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Full Length Research Paper

Microencapsulation and pharmacological evaluations for the anti-fertility effect of castor bean extract in female mice

Yi-ling Hou[1]*, Xiang Ding[2], Shuang-quan Duan[3], Zhi-rong Yang[2] and Ping Gao[2]

[1]Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), College of Life Science, China West Normal University. No. 44 Yuying Road, Nanchong 637002, China.
[2]Key Laboratory for Biological Resource and Ecological Environment of the Ministry of Education, College of Life Sciences, Sichuan University. No. 29, Wangjiang Road, Chengdu 610064, P. R. China.
[3]College of Sciences, Tibet University, Lhasa 850000, China.

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Castor Bean (*Ricinus communis*, Family Euphorbiaceae) ethanol extraction (CBE) possesses potent antifertility effect, but the absorption of it is limited. Therefore, this study was aimed to prepare CBE microparticles for targeted drug effect. Poly (toluene-2, 4-di-isocyanate and ethylene alcohol) (TDI-EA) microparticles loaded with CBE were prepared by Interracial polymerization, a semi-industrial technique capable of encapsulating fragile molecules maintaining their native properties. The effects of several parameters on the properties of the particles were investigated. Microparticles showed of CBE- TDI-EA microparticles following 200, 400 and 600 mg/kg with a content of 5 mg two mean diameters that were 4 and 120 µm, separately, which are suitable for their absorption. Entrapment efficiency of CBEM in TDI-EA microparticles was 98.3%. The drug efficiency ranged from 73.26 to 100% depending on the drug consumption. The optimal growth suppression of the Kunming strain female mice vital organs could be achieved by oral application CBE in 6 mg microparticles while the Control Group showed the normal growth of the vital organs and CBE at the same concentration in solution form could not suppress the growth of the vital organs to the same extent. Finally, a bioassay demonstrated that the *in vivo* CBE microparticles have a strong depression of fertility without significant adverse reaction.

Key words: Antifertility, castor bean extract, female mice, microencapsulation.

INTRODUCTION

Ricinus communis, Family Euphorbiaceae, is a common plant and cultivated throughout China as traditional Chinese medicine and horticultural plant. Its seed, Castor Bean, has been used in many countries of the world as an antileprotic agent and a cure for cancer, syphilis and rheumatic affections, such as lumbago, pleurodynia and sciatica (Scarpa and Guerci, 1982). The use of the beans as a contraceptive agent has also been reported (Dafallah and Al-Mutairy, 1994; Brondegaard, 1973; Malhi and Trivedi, 1972; Woo et al., 1981; Salhab and Issa, 1997) in India, Korea, Algiers, Egypt, China, etc., but the Castor beans usually cause coprorrhea, stomachalgia, emesia and other side effects by oral taken directly (Scarpa and Guerci, 1982). Thus, research on microencapsulation design in order to improve drug affectivity is of a great interest. In fact microspheres can provide various advantages such as sustained drug delivery, localized release, enhanced drug safety and efficacy and improvement of patient compliance. There have been a lot of reviews dealing with the different strategies on microencapsulation (Freitas et al., 2005; Sah, 1997) with all kinds of materials (Rish and Reineccius, 1988; Song et al., 2001). Among these compounds, toluene-2, 4-di-isocyanate and ethylene alcohol are interesting polymer with various potential

*Corresponding author. E-mail: biostart8083@yahoo.cn. Tel/Fax: +86 28 85251989.

applications in the formulation area (Huang et al., 1999; Li et al., 2000). Especially poly (toluene-2, 4-di-isocyanate and ethylene alcohol) (TDI-EA), being a flexible, hydrophilic, uncharged and highly hydrated polymer, soluble both in water and in DCM, presents all the basic requirements for efficient microencapsulation (Song et al., 2001). From morphology measurements to assess the structural changes of CBE induced by its exposition to interfaces created during the emulsification process, it was concluded that the addition of TDI-EA in the organic phase could optimize the reduction of the amount of non-covalent CBE aggregates and a subsequent loss of activity. Even if toluene-2, 4-di-isocyanate and ethylene alcohol have already been the focus of many interfacial characterization studies alone with drugs, the influence of the antifertility of the CBE by means of microcapulation in combination with Poly(toluene-2, 4-di-isocyanate and ethylene alcohol) (TDI-EA) using Interracial polymerization has not been investigated so far.

MATERIALS AND METHODS

Plant material

R. communis, Family Euphorbiaceae, were collected in the Emei country of Sichuan province, China. The plant was identified taxonomically by Dr. Xing-Jin He (Biology Department, Sichuan University, Sichuan China). Voucher specimen has been deposited in the herbarium of the Biology Department, Sichuan University.

Extraction, purification and identification

Ground unshelled and powdered castor beans (300 g) were extracted with 95 ethanol (400 ml) at 40℃ for 2 h under the supersonic wave, filtered, concentrated to give yellow brown residues (127 g yield 42.3%) and stored at 4℃ for subsequent use. After saponification and recrystalization, the crude extract of castorbean seeds (127 g) yielded 0.14% (w/w) of active fraction CBE, colorless crystals. The components were identified by gas chromatography and mass spectrometric detector (GC–MS) analysis using a capillary column OV-5 (30 × 0.25 × 0.25 mm), helium as carrier gas at a flow rate of 1 $mLmin^{-1}$, in electronic impact mode (70 eV) and split injection (1:20). The injector and GC/MS interface were kept at 250℃. The column temperature program was as follows: 285℃(1 min), heating at 5℃min^{-1} until 320℃ and remaining at this temperature for 30 min. Similarity comparison of the mass spectra obtained from NIST05.LIB with those of the samples, allowed the identification of the five compounds of activity fraction CBE.

Animals

Kunming strain female mice (30 ± 2 g) obtained from the Experimental Animal Center of Chengdu Chinese Medical Science University were used. Animals were raised in steel cages under a controlled temperature of 20 ± 2℃ in a 12 h light, 12 h darkness schedule (lights on 06.00 - 18.00 h). Food and water were offered *ad libitum*.

Microparticles preparation

Microparticles containing purified CBE were prepared by interracial polymerization method under orthogonal design experiment conditions. Briefly, the ethanol extract of castor beans was emulsified with 5% acacia solution. After this step, interracial polymerization happened by adding toluene-2, 4-di-isocyanate (TDI) and ethylene alcohol into the solution on thermostat-controlled water bath. Finally, the particles were stored at 0℃ (Shantha et al., 2006).

Microparticle characterization

The morphology and surface structure of the coacervate droplets and microcapsules particle size were observed using an optical microscope (Nikon, eclipse E600, Japan) and Laser granulometry (Mastersizer 2000, Malnern) (Ducel et al., 2004; Lamprecht et al., 2000a; 2000b). The results were expressed in volumetric mean diameter, which is the diameter that divides the volume distribution curve of the sampled microparticles in two equal parts. Samples were measured in triplicate. The measurement was done 1 h after having stopped the stirring. Encapsulation efficiency was determined by the reported method (Fu et al., 2005; Yin et al., 2004). Microcapsules were washed three times with double distilled water, grinded mixing with diatomite and then extracted by Soxhlet extraction method to get the encapsulated Castor Bean ethanol extraction.

The percentage of encapsulation efficiency was calculated with the following equation:

$$\text{Encapsulation efficiency (\%)} = (E/E_0) \times 100\%$$

where E is the amount of the encapsulated castor bean ethanol extraction and E_0 is the initial amount of the Castor Bean ethanol extraction.

Bioactivity studies

Study protocol

Kunming strain female mice of proven fertility were divided into four groups of 8 each. The mice were given oral feeding from day 1 and day 8 to investigate anti-implantation and anti-early pregnancy, respectively:

Control group: Mice receiving vehicle (distilled water, 0.5 ml/day, p. o.) for 6 days.
CBEM group: Mice treated with microcapsules of test extract (200, 400, 600 mg/kg, respectively, p. o.) for 6 days.

On day 20, ovaries, uterus, adrenal, spleen and thymus were removed, cleared of fat and connective tissue, weighed and kept at -20℃ until total assay completed.

Histological preparations

Tissues were fixed in Bouin's fluid. Paraffin sections were made and stained with hematoxylin and eosin or periodic acid shiff reagent (PAS). Cross-sections were examined through an optic microscope.

Fertility test

The mating exposure tests on control and treated groups were performed from day 7 to day 13 and day 1 to day 7, respectively. The proestrus female mice were cohabited with males in a ratio of 1:1. Vaginal plug and the presence of sperm in the vaginal smear were checked for positive mating (Qin et al., 2006).

Statistical analysis

The data were statistically analyzed and expressed as mean ± S.E.M. Statistical analysis of the variance between control and CBEM values was done by Student's *t*-test. $P < 0.05$ or less was considered to be significant.

RESULTS AND DISCUSSION

CBE purity assay

Main chemical components of castorbean seed has been reported previously (Cheng, 1998). However, no report on the CBE antifertility bioactive constituents of the seed has been published. According to GC–MS data and the similarity comparison, the colorless crystal, was a mixture of five compounds (Table 1), of which four kinds of phytosterols were ergost-5-en-3-ol (6.10%), stigmasterol (35.80%), γ-sitosterol (44.77%) and fucosterol (8.40%), and one of probucol analog (4.93%). The percentage of γ-sitosterol was the highest in the mixture. Previous studies showed that the main compounds of phytosterols in most vegetable oil were campesterol, stigmasterol, β-sitosterol and brassicasterol. This study showed that the main compounds of phytosterols in the colorless crystal were γ-sitosterol and stigmasterol. Structurally, β-sitosterol closely resembled cholesterol, which was a precursor to the reproductive sex-steroid hormones (Mellanen et al., 1996). Literature survey revealed that the β-sitosterol could affect the behavior of aquatic vertebrates (Ethan and Alison, 2006) and it significantly caused the increase of plasma vitellogenin levels of the exposed parent both females and males zebra fish, changed the sex ratio of the species (Tarja and Kirsti, 2003). But no report has been seen that γ-sitosterol possessed the inhibitory activity on the CBE *in vitro*. In the present study, the major component of the colorless crystal was γ-sitosterol which was structurally similar to β-sitosterol (Khan and Mlungwana, 1999). However, except for γ-sitosterol possessing cytotoxicity against artemia salina (Khan and Mlungwana, 1999), few reports about the functional γ-sitosterol has been seen. Stigmasterol, another main component of the constituent, possessed the function of surfactant (Gao et al., 2000). Therefore, we presumed that γ-sitosterol might be the main component contributing to inhibit the reduction on weight of the vital organ and the stigmasterol might act as an assistant. Whether the conjecture was correct and other compounds in the crystal possessed the same activity, needs further investigation. Investigations revealed that the components of phytosterol were of similar structures only due to the variations of the number and location of double bond and to the number of carbon atoms composing the side chain (Xu et al., 2002a, 2002b). Thus, their physical properties are so nearly identical as to make separation very difficult. The separation and the function of every component in the crystal will be studied further.

Microsphere preparation and characterization

Several attempts were made to overcome the critical steps of microsphere preparation process: core/wall ratio, dose of 5% acacia solution, stirring speed, reaction time and temperature (Figure 1). Attention was focused on the preservation of the CBE biological activity. Microencapsulation containing CBE were prepared by interracial polymerization (Hirech et al., 2003; Anna et al., 2002) using Poly (toluene-2, 4-di-isocyanate and ethylene alcohol) (TDI-EA). This procedure avoids shear stress produced by sonication and ultraturrax that normally would affect drug integrity and consequently, their biological activity (Rasenack and Muller, 2002, 2004; Steckel et al., 2003; Rasenack et al., 2003). Another advantage of TDI-EA over microparticle production through interracial polymerization methods is the ability to produce homogeneous batches on a semi-industrial scale. Consequently, this would be of great interest considering scaling-up and industrial issues. CBE and TDI were co-encapsulated with EA in order to stabilize the primary emulsion and to reduce drug-polymer interactions. Previous studies showed that poly (toluene-2, 4-di-isocyanate and ethylene alcohol) (TDI-EA) dissolved in the inner aqueous phase was a good candidate to protect CBE against denaturing by contact with the other organic phase during emulsification without modifying the microparticle structure. Also, TDI-EA limited CBE penetration in the interfilm of the primary emulsion and reduced the contact between the CBE and the organic phase. Furthermore TDI-EA promoted sufficient release of entrapped CBE. Subsequently, the TDI-EA loaded with CBE was a good kind of adjuvant to prepare particles by interracial polymerization method.

Particle size and morphological analysis

Particle size was measured by laser diffractometry. The microcapsulation results are shown in Figures 1, 2 and 3. All the microparticles exhibited a monomodal size distribution with two mean diameters that were 4 and 120 μm, separately, which compatible with an oral feeding. The mean particle size decreases when the nominal drug loading increases. Thus, at nominal CBE content of 3 mg, the mean particle size was around 40 µm independently of the polymer used. Surprisingly, when 5 mg of CBE were included in the inner water phase, the mean particle size decreased to 20 µm. Non-loaded microspheres prepared under the same conditions showed a mean particle size of 100 µm. The decrease in microparticle size could be ascribed to tension active properties of the CBE. The mean particle size was slightly influenced by the addition of TDI-EA. F-test and p-value indicated that

Table 1. Components of the fraction CBE-1.

Peak	Component	RT[a] (%)	RA[b]
1	Ergost-5-en-3-ol	7.272	6.10
2	Stigmasterol	7.470	35.80
3	γ-Sitosterol	7.998	44.77
4	Fucosterol	8.120	8.40
5	Probucol analog	14.007	4.93

Notes: [a]RT indicated the retention time on the column in minutes. [b]RA indicated relative area (peak area relative to the total peak area).

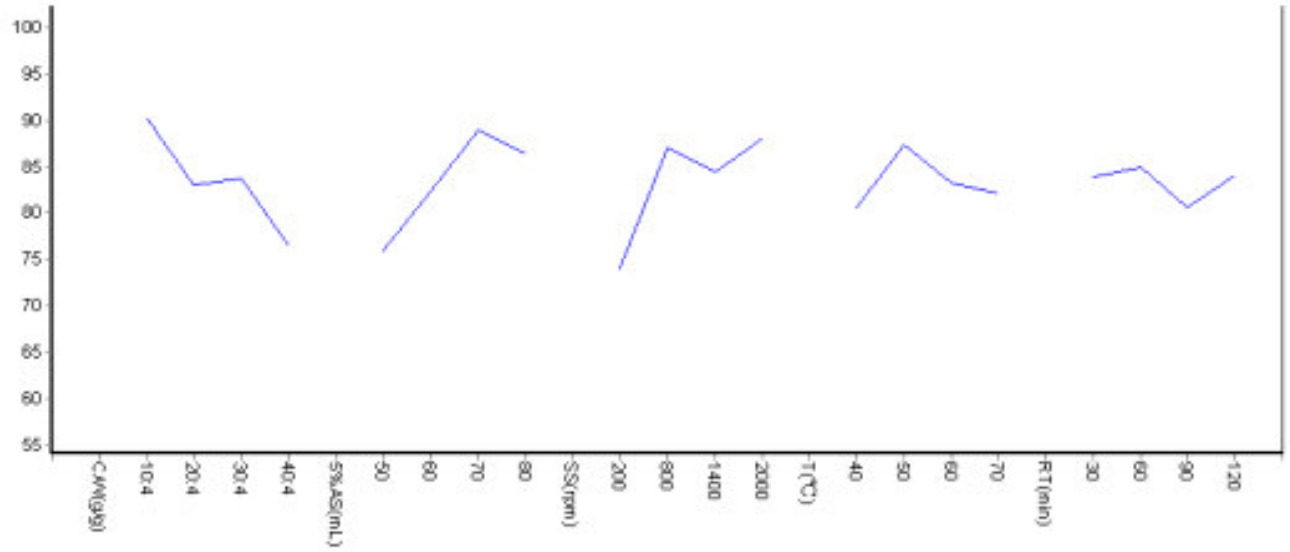


Figure 1. Effect of controllable factors on encapsulation efficiency. Y-axis: entrapment rate; X-axis: level of factor; C/W: core/wall (g/g); 5% A S: 5% acacia solution (ml); S S: stirring speed (rpm); T: temperature (□); R T: reaction time (min).

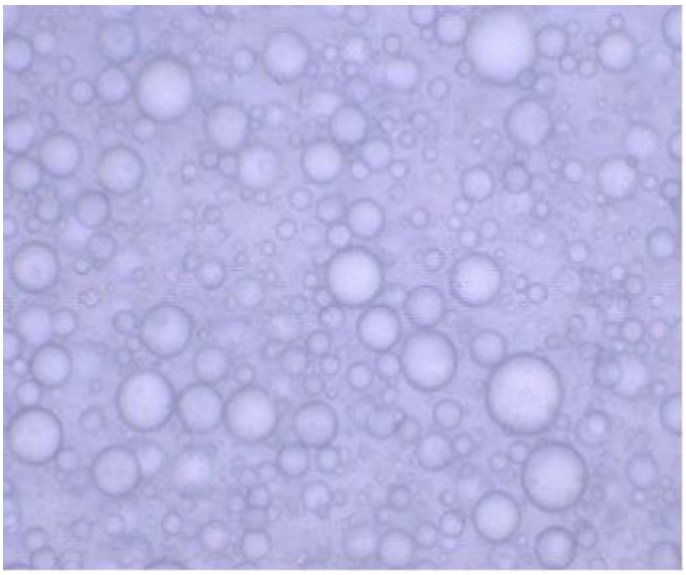
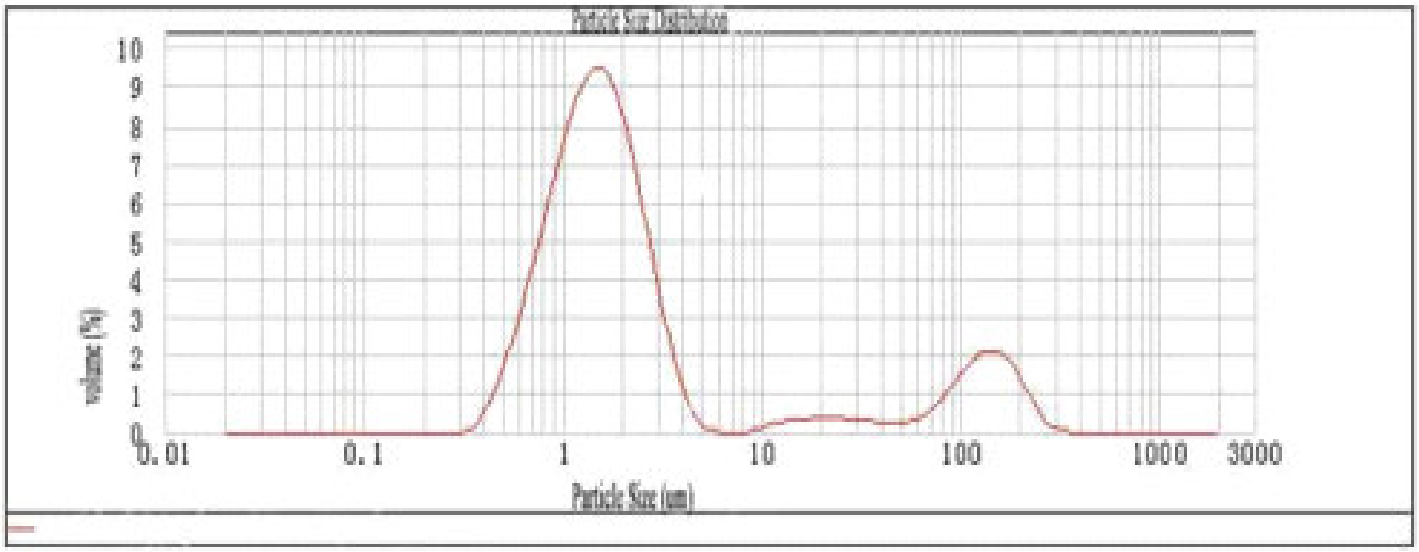


Figure 2. Photographs of microcapsules of Castor bean extract (× 400).

core/wall ratio, emulsifier dose and stirring speed had the main effect on microcapsulation. The optimal conditions were obtained at the core/wall ratio of 10:4, 5% acacia solution of 70 ml, stirring speed of 2000 rpm, reaction time of 60 min and temperature of 50℃ under which the encapsulation efficiency is 98.3%.

In vivo bioactivity assay

The weight and the Histological preparations of the vital organ in each female rat in the control group were compared to that in the treated group in order to measure the effect of the drug (Abdulazim et al., 1998). The drug effects shown by the reduction of the weight of the Kunming strain female mice vital organs on the untreated female mice are shown in Tables 2 and 3. CBE-TDI-EA microparticles exhibited the high activity as can be seen from the reduction of weight of the vital organ which was significantly different from that one of control mice group. Histology of uterus shows more irregularly arranged



Density :	Span Number :	Consistency :	Result Sort :
0.0018 × Volume	62.503	12.1	Volume
Specific Surface :	**Surface Area Mean Diameter D[3,2] :**	**Volume Mean Diameter D[4,3] :**	
4.07 (m^2/g)	1.473 (um)	20.743 (um)	
d(0.1):0.750 um	**d(0.5):1.631 um**	**d(0.9):102.689 um**	

Figure 3. Particle size and distribution in percentage of volume. D[4, 3] is the mean diameter in volume whereas D [3, 2] is the mean diameter in surface called “Sauter diameter”; D(v, 0.5) is the size for which 50% of the sample particles has a lower size and 50% has a upper size; D (v, 0.1) is the size for which 10% of the sample particles has a lower size and D (v, 0.9) is the particle size for which 90% of the sample particles has a lower size. The particle size distribution width is characterized by the relation: [D (0.9) – D (0.1)]/D (0.5) which defines the span number.

endometrial epithelial cells, destroyed and fewer gland cells, smaller uterine cavity, less endomembrane cilia and thinner endometrium with no hyperplasia in CBEM group than those in control group (Figure 4). Following the application of the various formulations containing CBE as active principle, the size of the mice vital organs was gradually reduced or remained in the original size whereas that of the control mice increased in size (Tables 2 and 3). It may be of some interest that the weights of spleen, adrenal and thymus were increased and positively correlated with the dose level of CBEM. Following 200, 400 and 600 mg/kg extract oral feeding for 6 days, a strong anti-implantation (inhibition 100%) at the dose level of 400 mg/kg and a strong anti-early pregnancy (inhibition 100%) at the dose level of 200 mg/kg were observed respectively. The potency pharmacological action of CBE-TDI-EA microparticles to inhibit the growth of the mice organs can be explained by their ability to deliver CBE gradually. Importantly, the orally tested CBEM did not cause coprorrhea, hyperspasmia, death or other side effect of treated mice. Using single extract of castor bean would increase the risk of coprorrhea, stomachalgia, emesia, etc. However, the combined use of castor bean extract and Microencapsulation could alleviate these syndromes.

Conclusion

We have clearly shown that the CBE displayed the inhibitory effect on the fertility of mice *in vitro*, mainly due to the presence of phytosterols (Table 1). Further studies are needed to confirm that the detailed functions of each component in the colorless crystal. The microcapsulation results are shown in Figures 1, 2 and 3. F-test and p value indicated that core/wall ratio, emulsifier dose and stirring speed had the main effect on microcapsulation. The optimal conditions were obtained at the core/wall ratio of 10:4, 5% acacia solution of 70 ml, stirring speed of 2000 rpm, reaction time of 60 min and temperature of 50℃ under which the encapsulation efficiency is 98.3%. We have developed novel TDI-EA for the oral delivery of CBE. We demonstrated the CBE-TDI-EA containing 5% acacia as surfactant and TDI-EA as cosurfactant

Table 2. Anti-implantation activity and effect of CBEM treatment on vital organ weight in female mice.

Treatment (day 1-day 6)	Vital organ weight (mg/100 g body weight)					No. of litters	No. of pregnancies	Inhibition (%)
	Uterus	Ovaries	Spleen	Adrenal	Thymus			
Control (distilled water, 0.5 ml)	2240 ± 2770	45 ± 8	738 ± 145	19 ± 7	244 ± 102	11.5 ± 2.14	8	Nil
CBEM 200 mg/kg	328 ± 73	45 ± 11	748 ± 219	27 ± 7	251 ± 63	2.13 ± 3.94	2	81.52 □**
CBEM 400 mg/kg	387 ± 106	42 ± 10	828 ± 262	30 ± 6	287 ± 86	1.00 ± 2.83	1	91.30 □**
CBEM 600 mg/kg	376 ± 105	47 ± 7	914 ± 119	244 ± 50	316 ± 78	0	0	100 □**

Values are mean ± S. E. M. (n = 8); *P < 0.05, **P < 0.001 vs. control; Student's t-test.

Table 3. Anti-early pregnancy activity and effect of CBEM treatment on vital organ weight in female mice.

Treatment (day 8-day 13)	Vital organ weight(mg/100g body weight)					No. of litters	No. of pregnancies	Inhibition (%)
	Uterus	Ovaries	Spleen	Adrenal	Thymus			
Control (distilled water, 0.5 ml)	5620± 4510	45 ±12	703 ±150	20 ± 3	130 ± 54	10.8 ± 5.00	8	Nil
CBEM 200 mg/kg	4210± 4580	48 ± 9	710 ±122	23 ± 9	184 ± 54	3.13 ± 3.64	3	73.26 □*
CBEM 400 mg/kg	315 ± 105	58 ± 8	908 ±236	25 ±12	185 ± 77	0	0	100 □**
CBEM 600 mg/kg	3140± 5100	47 ± 8	950 ±148	24 ±2	480 ±717	2.5 ± 4.90	2	75.60 □*

Values are mean ± S. E. M. (n = 8); *P < 0.05, **P < 0.001 vs. control; Student's t-test.

significantly. CBE-TDI-EA is a promising delivery system for the safe and effective delivery of poor oral bioavailability drugs with potential for application to human therapy by increasing the efficiency of oral administration. Anemulsification technique for the preparation of CBE-TDI-EA microparticles was developed resulting in a size range of 4 - 120 µm and hence suitable for oral feeding and the maximal loading degree with CBE was 83.3%. The anti-implantation and anti-early pregnancy effects are exibihited in Tables 2 and 3, respectively. Treated animals showed a notable depression of fertility. Following 200, 400 and 600 mg/kg extract oral feeding for 6 days, a strong anti-implantation (inhibition 100%) at the dose level of 400 mg/kg and a strong anti-early pregnancy (inhibition 100%) at the dose level of 200 mg/kg were observed respectively without obvious side effect. Although the release of CBE from the microparticles could not be measured directly it seems to be sufficient for a therapeutic effect. Further studies should be necessary to confirm its working mechanism responsible for the activity.

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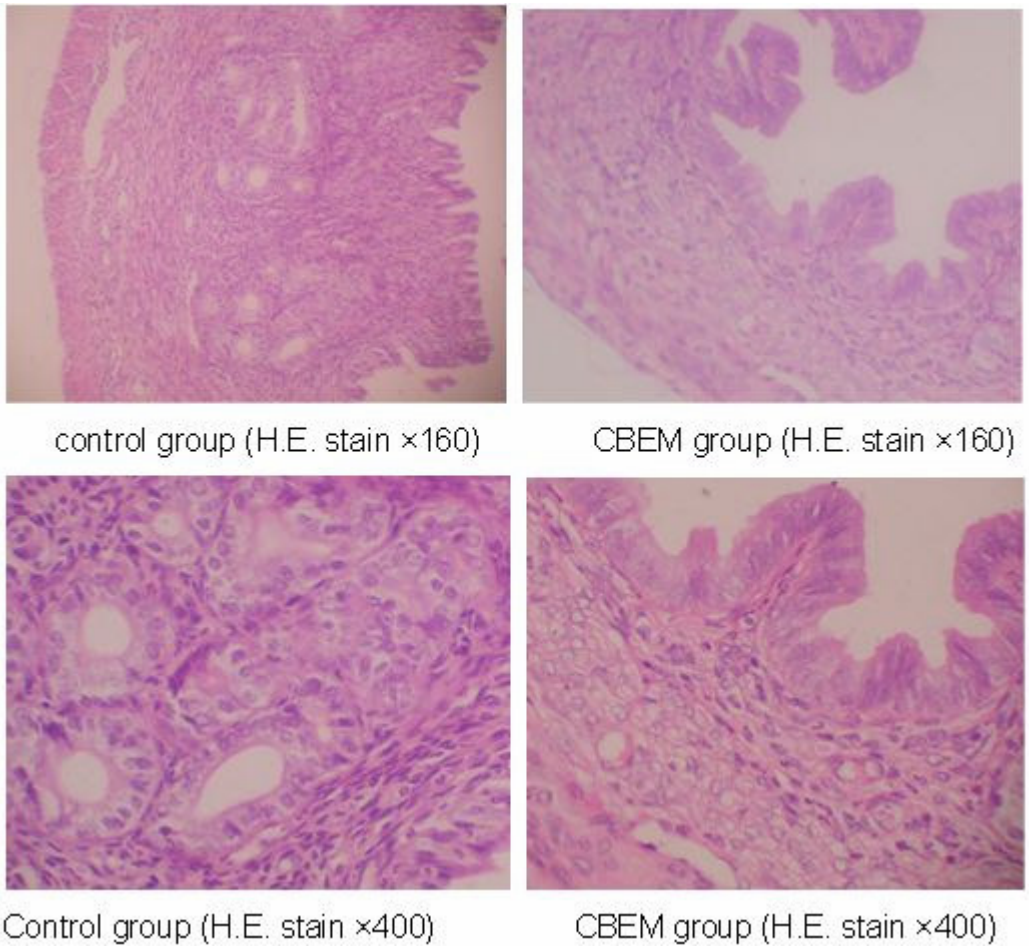

Figure 4. Histological preparations of the uterus in CBEM group compared with control group. Histology of uterus shows more irregularly arranged endometrial epithelial cells, destroyed and fewer gland cells, smaller uterine cavity, less endomembrane cilia and thinner endometrium with no hyperplasia in CBEM group than those in control group.

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