shows that propolis at dose of 200mg/kgBW/day evidently reduces the degree of inflammation and MDA level of tested animal (Prasetyo, Suparyanti and Guntur, 2013). In addition, propolis extract at doses of 9, 18 and 27mg/day for 30 days is able to improve spermatozoa parameter which decreases because of exposure to MSG (Nasihun, Widyatari and Anindiya Kusuma W, 2012). On the other hand, in cervical cancer cells (Hela cell line), propolis ethanol extract administration improves apoptosis rate mediated by reduced Bcl2 expression and increased p21 expression in cervical cancer cell culture (Susanto, Maryono and Purwanto, 2017).

Propolis may be extracted using ethanol solvent or water solvent. Propolis used in this research is one extracted using the Continuous Multistage Countercurrent Extraction (CMCE) technology method, thus it may easily be absorbed and improve its bioavailability. The result of antioxidant level measurement in CMCE propolis using the Oxygen Radical Absorbance Capacity (ORAC) method shows its relatively high level (21.921 µmol/gram) (Fauzan and Bagus, 2016). Therefore, CMCE propolis is expected to be used more effectively. The objective of this research is to evaluate whether CMCE propolis administration may increase Leydig cells and Sertoli cells counts and sperm parameter quality after MSG administration.

METHODS
This experimental research employed a Post Test Only Control Group Design. In this research, 18 three-month old male Wistar rats with body weight 200-250gram were divided into 3 groups: control group, only administered with MSG at dose of 140mgBW/day; treatment group, induced with MSG at dose of 140mg/BW/day and CMCE propolis extract 8.3mg/day (CM8-G) for 21 days; and treatment group induced with MSG at dose of 140mg/BW/day and CMCE propolis extract at dose of 10.8mg/day for 21 days. During the research, meal and drinking water were provided to all groups ad libitum. At the end of research (day 22), the rats were terminated and their sperm was taken from epididymis and ductus deferens, and tested for spermatozoa quality (count, motility, morphology). The Leydig cells and Sertoli cells counts were obtained from preparation stained with Hematoxylin Eosin (HE). Leydig cells and Sertoli cells counts were calculated using a microscope with 400x magnification. The research was conducted upon approval from the Ethics Commission of the Faculty of Medicine, Unissula.

CMCE Propolis Extract
CMCE propolis extract is propolis specially extracted using Continuous Multistage Countercurrent Extraction method, thus any wax and resin are removed, leaving only beneficial materials such as flavonoids, flavon, polyphenols and phenolic acid ester which are easily absorbed.

Propolis Dose
Propolis extract dose used in this research was
The doses used for conversion were 450mg and 600mg. From these doses, the conversion into those of the body weight 200gram were: 450mg x 0.018 = 8.3mg/head/day and 600mg x 0.018 = 10.8mg/head/day.

**Testis Preparation Making**

After testis tissue was taken, preparation was made in order from fixation with 10% buffer formalin, and thin slices then less than 4mm were made and stored in formalin. Paraffin block was made through dehydration with alcohol, clearing with xylene, and embedding with paraffin wax, slides of which were then made for observation under 400x magnification microscope.

**Statistical analysis**

The data were analyzed using one way Anova (p<0.05), followed with post hoc LSD. The analysis result was deemed significant if p < 0.05.

**RESULTS**

This research was conducted in examining the effect of CMCE propolis extract administration on the increase in heydig cells and sertoli cells counts and spermatozoa concentrations of MSG-induced male wistar rats for 21 days. The average Leydig cells and Sertoli cells counts are presented in table 1.

The research result shows that the highest Leydig cells and Sertoli cells counts and spermatozoa quality (count, motility and morphology) are with CM10-G group, followed with CM-G group, and the lowest is with MS-G group. The statistical Anova shows that Leydig cells and Sertoli cells counts and spermatozoa quality (count, motility and morphology) among the groups are significantly different, p < 0.05. In examining the difference between the two variables of the various groups above, further analysis is needed as described below.

**Leydig Cells Count**

The Post Hoc analysis result shows that the Leydig cells counts of CM8-G and CM10-G groups are significantly higher than that of MS-G group, p < 0.05. The Leydig cells count of CM10-G group is also significantly higher than that of CM8-G group, p < 0.05 (figure 1).

**Sertoli Cells Count**

The Post Hoc analysis result shows that the Sertoli cells counts of CM8-G and CM10-G groups are significantly higher than that of MS-G group, p < 0.05. The sertoli cells count of CM10-G group is also significantly higher than that of CM8-G group, p < 0.05 (figure 1).

**Sperm Quality**

Sperm quality is marked with concentration, motility and morphology of spermatozoa.

**Spermatozoa Concentration**

The Post Hoc analysis result shows that the spermatozoa concentrations of CM8-G and CM10-G groups are significantly higher than that of MS-G group, p < 0.05. The spermatozoa concentration of CM10-G group is also significantly higher than that of CM8-G group, p < 0.05 (figure 2).

**Spermatozoa Motility**

The Post Hoc analysis result shows that the percentages of spermatozoa motility of CM8-G and CM10-G groups are significantly higher than that of MS-G group, p < 0.05. The percentage of spermatozoa motility of CM10-G group is also significantly higher than that of CM8-G group, p < 0.05 (figure 2).

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**Table 1. Average leydig cells count, sertoli cells count and spermatozoa count, motility and morphology.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>MS-G (n=6 X ±SD)</th>
<th>CM8-G (n=6 X ±SD)</th>
<th>CM10-G (n=6 X ±SD)</th>
<th>P (anova)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leydig Cell number (Σ)</td>
<td>3.53±0.242</td>
<td>4.46±0.242</td>
<td>5.53±0.206</td>
<td>0.000</td>
</tr>
<tr>
<td>Sertoli Cell Number (Σ)</td>
<td>18.73±3.1</td>
<td>25.03±4.5</td>
<td>31.43±6.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Sperm Density (10¹⁰/ml)</td>
<td>2.56±0.914</td>
<td>3.63±0.258</td>
<td>4.91±0.136</td>
<td>0.000</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>41.33±1.211</td>
<td>53.83±1.472</td>
<td>65.33±1.633</td>
<td>0.000</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>54.50±2.174</td>
<td>65±1.414</td>
<td>76±2.191</td>
<td>0.000</td>
</tr>
</tbody>
</table>
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Figure 1. Leydig and Sertoli cells on Hematoxylin Eosin stain method identified by light microscope with 400x magnification in three groups. Post Hoc analysis: * p < 0.05

Figure 2. Post Hoc analysis on Leydig and Sertoli cells in three groups. *p < 0.05

Figure 3. Post Hoc analysis on sperm quality in each group. Sperm density, Sperm motility, sperm morphology in three groups: *p<0.05

Spermatozoa Morphology

The Post Hoc analysis result shows that the percentages of normal spermatozoa morphology of CM8-G and CM10-G groups are significantly higher than that of MS-G group, p < 0.05. The percentage of spermatozoa morphology of CM10-G group is also significantly higher than that of CM8-G group, p < 0.05 (figure 2).

DISCUSSION

The research result shows that MSG administered at dose of 140mg/BW/day for 21 days evidently reduces...
This is proven that the Leydig cells count, Sertoli cells count and sperm parameter of the group administered with MSG are significantly lower than those of the other groups administered with propolis extract at dose of 8.3 or 10mg/day. This research result is in line with some previous researches that MSG administration may damage some organs such as kidney, liver, epididymis and prostate (Onyema, Farombi and Emerole, 2006; Onyema and Alisi, 2012; Hanipah et al., 2018). Such damage to various organs is related to oxidative stress caused by increased ROS level and increased lipid peroxidation marked with increased malondialdehyde (MDA) level, reduced oxidized glutathione (GSH) level and increased glutathione transferase (Onyema and Alisi, 2012; Hanipah et al., 2018).

Moreover, excessive use of MSG may also cause oxidative stress to testis cells, hypophysis and hypothalamus, which thus interferes in the performance of hypothalamic, pituitary and gonadal axis (Dong and Robbins, 2015). Consequently, gonadotropin releasing hormone (GnRH) secretion by hypothalamus decreases, followed with reduced FSH and LH levels secreted by anterior hypophysis. Decreased FSH level will be followed with decreased spermatogenesis, while decreased LH will be followed with reduced testosterone level produced by testis Leydig cells (Dong and Robbins, 2015). In addition, decreased FSH also causes Sertoli cells to fail to form androgen binding protein (ABP) as protein which transports testosterone to seminiferous tubules for spermatozoa maturation. Therefore, generally, excessive use of MSG may reduce sperm quality.

Furthermore, the research result also shows that CMCE propolis extract administration for 21 days, both at doses 8.3 and 10.8mg/day, evidently increases Leydig cells count, Sertoli cells count and sperm quality. This research result is in line with another research stating that propolis administration at doses 9, 18 and 27mg/day for 30 evidently increases the concentration and percentage of normal spermatozoa morphology of male wistar rats induced with monosodium glutamate (Nasihun, Widyatmi and Anindiyau Kusuma W, 2012). As previously hypothesized, MSG administration will damage hypothalamic, pituitary and testis organ axis and reduce sperm quality because of increased ROS. Therefore, improved sperm quality because of propolis administration is related to improved function of hypothalamic, pituitary and testis organ axis. Consequently, LH, FSH and testosterone hormones production will also improve (Capucho et al., 2012; Dong and Robbins, 2015). The statement is pursuant to previous study showing that CMCE propolis administration at doses 8.3 and 10.8mg/day evidently increases LH, FSH and testosterone level.

CONCLUSION
Administration of continuous multistage countercurrent extraction (CMCE) propolis extract increases Leydig cells count, Sertoli cells count and sperm quality of male wistar rats induced with MSG.

REFERENCES
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