The Effect of Implant Origin Differences on Peritoneal Endometriosis in an Endometriosis Mouse Model

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Abstract

Objectives: This study aimed to observe the difference in the area of endometriosis lesions and the histopathology of inflammatory cells and granuloma masses in an endometriosis mouse model treated with endometrial cell implants, endometrioma capsules, and adenomyosis tissue.

Materials and Methods: This is an experimental study with posttest-only research design which was conducted with the control group. Thirty-two mice (*Mus musculus*) were injected with 0.2 mL/mice cyclosporin A and then were divided into three groups which were injected with endometrial tissue from the uterine cavity (group A), endometriosis from endometrioma capsule (group B), and endometriosis from adenomyosis (group C). The injection was done slowly into the peritoneal cavity, 0.1 mL each, and followed by intramuscularly Ethinyl estradiol, 0.2 µG/mice. On the 15th days, mice were dissected to observe the peritoneal endometriosis implant and microscopic examination with hematoxylin-eosin (HE) staining to determine the inflammatory cell infiltration and mass granuloma presence. Data were analyzed using SPSS, version 19.

Results: The study obtained that the area of implanted endometriosis lesions in group C covered a larger area of endometriosis implants than other groups ($P < 0.05$). The peritoneal damage in group C was the most severe based on the Klopfleisch method ($P < 0.05$), with mass granuloma and massive infiltration of inflammatory cells and fibrous connective tissue formation occurring in muscle tissue.

Conclusions: The implantation of adenomyosis cell tissue is the best method to develop mice model of endometriosis based on its inflammatory infiltration, the extent of lesion implant, and granuloma mass.

Keywords: Endometriosis, Granuloma mass, Implant area, Peritoneal damage

Introduction

Endometriosis is a disease that can occur in humans and some other primates. The pathophysiology of this disease has not been clearly understood although many theories are evolving and the existing research is continuously demonstrating contradictory results. Complaints, diagnostic processes, therapies, progressiveness, and recurrence are always associated with laparoscopic action leading to separate issues like financing, safety, and ethics. Therefore, research about endometriosis should be conducted on endometriosis animal subjects in order to minimize the cost, ethical, safety and legal issues (1).

Regarding the human subjects, research must be performed by laparoscopy and continually observe the condition of disease and the results of therapy and monitor its recurrence and to find the technical, financial, and legal constraints. In addition, new therapeutic explorations are unethical if directly tested on humans before being tested on experimental animals (2). Many studies used both primate and non-primate endometriosis models. Non-primate animals such as mice do not experience spontaneous endometriosis, but it can be induced by using

either autologous uterine or human endometrial tissues (3). However, primates may spontaneously experience endometriosis. Meanwhile, it is more challenging to continually observe endometriosis in the apes or baboons (4,5).

Although non-human primates are the most appropriate models for studying endometriosis, the procedure takes a long time and requires human-like diagnostic tools. Therefore, non-primate animals such as rats and mice are selected as alternatives to solve these constraints since they are more practical models of endometriosis (6,7). This study observed the ideal mice implants regarding endometriosis research. The model is already developed but no model could produce a high rate of success as endometriosis mice model. Using adenomyosis is the rational reason to increase the rate of success because adenomyosis is specific endometriosis that produces a complicated problem in a human setting. No design model of endometriosis mice used adenomyosis as the origin of the implant. The present study sought to demonstrate the significant differences in endometriosis lesion implants and histopathological features of inflammatory cells and

granuloma masses between endometriosis model mice receiving endometrial cells from the uterine cavity (A), endometrioma capsules (B), and endometriosis from adenomyosis (C). Further, it was attempted to investigate the best model for designing endometriosis model in mice.

Materials and Methods
Experimental Design
This experimental study used a posttest research design only with the control group. Thirty-two female mice (*Mus musculus*) weighing 20-30 g and aged 2-3 months were obtained from the Laboratory of Reproductive Physiology Embryology, Faculty of Veterinary Medicine, Airlangga University, Surabaya out of which 30 were used for the purpose of the study.

The Sampling of Endometrial Tissue
The endometrial cells from the uterine cavity, endometrioma capsules, and uterine adenomyosis were collected by the following steps: The endometrium cell was obtained by scraping the uterine cavity wall with a curette spoon to obtain the viable endometrial tissue. A wall biopsy/capsule (2x2 cm) was performed on endometrioma. Adenomyosis tissue (2x2 cm) was obtained from the female uterus with adenomyosis. Then, the tissue was further inserted into the tube containing the labeled formaldehyde solution and stored at a temperature of 20-250°C.

Experimental Treatments
After the mice underwent an adaptation process in the cage by receiving the same feed for 1 week, they were injected with 0.2 cc/mice cyclosporin A (8). The cyclosporine injection was used to suppress the immune status of the mice in order to facilitate the growth of endometriosis implant in the mice peritoneal cavity. Furthermore, the mice were classified into 3 groups each containing 10 mice employing the following experimental treatments: Mice in group A were injected with endometrial cells from the endometrial uterine cavity, those of group B recieved endometriosis from the capsule of endometrioma, and finally mice in group C were injected with endometriosis from adenomyosis tissue. The differences of this cell origin are based on the pathophysiology of endometriosis in which these three endometriosis forms differ in the complaint, clinical finding, diagnostic, and therapeutic process and recurrences.

The endometrial tissue of the uterine cavity, the endometriosis tissue from the capsule of endometrioma, and adenomyosis tissue were stored in phosphate buffer saline (PBS) and then centrifuged twice (2500 rpm). The pellet was removed, and then PBS was added along with 200 µG/mL of streptomycin and 200 IU/mL of penicillin (8). Each mouse was slowly injected with 0.1 mL of supernatant through the peritoneal cavity over 60 seconds. Then, the mice were injected with an ethinyl estradiol dose of 0.2 µGR/mice intramuscularly on the thighs using a disposable 1 mL syringe. On the day 15, the mice were dissected to calculate the extent of endometriosis implantation in the peritoneum and underwent histopathological examination employing hematoxylin-eosin (HE) staining.

Sampling Endometriosis Model Mice
Samples were collected immediately after the mice were euthanized. The abdominal wall and peritoneum were separated, then the peritoneum was excised and stretched on millimeter paper and documented using a photo to observe the extent of endometriosis. Next, the histopathological examination was prepared. Afterward, the result was recorded on the data collection sheets and analyzed statistically. The preparation for anatomical pathology examination was performed applying the reddest peritoneal tissue taken for preparation, which was then preserved with 10% formalin.

Extensive Examination of the Peritoneum
The peritoneum was examined using a Nikon H600L microscope equipped with a Fi2 300-megapixel DS digital camera and the Nikon image processing software (Nikon Corporation). The area of the endometriosis implant was macroscopically assessed in the area of hyperemia which was then confirmed by taking the samples in the most hyperemic areas to be examined for any endometriosis lesion. Measurements of the implantation of endometriosis lesions were made by calculating the red area lesion on the peritoneal wall by mm2 units calculated using Motic Image software, which is specific computer software for computing the certain area.

Histopathological Examination
The level of damage to the peritoneum was determined by examining the inflammatory cell infiltration and the presence of granuloma mass. Additionally, the degree of peritoneal damage was assessed using a scoring system according to the modified Klopfleisch method where the damage level was computed by summing up all the scores of the lesions (9). The scoring system contained two assessments based on inflammatory cell infiltration (Table 1) and granuloma mass (Table 2).

The staining used in this study included HE streptavidin and biotin (labeled as streptavidin-biotin-method/LSAB). Endometriosis spots in peritoneum were embedded in paraffin, then cut 4-6 µM. The tissue was deparaffinized in xylol two times (5 minutes each). Then, it was consecutively soaked in ethanol absolute (2 times for 3 minutes), ethanol 95% (two times 3 minutes each), and ethanol 70% (for 3 minutes). The tissue was washed with aquabides (2H20) and then sprayed with proteinase K solution for 5 minutes. Afterward, it was double washed with PBSsprayed with hydrogen peroxidase 3% (H_2O_2)

Table 1. Scoring Peritoneal Damage Level Based on Inflammatory Cell Infiltration (8)

Lesion	Score	Information
Inflammation cell infiltration	0	Not detected
	1	< 10 in 5 lp (400x)
	2	Between 11-50 in 5 lp (400x)
	3	Between 51-100 in 5 lp (400x)
	4	> 100 in 5 lp (400x)

Table 2. Scoring Degree of Peritoneal Damage Based on Mass Granuloma (8)

Lesion	Score	Information
Granuloma	0	Granuloma mass was not detected
	2	Granuloma mass was detected
	4	Granuloma mass was detected with abscess
	6	Granuloma mass was detected with abscess and muscle tissue necrosis
	8	Granuloma mass was detected with abscess, muscle tissue necrosis, and fibrosis

Note: Damage level is the total of the 2 above lesions which are between 0–12 intervals.

Figure 1. The calculation of hypervascularization/endometriosis implantation in endometriosis mice.

for 5 minutes, and then double washed using PBS 2 times.

Data Analysis
Data were analyzed using the following steps: conducting data normality test employing the Shapiro-Wilk test, comparative test using the independent sample t test (normally distributed data) or Mann-Whitney (when not normally distributed), and one-way ANOVA test (F-test) (if the data were normally distributed) or Kruskal Wallis test (if the data were not normally distributed). All calculations were performed using the statistical package for the social sciences (SPSS) software, version 19.

Results
Area of Implantation on the Peritoneum
Based on the size of the implanted endometriosis spots, it appears that group C had larger peritoneal endometriosis implants ($P < 0.05$) of 42.75 ± 3.28 mm^2 compared to other groups while group B had endometriosis with an implant area that was 10.68 ± 1.41 mm^2 smaller than that of the group A (Figure 1).

Macroscopic Overview Wide Implantation of Endometriosis
Macroscopically, group C had more hyperemic features, which indicated better hypervascularization/implantation compared to groups A and B (Figure 2).

Histopathology Degree of Peritoneal Damage
This analysis aimed at examining the level of damage to the peritoneum. Based on the calculation using the Klopfleisch scoring method, group C was found to have a higher score (9.1 ± 3.28; $P < 0.05$) than other groups
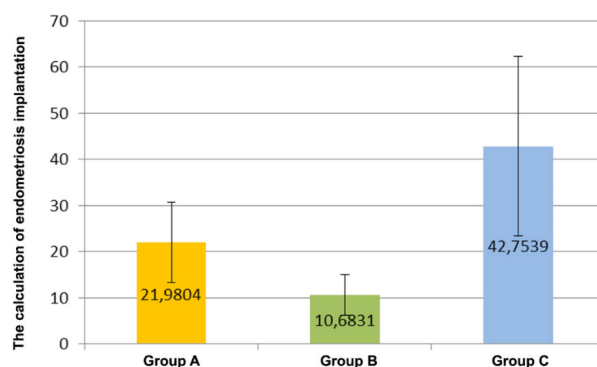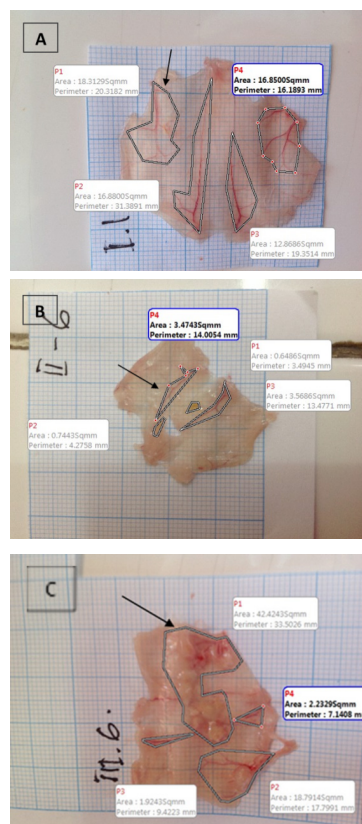

Figure 2. Measurement of Hypervascularization Area in Endometriosis Model of Mice.

(group A: 0.9 ± 0.88; group B: 1.7 ± 1.42), the differences of which are displayed in Figure 3 (9).

Histopathology Lesions Endometriosis
In the histopathological examination of endometriosis lesions formed in each group, it was observed that mice in groups A and B had inflammation. Generally, the inflammation score was between 1 and 5. Additionally, group C mice had severe inflammation which was followed by myocyte cell death and fibrous connective tissue formation in some cases. The comparison of
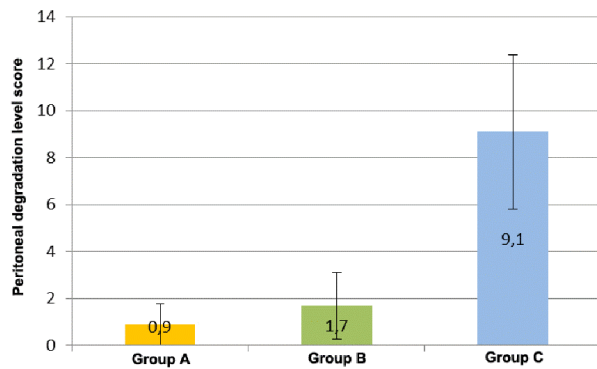
Figure 3. The Differences in Peritoneal Degradation Level.

histopathological examination is presented in Figure 4. In group C model mice, granuloma mass, inflammatory cell mass infiltration, and fibrous connective tissue formation occurred in muscle tissue (Figure 5).

Discussion

Endometriosis is defined as the endometrial tissue which is present outside the uterine cavity. The most normally affected areas are pelvic or peritoneal organs although other areas may either have the possibility to be affected. Clinical manifestations may be the lesions that are typically acquired on the peritoneal surface of the reproductive organs, but they may occur anywhere in the female organs. The size of the lesions varies considerably from microscopic to large invasive masses that erode the inside of the organ and cause extensive adhesion (10).

Following the macroscopic analysis, it was found that the implant tissue was the rounded nodules with varying sizes which were strongly attached to the peritoneal tissue underneath. The damage level in endometriosis nodules occurred as a result of experimental treatment mice varied between the groups. The damage level was determined by assessing the extent of the area of endometriosis implants formed in the peritoneum mice model. As shown in Figure 1, group C had a larger area of implanted endometriosis compared to groups A and B. Therefore, supernatant injection of the adenomyosis should be used to obtain the most endemic mouse model of the endometriosis. Even the heterologous model of a mouse model of endometriosis which was reported as a good alternative to make peritoneal endometriosis in mice for research purposes had a specific limitation (11)

In this study, histopathological examination was used to determine the damage level to the peritoneum based on inflammatory cell infiltration and the appearance of granuloma mass. The scoring method of the damage level uses a scoring system with the modified Klopfleisch method (9). Based on microscopic observation, group C had a mean value infiltration of inflammatory cells of about >100 cells in 5 viewing fields (M = 400x). In other words, group C had severe inflammation some of which was followed by myocyte cell death and fibrous connective tissue formation. In groups A and B, however, inflammation was present at the scores between 1 and 3. Greaves et al declared that the implantation of human endometrium tissue to peritoneal mice would produce similar characteristics with original tissue in a human setting which is suitable for endometriosis research purposes (12). Measurement size of the lesion proved that adenomyosis tissue could induce an inflammatory environment more severely than either endometrium or endometrioma capsule. In addition, worsen inflammation
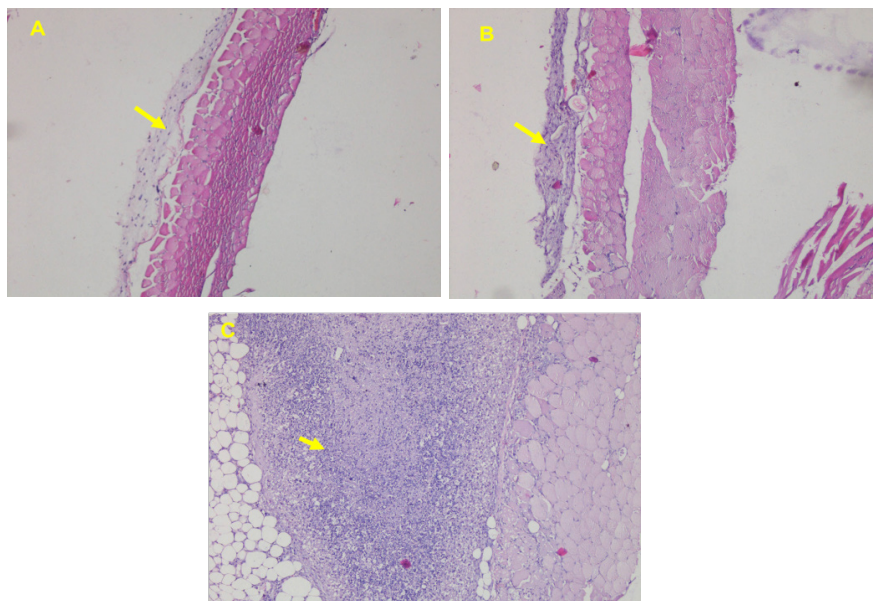

Figure 4. Histopathology Level of Peritoneal Damage. In A and B models, the inflammatory group scored between 1 and 3 whereas in group C (the treatment group) there was severe inflammation some of which were followed by the death of myocyte cells and fibrous connective tissue formation. Inflammation is illustrated by the arrows (M = 100x).
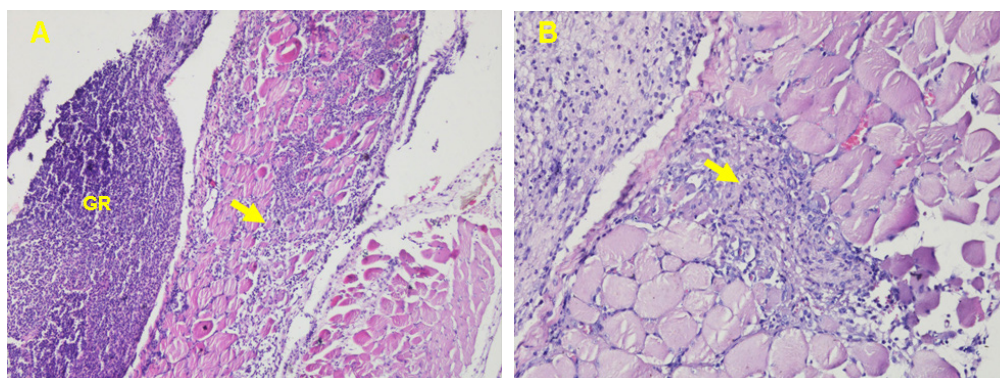
Figure 5. Description of Granuloma (GR) Mass and Infiltration of Inflammatory Cells and Connective Tissues in Muscle Tissue of Mice Receiving an Injection of Adenomyosis Tissue (group C). Note. (A) The form of granuloma and massive infiltration of inflammatory cells in muscle tissue (arrows). (B) The formation of fibrous connective tissue (arrows) between the muscle cells (M = 200x).

state of the intraperitoneal cavity would induce endometriosis more severely and vice versa (10).

Based on histopathological examination of the endometriosis lesions in the peritoneum, group C had the most severe damage level. The granuloma mass, massive infiltration of inflammatory cells, and fibrous connective tissue formation occurred in muscle tissue in this group. Conversely, mice injected with the supernatant of endometrial tissue did not develop granuloma masses (Figure 5). Further, no granuloma masses were detected in group A while it was observed only in one of the mice of group B. However, a granuloma mass was noted in almost all the mice of group C. That is, mouse model C was the best one through which inflammatory infiltration and granuloma mass simulation were obtained among the three treatment groups. Inflammatory mediators such as TNF-α and IL-6 up-regulated vascular endothelial growth factors and led to increased angiogenesis and inflammation reactions and stimulated the growth of nodule/ endometriosis spot in the gut, the muscle of the abdomen wall, liver, and adipose tissue surrounding abdominal organ (12).

The immune system which involved in the development of endometriosis includes humoral and cellular immunity. In patients with endometriosis, the occurrence of immune system disorders is characterized by the reduced T cells and a natural killer cell response (13). Furthermore, the disease indicates an increase in humoral immune response and macrophage activity (13). Endometriosis lesions secrete haptoglobin which affects the normal function of the macrophages. Moreover, the inflammatory mediator that can stimulate the cascade reaction with the end product includes increasing endometriosis cell proliferation, showing less response to apoptosis stimulus, increasing the formation of the new vascular vessel, and aggravating the development of endometriosis lesion (10). The deficient immune system in mice for a heterologous model of endometriosis is useful for studying the immune modulating drug in endometriosis (12,14)

According to the immunological theory, the adhesion of endometrial cells released onto the peritoneal surface and invasion of the subperitoneal involves the appearance of extracellular membrane adhesion molecules (ECAM) molecules and their co-receptor. Endometrial fragments may accumulate in certain places within the pelvic cavity and adhere to the peritory surface. A microscopic defect causes the endometrial cells to come into direct contact with the submesothelium matrix, which then proliferate, spread, grow, and sometimes invade down to the subperitoneal layer. Endometriosis is often found in women with low cellular immunity due to its inability to degrade the tissue of endometriosis that enters the peritoneum (10).

Macrophages and monocytes in the peritoneal fluid are vital elements of the immune system contained in the peritoneal fluid. The macrophage is the most common type of cell which is found in peritoneal fluids and is involved in the pathogenesis of endometriosis. Additionally, peritoneal macrophages and monocytes of the endometriosis have an increased effect of cytokine production, growth and angiogenic factors, and other substances that stimulate ectopic endometrial proliferation and decrease apoptosis. Increased cytokine production mediates a number of endometriosis symptoms such as infertility and pain in women of reproductive age (15,16).

The growth of the ectopic endometrium, facilitation of infiltration by the immune cells, and the increased production of pro-inflammatory cytokines as well as angiogenesis and growth factors are considered the entire picture of the inflammatory response detected in endometriotic implants. This condition leads to the mobilization of fibroblasts and the proliferation of the connective tissue as a homeostatic mechanism to isolate and cure the injury site. The emergence of fibroblasts and connective tissues plays an important role in the pathogenesis of this disease. However, it remains unclear whether these immunological abnormalities are the cause or consequence of endometriosis (15).

Ectopic endometrium growth stimulates excessive macrophage production, proinflammatory cytokine

products, and growth factors in peritoneal fluid, leading to further growth. The major pro-inflammatory cytokines, namely, TNF-αand IL-1β are released from peritoneal macrophages and endometriosis cells which subsequently activate transcription factors such as nuclear factor-kappa B (NF-kB) and protein activator 1 (AP-1). Active transcription factors bind to endometriotic cell DNA and stimulate subsequent gene transcription activity (13).

Our knowledge about the etiology of peritoneal endometriosis is limited. The most broadly accepted explanation is the "Sampson hypothesis" which suggests that peritoneal endometriosis occurs due to retrograde menstruation when the endometrial tissues pass through the fallopian tubes into the peritoneal cavity where the tissue undergoes implantation (17). Nevertheless, this mechanism cannot justify why endometriosis happens only in some women if retrograde menstruation is about to occur in about 90% women (18). Metaplasia of the colon as another usual hypothesis indicates that the epithelium can be converted into endometrium by metaplasia. However, this theory cannot account for the extreme rarity of endometriosis in men, its common localization in the abdominal cavity, and lack of increase with age as compared to other metaplasia (19).

The implantation theory offers that the endometriosis formation in the peritoneal cavity needs the endometrial or cell tissue in order to complete the adhesion, invasion, and proliferation process. Several studies examined whether pelvic peritoneum was involved in the endometriosis formation and maintenance or contained these changes in women with endometriosis. Some potential roles in the pathophysiology of peritoneal endometriosis were discussed and considered which include providing the of ectopic endometrium cell attachment sites, facilitation of endometrial cell invasion, transcendental epithelial-mesenchymal potential, changes in immune cell activation or recruitment, and the differential expression of inflammatory cytokines.

There are epithelium, stromal, and endometrial glands in endometriotic implants, the histology picture of which is similar to the eutopic endometrium (18,20). Microscopic analysis demonstrated that endometriosis encompass endometrial glands and stroma which are sometimes found in smooth muscle fibers and respond to hormonal circulation as was reported for eutopic and ectopic endometrium (21). Injecting endometriosis from human origin into mice peritoneal cavity increases the inflammatory reaction in peritoneal cavity organ and develops more nodule growth and adhesion intraperitoneally. In addition, it is a more practical method to develop the mice model of endometriosis (12,14).

Conclusions
The size of the implanted endometriosis lesions which were injected with supernatant from adenomyosis into the mice (group C) caused larger endometriosis implant areas and most severe damage to the peritoneum, granuloma mass, and massive infiltration of inflammatory cells and the formation of fibrous connective tissue in the muscle tissue. Generally, based on the results, implantation of the adenomyosis cell tissue is regarded as the best method for developing the mice model of endometriosis.

Ethical Issues
All the methods were approved by the Ethical Committee of Medicine Faculty, Brawijaya University with the ethical clearance No.197/EC/KEPK-S3/05/2017.

Conflict of Interests
Authors declare that they have no conflict of interests.

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None.

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