

Co-Supplementation of Vitamin E & *Nigella sativa* Improved the Endometrial Thickness in Mice

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ABSTRACT

Background: The thin endometrial lining has been reported to be associated with significant complications in maintaining a healthy pregnancy. Studies have been conducted to find ways to improve this condition, and one of the possibilities is through micronutrient intake. Vitamin E and *Nigella sativa* (NS) are the two potential sources of micronutrients that could play essential roles in improving endometrial thickness.

Purpose: The present study was conducted to determine the effect of vitamin E and NS supplementation on endometrial thickness in normal (not induced to any disease) mice.

Methods: Twenty-four balb/c mice were divided into four groups with six mice each; Group A as negative control (no treatment), Group B given with vitamin E (400 mg/kg), Group C given with NS (200 mg/kg) and Group D given with both vitamin E (400 mg/kg) and NS (200 mg/kg). The uterus histological sections from each group were evaluated for endometrial thickness and other structures of the uterus components were also observed.

Results: Co-supplementation of vitamin E and NS have a significantly higher endometrial thickness ($131.84 \pm 14.48\mu\text{m}$) compared to the control group ($88.08 \pm 11.49\mu\text{m}$) and other uterus structures were not disrupted.

Conclusion: The co-supplementation of vitamin E and NS could enhance the endometrial thickness without inducing any disruption to the other structures of the uterus.

KEYWORDS: Endometrial thickness; vitamin E; *Nigella sativa*; uterus histology.

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INTRODUCTION

The uterus plays an essential role in the reproductive system. Anatomically, the uterus is a hollow, muscular organ within the pelvic

cavity. The interior part of the uterus is lined with endometrium. The endometrium layer will shed and regrow each month in response to the changes in estrogen and progesterone levels during the ovarian cycle [1].

The middle layer in the uterus is known as the myometrium, composed of smooth muscle layers. Meanwhile, the outermost layer of the uterus is the perimetrium consisting of the serosal layer covering the uterus lining.

The primary function of the uterus is to prepare itself for any chances of pregnancy by thickening the endometrium during the menstrual cycle [2]. Menstruation occurs if there is no pregnancy. The uterus is an important part of the reproductive system in which any abnormalities could lead to serious problems in fertility and pregnancy. For example, a study reported that a thin endometrium in assisted reproductive technologies (ART) is linked to early and late pregnancy complications [3]. Besides, a thin endometrium also is found to have less vascular endothelial growth factor (VEGF) expression, which results in defective placentation [4]. Studies have been conducted to find ways to improve this condition, and one of the possibilities is through natural product-derived micronutrient intake.

Vitamin E is also known as the vitamin for reproduction, as its initial discovery by Evans and Bishop (1922) [5] was a result of a study on the reproductive system. However, vitamin E is more recognized as a powerful lipid-soluble antioxidant due to the extensive reports on its radical scavenging property [6]. Vitamin E is made up of tocopherols (TOCs) and tocotrienols (TCTs), with both TOCs and TCTs present in eight different homologs. These homologs are alpha (α)-TOC, beta (β)-TOC, gamma (γ)-TOC, delta (δ)-TOC and α -TCT, β -TCT, γ -TCT, and δ -TCT [7]. The effects of vitamin E on reproductive health have been reported (reviewed in [8]), however, its effects specifically on the uterine layers are limited. For instance, there is a study that reported vitamin E intake could increase endometrial thickness in women [6].

Nigella sativa (NS) is another natural resource that has gained attention as a possible beneficial supplementation for reproductive health [9]. *N. sativa* is a medicinal plant that consists of many active compounds like thymoquinone, alkaloids, saponins, flavonoids, proteins, fatty acids, and many

others contributing to its medicinal effects [10]. *N. sativa* has been reported to exert many therapeutic potentials as an anti-microbial, anti-inflammatory, anti-oxidant [11-13] including in the reproductive system [14], however its specific effect on the endometrial thickness is lacking. Hence, this study aimed to determine the effect of supplementation of vitamin E and NS on the changes in endometrium thickness using mice as the study model.

MATERIALS AND METHODS

Animal treatment: Research ethics approval was obtained from the University's Committee on Animal Research & Ethics (Ref. No.: 404/2023) prior to conducting the study. This study involved twenty-four female balb/c mice aged 6-8 weeks which were obtained from the Laboratory Animal Facility and Management (LAFAM), UiTM, Malaysia. Mice were held at room temperature (20–25°C) with a 12-hour dark/light cycle. Food and water were provided *ad libitum* and tissue rolls were used for enrichment purposes.

The mice were divided into four groups and force-fed with the following treatments for 16 consecutive days; Group A served as the control group and received no treatment (n=6), Group B was given vitamin E (400 mg/kg/day) (n=6), Group C was given NS (200 mg/kg) (n=6) and Group D was given both vitamin E (400 mg/kg) and NS (200mg/kg) (n=6). Both vitamin E and NS were purchased from a local supplier (Sigma-Aldrich (M) Sdn. Bhd., Malaysia).

Histology analysis: Upon completion of the treatments, all mice were euthanized and the uterus samples were removed. The samples were immediately washed with 0.9% normal saline solution to clean the blood remains on the surface of the uteruses. Following that, the samples were processed through the steps of fixation, dehydration, clearing, embedding, sectioning, staining, and mounting.

Fixation: The uterus samples were placed in Bouin's solution overnight in which the solution will coagulate protoplasm and harden the tissue. The volume of Bouin's solution used was 10 times greater than the volume of the

samples. The fixation step is crucial to prevent sample degeneration due to autolysis, self-digestion, or post-mortem.

Dehydration: Following fixation, the samples were placed successively in alcohol solutions of increasing strength, from alcohol 70%, 80%, 95%, and ending with a couple of passages through absolute alcohol. The purpose of dehydration is to remove all the water content inside the samples. The volume of each alcohol concentration used was 10 times greater than the volume of the samples. The samples were immersed in each alcohol solution for one hour.

Clearing: The samples were then immersed overnight in the toluene solution (a clearing agent) to clean the samples from any alcohol remains.

Paraffin moulding: In this step, all samples were embedded in paraffin. The volume of the paraffin used was three times the volume of the samples. This step is important to ensure that paraffin penetrates into the whole sample. The dehydration, clearing, and paraffin moulding steps were done using an automatic tissue processor.

Embedding: Heated paraffin wax was poured into a cast block (70mm L x 30mm H x 30mm W), and the samples were placed into the block (one sample in a block). The block was then left on a cold platform for an hour to solidify. The embedding step is done to hold the samples during the sectioning.

Sectioning: Sectioning refers to the process of cutting the paraffin block into thin slices using a microtome to a thickness between 4µm – 5µm. Following sectioning, the obtained ribbons were transferred onto a clean microscope slide in which a drop of water has been smeared. Then, the slides were placed on a heater to dry the ribbons.

Staining and mounting: Slides with the sample sections were next processed for staining using the Hematoxylin-Eosin (H&E) method. The steps taken in this step are shown in Table 1. After staining is completed, the slides were covered with coverslips and allowed to dry. Staining is important as it enhances the visibility of the sample when observed under

the microscope.

Table 1: Hematoxylin-Eosin (H&E) staining procedure.

Step	Solution	Time (min)
1	Xylene 1	10
2	Xylene 2	5
3	Pure alcohol : Xylene (1:1)	5
4	Pure alcohol	3
5	95% Alcohol	3
6	80% Alcohol	3
7	70% Alcohol	3
8	Distilled water	5
9	Hematoxylin	5
10	Tapping Water	5
11	Eosin	4
12	95% Alcohol	2 times immerse
13	95% Alcohol	1 time immerse
14	Pure Alcohol	3
15	Pure Alcohol : Xylene (1:1)	5
16	Xylene 3	10
17	Xylene 4	15

Slide observation and data analysis: Stained samples were observed under the microscope. The uterus section from each study group was observed for the differences in the structural changes between the studied groups. Obtained data were statistically analyzed using one-way ANOVA (SPSS 28.0) and data are expressed as mean ± S.E.M, with a p-value less than 0.05 is considered significant.

RESULTS AND DISCUSSION

The present study aimed to determine the effect of vitamin E and NS supplementation on endometrial thickness in normal mice. The obtained results showed that the average endometrial thickness in Group A is $88.08 \pm 11.49 \mu\text{m}$, Group B is $88.16 \pm 5.31 \mu\text{m}$, Group C is $95.05 \pm 6.97 \mu\text{m}$ and Group D is $131.84 \pm 14.48 \mu\text{m}$, showing a significant ($p = 0.002$) increase in the endometrial thickness in Group D (co-supplemented with vitamin E and NS) in comparison to the control group (Group A). Statistical analysis also showed a significant increase in the endometrial thickness in Group D in comparison to Group B ($p = 0.003$) and Group C ($p = 0.01$), showing that co-supplementation of vitamin E and NS is also beneficial compared to the separate treatments. The histological photomicrographs of the differences in the endometrial thickness are shown in Figure 1.

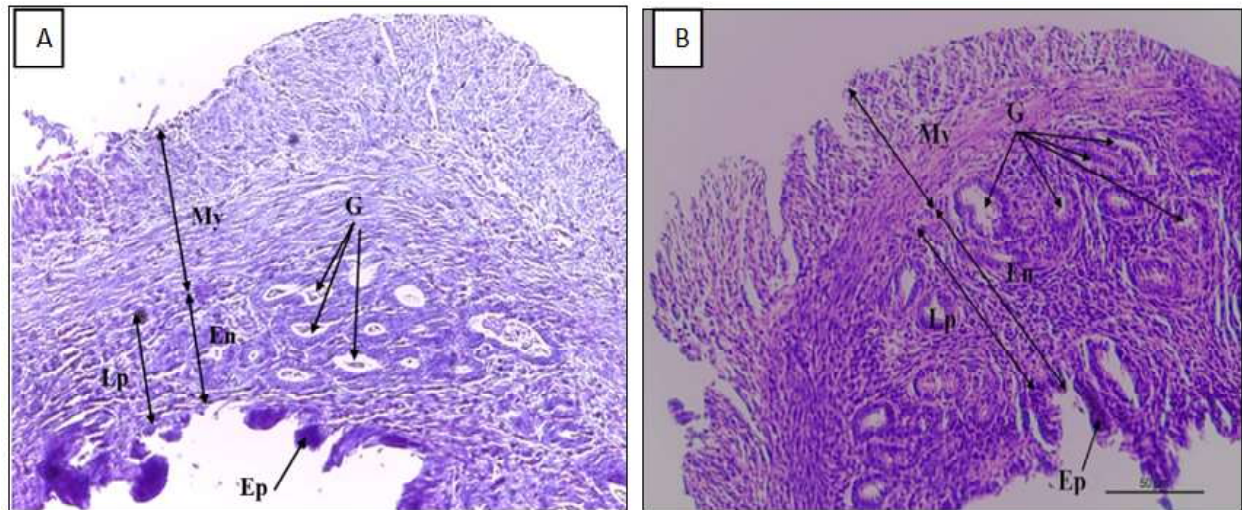


Fig. 1: The differences in histological results of the endometrial thickness. (A) The average endometrial thickness was observed in Group A (control, no treatment), Group B (vitamin E (400 mg/kg)) and Group C (*Nigella sativa* (200 mg/kg)). (B) The increased thickness in the endometrial layer in Group D (co-supplementation with vitamin E (400 mg/kg) and *N. sativa* (200 mg/kg)).

Abbv: En = endometrium; Ep = epithelial surface lining; G = uterine glands; Lp = lamina propria; My = myometrium

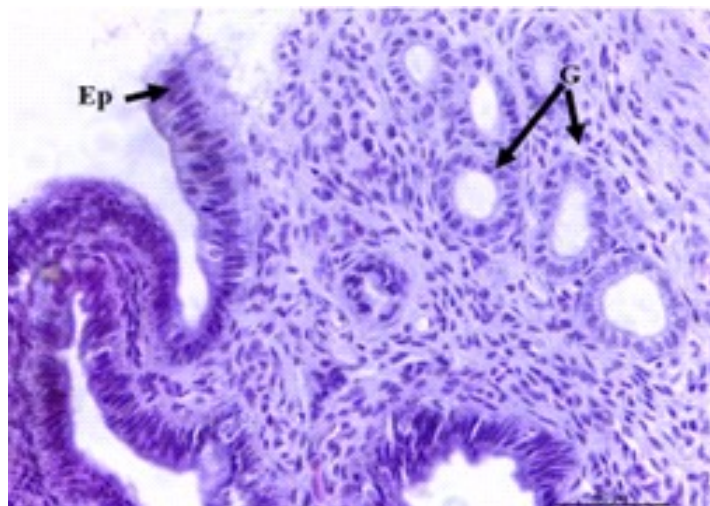


Fig. 2: An intact epithelial lining (Ep) on the internal surface of the uterus and an intact columnar epithelium lining on the uterine glands (G) were observed in the uterus samples of all groups

Besides the endometrial thickness, the structures of the uterus components were also observed. The findings indicated a normal well-distinguishable presence of the endometrium and myometrium layers, intact epithelial lining on the internal surface of the uterus, and intact columnar epithelium lining on the uterine glands (Figure 2). These results indicate that the treatments with vitamin E and NS alone or in combination did not induce any structural disruption to the uterus. Findings from the present study showed that the co-supplementation of vitamin E and NS in normal mice resulted in a significantly higher endometrial thickness ($131.84 \pm 14.48 \mu\text{m}$) compared to the control group ($88.08 \pm 11.49 \mu\text{m}$). Endometrial thickness is usually used as a marker of endometrial receptivity.

Based on previous studies, the higher endometrial thickness increases the chances of successful embryo implantation [15].

The endometrium is known as the site of implantation and having thin endometrium reduces the chances of successful embryo attachment which could lead to miscarriage. Present data also showed that the supplementation with vitamin E and NS alone did not induce any changes in the endometrial thickness in normal mice, as the obtained results were similar to the control group.

Previous studies on the effects of vitamin E and NS on endometrium thickness in normal mice are not available, hence, similar comparisons to the present results could not be discussed. However, there are previously reported studies on subjects with infertility

and implantation failure. A recent study by Hashemi et al. (2019) [16] reported that a 12-week intervention with vitamin E supplementation in women with implantation failure resulted in a significant increase in endometrial thickness. Another study by Pala et al. (2016) [17] on the protective effects of vitamin E on hysterosalpingography (HSG)-induced epithelial degeneration and proliferation in rat endometrium suggested that vitamin E may exert protection against radiation injury. There is also a study that provided evidence of the promising effect of vitamin E supplementation on endometrial thickness in women with infertility problems [6]. The effect of *Nigella sativa* supplementation is also supported by a previous study that reported the crude aqueous extract of the NS seeds might possibly have an estrogen and/or progesterone-like action on rats' endometrium which resulted in endometrial thickening [18]. Another related study also reported on the protective effect of NS against cimetidine-induced damage in the uterus [19].

Present results also indicated that the treatments with vitamin E and NS alone or in combination did not induce any structural damage to the uterus. Obtained results showed the presence of normal and intact cells of the uterus. These findings are in line with a previous study that reported on the importance of the uterine secretory luminal and glandular epithelial cells in sustaining a healthy pregnancy through the presence of alpha-tocopherol transfer protein (α -TTP), suggesting the constant vitamin E supply is essential for pregnancy [20]. Another study by [18] also reported on the regular columnar epithelial cells lining the uterine lumen and glands following treatment with the aqueous extract of the seeds of NS.

CONCLUSION

Co-supplementation of vitamin E and *N. sativa* could enhance the endometrial thickness in normal mice without exerting any harmful effects on the histological structures of the uterus. Hence, this may help to increase the chances of successful implantation and pregnancy. Further studies are needed to

understand the molecular mechanism of actions of both vitamin E and NS on their effects on the regulation of reproductive functions.

Conflicts of Interests: The authors declare that there are no conflicts of interest.

Author Contributions

Siti Syairah worked on the conception and design of the study. **Sabrina, Nurul Hannah, and Siti Syairah** carried out the laboratory work. All authors have been involved in analyzing the data and writing the manuscript. All authors read and approved the final version of the manuscript.

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