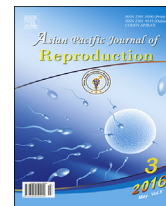




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Effects of honey to mobilize endogenous stem cells in efforts intestinal and ovarian tissue regeneration in rats with protein energy malnutrition

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ABSTRACT

Objective: Auto-regeneration of the intestinal and ovarian tissue are experiencing degenerative due to protein energy malnutrition (PEM) through of auto-mobilization, increase of immune response and differentiation of endogenous stem cells.

Methods: Female rat model of PEM obtained through fasting meals for 5 d and only to drink, causing malnutrition and damage of the intestinal and ovarian tissue. Furthermore, the therapy of honey with a dose of 30% water (T1) and 50% water (T2), respectively for 5 d and compared with the positive control, were fasted without being given honey (T0+) and negative control, not fasted and without being given honey (T0-). Observations on: auto-mobilization, increased immune response, differentiation of stem cells, and regeneration of the intestinal and ovarian tissue with HE staining.

Results: Auto-mobilization of stem cells based on the expression of CD34+ and CD45+, which is a marker of endogenous stem cells (hematopoietic stem cells/HSCs). Increased immune response is based on Hsp70 expression and PGE2 in intestinal tissue. Differentiation of stem cells into progenitor cells that expected based expression of growth differentiation factor-9 (GDF-9) by immunohistochemistry in ovarian tissue.

Conclusions: Expression of CD34+ and CD45+, which significantly different in treatment 2 (2). Furthermore, increase of immune response (decrease Hsp70 expression and increased PGE2) in intestinal tissue. Increased immune response causes expression of GDF-9 in ovarian tissue. Decreased of Hsp70 expression, increased PGE2 and increased GDF-9 followed the process of regeneration of the intestinal and ovarian tissue.

1. Introduction

Protein energy malnutrition (PEM) has become one of the causes of immune deficiency. Immune deficiency conditions PEM cause a decrease in the number of immune cells such as B-IgA+, population of T-CD5+, CD4+ cells, CD8 α +, CD8 β +, TCR α β +, and TCR γ δ in the lamina propria and intraepithelial villi intestine [1]. PEM can cause macrophage dysfunction [2]. Besides, the condition of protein energy malnutrition is the most common cause of secondary immune deficiency [3], thus opening incidence of opportunistic infections of intestinal parasites such as *Cryptosporidium* [4–9]. In the United States

and Western Europe, the prevalence of cryptosporidiosis in patients with immune deficiency was about 10%–20%, and in the developing countries of Africa and Latin America it reached 50% [4].

Until now, malnutrition is still a health problem in Indonesia. The prevalence of protein energy malnutrition in Indonesia has shown an increase since 2000. Death of nutrient deficiency in children is more than 50% due to protein energy malnutrition, and the cause of death of nutrient deficiency in children increased mortality due to diarrheal diseases. The result of the Direktorat Bina Gizi Masyarakat Ministry of Health, East Java was included in the category of ten provinces with the highest protein energy malnutrition cases in 2005. In 2009, East Java occupied the top position of national cases of severe malnutrition. This year, the number of PEM patients under 5 years old in East Java reached 77 500, the figure reached 2.5% out of the 3.1 million. Even the number of nutrient deficiency of children under 5 years is 527 000 children, or 17% of the total children

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under five year which is much higher [10]. In trial animals who have reached the age of puberty, protein energy malnutrition caused of the degeneration of the testes and ovaries cases so that the animals become infertile [11].

Interest in stem cell therapy today and the next few decades greatly increased sharply. This is because the potential of stem cell is very promising to be used as a treatment of various diseases. Stem cell transplantation provide new hope in the treatment of various diseases including immune deficiency diseases and infertility due degenerative conditions of the gonads can not be cured through treatment and operative measures [12–15].

However, due to the complexity of the method of isolation, culture *in vitro* and transplant process with the high cost of transplantation of stem cells, it would require an innovation in an effort to auto-mobilize and increase immune response is accompanied by differentiation of endogenous stem cells without going through the transplant process. Auto-mobilization and increased immune response accompanied differentiation was achieved through the provision of food or beverages derived from natural materials [14].

In this research through the provision of honey, it is expected there will be an auto-mobilization and increased immune response and differentiation of the patient's with degeneration of intestine and ovary [16]. The presence of auto-mobilization and increased immune response and differentiation of stem cells is accompanied sourced from the body itself will take place regeneration of lamina propria and epithelial intestinal villi and follicle and corpus luteum of the ovary.

The regeneration can be proven both histopathological and molecular. The histopathologically will occur regeneration of intestinal and ovarian tissue. Molecularly proven through several expressions such as expression of CD34+ and CD45+ of hematopoietic stem cells (HSCs), Hsp70 and PGE2 intestinal tissue and growth differentiation factor-9 (GDF-9) of the ovary [17].

2. Material and methods

2.1. PEM modeling causes intestinal and ovarian degeneration

This study begins by modeling PEM causes the intestine and ovaries degeneration in female mice were fasted for 5 d without food, only water to drink every 8 h per sonde [10]. Animals used in this study were Wistar strain female rats, aged 10–12 weeks with a weight of 200–250 g, in a healthy condition characterized by active movement. Mice kept in a plastic cage space per individual in laboratory animals experiments in Veterinary Medicine Faculty Airlangga University with adequate ventilation.

2.2. Treatment

The study were divided into 4 groups, each containing 6 replicates: The control group– (T0–): rats not fasted and without honey; The control group+ (T0+): rats were fasted for 5 d and without honey; The treatment group (T1): rats were fasted for 5 d, then given a 30% (v/v) honey in the drinking water for the next 5 d; The treatment group (T2): mice were fasted for 5 d, then given a 50% (v/v) honey in the drinking water for the next 5 d.

2.3. Flowcytometric observation of HSCs mobilization based on expression of CD34+ and CD45+

After the rats were treated, further examination of whole blood as a sample is taken via cardiac puncture and inserted into the tube heparin to prevent coagulation. Further observations were done on the expression of CD34+ and CD45+ by flowcytometry.

Flowcytometric method, starting with the preparation of whole blood centrifugation in a temperature of 4 °C, with a speed of 6000 r/min for 15 min. Results centrifuging the cell in the form of sludge mixed with cytoperm/cytofix amount of 2 times the number of cells are obtained. A mixture of cells and cytoperm/cytofix centrifuged to obtain a supernatant and a pellet. BD then added to wash the pellet amount 4 times the number of cells obtained in the first centrifugation.

Furthermore add lysis buffer amounting to 2 times the amount of the initial cells were obtained. After that add labeled antibody conjugate to each sample, five tubes are prepared and processed in parallel. (1) Single staining with CD34 PE added to the wash tube. (2) Double staining with CD34 PE and CD45 PerCP and CD44 FITC wash tube. (3) Double staining with CD34 PE and CD45 PerCP truocount tube. The entire sample was then stored at 4 °C in the dark and analyzed using flowcytometry for 1 h [17].

2.4. Immunohistochemical (IHC) methods observation of HSP70, PGE2 and GDF-9

Immunohistochemical observation was performed to determine the expression of HSP70, PGE2 dan GDF-9. Before to IHC methods were made histological preparation, by way of an incision is made transversely intestine and ovarium tissue from paraffin blocks. Further examination was performed by making outward through immunohistochemical techniques using monoclonal antibodies. This is done to determine the expression of HSP70, PGE2 and GDF-9. Observations of HSP70, PGE2 and GDF-9 were made using a light microscope with a magnification of 200 times and the expression of each variable is indicated by the number of cells with brownish discoloration chromogen in each incision [18].

2.5. Histopathology anatomy observation of ovarium

Regeneration identified of intestine and ovarium tissue through histopathological examination begins with the making of histological preparations. Histological preparations such as the following: rat intestine and ovarium fixation in 10% buffer formalin. Subsequently, rat intestine and ovarium dehydrated in alcohol solution with a higher concentration, i.e., from 70%, 80%, 90%, 96% (absolute). Then do the clearing in the intestine and ovarium of rat in xylol solution or chloroform or benzene. Furthermore performed embedding using liquid paraffin and rat ovarium were put into molds containing liquid paraffin. Before stained and sectioning performed, an incision using a microtome and mounted on glass objects. Furthermore is done the staining by removing of paraffin with xylol then put into a solution of alcohol with decreased concentration and then put into stain matter. The last stage after stained is done mounting, put into water or alcohol to remove excess stain. Then put into a solution of alcohol with increasing concentration, and then put into xylol.

Preparations then covered with a cover glass and mounted with Canada balsam or anthelan [19].

After making preparations histopathologic like the above examination using a light microscope with a magnification of 200 times. Observations identification of ovarium regeneration is based on the existing histological description.

2.6. Statistical analysis

Expression of CD34, CD45, HSP70, PGE2 and GDF-9 were statistically analyzed using SPSS 15 for Windows XP with the level of significance 0.05 ($P = 0.01$) and the confidence level of 99% ($\alpha = 0.01$). Steps comparative hypothesis testing is as follows: test data normality with the Kolmogorov–Smirnov test, homogeneity of variance test, Analysis of variants (ANOVA) factorial, Post hoc test (Least Significant Difference test) using the Tukey HSD 5%.

3. Results

Data were collected from 24 female rats which were divided into four treatments: negative control group (T0–) is normal intestine and ovary without bee honey treatment; positive control group (T0+) is degenerative intestine and ovary without bee honey treatment; (T1) group is degenerative intestine and ovary + 30% (v/v) honey in drinking water for 5 d; (T2) group is degenerative intestine and ovary + 50% (v/v) honey in drinking water for 5 d. In detail, the results of the study are as follows: the

effectively of bee honey was based on: mobilization of endogenous stem cells (HSCs), HSP70, PGE2 and GDF-9 expressions and regeneration of intestine and ovarium tissue.

Mobilization of HSCs was analyzed by flow cytometry based on increased concentration of CD34 and CD45. The analysis showed that: either a negative control group (T0–), positive control group (T0+) or group (T1) showed no mobilization of HSCs, based on the percentage of CD34 and CD45 which are at a percentage of less than 25% (Figure 1A,B, and C), whereas in the group (T2) showed mobilization of HSCs is based on the percentage of CD34 and CD45 which is located on the percentage of over 70% (Figure 1B). Based on statistical calculations T2 groups was significantly different ($P < 0.05$) than the other three treatments (T0–, T0+ and T1), whereas among the three treatments no significant difference ($P > 0.05$) (Table 1).

Furthermore, increased of immune response based on Hsp70 expression, in the normal control group (T0–) was on the score 0.17 ± -0.78 (Hsp70 expression between 1% and 5%). The group of intestine degenerative (T0+) was on the score 2.83 ± 0.45 (Hsp70 expression $>0.50\%$). The group 30% (v/v) honey (T1) was on the score 2.33 ± 0.37 (Hsp70 expression 25%–50%). The group use 50% (v/v) honey (T2) was on the score 0.67 ± -0.18 (Hsp70 expression between 6% and 25%) (Table 1).

Furthermore, the increased of immune response based on PGE2 expression, in the normal control group (T0–) was on the score 1.00 ± 0.00 (PGE2 expression between 6% and 25%). The group of intestine degenerative (T0+) was on the score

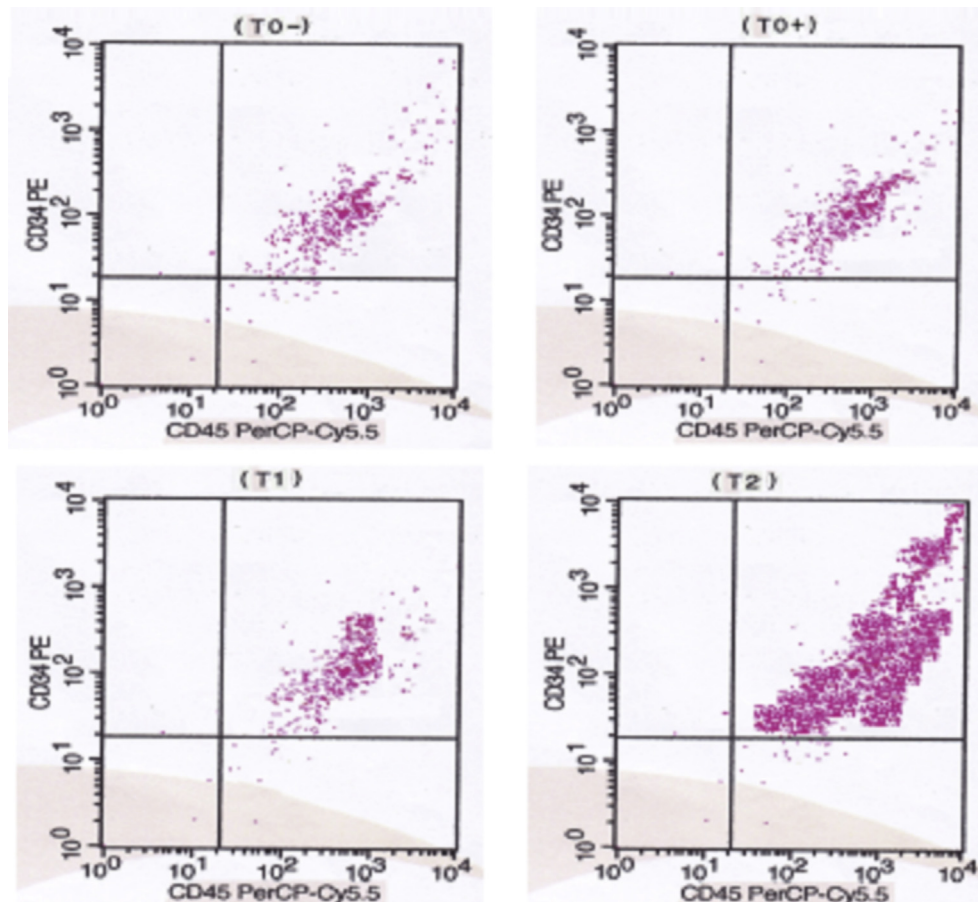


Figure 1. Flowcytometric analysis of endogenous stem cells mobilization (HSCs).

A. Control negative group (T0–): expression of CD34 and CD45 of 22.33 ± 1.35 ; B. Control positive group (T0+): expression of CD34 and CD45 of 23.66 ± 1.37 ; C. T1 group: expression of CD34 and CD45 of 24.83 ± 1.39 ; D. T2 group: expression of CD34 and CD45 of 74.83 ± 1.87 .

Table 1

Analysis of average percentage of CD34 and CD45 by flowcytometry, expression score of Hsp70, PGE2 and GDF-9 by immunohistochemical method.

Treatments	Average CD34 and CD45 (%)±sd	Average Hsp70 expression score ± sd	Average PGE2 expression score ± sd	Average GDF-9 expression score ± sd
Control negative group (T0-) with normal intestine and ovarium without honey	22.33 ± 1.35 ^a	0.17 ± -0.78 ^a	1.00 ± 0.00 ^b	2.83 ± 0.40 ^c
Control positive group (T0+) with degenerative intestine and ovarium without honey	23.66 ± 1.37 ^a	2.83 ± 0.45 ^b	0.17 ± -0.78 ^a	0.00 ± 0.00 ^a
(T1) group is degenerative intestine and ovarium + 30% honey in drinking water for 5 d	24.83 ± 1.39 ^a	2.33 ± 0.37 ^b	0.33 ± -0.48 ^a	0.33 ± -0.48 ^a
(T2) group is degenerative intestine and ovarium + 50% honey in drinking water for 5 d	74.83 ± 1.87 ^b	0.67 ± -0.18 ^a	2.83 ± 0.45 ^c	2.00 ± 0.00 ^b

^{a,b,c}Values in the same column with different superscripts indicate significant difference at $P < 0.005$ ($n = 6$).

0.17 ± -0.78 (PGE2 expression between 1% and 5%). The group 30% (v/v) honey (T1) was on the score 0.33 ± -0.48 (PGE2 expression between 1% and 5%). The group use 50% (v/v) honey (T2) was on the score 2.83 ± 0.45 (PGE2 expression > 50%) (Table 1). Honey treatment in this study proved to increase the immune response and intestinal motility via decreased expression of Hsp70 and increased PGE2 in the intestinal tissue.

Furthermore effectiveness of honey is based on GDF-9 expression as a result of the differentiation of the progenitor cells. Expression of GDF-9 in the group use 50% (v/v) honey (T2) was on the score 2.00 ± 0.00 (GDF-9 expression between 25% and 50%) (Figure 2D). Although the score was below the normal control group (T0-) was on the score 2.83 ± 0.43 (GDF-9 expression > 50%) (Figure 2A), but the percentage was still well above the group 30% honey (T1) was on the score 0.33 ± -0.48 (GDF-9 expression between 1% and 5%) (Figure 2C) and a group of ovarium degenerative (T0+) was not expressed at all 0.00 ± 0.00 (GDF-9 expression 0%) (Figure 2 and Table 1).

In this study, the regeneration of the ovarium can be observed through the method of histopathology with hematoxylin eosin (HE) staining. Microscopic examination showed that the group of 50% honey (T2), leading to the occurrence of ovarium tissue repair. Improvements are identified based on the regeneration of ovarium with growing follicle expression. Overview of these improvements can be compared with a control negative group (T0-) who did not experience ovarium degeneration, which remains in normal condition with growing follicle expression. As for the group of 30% (v/v) honey (T1) didn't indicate the occurrence of ovarium tissue repair. Not the improvement in the form of ovarium that are no longer intact (broken). Picture of the damage can be compared with control positive of rat (T0+) rat with ovarian degeneration. The group of ovarian degenerative (T0+), ovarly congested and hemorrhagic extensive, also visible hemosiderin due to blood cell lysis (brownish yellow color) accompanied by fibrin deposition indicating that chronic congestion has occurred. The group use 30% (v/v) honey (T1), ovarly does not regenerate, it appears there is congestion along hemosiderin expression and are still widely hemorrhagic (Figure 3).

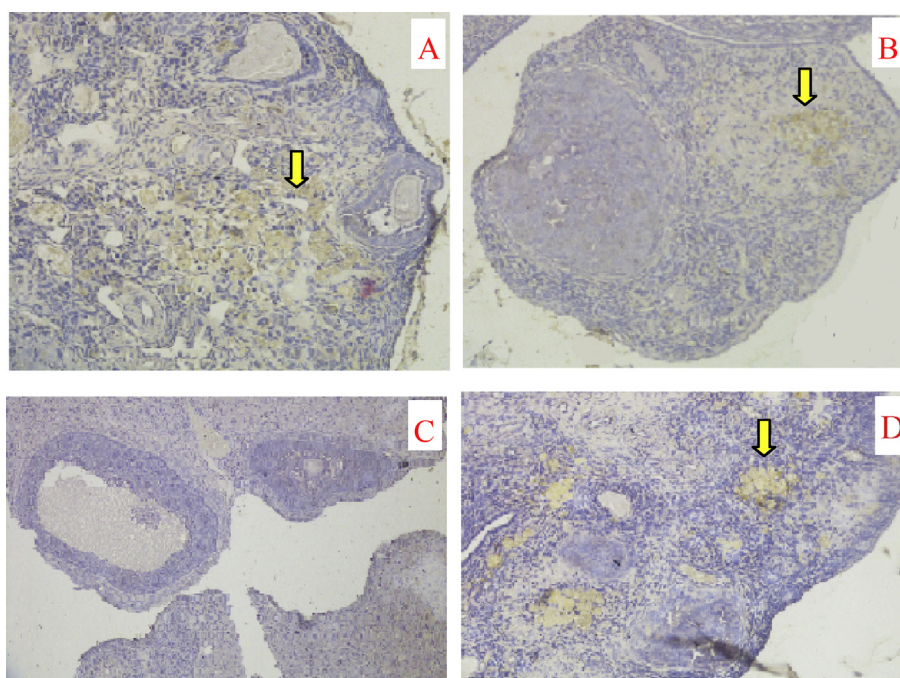


Figure 2. GDF-9 expression in rat ovarian tissue through immunohistochemical methods on several treatments.

A. The normal control group (T0-), score GDF-9 expression = 2.83 ± 0.43; B. The group use 30% honey (T1), score GDF-9 expression = 0.33 ± -0.48; C. The group of ovarium degenerative (T0+), score GDF-9 expression = 0.00 ± 0.00; D. The group use 50% honey (T2), score GDF-9 expression = 2.00 ± 0.00.

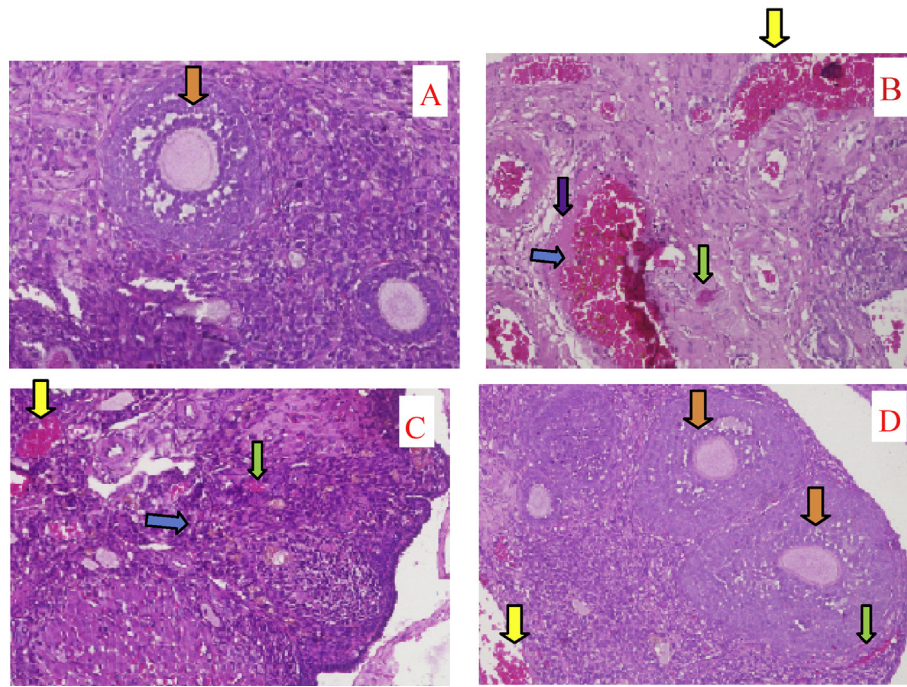


Figure 3. The regeneration of ovarian tissue through the method immunohistopathology anatomy (HP) with hematoxylin eosin (HE) staining in rat ovarian tissue in a few treatments.

A. The normal control group (T0–), was sowed growing follicle (↘); B. The group of ovarium degenerative (T0+), congestion of ovarian (↘) and widely hemorrhagic (↘), also visible hemosiderin (↔) due to blood cell lysis (brownish yellow color) with deposition of fibrin (↘) indicating that chronic congestive has occurred; C. The group use 30% honey (T1), ovary does not regenerate, it appears there is congestion along hemosiderin expression and are still widely hemorrhagic; D. The group use 50% honey (T2), ovaries begin to regenerate so it looks intact, although it still looks a bit happening hemorrhagic and congestion in some areas, but has seen growing follicles.

4. Discussion

The present study showed that giving honey by 50% (v/v) for 5 d in treatment (T2) can used for the treatment of female rat model of PEM. The effectivity of honey was based on: mobilization of endogenous stem cells (HSCs), HSP70, PGE2 and GDF-9 expressions, and regeneration of intestine and ovary tissue.

Mobilization of endogenous stem cells can occur due to undergo induction of stem cells so mobilized toward the defect. The process of mobilization can occur in several ways, one of which is an increase due to the immune response induced inflammatory reaction due to injury signals (Cytokines, NFκB, Wnt through β catenin) of tissue damage [20]. In this study, injury due to malnutrition signal causes an increase in Hsp70 and decreases PGE so that damage to the intestinal tissue can not be inevitable. Damage to the intestinal tissue is the cause of the disruption of the absorption of food that is needed by all body tissues including the ovary as the primary network of the female reproductive system [21].

This condition was need of repair, especially improvements in intestinal tissue as the tissue is responsible for the absorption of food. PGE2 have long been known to have cytoprotective effects on the gastrointestinal epithelium. Their cytoprotective effect appears to result from a complex ability to stimulate mucosal mucus and bicarbonate secretion to increase mucosal blood flow and particularly in the stomach, to limit back diffusion of acid into the epithelium [22].

Furthermore, this causes endogenous stem cells that had been induced to be will give support continual replenishment of gastrointestinal epithelium reside in the middle of gastric pits

and within the crypts of the small and large intestine. These stem cells proliferate continually to supply cells that then differentiate into absorptive enterocytes, mucus-secreting goblet cells, enteroendocrine cells and Paneth cells. Except for Paneth migrate up from the crypts to replace cells extruded from the tips villi. This migration takes 3–6 d [21].

Furthermore, the improvement of the intestine is eventually causes the ovarian tissue repair through GDF-9 expression. GDF-9 which is progenitor cells of germ line stem cells will stimulate ovarian cortex cell proliferation. This is in accordance with the opinion, that honey will cause the stem cells develop rapidly differentiate into cells that are needed as a response of the defect and enhancement of the immune response [23].

Regeneration of ovarium tissue, such as: intact of ovarium tissue with growing follicle expression is the third identification of the effectiveness from the use of honey. In this study, the regeneration of the ovarium can be observed through the method of histopathology anatomy (HPA) with HE staining. Microscopic examination showed that the group of 50% (v/v) honey (T2), leading to the occurrence of ovarium tissue repair. Improvements are identified based on the regeneration of ovarium with growing follicle expression. Overview of these improvements can be compared with a control negative group (T0–) who did not experience ovarium degeneration, which remains in normal condition with growing follicle expression. As for the group of 30% (v/v) honey (T1) does not indicate the occurrence of ovarium tissue repair. Not the improvement in the form of ovarium that are no longer intact (broken). Figure of the damage can be compared with control positive of rat (T0+) with ovarium degeneration. The group of ovarium degenerative (T0+), ovary was congested and hemorrhagic extensive, also visible hemosiderin (yellow brown) due to blood cell lysis with fibrin

deposition (baby color pink) indicating that chronic congestion has occurred. The group use 30% (v/v) of honey (T1), the ovary does not regenerate, it appears there is congestion along hemosiderin expression and are still widely hemorrhagic.

Treatment of 50% concentration honey for 5 d in female rat with degenerated intestine and ovary can show (i) mobilization of endogenous stem cells (HSCs), in the form of markers CD34 and CD45 are expressed in blood serum; (ii) increased immune response in the form of a decrease in the expression of Hsp70 and increased PGE2 by immunohistochemistry in tissue intestine and GDF-9 expression by immunohistochemistry in ovarian tissue; (iii) regeneration of ovarian tissue, such as: intact of ovarian tissue with growing follicle expression, although there is still little hemorrhagic and congestion but hemosiderin expression and fibrin deposition has not looked back.

Conflict of interest statement

We declare that we have no conflict of interest.

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