THE EFFECT OF "DONG TRUNG HA THAO SAPA" HARD CAPSULES IN THE UTERUS OF OVARIECTOMIZED MICE

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"Dong trung ha thao Sapa" (DTHT) capsules, prepared from Cordyceps militaris powder, are intended to be used in relieving menopausal signs and symptoms. The current study aimed to evaluate the estrogenic properties of DTHT capsules on experimental animals. Female mice from 6 to 8 weeks were used for the study. Mice were divided into the following groups: group I - normal control without being ovariectomized (saline-treated); group II to V were ovariectomized, group II (saline-treated); group III (Ethinyl estradiol-treated), group IV (low dose DTHT capsules) and group V (high dose DTHT capsules). The potential effect of DTHT capsules was evaluated by measuring both the wet and blotted uterus weights. The uterus was then fixed in 10% neutral buffered formalin, HE(Haematoxylin & Eosin) stained and examined histopathologically. The results suggest that DTHT capsules show estrogenic activity in ovariectomized mice by increasing wet uterus and blotted uterus weight and improving the uterus's histology. DTHT capsules appear to be an effective therapeutic drug for the treatment of menopausal symptoms.

Keywords: DTHT capsules, Cordyceps militaris, menopausal, female mice, ovariectomized, Ethinyl estradiol.

I. INTRODUCTION

Women have menopause at a mean age of 51 years, with 95 percent having their final menstrual period between the ages of 45 to 55 years. Menopause is associated with a marked decrease in ovarian estrogen production. In most women, this results in low serum estradiol concentrations, vasomotor symptoms (hot flashes), and menopausal symptoms such as urogenital atrophy and osteoporosis.[1]

Hormone therapy remains the most effective treatment for vasomotor and genitourinary menopause symptoms and to prevent bone loss and fracture. The term hormone therapy encompasses estrogen therapy (ET) and estrogen-progestogen therapy (EPT) when outcomes are not specific to one or the other treatment. However, the different effects of ET, EPT, and estrogen-receptor (ER) agonists or antagonists are included whenever possible.[2] Estrogen plays vital role in menopausal hormone therapy (MHT). However, before prescribing MHT, one must be conscientious of its adverse effects on venous thromboembolism, stroke, breast cancer and endomentrial cancer. Thus, there is a current popular trend to discover and study medicinal products derived from traditional herbs.[2]

Cordyceps species, including *Cordyceps sinensis (CS)*, *Cordyceps militaris* are

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valuable traditional medicinal materials from *Ascomycetes* fungus parasitic to *Lepidoptera larvae*. *CS* has been traditionally used to enhance sexual performance and restate impaired sexual function in Chinese society.[3] Due to overexploitation process, *CS* is now in danger of extinction. While *Cordyceps militaris* is considered as containing the same nutritional and medicinal ingredients as *CS*, no study has provided reliable shreds of evidence of their effects on sexual and reproductive functions. Therefore, the purposes of this study were to evaluate impact of *Dong trung ha thao Sapa (CM)* in the uterus of ovariectomized mice.

II. MATERIALS AND METHODS

1. Research sample

DTHT hard capsules were provided by Traphaco Sapa Company. These were developed in the form of hard capsules, and each capsule contained 350 mg of *Cordyceps militaris* powder combined with several excipients including PVP, Amidon and stearate. Cordycepin and adenosine are effective components isolated from *Cordyceps militaris.* Adenosine consists of adenine attached to a ribose via a β-N9-glycosidic bond. And Cordycepin, 3'-deoxyadenosine, is also known as an adenosine analog. Cordycepin from the Cordyceps militaris has been reported to have acute anti-inflammatory, anti-nociceptive, anti-angiogenesis, and immunoregulatory activities.[4] DTHT hard capsule contain both components at 4.68 and 0.67 mg, respectively.

DTHT hard capsules were stored in dry environment, between 15-30 °C. Capsules shells were discarded and the ingredients were dissolved in water.

Research location: The study was conducted in the Laboratory of the Department of Pharmacology, Hanoi Medical University. Members in Laboratory of the Department of Pharmacology removed the ovaries in mice. Histological analysis were performed at Department of Pathology, Ha Noi Medical University.

2. Experimental animals

Female *Swiss* mice between 6 and 8 weeks of age, were used in this study. The ovariectomy procedure began with the animal in ventral recumbency after the animal had been adequately anesthetized. The incision opening the dorsolateral abdominal wall was approximately 1 cm lengthways at the midpoint between the inferior costal border and the iliac crest and a few millimeters lateral to the lateral margin of the lumbar muscle. The ovary was removed from the abdominal cavity onto an aseptic field. The ovary was disconnected at the junction of the oviduct and the uterine body. After confirming that no massive bleeding occurred, the abdominal wall was closed by a suture and the skin closed by autoclips or appropriate suture. 14 days from ovariectomy, mice were given water and drug.[5]

All procedures conformed to all local standards of laboratory animal care. The temperature in the experimental animal room was 22°C ± 3°C. The relative humidity was at a minimum of 30% and preferably would not exceed a maximum of 70%, other than during room cleaning. Relative humidity of 50- 60% would be considered as an ideal condition. The daily artificial lighting sequence would be 12 hours light /dark. Water was provided ad libitum.

3. Experimental design

Fifty mice were divided into five groups. Group I was not ovariectomized. Ovariectomy was done from group II to V.

Group I: Control - The mice received orally distilled water (10 ml/kg b.wt.)

Group II: The mice were given orally distilled water (10 ml/kg b.wt.)

Group III: Positive control - The mice were given Ethinyl estradiol orally at 0.9 mg/kg b.wt./day

Group IV: DTHT capsules were given orally at 504 mg/kg b.wt./day

Group V: DTHT capsules were given orally at 1512 mg/kg b.wt./day

The experiment lasted for seven days. On day 8 (24 hours after the last dose), mice were weighed and then necropsied. The study objective was to measure both the wet and blotted uterus weights. The wet weight included the uterus and the luminal fluid contents. The blotted weight was measured after the luminal contents of the uterus had been expressed and removed. After weighing, the uterus was fixed in 10% neutral buffered formalin to be examined histopathologically after Haematoxylin & Eosin (HE)-staining.

4. Statistical analysis

Data were analyzed by the T-test using Microsoft Excel software version 2010. Data were presented as a mean ± standard deviation. A p-value of less than 0.05 is statically significant.

III. RESULTS

1. The uterus weight

The wet uterus weight

Table 1. Effect of DTHT capsules on wet uterus weight

Group	Wet uterus weight (mg/10g b.wt)
Group I	15.28 ± 4.54
Group II	4.50 ± 0.97
p2-1	**p < 0.001**
Group III (Ethinyl estradiol)	23.87 ± 5.42
p3-2	**p < 0.001**
Group IV (DTHT capsules at dose of 504 mg/kg/day)	6.33 ± 2.42
p4-2	p > 0.05
Group V (DTHT capsules at dose 1512 mg/kg/day)	8.44 ± 3.10
p5-2	**p < 0,001**

The weight of the wet uterus in group II (ovariectomized mice) was significantly decreased compared with the control group (non- ovariectomized mice) ($p < 0.001$). Ingroup III- positive control- wet uterus weight increased significantly compared with group II ($p < 0.001$). Ingroup IV, wet uterus weight tended to increase compared with group II but the difference was not statistically significant ($p > 0.05$). In group V there was a statistically significant increase in wet uterus weight compared with group II ($p < 0.001$).

The blotted uterus weight

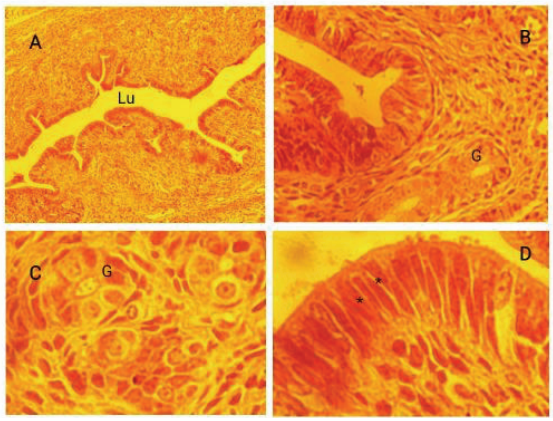
Table 2. Effect of DTHT capsules on blotted uterus weight

Group	Blotted uterus weight (mg/10g b.wt)
Group I	10.82 ± 3.37
Group II	4.00 ± 1.63
p2-1	**p < 0.01**
Group III (Ethinyl estradiol)	21.12 ± 5.00
p3-2	**p < 0.001**
Group IV (DTHT capsules at dose of 504 mg/kg/day)	5.09 ± 2.61
p4-2	p > 0.05
Group V (DTHT capsules at dose 1512 mg/kg/day)	7.16 ± 2.64
p5-2	**p < 0.05**

As shown in Table 2, the weight of blotted uterus in group II (ovariectomized mice) decreased significantly compared with the control group (non- ovariectomized mice) ($p < 0.01$). In group III given Ethinyl estradiol at the dose of 0.9 mg/kg/day, the blotted uterus weight increased significantly compared with group II ($p < 0.001$). In group IV using DTHT capsules at the dose of 504 mg/kg/day, there was an increase of blotted uterus weight when compared with group II but the difference was not statistically significant ($p > 0.05$). In group V, using DTHT capsules at the dose of 1512 mg/kg/day, there was a statistically significant increase in blotted uterus weight when compared with group II ($p < 0.05$).

2. Histopathological changes after one week of taking DTHT capsule

Group I: Control (non- ovariectomized mice received orally distilled water)



A: HE-stained mouse uterus (objective x10), Lu: uterine lumen

B: Mouse uterine lining (objective x40), G: submucosal gland

C: Mouse uterine submucosal layer (objective x40)

D: Mouse uterine lining, (*) simple columnar epithelial cells

Figure 1. Uterus hispathology of group I

The uterus was tubular. The tubular wall was composed of two layers: the inner layer was the mucosal layer and the outer layer was the muscular layer (Figure 1). The outer layer of the uterus was covered with a sheath of fibrous connective tissue. The epithelium covering the mucosa was a simple columnar epithelium. Underneath the epithelium was a cushion layer with many submucosal glands, the glandular wall was also covered by a simple columnar or cuboidal epithelium, and the outer layer was a basement membrane. The nucleus of the epithelial cells was globose, bright in color.

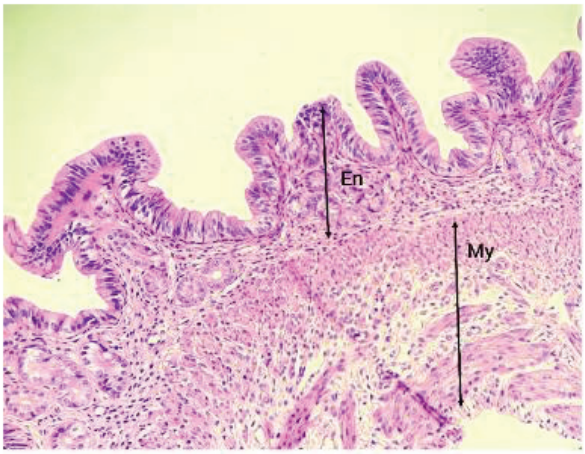


Figure 2. The uterus structure of group I (objective x20)

En: Endometrial layer; consists of simple columnar epithelial cells and the submucosa is sparse connective tissue and submucosal glands. Observed many villi grow into the uterine lumen.

My: The muscle layer; is composed of 2 layers of smooth muscle with a rich network of soft muscle fibers, interspersed with many blood vessels.

The muscle layer was quite developed, composed of smooth muscle fibers running in two main directions: the inner circular layer and the outer longitudinal layer. There were many blood vessels between the two muscle layers (Figure 2).

Group II (ovariectomized mice received orally distilled water)

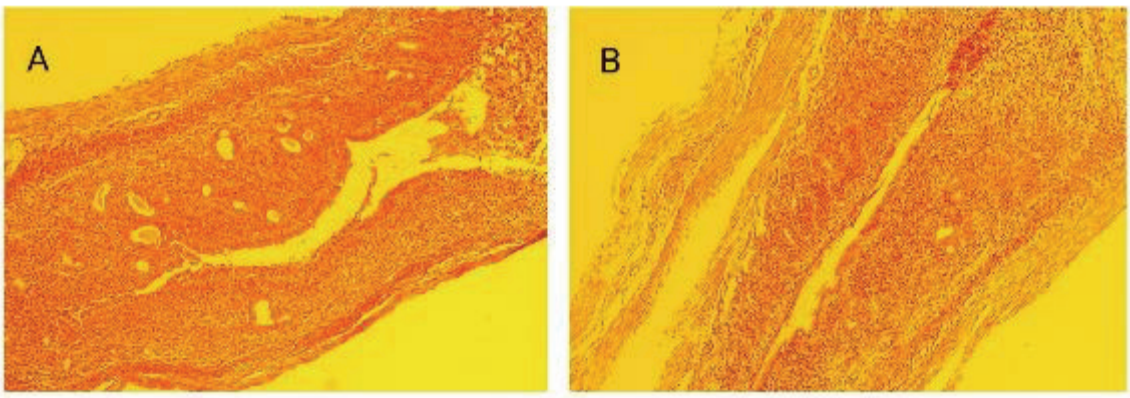


Figure 3. The uterus hispsthology of group II (objective x10)

We notice uterine structure changes in all mice (Figure 3). The uterine lumen tended to be minor, narrow. The uterine wall was thinner than the control group, the mucosal layer lacked villi, a thin muscle layer.

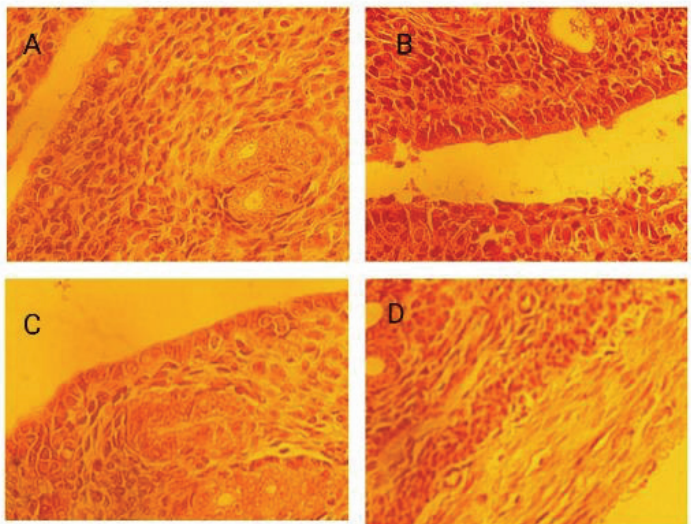


Figure 4. The uteru hispathology of group II (objective x40)

A, B, C Mucosal and submucosal layers

D: Muscle layer

When observing at the larger, x 40 objective, in the mucosal layer, the epithelial cells in this group belonged to the low columnar or simple cuboidal type. The submucosal glands were poor, also covered with simple cuboidal or simple squamous epithelium. The submucosa's vascular appearance was lacking (Figures 4 A, B, C). In the muscular layer, thin, smooth muscle cells had little sarcoplasm (Figure 4D). In particular, there was slight congestion and slight swelling of the mucosa (Figure 5).

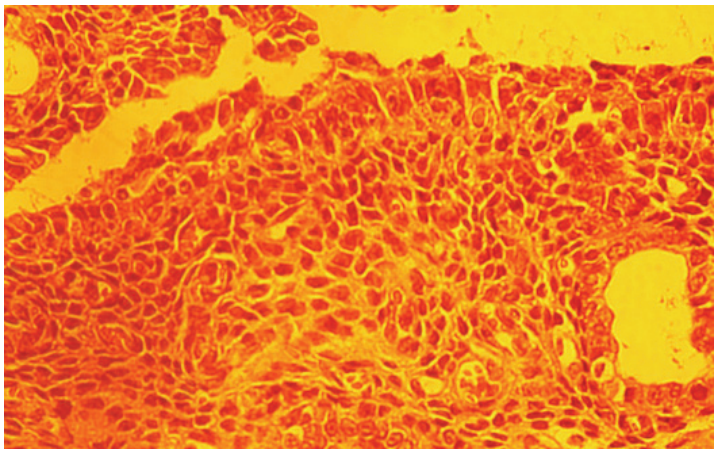


Figure 5. The uterus hispathology of group II (objective x40)

Arrows show epithelium sliding off the surface.

Group III (ovariectomized mice received Ethinyl estradiol orally)

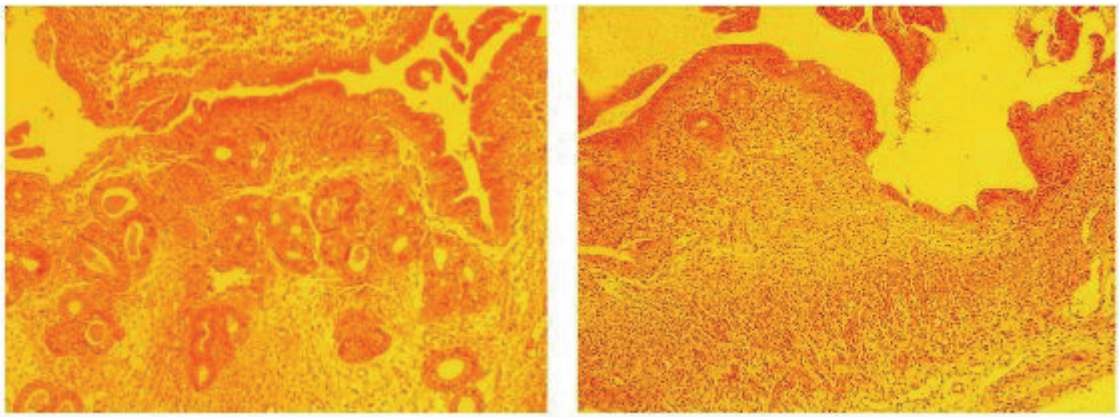


Figure 6. The uterus hispathology of group III (objective x10)

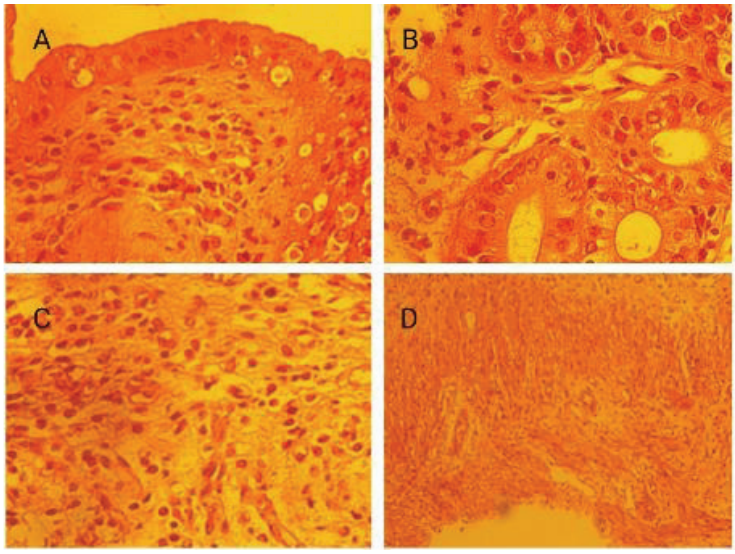


Figure 7. The uterus hispathology of group III

A: mucosal and submucosal layers (objective x40)

B, C: Submucosal layer (objective x40)

D: Muscle layer (objective x10)

In this group, it was observed that the uterine cavity of the mouse dilated again. The uterine wall was thick; the mucosal layer had many villi with high columnar epithelial and mucus-secreting cells. The submucosal layer of glands was rich, the gland contained much fluid. (Figure 6, figure 7). A thick layer of muscle, rich sarcoplasm (Figure 7).

Group IV (DTHT capsules at dose the 504 mg/kg/day)

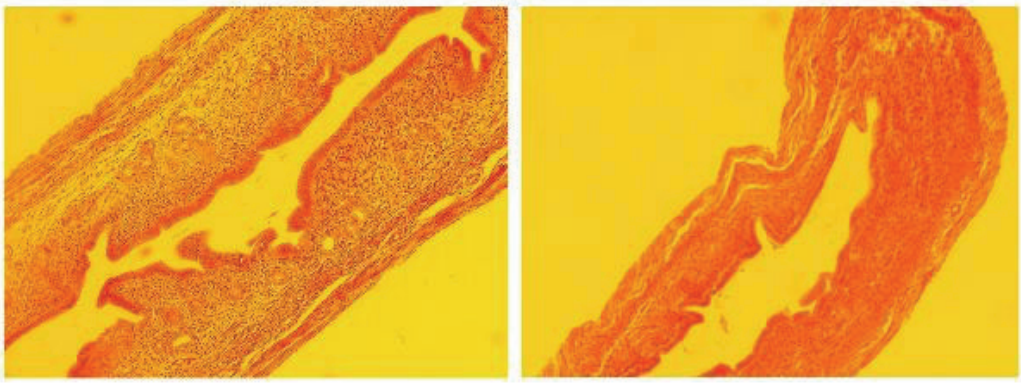


Figure 8. The uterus hispathology of group IV using DTHT capsules at a dose the 504 mg/kg/day (objective x10)

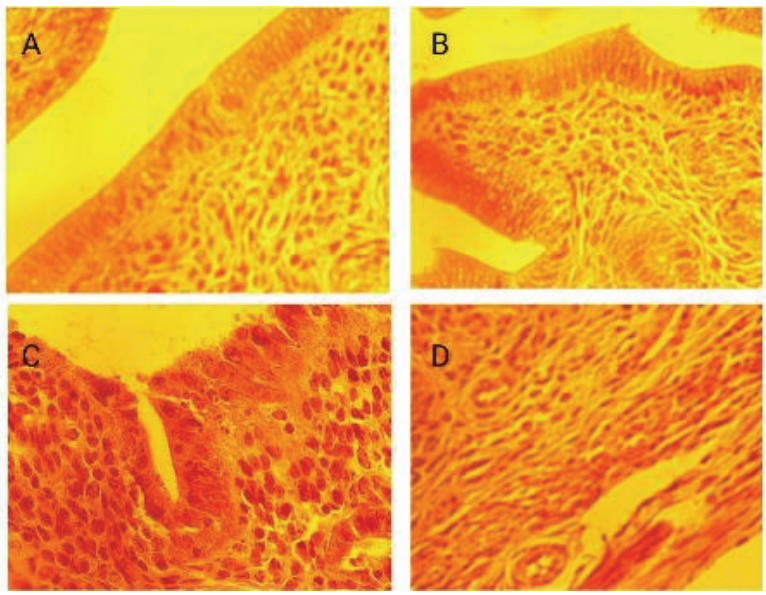


Figure 9. The uterus hispathology of group IV using DTHT capsules at a dose the 504 mg/kg/day (objective x40)

We observe uterus change compared to group II: the uterine cavity was more expansive and the uterine wall was thicker (Figure 8). Endometrial lining: tall columnar cells, some microvilli were observed. The submucosal layer had many glands; the gland wall was also covered with simple columnar or cuboidal cells (Figure 9 A, B, C). The muscle layer did not change much (Figure 9 D).

Group V (DTHT capsules at dose the 1512 mg/kg/day)

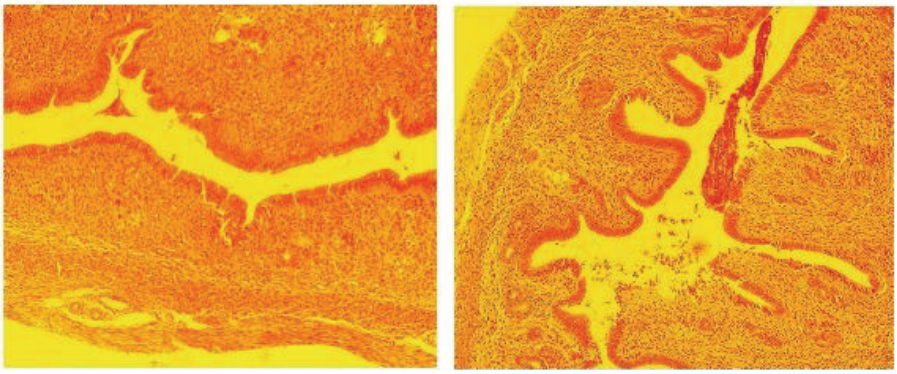


Figure 10. The uterus hispathology of group V using DTHT capsules at a dose the 1512 mg/kg/day (objective x10)

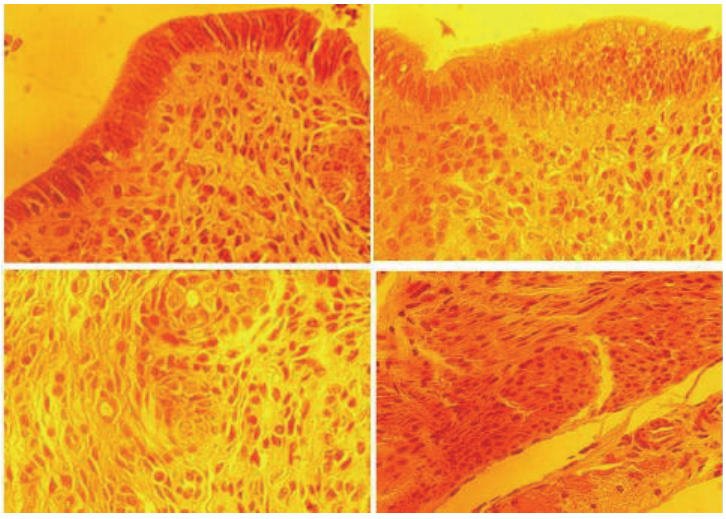


Figure 11. The uterus hispathology of group V using DTHT capsules at a dose the 1512 mg/kg/day (objective x40)

In this group, it was observed that the uterus changed significantly compared to group II; there was enlarged gland and thick glandular wall; the mucosa had many microvilli, and the submucosa was thick. The muscle layer was relatively thick (Figure 10), and the epithelial cells were tall, glandular and abundant (Figure 11).

IV. DISCUSSION

The model was carried out in ovariectomized female experimental animals according to OECD 440. Rat and mouse have been routinely used in the Uterotrophic Bioassay since the 1930s and the data obtained in rats and mice correspond well with each other.[6] In this study, we chose mice and did ovariectomy according to the guidelines of OECD 440. When the ovaries were removed, that means eliminating endogenous estrogen and the hypothalamic-pituitary-ovarian axis; eliminating the difference in estrogen levels between mice, thereby detecting substances with estrogenic properties.[3]

The study used Ethinyl estradiol orally at a dose of 0.9 mg/kg/day as a positive control

for seven days. The study results showed that Ethinyl estradiol at this dose showed estrogenic properties in ovariectomized mice compared with mice that did not receive Ethinyl estradiol.

Study data included wet, blotted uterus weights and histopathological changes. This is an organ that depends on estrogen. The results show that DTHT capsules at the dose of 504 mg/kg/day (equivalent clinical dose) given orally for seven days tend to increase wet uterus weight and blotted uterus weight in ovariectomized female mice, however the difference was not statistically significant. On the uterine histology, there was an improvement in the wider uterine lumen and thicker uterine wall; the uterine lining had high columnar cells, some microvilli were observed; the submucosal layer had many glands, the gland wall was also covered by simple columnar or cuboidal cells; the muscle layer had not seen much change.

DTHT capsules at the dose of 1512 mg/kg/day (dose equivalent to 3 times the usual clinical dose) given orally for seven days show estrogenic activity in ovariectomized mice model: markedly increase the wet uterus weight and the blotted uterus weight (squeeze out the luminal fluid). There was a marked improvement in the histology of the uterus: the gland was dilated; thick glandular wall; the mucosal layer had many microvilli, the submucosa was thick; relatively thick muscular layer; epithelial cells were tall, glandular, abundant.

In general, a test for estrogenicity should be considered positive if there is a statistically significant increase in uterine weight ($p< 0.05$) at least at the high dose level as compared to the solvent control group.[6] Thus, DTHT capsules at the dose of 1512 mg/kg/day exhibited estrogenic properties in ovariectomized mature mice. Based on the above results, the selected dose of DTHT capsules in mice for further studies was 1512 mg/kg. The recommended human dose of DTHT capsules for adult male patients is 18 capsules per day.

What mechanism does DTHT capsule has estrogenicity?

With the first mechanism, is DTHT capsule an exogenous estrogen or not? According to Da-wei Zhang *et al.* (2014), isoflavones from *Cordyceps* are naturally occurring plant chemicals belonging to the “phytoestrogen” class, that improve osteoporosis, increase the osteocalcin and estradiol level in ovariectomized osteopenic rats.[7]

The biologically active estrogen estradiol is produced in at least three major sites:

1) Direct secretion from the ovary in reproductive-age;

2) By conversion of circulating androstenedione of adrenal and/or ovarian origins to estrone in peripheral tissues; and

3) By conversion of androstenedione to estrone in estrogen-target tissues. In the latter two instances, estrogenically weak estrone is further converted to estradiol within the same tissue. The presence of the enzyme aromatase and 17β-HSD is critical for estradiol formation at these sites.[8] According to Huang BM (2004), *Cordyceps sinensis* induced estradiol production by granulosa-lutein cells in a dose- and time-dependent manner and that a 3-h treatment with *Cordyceps sinensis* induced increased levels of mRNAs coding for the P450 side chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and aromatase.[9] In another study, on the protective effect of *Cordyceps militaris* against bisphenol A-induced reproductive damage, bisphenol A is considered a potent endocrine disruptor by inhibiting CYP11A1, 3β-HSD, CYP17A1 and 17β-HSD. The results of this study suggest that *C. militaris* can directly

modulate CYP11A1, 3β-HSD and CYP17A1 expression.[10] Thus, DTHT capsules can cause estrogenicity through this mechanism.

We recommend future extensive studies on the mechanism of DTHT capsules to strengthen the basis for its use in menopausal treatment should be performed.

V. CONCLUSION

DTHT capsules at the dose of 1512 mg/kg/day given orally for seven days shows estrogenic properties in ovariectomized mice model: increased the wet uterus weight and the blotted uterus weight and have a statistical difference compared with the control group. There was also a marked improvement in the histology of the uterus. The result from this study would suggest the further clinical application of DTHT for treating menopausal women.

Further studies should be done to investigate the impact mechanisms of the product. It can be directly affected the tissue (uterus) or indirectly in another way (endocrine or adipose tissue).

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