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AUTOREGENERATION OF MICE TESTICLE SEMINIFEROUS TUBULES DUE TO MALNUTRITION BASED ON STEM CELLS MOBILIZATION USING HONEY

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AUTOREGENERATION OF MICE TESTICLE SEMINIFEROUS TUBULES DUE TO MALNUTRITION BASED ON STEM CELLS MOBILIZATION USING BEE HONEY

Key words : Seminiferous tubule autoregeneration, Malnutrition, Stem cell mobilization, Bee honey.

ABSTRACT

Objective: The purpose of this study is to find out a novel therapy based on mobilization and differentiation of own body-derived stem cells using natural bee honey. Administration of 50% bee honey in this study causes autoregeneration of seminiferous tubules cells through stem cells mobilization with so differentiation process that degenerative cells undergo regenerate. **Methods:** Testicle degeneration model of mouse is available through food fasting 5 days long that causes malnutrition and affect the testicle. Then administration of 30% (T1) and 50% bee honey (T2) each for five days long and compared to the positive control, fast without bee honey (T0+) and negative control, feed but without bee honey (T0-). Subsequently observation of Hematopoietik Stem Cells (HSCs) mobilization according to the CD34 and CD45 expressions performed using flowcytometry method. Besides, identification of differentiation is examined through the expression of spermatogonial stem cells (SSCs) using immunohistochemistry technique, while the seminiferous tubules cell regeneration through H&E staining microscopic examination. **Results**: Stem cells mobilization based on the expression of CD34 and CD45, which is a marker of HSCs. Differentiation of stem cells into progenitor cells that expected based expression of SSCs in testicle tissue. SSCs increase causes regeneration testicle seminiferous tubules. **Conclusions**: of this study revealed a significantly different of C34 and CD45 expressions between groups, also an increase SSCs expression and testicle seminiferous tubules cells regeneration as well.

1. Introduction

The regenerative-medicine in this decade could be a basis of many diseases treatment of the future, mainly the degenerative disease that cured through neither medical therapy nor surgery [1], especially testicle degeneration [2, 3]. Testicular degeneration causes azoospermia. Azoospermia is a condition which is no spermatozoon produced by testicular seminiferous tubules, and makes infertility in the male and it means no offspring [3].

Testicular degeneration is the main cause of infertility in the male, the etiology is vary such as genetic alteration, mechanical trauma, neoplastic changes and aging or senility. Current stem cell therapy usually is through stem cell transplantation which is cultured in vitro previously. This is obviously very expensive. Therefore, it is needed a novel therapy based on mobilization and differentiation of own body-derived stem cells using natural bee honey.

Rapidly mobilized stem cells in an adequate number towards defected tissue (testis), in turn will differentiate to be certain cells (seminiferous tubules) from a certain tissue (testis) and will replace the damaged and apoptotic cells due to degenerative diseases, in this case, the differentiation becoming seminiferous tubules, sertoli and interstitial cells of Leydig.

It is important to find out an innovation of therapy through auto-regeneration-induced of seminiferous tubule cells using beneficial of natural bee honey in reproducing of spermatozoa. Regeneration of seminiferous tubule and subsequently Sertoli cells will provide support, nutrients and other environmental factors for young spermatozoa and male behavior to allow spermatogenesis and the ability of conception to happen.

Natural bee honey, a nutrient source from bee [4, 5] has antibacterial and antioxidant potencies [5, 6, 7]. Antioxidant is an important substance in protecting individual against free radicals. An adequate antioxidant consumption can reduce the prevalence of cancers, cardiovascular disease, cataract, digestive tract disorder and other degenerative diseases [8]

and in present study, the testicular degeneration. Bee Honey consumption will improve the digestive system, has a good result in diarrhea therapy [5] and reproductive system disorder as well [9]. As conceptual solution, it is needed further study to explore the beneficial of natural bee honey to induce auto-regeneration of testicular seminiferous tubule as a source of spermatozoa-producing tissue.

2. Material and Methods

2.1. Testicular Degeneration Model and Treatment

Testicular degeneration model of male mice is available through food fasting 5 days long, but drinking water is still administered [10, 11]. The experiment animals used in this study are healthy 8-10 week-old Balb/C male mice and 20-25g bodyweight each. They are placed in an individual plastic cage in Experimental Animal Laboratory at Veterinary Medicine Faculty, Airlangga University. They are divided into 4 groups of 8 mice each :

1. Negative control group (T0-) : feed but without bee honey
2. Positive control group (T0+) : fast but without bee honey
3. Trial group 1 (T1) : fast with 30% bee honey for 5 days
4. Trial group 2 (T2) : fast with 50% bee honey for 5 days

2.2. *Flowcytometri* Observation of HSCs Mobilization Based on Expression of CD34 and CD45

After the treatment, *whole blood* collected from cardiac puncture placed in *heparin* tube to prevent coagulate. Flowcytometri observation reveal the expressions of CD34 and CD45.

Flowcytometri method begins with *whole blood* centrifugation in 4°C temperature, 6000 rpm for 15 minutes. Cellular precipitation as a result of centrifugation then mixed with cytoperm/cytofix in amount of 2 times of obtained cell number. This mixture then centrifuged again and formed supernatant and pellet. *BD wash* added to the pellet in the amount of 4

times of obtained cell number from the first centrifugation. Centrifuge the mixture then added *lisis buffer* in the amount of 2 times of the first obtained cell number. Subsequently labeled antibody added to each sample, five tubes are arranged and processed parallel. (1) single staining with CD34 PE added to the *wash tube*. (2) double staining with CD34 PE and CD45 PerCP. (3) double staining with CD34 PE and CD45 PerCP and CD105 FITC-*trucount tube*. All the samples then stored in 4°C and dark room and analyzed with flowcytometri for 1 hour [12].

2.3. Imunohistochemical (IHC) Technique Observation of SSCs

Immunohistochemical observation was performed to determine the expression of SSCs. Before to IHC methods were made histological preparation, by way of an incision is made transversely testicular tissue from paraffin blocks. Further examination by making outward through immunohistochemical techniques using monoclonal antibody. This is done to determine the expression of SSCs. Observations of SSCs were made with a 40x microscope objective and the expression of each variable is indicated by the number of cells with brownish chromogen discoloration in each incision [13].

2.4. Histopathology Observation of Seminiferous Tubules

Regeneration identification of seminiferous tubule cells of the testes through histopathological examination begins with the making of histological preparations. Histological preparations such as the following: Mice testicular fixation in 10% formalin, 1 hour later injected formalin 10% in mid-testis. Subsequently mice testes dehydrated in alcohol solution with a higher concentration gradually, *ie* from 70%, 80%, 90%, 96% (absolute). Then do the clearing in the testes of mice in xylol solution or chloroform or benzene. Furthermore performed embedding using liquid paraffin and mice testes 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 H&E staining procedure. 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 Entelan [14].

Histopathological examination is done using a light microscope with a magnification of 400 times. Observations and identification of seminiferous tubules regeneration is based on the existing histological descrition

2.5. Statistical analysis

Expression of CD34 and CD45 and SSCs were statistically analyzed using SPSS 15 for Windows XP with the level of significance 0.01 ($p = 0.01$) and the confidence level 99% ($\alpha = 0.01$). Steps of comparative hypothesis testing are as follows: Test data normality with the Kolmogorof Smirnov test, homogeneity of variance test, Analysis of variance (ANOVA) factorial, Post hoc test (Least Significant Difference test) using the Tukey HSD 5%.

3. Results

Data collected from 32 male mice, divided into 4 trials : negative control group (T0-) is normal testis without bee honey; positive control group (T0+) is degenerative testis without bee honey; (T1) group is degenerative testis + 30% bee honey in drinking water for 5 days; (T2) group is degenerative testis + 50% bee honey in drinking water for 5 days. In detail, the results of the study are as follows : The effectively of bee honey was based on : 1. Mobilization of HSCs, 2. SSCs formation, 3. Regeneration of testicular tissue (such as: intact of seminiferous tubule tissue).

Mobilization of HSCs analyzed with Flowcytometry based on increased CD34 and CD45 concentrations. The result shows there was no HSCs mobilization in the three groups (T0-, T0+ and T1 groups) based on CD34 and CD45 percentage, which is less than 25%. On the other hand, those percentage in T2 is more than 70%, indicated HSCs mobilization (Figure 1-4). Statistically, there is a significantly different ($p < .05$) between T2 with the three other treatments (T0-, T0+ and T1), but not different ($p > .05$) between those three groups (T0-, T0+ and T1) (Table 1).

Furthermore effectiveness of bee honey is based on SSCs formation as a result of the differentiation of the spermatogonia. Expression of SSCs in the group use 50% bee honey (T2) is $48,33^{b} \pm 0,226$ (< 50%). Although the percentage is below the normal control group (T0-) is $86,66^{a} \pm 1,938$ (> 80%), but the percentage is still well above the group 30% bee honey (T1) of value $8,33^{c} \pm 0,921$ (< 10%) and a group of degenerative testis (T0 +) were not expressed at all $0^{d} \pm 0$ (0%) (Figure 5 and Table 1).

In this study, the regeneration of the testes can be observed through the method of histopathology with hematoxylin and eosin (H&E) staining. Microscopic examination showed that the group of 50% bee honey (T2), leading to the occurrence of testicular tissue repair. Improvements are identified based on the regeneration seminiferous tubules regenerate intact. Overview of these improvements can be compared with a negative control group of non infertile (T0-) who did not experience testicular degeneration, which remains in normal condition (Figure 6). As for the group of 30% bee honey (T1) does not indicate the occurrence of testicular tissue repair. Not the improvement in the form of seminiferous tubules that are no longer intact (broken). Picture of the damage can be compared with positive control of mice (T0 +) mice with testicular degeneration (Figure 6).

4. **Disscusion**

The present study showed that giving 50 % bee honey for 5 days are effectiveness for the treatment of degenerative testes of male mice models. The Effectively of bee honey was

based on: 1. Mobilization of HSCs , 2. SSCs formation , and 3. Regeneration of testicular tissue, such as: intact tissue of the seminiferous.

Mobilization of HSCs can occure because induction of stem cells so mobilized towards defect place. The process of mobilization can occur through number ways : 1. Induction proteolytic from microenvironment of bone marrow inside (induction of pharmacological agents such as G - CSF or Cyclophosphamide); 2. Blockade Of CXCR4 or VLA - 4 by specific blocking molecules (AMD3100 OR BIO4860) ; 3. Effect neural mediators (dopamine and receptors b2 - adrenergic); 4. Elements modulation from the coagulation of cascade ; 5. The immune response or inflammatory reaction causes injury signals induction (cytokines , NFkB , β catenin through Wnt) from the tissue damage; 6. Homing signals like - SDF 1 , CXCL12 , VEGF , HGF , PDGF and Integrin that appears and to be act as recruitment of stem cells [15].

Bee honey according [16] contains a variety of biologically active components like melittin and phospholipase-A2 (PLA2). Previous observations have shown that bee honey or its components are effective in proliferation, survival and differentiation of the cells [17].
The role of honey in this study provide suport niche through triger process of Vascular Endothelial Growth Factor - 1 (VEGF - 1) which is homing signal. Furthermore, VEGF - 1 binds to VEGF Receptor - 1 (VEGFR - 1) . VEGF is a component of Extra Cellular Matric (ECM) from stem cells have a role in supporting for a conducive microenvironment for stem cells [18]. Triger presence of VEGF - 1 - VEGFR - 1 will pass a series of signaling that activates Stem Cell Factor (SCF) intersisiel . SCF is a mechanic in the niche signaling protein that is physiologically will happen further communication [19]. The presence of SCF intersisiel be recognized SCF receptor complex and enter the cell nucleus so that expression of nuclear β1 - integrine for activating Octamer4 (OCT4). OCT4 is a member of the POU family of transcription factors , which have a major role in the proliferation of stem cells ,

self- renewal and differentiation. The existence of this proliferation causes HSCs change of shape from quiescenct into a cycling state, so that the HSCs located in the central endosteum area toward marrow. This suggests that cycling HSCs occurred outside of their niche and mobilized to the peripheral circulation [20].

Furthermore effectiveness of bee honey is based on SSCs formation as a result of the differentiation of stem cells are mobilized . The SSCs are progenitors of germline stem cells are formed by differentiation of Stem Cells. In this study formed SSCs can be identified through immunohistochemical methods.

Expression of SSCs in the group use 50% bee honey (T2) is approximately 50% (Figure 5.B and Table 1). Although the percentage is below the control group (P0-) greater than 80% (Figure 5.A and Table 1), but the percentage is still well above the group of testis degenerative + 30% bee honey (T1) of value <10% (Figure 5.C and Table 1) and a group of testis degenerative without use bee honey (P0+) were not expressed at all (0%) (Figure 5.D and Table 1). This is in accordance with the opinion of [21], that bee honey can causes proliferation of stem cells fastly and then differensiated to be cells that needed as response from defect.

Regeneration of testicular tissue, such as: intact of tubulus seminiferus tissue is the third identification of the effectiveness for using bee honey. In this study, the regeneration of the testes can be observed through the method of histopathology anatomy (HPA) with hematoxylin and eosin (HE) staining. Microscopic examination showed that the use 50% bee honey (T2), leading to the occurrence of testicular tissue repair. Improvements are identified based on the regeneration of seminiferous tubules cell (intact). Overview of these improvements can be compared with a control group of normal testis (T0-) who did not experience testicular degeneration, which remains in normal condition (Figure 6.A and 6.B). As for the group of testis degenerative + 30% bee honey (T1) does not indicate the occurrence of testicular tissue repair (Figure 6.C). Not the improvement in the form of cell degeneration seminiferous tubules that are

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Conflict of interest statement

We declare that we have no conflict of interest

Acknowledgments

The study was supported by funding from the Directorate General of Higher Education (DIKTI) 2015. The National Education Ministry. Republic of Indonesia. The authors are also grateful to LPPM chairman Airlangga University Prof. Dr. Djoko Agus Purwanto, Apt., M.Si. This project was done in laboratory of Stem Cells in Institute of Tropical Disease Airlangga University and laboratory of Pathology in Faculty of Veterinary Med, Airlangga University and the authors wish to thanks Prof. Fedik Abdul Rantam for his support.

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Table 1. Percentage of CD34 and CD45 with Flowcytometry and SSCs Expression in Mice Testes Tissue on Several Treatments

Treatments	Average CD34 and CD45 (%) ± SD	Average SSCs Expression (%) ± sd
Negative Control group (T0-) with normal testis without bee honey	$21,07^{a}$ ± 1,32	$86,66^{a}$ ± 1,938
Positive control group (T0+) with degenerative testis without bee honey	$23,01^{a}$ ± 1,362	0^{d} ± 0
(T1) group is degenerative testis + 30% bee honey in drinking water for 5 days	$24,36^{a}$ ± 1,37	$8,33^{c}$ ± 0,921
(T2) group is degenerative testis + 50% bee honey in drinking water for 5 days	$72,18^{b}$ ± 1,86	$48,33^{b}$ ± 0,226

^{a,b,c}Values in the same column with different superscripts indicate significant difference at P<0,005 (n=8)

Figure. 1. Flowcytometric analysis of HSCs mobilization in control negative group (T0-) with normal testis without bee honey showing positive expression of CD34 and CD45 in amount of 21.31%

Figure. 2. Flowcytometric analysis of HSCs mobilization in control positive group (T0+) with degenerative testis without bee honey showing positive expression of CD34 and CD45 in amount of 23.37%

Figure 3. Flowcytometric analysis of HSCs mobilization in (T1) group with degenerative testis + 30% bee honey in drinking water for 5 days showing positive expression of CD34 and CD45 in amount of 24.46%.

Figure 4. Flowcytometric analysis of HSCs mobilization in (T2) group with degenerative testis + 50% bee honey in drinking water for 5 days showing positive expression of CD34 and CD45 in amount of 72.54%.

Figure 5. SSCs Expression in Mice Testes Tissue with immunohistochemistry technique.
A. The normal control group (T0-), average SSCs expression = $86,66^{a}$ ± 1,938;
B. The group use 50% bee honey (T2), average SSCs expression = $48,33^{b}$ ± 0,226;
C. The group use 30% bee honey (T1), average SSCs expression = $8,33^{c}$ ± 0,921;
D. The group of degenerative testis (T0 +), average SSCs expression = 0^{d} ± 0

Figure 6. The regeneration of testicular seminiferous tubule microscopically with hematoxylin eosin (HE) staining in Mice Testes Tissue in some treatments. A. The normal control group (T0-), tubulus seminiferous seen intact; B. The group use 50% bee honey (T2), tubulus seminiferous undergoing regeneration; C. The group use 30% bee honey (T1), there is no regeneration; D. The group of degenerative testis (T0 +), there is no regeneration of seminiferous tubule.

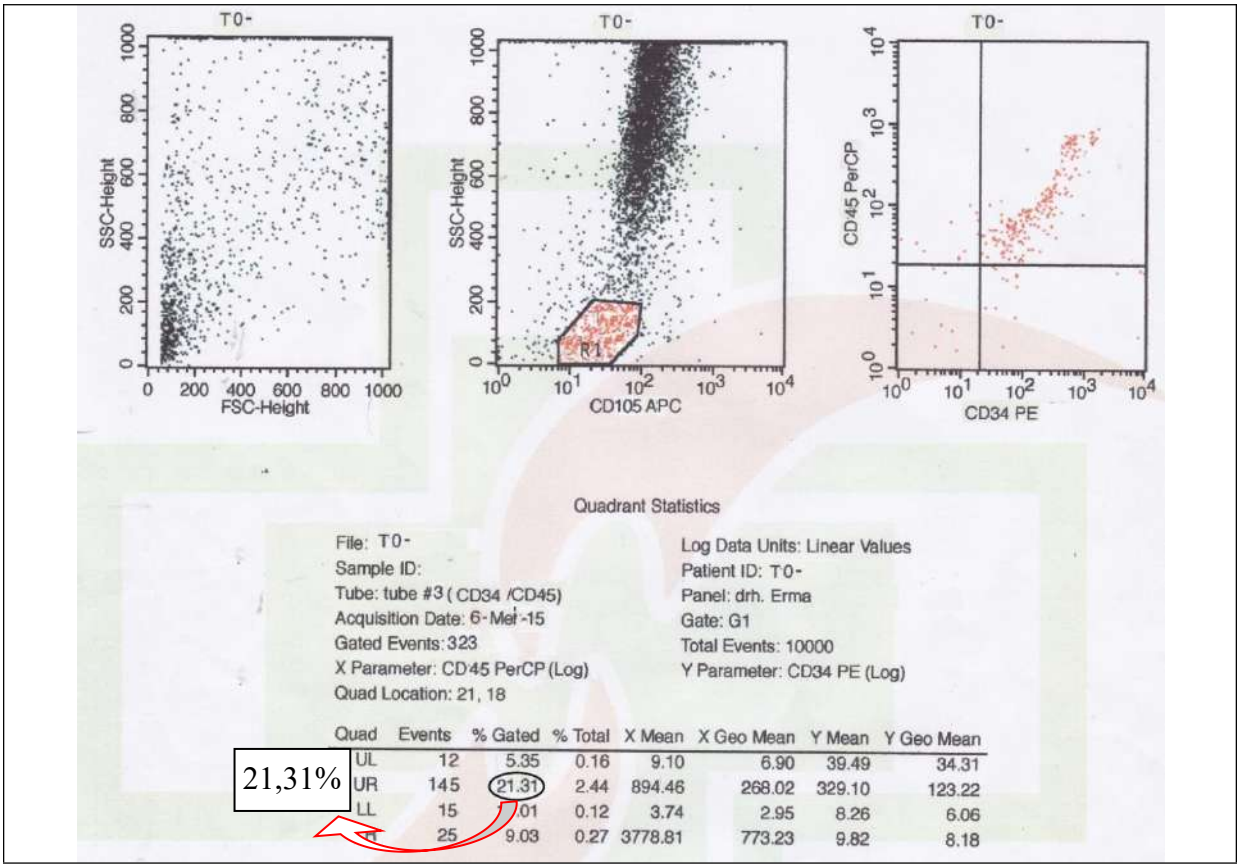


Figure. 1.

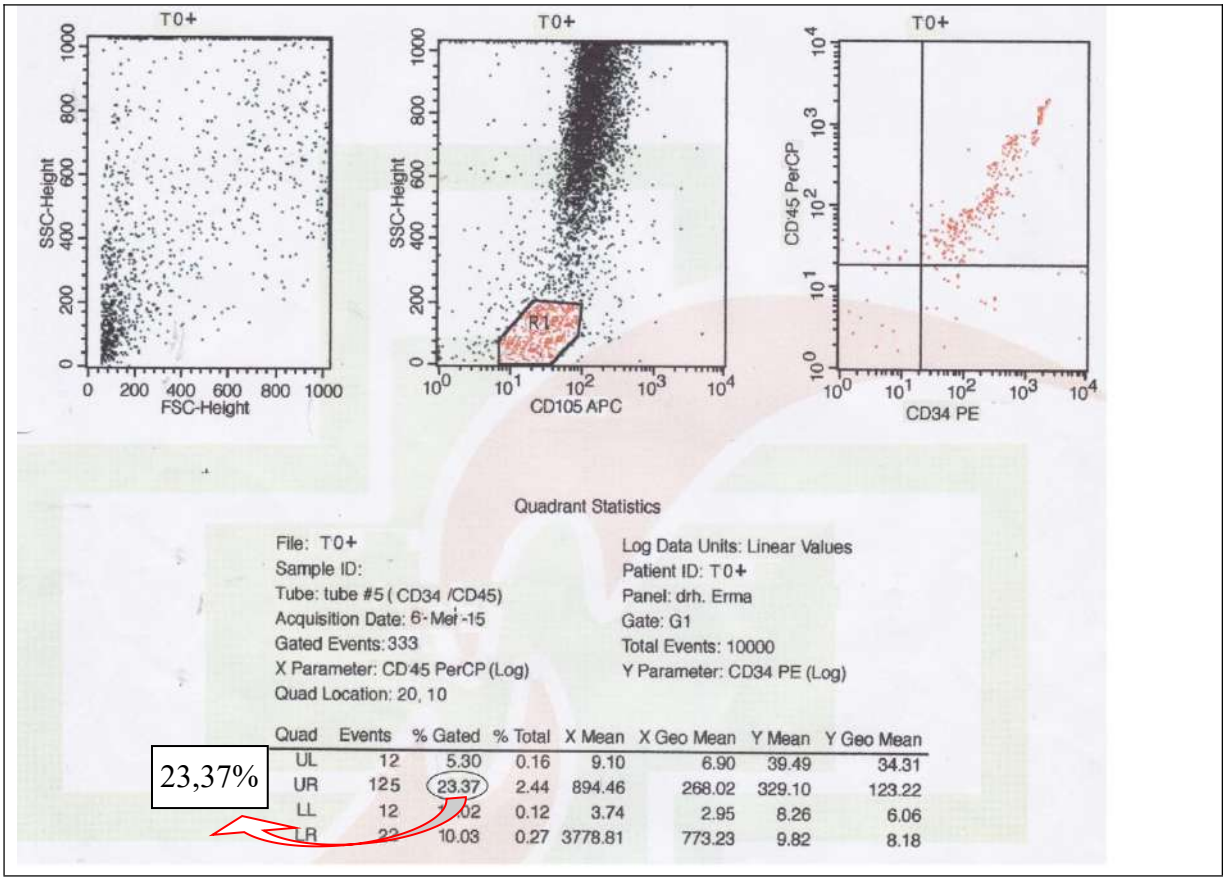
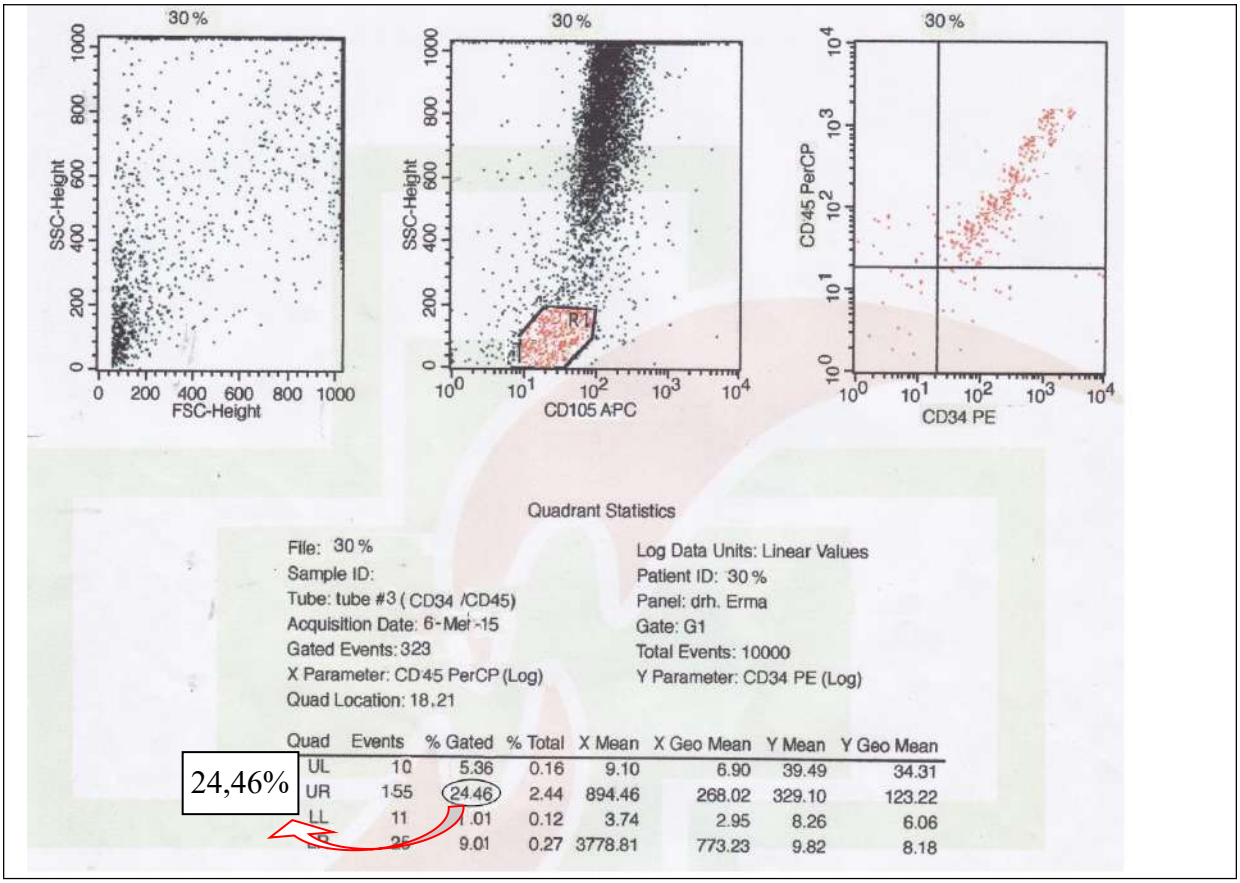


Figure. 2.



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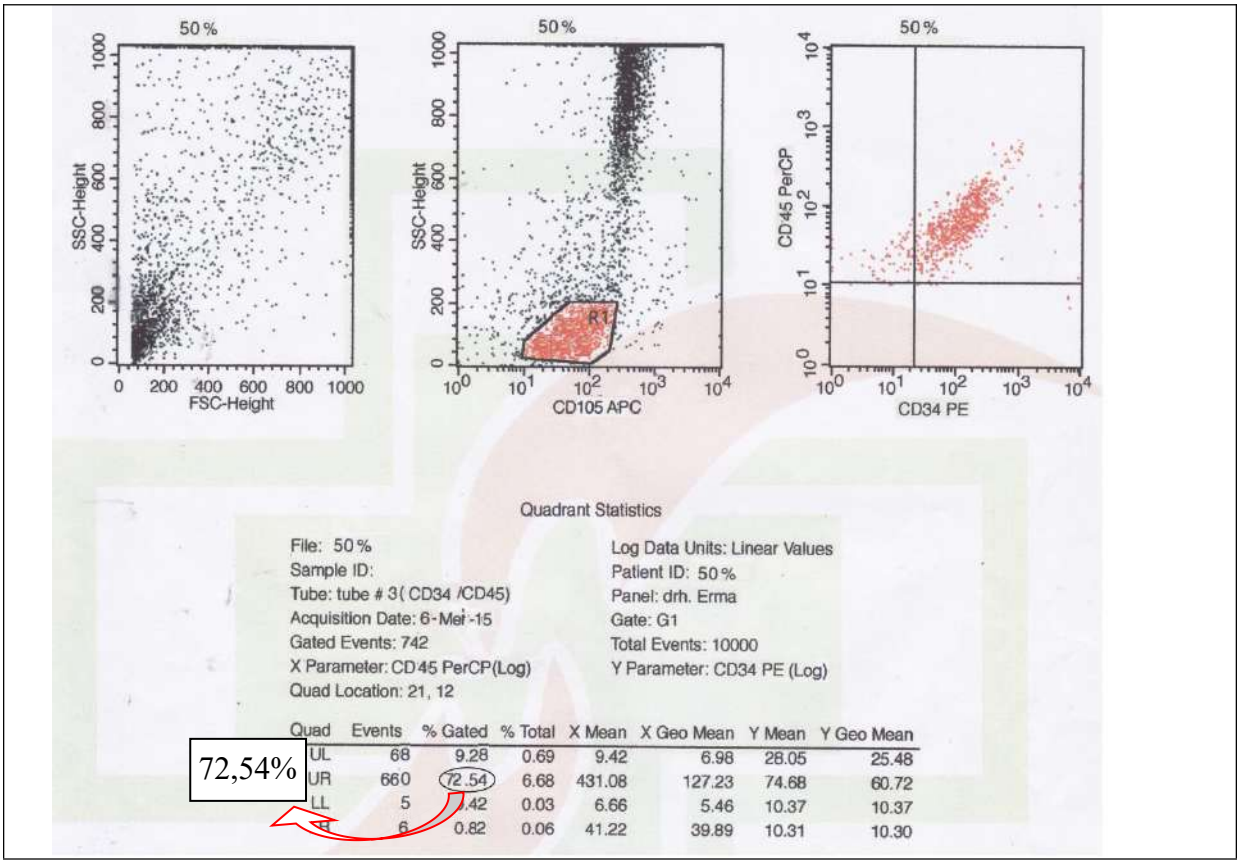

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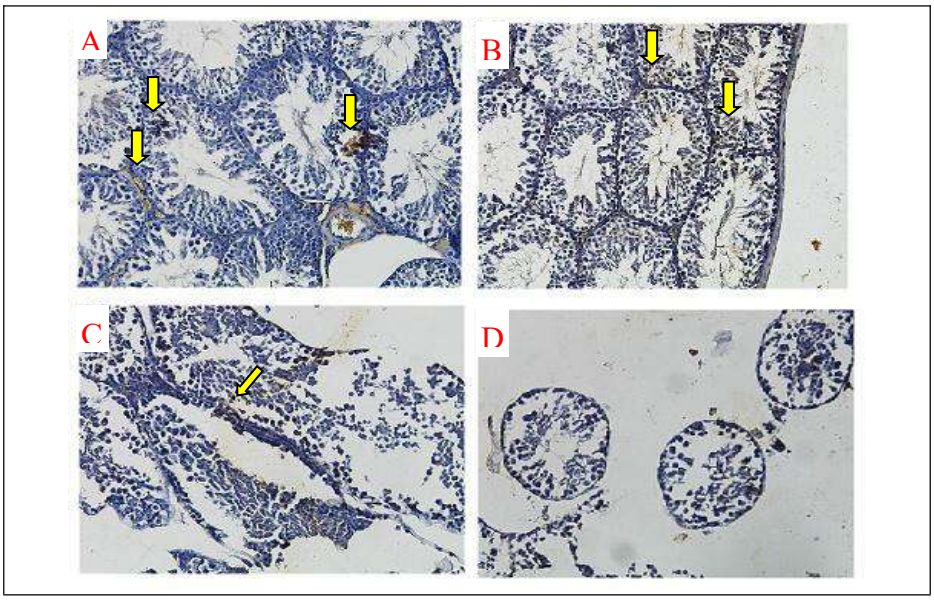

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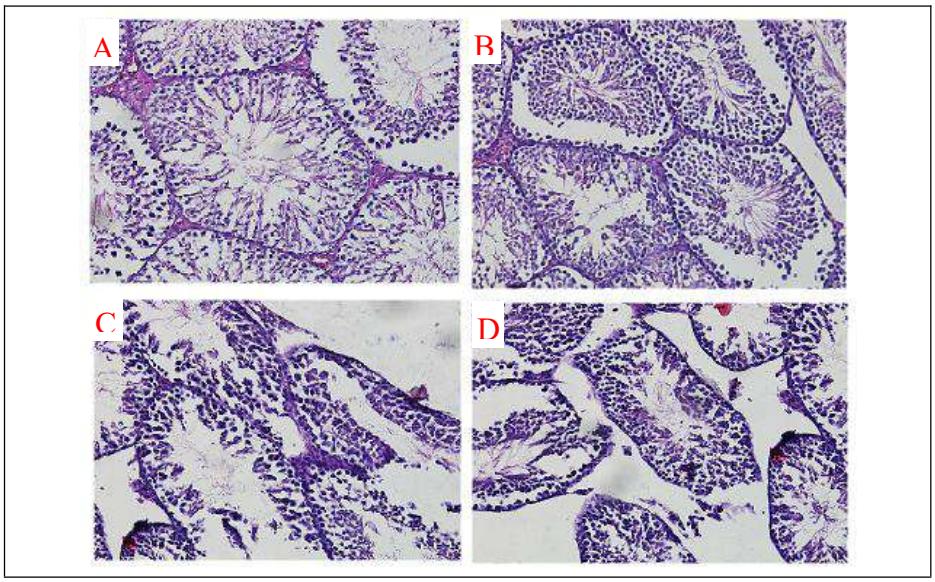

Figure 6

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To: apjr2012@163.com

Date: Monday, October 19, 2015 at 06:38 PM GMT+7

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To: apjr2012@163.com

Date: Wednesday, October 21, 2015 at 09:40 AM GMT+7

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> On Wednesday, October 21, 2015 7:32 AM, apjr2012 <apjr2012@163.com> wrote:
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Autoregeneration of mice testicle seminiferous tubules due to malnutrition based on stem cells mobilization using honey

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Seminiferous tubule autoregeneration
Malnutrition
Stem cell mobilization
Bee honey

ABSTRACT

Objective: To find out a novel therapy based on mobilization and differentiation of own body−derived stem cells using natural bee honey. **Methods:** Testicle degeneration model of mouse is available through food fasting 5 days long that causes malnutrition and affect the testicle. Then administration of 30% (T1) and 50% bee honey (T2) each for five days long and compared to the positive control, fast without bee honey (T0+) and negative control, feed but without bee honey (T0−). Subsequently observation of Hematopoietik Stem Cells (HSCs) mobilization according to the CD34 and CD45 expressions performed using flowcytometry method was conducted. Besides, identification of differentiation is examined through the expression of spermatogonial stem cells (SSCs) using immunohistochemistry technique, while the seminiferous tubules cell regeneration through H&E staining microscopic examination. **Results:** Stem cells mobilization based on the expression of CD34 and CD45, which is a marker of HSCs. Differentiation of stem cells into progenitor cells that expected based expression of SSCs in testicle tissue. SSCs increase causes regeneration testicle seminiferous tubules. **Conclusions:** Results of this study revealed a significantly different of C34 and CD45 expressions between groups, also an increase SSCs expression and testicle seminiferous tubules cells regeneration as well.

1. Introduction

The regenerative-medicine in this decade could be a basis of many diseases treatment of the future, mainly the degenerative disease that cured through neither medical therapy nor surgery[1], especially testicle degeneration[2, 3]. Testicular degeneration causes azoospermia. Azoospermia is a condition which is no spermatozoon produced by testicular seminiferous tubules, and makes infertility in the male and it means no offspring[3].

Testicular degeneration is the main cause of infertility in the male, the etiology varies such as genetic alteration, mechanical trauma, neoplastic changes and aging or senility. Current stem cell therapy usually is through stem cell transplantation which is cultured in vitro previously. This is obviously very expensive. Therefore, it is needed a novel therapy based on mobilization and differentiation of own body-derived stem cells using natural bee honey.

Rapidly mobilized stem cells in an adequate number towards defected tissue (testis), in turn will differentiate to be certain cells (seminiferous tubules) from a certain tissue (testis) and will replace the damaged and apoptotic cells due to degenerative diseases, in this case, the differentiation becoming seminiferous tubules, sertoli and interstitial cells of Leydig.

It is important to find out an innovation of therapy through auto-regeneration-induced of seminiferous tubule cells using beneficial of natural bee honey in reproducing of spermatozoa. Regeneration of seminiferous tubule and subsequently Sertoli cells will provide

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support, nutrients and other environmental factors for young spermatozoa and male behavior to allow spermatogenesis and the ability of conception to happen.

Natural bee honey, a nutrient source from bee[4, 5] has antibacterial and antioxidant potencies[5–7]. Antioxidant is an important substance in protecting individual against free radicals. An adequate antioxidant consumption can reduce the prevalence of cancers, cardiovascular disease, cataract, digestive tract disorder and other degenerative diseases[8] and in present study, the testicular degeneration. Bee Honey consumption will improve the digestive system, has a good result in diarrhea therapy[5] and reproductive system disorder as well[9]. As conceptual solution, it needs further study to explore the beneficial of natural bee honey to induce auto-regeneration of testicular seminiferous tubule as a source of spermatozoa-producing tissue.

2. Material and methods

2.1. Testicular degeneration model and treatment

Testicular degeneration model of male mice is available through food fasting 5 days long, but drinking water is still administered [10, 11]. The experiment animals used in this study are healthy 8-10 week-old Balb/C male mice and 20-25 g bodyweight each. They are placed in an individual plastic cage in Experimental Animal Laboratory at Veterinary Medicine Faculty, Airlangga University. They are divided into 4 groups of 8 mice each :

1. Negative control group (T0-) : feed but without bee honey; 2. Positive control group (T0+) : fast but without bee honey; 3. Trial group 1 (T1) : fast with 30% bee honey for 5 days; 4. Trial group 2 (T2) : fast with 50% bee honey for 5 days

2.2. Flowcytometri observation of hscs mobilization based on expression of CD34 and CD45

After the treatment, whole blood collected from cardiac puncture placed in heparin tube to prevent coagulate. Flowcytometri observation reveal the expressions of CD34 and CD45.

Flowcytometri method begins with whole blood centrifugation in 4 ℃ temperature, 6 000 rpm for 15 minutes. Cellular precipitation as a result of centrifugation then mixed with cytoperm/cytofix in amount of 2 times of obtained cell number. This mixture was then centrifuged again and formed supernatant and pellet. BD wash added to the pellet in the amount of 4 times of obtained cell number from the first centrifugation. Centrifuge the mixture then added lisis buffer in the amount of 2 times of the first obtained cell number. Subsequently labeled antibody added to each sample, five tubes are arranged and processed parallel. (1) single staining with CD34 PE added to the wash tube. (2) double staining with CD34 PE and CD45 PerCP. (3) double staining with CD34 PE and CD45 PerCP and CD105 FITC-trucount tube. All the samples were then stored in 4 ℃ and dark room and were analyzed with flowcytometri for 1 hour [12].

2.3. Imunohistochemical (IHC) technique observation of SSCs

Immunohistochemical observation was performed to determine the expression of SSCs. Before to IHC methods were made histological preparation, by way of an incision is made transversely testicular tissue from paraffin blocks. Further examination by making outward through immunohistochemical techniques using monoclonal antibody. This is done to determine the expression of SSCs. Observations of SSCs were made with a 40× microscope objective and the expression of each variable is indicated by the number of cells with brownish chromogen discoloration in each incision[13].

2.4. Histopathology observation of seminiferous tubules

Regeneration identification of seminiferous tubule cells of the testes through histopathological examination begins with the making of histological preparations. Histological preparations such as the following: Mice testicular fixation in 10% formalin, 1 hour later injected formalin 10% in mid-testis. Subsequently mice testes dehydrated in alcohol solution with a higher concentration gradually, ie from 70%, 80%, 90%, 96% (absolute). Then do the clearing in the testes of mice in xylol solution or chloroform or benzene. Furthermore performed embedding using liquid paraffin and mice testes were put into molds containing liquid paraffin. Before stained and sectioning performed, an incision using a microtome and mounted on glass objects. Furthermore the staining was done by removing of paraffin with xylol then put into a solution of alcohol with decreased concentration and then put it into H&E staining procedure. The last stage after stained is done mounting, put into water or alcohol to remove excess stain. Then it was 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 Entelan[14].

Histopathological examination is done using a light microscope with a magnification of 400 times. Observations and identification of seminiferous tubules regeneration is based on the existing histological descrition

2.5. Statistical analysis

Expression of CD34 and CD45 and SSCs were statistically analyzed using SPSS 15 for Windows XP with the level of significance 0.01 (P=0.01) and the confidence level 99% (α = 0.01). Steps of comparative hypothesis testing are as follows: Test data normality with the Kolmogorof Smirnov test, homogeneity of variance test, Analysis of variance (ANOVA) factorial, Post hoc test (Least Significant Difference test) using the Tukey HSD 5%.

3. Results

Data collected from 32 male mice were divided into 4 trials: negative control group (T0-) is normal testis without bee honey;

positive control group (T0+) is degenerative testis without bee honey; (T1) group is degenerative testis + 30% bee honey in drinking water for 5 days; (T2) group is degenerative testis + 50% bee honey in drinking water for 5 days. In detail, the results of the study are as follows: The effectively of bee honey was based on : 1. Mobilization of HSCs, 2. SSCs formation, 3. Regeneration of testicular tissue (such as: intact of seminiferous tubule tissue).

Mobilization of HSCs was analyzed with Flowcytometry based on increased CD34 and CD45 concentrations. The result showed there were no HSCs mobilization in the three groups (T0-, T0+ and T1 groups) based on CD34 and CD45 percentage, which were less than 25%. On the other hand, those percentage in T2 was more than 70%, indicated HSCs mobilization. Statistically, there was a significantly difference (P<0.05) between T2 and the three other treatments (T0-, T0+ and T1), but no difference (P>0.05) between those three groups (T0-, T0+ and T1) was observed (Table 1).

Table 1

Percentage of CD34 and CD45 with flowcytometry and SSCs expression in mice testes tissue on several treatments (Mean ± SD, %).

Treatments	Average CD34 and CD45 (%)	Average SSCs Expression
Negative Control group (T0–) with normal testis without bee honey	21.070±1.320[a]	86.660±1.938[a]
Positive control group (T0+) with degenerative testis without bee honey	23.010±1.362[a]	0.000±0.000[d]
(T1) group is degenerative testis + 30% bee honey in drinking water for 5 days	24.360±1.370[a]	8.330±0.921[c]
(T2) group is degenerative testis + 50% bee honey in drinking water for 5 days	72.180±1.860[b]	48.330±0.226[b]

a,b,cValues in the same column with different superscripts indicate significant difference at P<0.005 (n=8)

Furthermore effectiveness of bee honey is based on SSCs formation as a result of the differentiation of the spermatogonia. Expression of SSCs in the group use 50% bee honey (T2) was 48.330 ± 0.226 (< 50%). Although the percentage was below the normal control group (T0-) [86.660a± 1.938 (> 80%)], but the percentage was still well above the group 30% bee honey (T1) [8.330c ± 0.921 (< 10%)] and a group of degenerative testis (T0 +) were not expressed at all (Table 1, Figure 1).

In this study, the regeneration of the testes can be observed through the method of histopathology with hematoxylin and eosin (H&E) staining. Microscopic examination showed that the group of 50% bee honey (T2), leading to the occurrence of testicular tissue repair. Improvements were identified based on the regeneration seminiferous tubules regenerate intact. Overview of these improvements can be compared with a negative control group of non infertile (T0-) who did not experience testicular degeneration, which remains in normal condition (Figure 2). As for the group of 30% bee honey (T1) didn't indicate the occurrence of testicular tissue repair. Not the improvement in the form of seminiferous tubules that are no longer intact (broken). Picture of the damage can be compared with positive control of mice (T0+) mice with testicular degeneration (Figure 2).

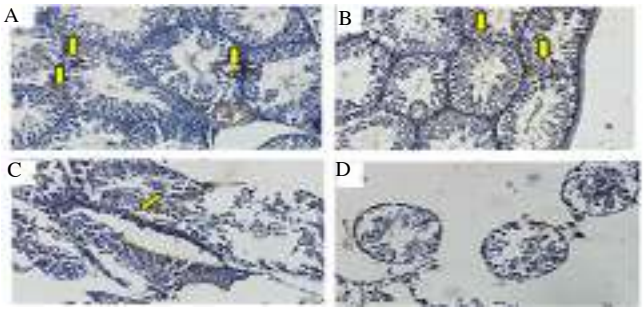

Figure 1. SSCs Expression in Mice Testes Tissue with immunohistochemistry technique.

A. The normal control group (T0-), B. The group use 50% bee honey (T2), C. The group use 30% bee honey (T1), D. The group of degenerative testis (T0 +)

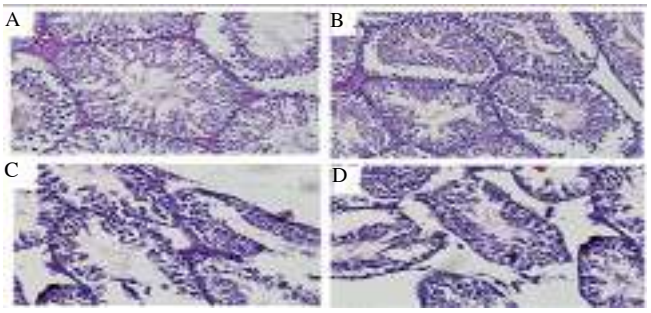

Figure 2. The regeneration of testicular seminiferous tubule microscopically with hematoxylin eosin (HE) staining in mice testes tissue in some treatments.

A. The normal control group (T0-), tubulus seminiferous seen intact; B. The group use 50% bee honey (T2), tubulus seminiferous undergoing regeneration; C. The group use 30% bee honey (T1), there is no regeneration; D. The group of degenerative testis (T0 +), there is no regeneration of seminiferous tubule.

4. Disscusion

The present study showed that giving 50 % bee honey for 5 days are effectiveness for the treatment of degenerative testes of male mice models. The effects of bee honey was based on: 1. Mobilization of HSCs, 2. SSCs formation, and 3. Regeneration of testicular tissue, such as: intact tissue of the seminiferous.

Mobilization of HSCs could occure because induction of stem cells mobilized towards defect place. The process of mobilization can occur through number ways: 1. Induction of proteolytic from microenvironment of bone marrow inside (induction of pharmacological agents such as G - CSF or Cyclophosphamide); 2. Blockade of CXCR4 or VLA - 4 by specific blocking molecules (AMD3100 OR BIO4860); 3. Effect neural mediators (dopamine

and receptors b2-adrenergic); 4. Elements modulation from the coagulation of cascade; 5. The immune response or inflammatory reaction causes injury signals induction (cytokines, NFkB, β catenin through Wnt) from the tissue damage; 6. Homing signals like - SDF 1, CXCL12, VEGF , HGF , PDGF and Integrin that appeared and to be act as recruitment of stem cells [15].

Bee honey contains a variety of biologically active components like melittin and phospholipase-A2 (PLA2) [16]. Previous observations have shown that bee honey or its components are effective in proliferation, survival and differentiation of the cells[17].

The role of honey in this study provide supportive niche through triger process of Vascular Endothelial Growth Factor-1 (VEGF - 1) which is homing signal. Furthermore, VEGF - 1 binds to VEGF Receptor - 1 (VEGFR - 1). VEGF is a component of Extra Cellular Matric (ECM) from stem cells have a role in supporting a conducive microenvironment for stem cells[18]. Triger presence of VEGF - 1 - VEGFR - 1 will pass a series of signaling that activates Stem Cell Factor (SCF) intersisiel. SCF is a mechanic in the niche signaling protein that is physiologically will happen further communication[19]. The presence of SCF intersisiel be recognized SCF receptor complex and enter the cell nucleus so that expression of nuclear β1 - integrine for activating Octamer4 (OCT4). OCT4 is a member of the POU family of transcription factors, which have a major role in the proliferation of stem cells , self- renewal and differentiation. The existence of this proliferation causes HSCs change of shape from quiescenct into a cycling state, so that the HSCs located in the central endosteum area toward marrow. This suggests that cycling HSCs occurred outside of their niche and mobilized to the peripheral circulation[20].

Furthermore effectiveness of bee honey is based on SSCs formation as results of the differentiation of stem cells are mobilized. The SSCs are progenitors of germline stem cells formed by differentiation of stem cells. In this study formed SSCs can be identified through immunohistochemical methods.

Expression of SSCs in the group use 50% bee honey (T2) is approximately 50%. Although the percentage was lower than that of the control group (P0-) (greater than 80%), but the percentage was still higher than that of the group of testis degenerative + 30% bee honey (T1) (<10%) and a group of testis degenerative without use bee honey (P0+) were not expressed at all (0%). This is in accordance with the opinion of other study[21], that bee honey can causes proliferation of stem cells quickly and then differentiated to be cells that needed as response from defect.

Regeneration of testicular tissue, such as: intact of tubulus seminiferus tissue is the third identification of the effectiveness for using bee honey. In this study, the regeneration of the testes can be observed through the method of histopathology anatomy (HPA) with hematoxylin and eosin (HE) staining. Microscopic examination showed that the use of 50% bee honey (T2), leading to the occurrence of testicular tissue repair. Improvements are identified based on the regeneration of seminiferous tubules cell (intact). Overview of these improvements can be compared with a control group of normal testis (T0-) who did not experience testicular degeneration, which remains in normal condition. As for the group of testis degenerative + 30% bee honey (T1) does not indicate the occurrence of testicular tissue repair. Not the improvement in the form of cell degeneration seminiferous tubules that are no longer intact (still broken). Picture of the damage can be compared with control + of group of testis degenerative without use bee honey (T0 +) mice with testicular degeneration (Figure 6.D).

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The study was supported by funding from the Directorate General of Higher Education (DIKTI) 2015. The National Education Ministry. Republic of Indonesia. The authors are also grateful to LPPM chairman Airlangga University Prof. Dr. Djoko Agus Purwanto, Apt., M.Si. This project was done in laboratory of Stem Cells in Institute of Tropical Disease Airlangga University and laboratory of Pathology in Faculty of Veterinary Med, Airlangga University and the authors wish to thanks Prof. Fedik Abdul Rantam for his support.

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Auto-regeneration of mice testicle seminiferous tubules due to malnutrition based on stem cells mobilization using honey

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ARTICLE INFO

Article history:
Received 10 Sep 2015
Received in revised form 2 Nov 2015
Accepted 16 Nov 2015
Available online xxx

Keywords:
Seminiferous tubule auto-regeneration
Malnutrition
Stem cell mobilization
Bee honey

ABSTRACT

Objective: To find out a novel therapy based on mobilization and differentiation of own body-derived stem cells using natural bee honey.

Methods: Testicle degeneration model of mouse is available through food fasting 5 days long that causes malnutrition and affects the testicle. Then administration of 30% (T1) and 50% bee honey (T2) each for five days long and compared to the positive control, fast without bee honey (T0+) and negative control, feed but without bee honey (T0−). Subsequently observation of Hematopoietik Stem Cells (HSCs) mobilization according to the CD34 and CD45 expressions performed using flowcytometry method was conducted. Besides, identification of differentiation is examined through the expression of spermatogonial stem cells (SSCs) using immunohistochemistry technique, while the seminiferous tubules cell regeneration through H&E staining microscopic examination.

Results: Stem cells mobilization based on the expression of CD34 and CD45, which is a marker of HSCs. Differentiation of stem cells into progenitor cells that expected based expression of SSCs in testicle tissue. SSCs increase causes regeneration testicle seminiferous tubules.

Conclusions: Results of this study revealed a significantly different of C34 and CD45 expressions between groups, also an increase SSCs expression and testicle seminiferous tubules cells regeneration as well.

1. Introduction

The regenerative-medicine in this decade could be a basis of many diseases treatment of the future, mainly the degenerative disease that cured through neither medical therapy nor surgery [1], especially testicle degeneration [2,3]. Testicular degeneration causes azoospermia. Azoospermia is a condition which is no spermatozoon produced by testicular seminiferous tubules, and makes infertility in the male and it means no offspring [3].

Testicular degeneration is the main cause of infertility in the male, the etiology varies such as genetic alteration, mechanical trauma, neoplastic changes and aging or senility. Current stem cell therapy usually is through stem cell transplantation which is cultured *in vitro* previously. This is obviously very expensive. Therefore, it is needed a novel therapy based on mobilization and differentiation of own body-derived stem cells using natural bee honey.

Rapidly mobilized stem cells in an adequate number towards defected tissue (testis), in turn will differentiate to be certain cells (seminiferous tubules) from a certain tissue (testis) and will replace the damaged and apoptotic cells due to degenerative diseases, in this case, the differentiation becoming seminiferous tubules, sertoli and interstitial cells of Leydig.

It is important to find out an innovation of therapy through auto-regeneration-induced of seminiferous tubule cells using beneficial of natural bee honey in reproducing of spermatozoa. Regeneration of seminiferous tubule and subsequently sertoli

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cells will provide support, nutrients and other environmental factors for young spermatozoa and male behavior to allow spermatogenesis and the ability of conception to happen.

Natural bee honey, a nutrient source from bee [4,5] has antibacterial and antioxidant potencies [5–7]. Antioxidant is an important substance in protecting individual against free radicals. An adequate antioxidant consumption can reduce the prevalence of cancers, cardiovascular disease, cataract, digestive tract disorder and other degenerative diseases [8] and in present study, the testicular degeneration. Bee honey consumption will improve the digestive system, has a good result in diarrhea therapy [5] and reproductive system disorder as well [9]. As conceptual solution, it needs further study to explore the beneficial of natural bee honey to induce auto-regeneration of testicular seminiferous tubule as a source of spermatozoa-producing tissue.

2. Material and methods

2.1. Testicular degeneration model and treatment

Testicular degeneration model of male mice is available through food fasting 5 days long, but drinking water is still administered [10,11]. The experiment animals used in this study are healthy 8–10 week-old Balb/C male mice and 20–25 g bodyweight each. They are placed in an individual plastic cage in Experimental Animal Laboratory at Veterinary Medicine Faculty, Airlangga University. They are divided into 4 groups of 8 mice each:

1. Negative control group (T0–): feed but without bee honey; 2. Positive control group (T0+): fast but without bee honey; 3. Trial group 1 (T1): fast with 30% bee honey for 5 days; 4. Trial group 2 (T2): fast with 50% bee honey for 5 days.

2.2. Flowcytometry observation of hscs mobilization based on expressions of CD34 and CD45

After the treatment, whole blood collected from cardiac puncture placed in heparin tube to prevent coagulate. Flowcytometry observation reveals the expressions of CD34 and CD45.

Flowcytometry method begins with whole blood centrifugation in 4 °C temperature, 6000 rpm for 15 min. Cellular precipitation as a result of centrifugation then mixed with cytoperm/cytofix in amount of 2 times of obtained cell number. This mixture then centrifuged again and formed supernatant and pellet. BD wash added to the pellet in the amount of 4 times of obtained cell number from the first centrifugation. Centrifuge the mixture then added lysis buffer in the amount of 2 times of the first obtained cell number. Subsequently labeled antibody added to each sample, five tubes are arranged and processed parallel. (1) Single staining with CD34 PE added to the *wash tube*. (2) Double staining with CD34 PE and CD45 PerCP. (3) Double staining with CD34 PE and CD45 PerCP and CD105 FITC-*trucount tube*. All the samples were then stored in 4 °C and dark room and were analyzed with flowcytometry for 1 h [12].

2.3. Immunohistochemical (IHC) technique observation of SSCs

Immunohistochemical observation was performed to determine the expression of SSCs. Before to IHC methods were made histological preparation, by way of an incision is made transversely testicular tissue from paraffin blocks. Further examination by making outward through immunohistochemical techniques using monoclonal antibody. This is done to determine the expression of SSCs. Observations of SSCs were made with a 40× microscope objective and the expression of each variable is indicated by the number of cells with brownish chromogen discoloration in each incision [13]. Q3

2.4. Histopathology observation of seminiferous tubules

Regeneration identification of seminiferous tubule cells of the testes through histopathological examination begins with the making of histological preparations. Histological preparations such as the following: mice testicular fixation in 10% formalin, 1 h later injected formalin 10% in mid-testis. Subsequently mice testes dehydrated in alcohol solution with a higher concentration gradually, *i.e.* from 70%, 80%, 90%, 96% (absolute). Then do the clearing in the testes of mice in xylol solution or chloroform or benzene. Furthermore performed embedding using liquid paraffin and mice testes were put into molds containing liquid paraffin. Before stained and sectioning performed, an incision using a microtome and mounted on glass objects. Furthermore the staining was done by removing of paraffin with xylol then put into a solution of alcohol with decreased concentration and then put it into H&E staining procedure. The last stage after stained is done mounting, put into water or alcohol to remove excess stain. Then it was 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 Entellan [14].

Histopathological examination is done using a light microscope with a magnification of 400 times. Observations and identification of seminiferous tubules regeneration is based on the existing histological description.

2.5. Statistical analysis

Expressions of CD34 and CD45 and SSCs were statistically analyzed using SPSS 15 for Windows XP with the level of significance 0.01 (P = 0.01) and the confidence level 99% (α = 0.01). Steps of comparative hypothesis testing are as follows: Test data normality with the Kolmogorov Smirnov test, homogeneity of variance test, Analysis of variance (ANOVA) factorial, Post hoc test (Least Significant Difference test) using the Tukey HSD 5%.

3. Results

Data collected from 32 male mice were divided into 4 trials: negative control group (T0–) is normal testis without bee honey; positive control group (T0+) is degenerative testis without bee honey; (T1) group is degenerative testis +30% bee honey in drinking water for 5 days; (T2) group is degenerative testis +50% bee honey in drinking water for 5 days. In detail, the results of the study are as follows: The effectively of bee honey was based on: 1. Mobilization of HSCs, 2. SSCs formation, 3. Regeneration of testicular tissue (such as: intact of seminiferous tubule tissue).

Mobilization of HSCs was analyzed with flowcytometry based on increased CD34 and CD45 concentrations. The result

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Q7

Table 1

Percentage of CD34 and CD45 with flowcytometry and SSCs expression in mice testes tissue on several treatments (Mean ± SD, %).

Treatments	Average CD34 and CD45	Average SSCs expression (%)
Negative control group (T0−) with normal testis without bee honey	21.070 ± 1.320[a]	86.660 ± 1.938[a]
Positive control group (T0+) with degenerative testis without bee honey	23.010 ± 1.362[a]	0.000 ± 0.000[d]
(T1) group is degenerative testis +30% bee honey in drinking water for 5 days	24.360 ± 1.370[a]	8.330 ± 0.921[c]
(T2) group is degenerative testis +50% bee honey in drinking water for 5 days	72.180 ± 1.860[b]	48.330 ± 0.226[b]

[a,b,c]Values in the same column with different superscripts indicate significant difference at $P < 0.005$ ($n = 8$).

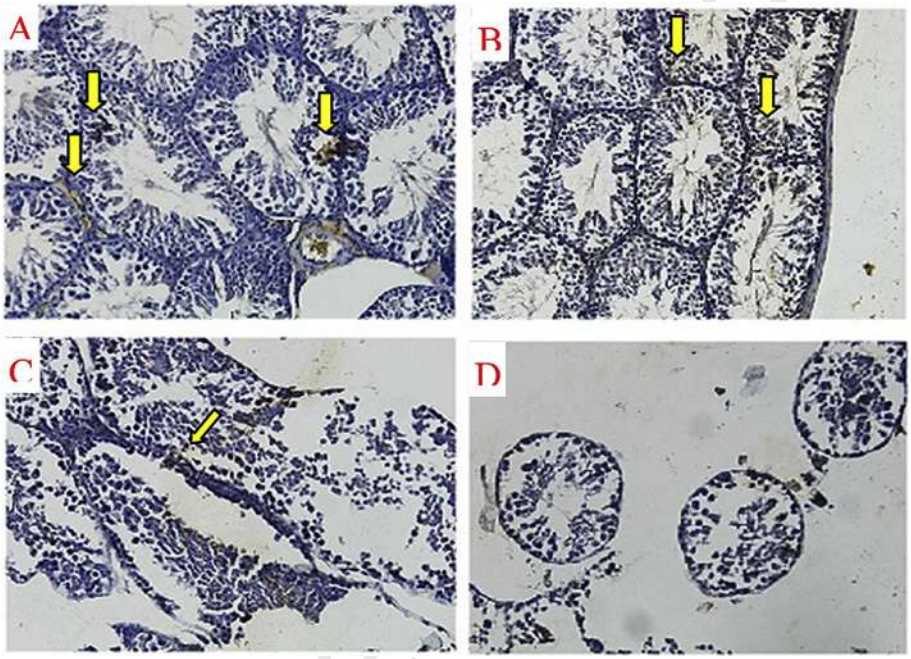

Figure 1. SSCs expression in mice testes tissue with immunohistochemistry technique.
A. The normal control group (T0−); B. The group use 50% bee honey (T2); C. The group use 30% bee honey (T1); D. The group of degenerative testis (T0+).

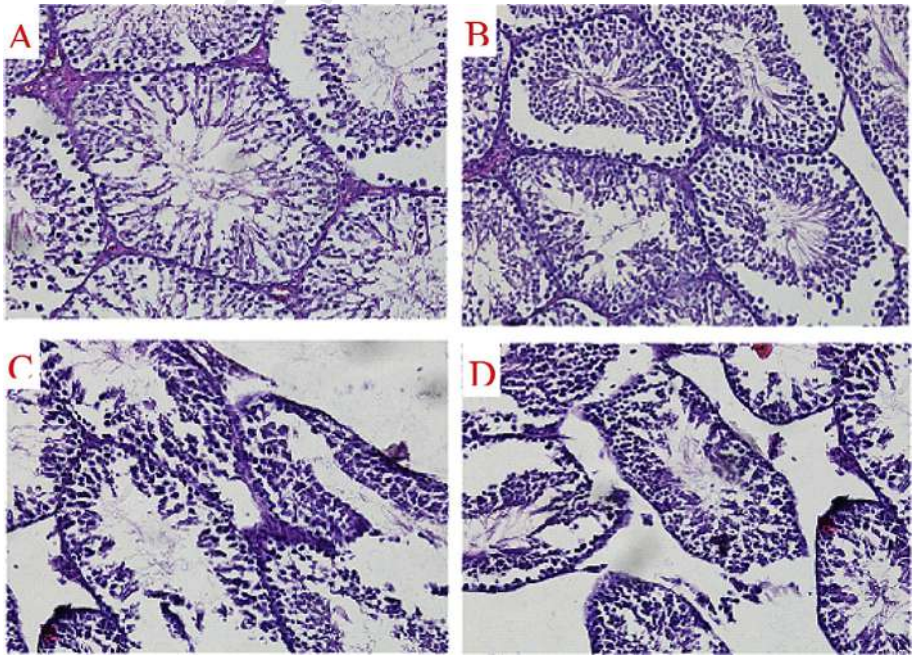

Figure 2. The regeneration of testicular seminiferous tubule microscopically with hematoxylin eosin (HE) staining in mice testes tissue in some treatments.
A. The normal control group (T0−), tubulus seminiferous seen intact; B. The group use 50% bee honey (T2), tubulus seminiferous undergoing regeneration; C. The group use 30% bee honey (T1), there is no regeneration; D. The group of degenerative testis (T0+), there is no regeneration of seminiferous tubule.

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showed there were no HSCs mobilization in the three groups (T0−, T0+ and T1 groups) based on CD34 and CD45 percentage, which were less than 25%. On the other hand, those percentage in T2 was more than 70%, indicated HSCs mobilization. Statistically, there was a significantly difference ($P < 0.05$) between T2 and the three other treatments (T0−, T0+ and T1), but no difference ($P > 0.05$) between those three groups (T0−, T0+ and T1) was observed (Table 1).

Furthermore effectiveness of bee honey is based on SSCs formation as a result of the differentiation of the spermatogonia. Expression of SSCs in the group use 50% bee honey (T2) was 48.330 ± 0.226 (<50%). Although the percentage was below the normal control group (T0−) [86.660a ± 1.938 (>80%)], but the percentage was still well above the group 30% bee honey (T1) [8.330c ± 0.921 (<10%)] and a group of degenerative testis (T0+) were not expressed at all (Table 1, Figure 1).

In this study, the regeneration of the testes can be observed through the method of histopathology with hematoxylin and eosin (H&E) staining. Microscopic examination showed that the group of 50% bee honey (T2), leading to the occurrence of testicular tissue repair. Improvements were identified based on the regeneration seminiferous tubules regenerate intact. Overview of these improvements can be compared with a negative control group of non infertile (T0−) who did not experience testicular degeneration, which remains in normal condition (Figure 2). As for the group of 30% bee honey (T1) didn't indicate the occurrence of testicular tissue repair. Not the improvement in the form of seminiferous tubules that are no longer intact (broken). Picture of the damage can be compared with positive control of mice (T0+) mice with testicular degeneration (Figure 2).

4. Discussion

The present study showed that giving 50% bee honey for 5 days are effectiveness for the treatment of degenerative testes of male mice models. The effects of bee honey were based on: 1. Mobilization of HSCs, 2. SSCs formation, and 3. Regeneration of testicular tissue, such as: intact tissue of the seminiferous.

Mobilization of HSCs could occur because induction of stem cells mobilized towards defect place. The process of mobilization can occur through number ways: 1. Induction of proteolytic from microenvironment of bone marrow inside (induction of pharmacological agents such as G – CSF or Cyclophosphamide); 2. Blockade of CXCR4 or VLA-4 by specific blocking molecules (AMD3100 OR BIO4860); 3. Effect neural mediators (dopamine and receptors b2-adrenergic); 4. Elements modulation from the coagulation of cascade; 5. The immune response or inflammatory reaction causes injury signals induction (cytokines, NFkB, β catenin through Wnt) from the tissue damage; 6. Homing signals like – SDF 1, CXCL12, VEGF, HGF, PDGF and integrin that appeared and to be act as recruitment of stem cells [15].

Bee honey contains a variety of biologically active components like melittin and phospholipase-A2 (PLA2) [16]. Previous observations have shown that bee honey or its components are effective in proliferation, survival and differentiation of the cells [17].

The role of honey in this study provides supportive niche through trigger process of Vascular Endothelial Growth Factor-1 (VEGF-1) which is homing signal. Furthermore, VEGF-1 binds to VEGF Receptor-1 (VEGFR-1). VEGF is a component of Extra Cellular Matrix (ECM) from stem cells has a role in supporting a conducive microenvironment for stem cells [18]. Trigger presence of VEGF-1–VEGFR-1 will pass a series of signaling that activates Stem Cell Factor (SCF) intersisiel. SCF is a mechanic in the niche signaling protein that is physiologically will happen further communication [19]. The presence of SCF intersisiel be recognized SCF receptor complex and enter the cell nucleus so that expression of nuclear β1 – integrin for activating Octamer4 (OCT4). OCT4 is a member of the POU family of transcription factors, which have a major role in the proliferation of stem cells, self-renewal and differentiation. The existence of this proliferation causes HSCs change of shape from quiescent into a cycling state, so that the HSCs located in the central endosteum area toward marrow. This suggests that cycling HSCs occurred outside of their niche and mobilized to the peripheral circulation [20].

Furthermore effectiveness of bee honey is based on SSCs formation as results of the differentiation of stem cells are mobilized. The SSCs are progenitors of germline stem cells formed by differentiation of stem cells. In this study formed SSCs can be identified through immunohistochemical methods.

Expression of SSCs in the group use 50% bee honey (T2) is approximately 50%. Although the percentage was lower than that of the control group (P0−) (greater than 80%), but the percentage was still higher than that of the group of testis degenerative +30% bee honey (T1) (<10%) and a group of testis degenerative without use bee honey (P0+) were not expressed at all (0%). This is in accordance with the opinion of other study [21], that bee honey can causes proliferation of stem cells quickly and then differentiated to be cells that needed as response from defect.

Regeneration of testicular tissue, such as: intact of tubulus seminiferous tissue is the third identification of the effectiveness for using bee honey. In this study, the regeneration of the testes can be observed through the method of histopathology anatomy (HPA) with hematoxylin and eosin (HE) staining. Microscopic examination showed that the use of 50% bee honey (T2), leading to the occurrence of testicular tissue repair. Improvements are identified based on the regeneration of seminiferous tubules cell (intact). Overview of these improvements can be compared with a control group of normal testis (T0−) who did not experience testicular degeneration, which remains in normal condition. As for the group of testis degenerative +30% bee honey (T1) does not indicate the occurrence of testicular tissue repair. Not the improvement in the form of cell degeneration seminiferous tubules that are no longer intact (still broken). Picture of the damage can be compared with control + of group of testis degenerative without use bee honey (T0+) mice with testicular degeneration.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The study was supported by funding from the Directorate General of Higher Education (DIKTI) 2015, The National Education Ministry and Republic of Indonesia. The authors are also grateful to LPPM chairman Airlangga University Prof. Dr. Djoko Agus Purwanto, Apt., M.Si. This project was done in

laboratory of Stem Cells in Institute of Tropical Disease Airlangga University and laboratory of Pathology in Faculty of Veterinary Med, Airlangga University and the authors wish to thanks Prof. Fedik Abdul Rantam for his support.

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