Hormone-like activities of Kuntai capsule in the uteri of ovariectomized rats and immature rabbits

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Abstract

OBJECTIVE: To investigate the hormone-like activities of Kuntai capsule (KTC) in the uteri of ovariectomized rats and immature rabbits.

METHODS: Following bilateral ovariectomy, rats were randomly divided into six groups including sham group, control group, estradiol valerate group, KTC 0.24, 0.6, and 1.5 g/kg groups. The rats were treated with 0.5% CMC-Na, estradiol valerate and KTC (0.24, 0.6, and 1.5 g/kg), respectively for 28 consecutive days. Then the estrous cycle, uterine changes and pathological changes were examined. Serum levels of estradiol (E₂), and progesterone (P₄) were measured by enzyme-linked immunosorbent assay (ELISA). Protein levels of estradiol receptor (ER), progesterone receptor (PR), vascular endothelial growth factor (VEGF), proliferating cell nuclear antigen (PCNA) and nuclear-associated antigen-67 (Ki-67) in uterine tissues were detected by western blot. Immature rabbits were estrogen-primed prior to intragastric administration with KTC for 6 d consecutively. Then, the uteri underwent hematoxylin-eosin staining to observe endometrial transformation.

RESULTS: Compared with the control group, 0.6 and 1.5 g/kg KTC markedly decreased the uterine organ coefficient and endometrial thickness (P < 0.05). The serum level of P₄ was increased in the KTC 0.6 g/kg group (P < 0.05). There were no significant variations in the serum level of E₂ in the KTC groups compared with the control group. ERβ, but not ERα, was markedly upregulated after KTC administration (P < 0.05). Furthermore, 1.5 g/kg KTC significantly decreased the protein level of PRA (P < 0.05) and 0.6 g/kg KTC increased the protein level of PRB in the uteri (P < 0.05). VEGF was highly expressed after treatment with 0.24 and 0.6 g/kg KTC, and Ki-67 was markedly reduced in ovariectomized rats treated with 1.5 g/kg KTC. No difference was found in the expression of PCNA. KTC 0.24 and 0.6 g/kg promoted endometrial transformation in immature rabbit uteri.

CONCLUSION: KTC does not demonstrate obvious estrogen-like effect on uteri after ovariectomy, but it does exhibit weak progestogen-like effect, by which mechanism of action is yet to be further investigated.

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Keywords: Kuntai capsule; Ovariectomy; Endometritis; Receptors, progesterone; Receptors, estradiol; Vascular endothelial growth factors; Allen-Doisy bioassay; McPhail score
INTRODUCTION

The Allen-Doisy bioassay, the most well-established and widely used method to detect estrogenic potency, has been widely employed to determine estrogen activity based on the change in uterus wet weight. Furthermore, a modified Allen-Doisy bioassay with a higher specificity bioassay for estrogen activity has been used to assess the response of the vaginal epithelium and the uterus (weight increase) in ovariectomized (OVX) adult rats. The McPhail model evaluates endometrial transformation in estrogen-primed rabbits and is a traditional and classical method to examine the progestogen activity of drugs. Because the proliferation and differentiation of the uterine epithelium are stimulated by progesterone or synthetic progestins, this semi-quantitative method allows the user to screen out compounds with progesterone receptor agonist and antagonist activity based on endometrial histological changes. The McPhail scores are determined based on a scale of 0-4 with 0 indicating no postovulatory response and 4 being the maximum response.

Kuntai capsule (KTC), a traditional Chinese herbal formula, consists of Dihuang (Radix Rehmanniae), Huangqin (Radix Scutellariae Baicalensis), Huanglian (Rhizoma Coptidis), Fuling (Poria), Baishao (Radix Paeoniae Alba) and Ejiao (Colla Corii Asini). KTC has been widely used to improve female menopause-related symptoms in China. Previous studies revealed that KTC alleviated breast pain and vaginal bleeding in menopausal women. Du et al. reported that KTC has fewer adverse reactions for patients with perimenopausal syndrome than hormone replacement therapy. In addition, KTC promoted embryo implantation by improving the endometrial receptivity and reducing cervical mucus. Although the clinical efficacy of KTC is accepted in China, there is little information on the estrogenic or progestogen activity of KTC. In the present study, we utilized the Allen-Doisy bioassay and McPhail bioassay to evaluate whether KTC has an effect on the uterus, revealing its potential hormone-like activities.

MATERIALS AND METHODS

Reagents

KTC (lot No.130705) is a product of Guiyang Xin-Tian Pharmaceutical Limited Company (Guiyang, China). E2 and P4 ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The BCA Protein Assay Kit (P0012S) was purchased from Beyotime Biotechnology (Shanghai, China). Rabbit polyclonal antibodies to nuclear-associated antigen-67 (Ki-67) (ab66155) and vascular endothelial growth factor (VEGF) (ab46154) were purchased from Abcam Biotechnology (Cambridge, USA). PCNA (BM0104) was obtained from Boster Biotechnology (Wuhan, China). An enhanced chemiluminescence (ECL) detection kit was purchased from Boster (Wuhan, Hubei, China).

Preparation of KTC

To prepare KTC, 300 g Huangqin (Radix Scutellariae Baicalensis) was poured into boiling water for 1.5 h and the filtrate was collected. This step was repeated once. Then the filtrate was merged. Next, 600 g Dihuang (Radix Rehmanniae), 300 g Huangqin (Radix Scutellariae Baicalensis) and 300 g Baishao (Radix Paeoniae Alba) were soaked overnight with water. Then, the mixes were decocted 2 times for 1.5 h. The filtrate was collected each time. Filtrate from the mixture and Huangqin (Radix Scutellariae Baicalensis) were mixed and concentrated to form a clear cream with a relative density of 1.10 at 70 °C. The powder was generated from the clear cream using a spray-drying process. Then, 100 g Fuling (Poria) and 100 g gelatin were mixed and made them powder-like. These were mixed and packed into 1000 capsules. The dosage is 0.5 g per capsule.

Animals

Overall, sixty female Sprague-Dawley rats weighing 180–200 g and thirty-six immature New Zealand white rabbits weighing 700–900 g were purchased from Sino-British Experiment Animals (Shanghai, China). The animals were treated in accordance with protocols approved by the Laboratory Animal Ethics Committee at the Shanghai Institute of Planned Parenthood Research (Approval No. 2015-05). Five rats were housed per cage and one rabbit was housed per cage under a 12-h/12-h light/dark cycle with free access to food and tap water. All animals were weighed once per week.

Modified Allen-Doisy bioassay

Sixty rats were randomly assigned to two groups, ten rats were used as the sham-operated control and fifty rats were bilateral ovariectomized. All rats in the ovariectomized group were subjected to removal of bilateral ovaries under sterile conditions. Rats were anesthetized with 3% pentobarbital sodium and a small incision was made in the middle of the abdomen. Ligation was performed on the bilateral uterine horn and ovaries were removed. The incision was sutured with a 4-0 medical suture line. Sham-operated rats were subjected to removal of a segment of fat around the ovaries. One week post-recovery from surgical damage, the castrated rats were randomly assigned to five subgroups according to their weight (n = 10) and administered as follows: sham group: 0.5% sodium carboxymethylcellulose (CMC-Na); control group: ovariectomy + 0.5% CMC-Na; estradiol valerate group: ovariectomy + estradiol valerate; KTC (L) group: ovariectomy + 0.24 g · kg⁻¹ · d⁻¹ KTC; KTC (M) group: ovariectomy + 0.6 g · kg⁻¹ · d⁻¹ KTC; and KTC (H) group: ovariectomy + 1.5 g · kg⁻¹ · d⁻¹ KTC.
Animals were administered drugs by oral gavage once daily for 28 consecutive days accompanied with a vaginal smear. A drop of vaginal washing was placed on a glass slide and examined by light microscopy to detect the presence or absence of cornified epithelial cells. Twenty-four hours after the final treatment, an abdominal incision was made and the uterus was carefully excised and weighed. The uterine organ coefficient was calculated using the formula: uterine organ coefficient = (uteri weight/body weight) × 100. One side of the rat’s uterus was immediately fixed with 4% paraformaldehyde solution and the other side of the uterus was stored at −80 °C. The serum drawn from the abdominal aortic blood was stored at −20 °C until further detection.

**Hematoxylin-eosin (HE) staining**

The uterus tissues embedded in paraffin were sliced into 4-μm-thick sections and stained with HE for histological examination under a light microscope. Uterine morphological changes, uterus cavity area and ectopic endometrial thickness were observed under an optical microscope (Leica DM3000, Leica Microsystems Inc., Heidelberg, Germany).

**Measurement of E₂, P⁴, FSH and LH**

After KTC treatment, serum levels of E₂, P⁴, FSH and LH were detected by ELISA according to the manufacturer’s instructions to monitor the KTC treatment effects. The absorbance was detected using a Biotek Synergy 2 multi-mode microplate reader (Biotek Instruments, Inc., Vermont, USA) at wavelength of 450 nm.

**Western blot analysis**

Total protein was extracted from uterus tissues as previously described. The protein concentration was determined using the bicinchoninic acid (BCA) assay following the manufacturer’s instructions. Eighty micrograms of protein from each sample loaded onto a gel containing acrylamide:bisacrylamide (29:1) for 12% and 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The primary antibody dilutions included VEGF (diluted in 1:1500), Ki-67 (1:1500), PR (1:2000), ERα (1:400), ERβ (1:400) and PCNA (1:400), which were separately incubated with the membrane overnight at 4 °C. The relative protein levels were semi-quantitatively determined by the band intensity of the target protein against that of β-actin using Image lab software (version 4.0, Bio-Rad Laboratories Inc., California, USA). Five uteri tissues were measured in each group.

**Endometrial transformation**

The assay was performed as described by DeManno. Immature New Zealand white rabbits were primed with 5 μg estradiol benzoate subcutaneously per animal per day on study days 1-6. Rabbits were subsequently matched for body weight and randomly assigned to six groups (n = 6) and treated as follows: sham group: vehicle; control group: estradiol benzoate + vehicle; progesterone group: estradiol benzoate + progesterone; KTC (L): estradiol benzoate + 0.16 mg·kg⁻¹·d⁻¹ Kuntai capsule; KTC (M): estradiol benzoate + 0.4 mg·kg⁻¹·d⁻¹ Kuntai capsule; and KTC (H): estradiol benzoate + 1.0 mg·kg⁻¹·d⁻¹ Kuntai capsule. Progesterone was administered subcutaneously and different doses of KTC were administered intragastrically for 7 d in the 7 to 13 d. Twenty-four hours after the final administration, the rabbit was euthanized and the uterus was rapidly excised and fixed in Bouin’s solution, embedded in paraffin and sliced into 4-μm-thick sections for HE staining to examine morphological and histological changes under a light microscope.

**Statistical analysis**

All data were analyzed with SPSS 17.0 software (SPSS Inc., Released 2008. SPSS Statistics for Windows, Version 17.0, Chicago, IL, USA). Comparisons of variables of interest between groups were determined by one-way analysis of variance and followed by least squares difference tests or Dunnett’s T3 tests. Data are presented as the mean ± standard deviation (x ± s). A level of P < 0.05 was considered statistically significant.

## RESULTS

**Uterine coefficient and estrous cycle**

Changes of the uterine organ coefficient in O VX-rats were analyzed after KTC treatment. As shown in Table 1, the uterine organ coefficient was significantly decreased in the OVX control group (P < 0.05) compared with the sham group. Compared with the control group, the uterine organ coefficient in the estradiol valerate group increased, although it was not statistically significant (P > 0.05). Moreover, the uterine coefficient was significantly decreased in the KTC group in a dose-dependent manner (P < 0.05).

**Estrous cycle**

Vaginal smears were taken to assess the estrous cycle of rats. The results showed that the estrous cycle in the sham group was normal (data not shown). After ovariectomy, the dioestrus was prolonged without a estrum in the control group and KTC 0.24, 0.6, and 1.5 g/kg groups. Rats in the estradiol valerate group had many keratinized epithelial cells in the last 2-week administration period. It means that rats are at the phase of estrus.

**Endometrium thickness and uterine cavity area**

Compared with the sham group, the thickness of the endometrium in the control group was significantly decreased (P < 0.05). Compared with the control group, there were no changes in the endometrium of the estradiol valerate and KTC 0.24 g/kg groups. The thickness was significantly increased in the KTC group in a dose-dependent manner (P < 0.05).
of the endometrium in the KTC 0.6 and 1.5 g/kg groups was significantly reduced (P < 0.05) compared to that of control group. Compared with the estradiol valerate group, the thickness of the endometrium in the KTC 0.6 and 1.5 g/kg groups was significantly decreased (P < 0.05).

As expected, the endometrium thickness was significantly decreased after ovariectomy compared with the sham group (Figure 2A, P < 0.05). Treatment with estradiol valerate and low-dose KTC had no effect on endometrium thickness, which was decreased in the KTC 0.6 and 1.5 g/kg groups when compared with the control group (P < 0.05). The endometrium was less thick when OVX-rats were intragastrically fed with middle- and high-dose KTC compared with the estradiol valerate rats (Figure 2B, P < 0.05).

The uterine cavity area of OVX-rats was smaller than in the sham-rats (Figures 1 and 2A, P < 0.05). However, the cavity area increased in the estradiol valerate-treated group (Figure 1), although this was not statistically significant compared with the control group (Figure 2A). In contrast, the uterine cavity area was reduced in a dose-dependent manner after KTC treatment (Figures 1 and 2A, P < 0.05).

**Table 1** Effect of KTC on uterine organ coefficient in ovariectomized rats (± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg)</th>
<th>Number</th>
<th>Uterine organ coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>-</td>
<td>10</td>
<td>0.152±0.028 a</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>10</td>
<td>0.04±0.013 a</td>
</tr>
<tr>
<td>Estradiol valerate</td>
<td>0.0001</td>
<td>10</td>
<td>0.066±0.015 a</td>
</tr>
<tr>
<td>KTC(L)</td>
<td>0.24</td>
<td>10</td>
<td>0.03±0.009 a</td>
</tr>
<tr>
<td>KTC(M)</td>
<td>0.6</td>
<td>10</td>
<td>0.03±0.009 a</td>
</tr>
<tr>
<td>KTC(H)</td>
<td>1.5</td>
<td>10</td>
<td>0.02±0.004 a</td>
</tr>
</tbody>
</table>

Notes: sham group: 0.5% CMC-Na; control group: ovariectomy + 0.5% CMC-Na; estradiol valerate group: ovariectomy + estradiol valerate; KTC (L) group: ovariectomy + 0.24 g·kg⁻¹·d⁻¹ KTC; KTC (M) group: ovariectomy + 0.6 g·kg⁻¹·d⁻¹ KTC; KTC (H) group: Ovariectomy + 1.5 g·kg⁻¹·d⁻¹ KTC. *P < 0.05, compared with the sham group; "P < 0.05, compared with the control group; "P < 0.05, compared with the estradiol valerate group.

**Figure 1** Morphological changes in the uterus after KTC treatment for 28 d (HE, × 20)

A: sham group (0.5% CMC-Na); B: control group (ovariectomy + 0.5% CMC-Na); C: estradiol valerate group (ovariectomy + estradiol valerate); D: KTC (L) group (ovariectomy + 0.24 g·kg⁻¹·d⁻¹ KTC); E: KTC (M) group (ovariectomy + 0.6 g·kg⁻¹·d⁻¹ KTC); F: KTC (H) group (ovariectomy + 1.5 g·kg⁻¹·d⁻¹ KTC); HE: hematoxylin-eosin; CMC-Na: carboxymethylcellulose. The figure shows the gross appearance and histological changes of endometrial tissues and uterine cavity area after KTC treatment.

**Serum levels of E₂, P₄, FSH and LH**

As shown in Figure 3A, serum E₂ in the ovariectomized rats receiving 0.5% CMC-Na was decreased compared with the sham group (P < 0.05), while E₂ was significantly increased after OVX-rats were fed estradiol valerate (P < 0.05). The serum levels of E₂ in ovariectomized rats treated with KTC 0.24, 0.6, or 1.5 g/kg were decreased compared with the control group, but there was no significant variation (P > 0.05).

P₄ levels in the plasma of control group were significantly suppressed after ovariectomy compared with the sham group (P < 0.05). Estradiol valerate treatment had no influence on P₄ production (Figure 3B). In contrast, the serum levels of P₄ in the KTC 0.6 g/kg group increased compared with the control group (P < 0.05), while no changes were observed in either the middle- or high-dose groups (Figure 3B).

Serum levels of FSH and LH were also detected. Compared with the control group, there was no difference in serum FSH levels in either the estradiol valerate or KTC groups (Figure 3C). The serum levels of LH were all below the detection limit (< 0.1 mIU/mL).

![Image of morphological changes in the uterus after KTC treatment for 28 d (HE, × 20)](image-url)
protein level of PRB in the uterus ($P < 0.05$). KTC ($P < 1.0$) g/kg significantly reduced ($P < 0.05$). Treatment with estradiol valerate upregulated ($P > 0.05$). KTC (M) group; ovariectomy + 0.6 g·kg$^{-1}$·d$^{-1}$ KTC; KTC (H) group; ovariectomy + 1.5 g·kg$^{-1}$·d$^{-1}$ KTC. KTC: Kuntai capsule; CMC-Na: carboxymethylcellulose. $P < 0.05$, compared with the control group; $^{a,b}$, compared with the estradiol valerate group; $P < 0.05$, compared with the sham group.

**Protein levels of ERα, ERβ, progesterone receptor A (PRA) and PRB**

The protein levels of ERα were lower in the control group compared with the sham group, but there was not statistically significant ($P > 0.05$). Treatment with estradiol valerate induced the upregulation of ERα compared with the control group ($P < 0.05$). There was no significant effect on ERα expression in the KTC 0.24, 0.6, and 1.5 g/kg groups when compared with the control group. Ovariectomy slightly decreased the protein level of ERβ compared with the sham group, but no statistically significant was observed. Treatment with KTC 0.24, 0.6, or 1.5 g/kg markedly upregulated ERβ ($P < 0.05$) and the maximum increase was observed in the KTC 0.6 g/kg group. Ovariectomy had no effect on PRA or PRB protein levels in the uterus compared with the sham group ($P > 0.05$). Treatment with estradiol valerate upregulated PRA levels and KTC 1.5 g/kg significantly reduced PRA levels compared with the control group ($P < 0.05$). KTC 0.6 g/kg group significantly increased the protein level of PRB in the uterus ($P < 0.05$).

**Protein levels of VEGF, PCNA and Ki-67**

Figure 5 shows the protein expressions of VEGF, PCNA, and Ki-67 in uterus from each group. VEGF expression decreased compared with the sham group (Figure 5A and B, $P < 0.05$). Compared with the control group, treatment with estradiol valerate, KTC 0.24 or 0.6 g/kg increased VEGF protein levels (Figure 5A and B, $P < 0.05$).

The protein level of Ki-67 in the control group was significantly decreased after ovariectomy (Figure 5A and C, $P < 0.05$) compared with the control group. Treatment with estradiol valerate or KTC 1.5 g/kg significantly reduced the protein level of Ki-67 compared with the control group in the tissues investigated (Fig-
and did not contact internal mesenchymal cells. In addition, the number of glands increased in the KTC 0.24 and 0.6 g/kg groups compared with the control group and the McPhail scores of the KTC 0.24 and 0.6 g/kg group were 1.17 and 1.67, respectively (P < 0.05). However, there was no significant variation in glands in the KTC 1.5 g/kg group, which had a McPhail score of 0.33.

**DISCUSSION**

This study investigated the hormone-like activities of KTC on the uterus in ovariectomized rats and estradi-
ol-primed immature rabbits. There are two parts to the modified Allen-Doisy bioassay: the wet weight of the uterus and vaginal epithelial cell cornification. It was previously reported that the effect of oral estradiol increased the uterus weight and vaginal epithelial cell cornification after 7 d.1 The effect of estradiol valerate on ovariectomized rats in our study was consistent with the previous study. We found that KTC inhibited the increase of uterus weight in a dose-dependent manner while there was an opposite effect compared with estradiol valerate treatment. In addition, there was no keratinosis in the vagina epithelia of SD rats after treatment with different doses of KTC (data not shown).

Other studies showed that estrogen increased the uterine epithelial cell thickness.12,15 Kang et al15 showed evidence that 17-ethinyl estradiol at doses of 3.0 and 10.0 mg·kg⁻¹·d⁻¹ increased uterine thickness. In our study, the uterine epithelial thickness showed no variation because of the smaller dose of estradiol valerate used. Compared with the control group, the endometrial thickness was decreased in the KTC 0.6 and 1.5 g/kg rats. Further studies demonstrating KTC has no obvious estrogenic activity on rats are required. We also observed no weight loss or abnormal activities in rats, which indicated that KTC has no apparent toxicity after its administration. These two indexes indicate that KTC has no estrogenic activity.

Serum E₂, P₄, FSH and LH levels were also measured. Compared with the control group, the level of serum E₂ was markedly decreased in the estradiol valerate group, consistent with a study by Wei et al.14 There were no differences in serum E₂ levels between the control group and KTC group, suggesting KTC may not have mediate an estrogen-like effect. The level of serum P₄ in the KTC 0.6 g/kg group was higher than in the control group. As a result, we consider that KTC is conductive to increase of progesterone. In addition, there were no changes in the serum FSH and LH levels, indicating KTC had no influence on hypophysis. Therefore, serum gonadotropin-releasing hormone released by hypothalamus was not detected in this study. Paruthiyil et al17 found that ERα and ERβ produced opposite effects where ERα enhanced the proliferation of breast cancer in response to estrogens while ERβ inhibited this effect. Moreover, ERβ had an anti-proliferative role by modulating the interactions between ERα and ERβ in the immature uterus.12 Similarly, we found that the expression of ERβ, but not ERα, was highly
induced in the uterus after KTC treatment, suggesting KTC might bind to ERβ with a higher selectivity. PR has several isoforms including PRA and PRB. In general, PRB has a greater ability for transactivation compared with PRA, which dominantly inhibits PRB expression.\(^1\)\(^–\)\(^2\) In our study, the protein levels of PRA and PRB in the uterus were detected by western blot assay. Our results demonstrated that KTC downregulated the expression of PRA and that 0.6 g/kg KTC upregulated the protein levels of PRB.

We also analyzed the expression of PCNA, VEGF and Ki-67 in the uterus of OVX-rats treated with KTC by western blot. Ki-67, an easily assessable and reproducible proliferation marker, was differently expressed at each phase of the cell cycle (G\(_1\), S, G\(_2\), and M).\(^1\)\(^9\) The level of Ki-67 in the uterus was significantly increased in the estradiol valerate-treated OVX-rats compared with the vehicle-treated OVX-rats, consistent with a previous study,\(^1\)\(^9\) suggesting KTC does not activate the Ki-67 gene. Previously, it was shown that VEGF synthesis was induced when exposed to estrogens.\(^1\)\(^1\)\(^–\)\(^1\)\(^2\) In our study, we observed similar results in the estradiol-treated group. The level of VEGF was increased after treatment with KTC 0.24 or 0.6 g/kg whereas KTC 1.5 g/kg decreased the levels compared with the control group. These results suggest that different doses of KTC might have different effects on VEGF.

PCNA, a nuclear protein expressed in proliferating cells during the S phase, is accepted as a potential prognostic marker for cervical and endometrial cancer.\(^1\)\(^3\)\(^–\)\(^2\)\(^4\) In our study, there was no difference in the level of PCNA between the KTC and control groups, suggesting KTC does not activate the PCNA gene. These expressions of proteins indicated that KTC stimulates the growth of the uterus in ovarioectomized rats.

P\(_4\) and synthetic progestins stimulate both proliferation and differentiation of the rabbit uterine epithelium in immature rabbits.\(^3\) The McPhail index reflects the differentiation extension. Our study showed that the uterine gland numbers were increased and glandular cavity was extended after treatment with KTC 0.24 or 0.6 g/kg. However, KTC 1.5 g/kg had no effect on rabbits after its administration. This result implies that a different dose of drug might induce different biological activity.

In conclusion, KTC had no obvious estrogenicity on uteri in ovarioectomized rats. However, KTC promoted uterine differentiation in immature rabbits, and demonstrating weak progestogen activity.

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