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Original Article

Effect of Aqueous Extract of *Physalis Alkekengi* Fruits on the Activity of Ovarian 3beta- and 20alpha-Hydroxysteroid Dehydrogenases in Late Pregnancy in Rat

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Abstract

Background: Aqueous extract of winter cherry (*Physalis alkekengi* L; family of Solanaceae) fruits (WCF) has long been recommended for fertility control by herbalists in Iran. The effect of this extract on lowering serum progesterone levels has been reported previously.

Objective: To study the effects of WCF extract on the activities of ovarian 3β-hydroxysteroid dehydrogenase (3β-HSD) responsible for the synthesis of progesterone and 20α-HSD responsible for its degradation in rats.

Methods: One ml aliquots of the aqueous extract of WCF (containing 400 mg of dried extract) were intraperitoneally injected for 8 consecutive days to rats from day 8 of pregnancy. Rats were then sacrificed 21 days and 11 hours after the day of observing sperm positive vaginal smears. Blood was collected for the determination of serum progesterone and the total and the live numbers of embryos were counted. Ovaries were also used for the measurement of the activities of 3β- and 20α-HSD.

Results: The extract of WCF decreased the ovarian 3β-HSD specific activity by 47%, serum progesterone concentration by 30% and the number of live embryos by 67%, but it had no effect on the specific activity of ovarian 20α-HSD.

Conclusion: The aqueous extract of WCF, containing steroidal compounds with known estrogen antagonistic properties, probably interferes with the function of estradiol in inducing ovarian 3β-HSD synthesis. It may also contain components which inhibit this enzyme, thus reducing progesterone synthesis that is required for maintaining pregnancy. Such natural compounds, if purified, might be beneficial for control of fertility.
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Keywords ● Physalis alkekengi ● Hydroxysteroid dehydrogenases ● Progesterone ● Fertility ● Rat

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Introduction

One of the ideal targets for fertility control is reducing the production of progesterone by enzyme inhibition. Trilostane, a 3β-hydroxysteroid dehydrogenase (3β-HSD)

inhibitor, which is a synthetic steroid compound has used successfully to reduce progesterone production as a pretreatment prior to prostaglandin therapy in mid-trimester termination of pregnancy in women.[1]

20α-hydroxysteroid dehydrogenase (20α-HSD), an enzyme responsible for progesterone inactivation, is undetectable in luteal tissue of normal pregnant rats between day-12 and day-18 of pregnancy. Hypophysectomy and hysterectomy results in its slow appearance on day 12. Treatment of these animals with estradiol delays this increase in 20α-HSD until day-17, at which time a rapid induction of this enzyme occurs. The activity of 3β-HSD, the enzyme which synthesizes progesterone from pregnenolone in luteal tissue of pregnant rats, increases between the days 2 and 18 of pregnancy and is under the control of estradiol.[2] Serum progesterone and estradiol concentration in normal pregnant rats also increases during this period.[3] The antiovulatory effects of onapristone, another synthetic steroid derivative and an antiprogestin, is also related to the down regulation of intra-ovarian progesterone by reducing the activity of 3β-HSD and decreasing progesterone receptor expression.[4]

Using subcutaneous injections of RU486 (mifepristone, Company), a synthetic steroid derivative and a progesterone antagonist, to pregnant rats on day-18 of pregnancy, a significant decrease in the concentration of serum progesterone and luteal activity of 3β-HSD and a significant increase in 20 α-HSD activity was observed on day 21 of pregnancy. In addition, RU486 also induced preterm delivery in pregnant rats.[5]

The aqueous extract of winter cherry (P*hysalis alkekengi* L.; family Solanaceae) fruits (WCF) is used for fertility control in some rural areas of Iran. In rat, it is shown that WCF has no effect on ovum implantation, but it significantly reduces the number of pups born.[6] Both aqueous and the ethanol extracts of WCF contain steroidal compounds.[7,8] The structure of few cyclosteroid constituents (physalins) in both organic and aqueous extracts of this fruit has been elucidated.[9,10] The estrogen antagonistic properties of this extract on lowering the activities of several estrogen inducible enzymes in the uterus,[6,11] liver,[7] the pituitary and hypothalamus,[12-13] were also previously reported.

In this study the influence of aqueous extract of P*hysalis alkekengi* fruits, intraperitoneally injected (I.P) to the rat during the second week of pregnancy (day-8 to day-15), on the level of serum progesterone, the activity of ovarian 3β-HSD and 20α-HSD and the number of live embryos of late pregnant rats are investigated.

Materials and methods

Preparation of winter cherry fruit extract

Winter cherry fruits were authenticated and extracted with water as described previously.[7] 200 ml of distilled water was added to 30 g of dried ground powder of winter cherry fruits and the suspension was brewed over boiling water for 8 hrs. The suspension was then centrifuged at 27000 x g for 15 min. The supernatant was dried in a flush evaporator at 45^{o}C and then dissolved in 20 ml of distilled water. This solution which contained 400 mg of the water extract (equivalent of 1.5 g of dried fruit powder) per ml was used as “aqueous extract” through the experiment.

Experiments

Adult female Sprague-Dawley rats (180-200 g), obtained from Shiraz University of Medical Sciences Animal House, were kept under standard conditions of room temperature and 12 h (06.00-18.00) light/dark cycle.[6] Animals were housed individually in plastic cages (Iran) and had access to food (rat chow; Pars Dam, Tehran, Iran) and water *ad libitum*.

The stages of the estrus cycle were defined through vaginal smears prepared and examined between 09:00-11:00 daily. Rats showing a minimum of three consecutive 4-day cycles were included in the study. Each female rat was housed with a 4-month-old adult male rat on the day of proestrus. The first day of sperm positive vaginal smears was considered as day zero of pregnancy. From day-8 of pregnancy, the experimental animals (n=5) received an intraperitoneal (ip) injection of 1 ml of the aqueous extract of WCF (400 mg/ml equal to 2 g of dried extract/kg) every day at 09:00 for 8 consecutive days. The control group (n=5) received daily ip injection of one ml normal saline at the same time and for the same period as of experimental group.

In a preliminary experiment, it was noticed that the activity of 20α-HSD in ovaries of the pregnant rats increased gradually from day-21 of pregnancy and reached to its maximum level after 21 days and 11 hrs from the day of sperm positive vaginal smears. Therefore, pregnant rats were decapitated at the same time as above and blood samples were collected from the jugular vein for the determination of serum progesterone. T he ovaries were removed for the determination of 20 α-HSD and 3β-HSD activities and the total number of embryos, as well as live embryos, was counted.

Preparation of ovarian extracts

Ovaries were freed of fat, weighed and homogenized with 0.7 ml of 0.1 M Tris-HCl buffer containing 1 mM sodium-EDTA (pH 8.0) at 4ºC in a glass-glass homogenizer. The suspension formed was centrifuged (Beckman Optima TLX ultracentrifuge) at 105,000 X g for one hr at 4ºC. The clear supernatant solution was immediately used for the measurement of 20α-HSD activity. The precipitate was resuspended in 0.7 ml of 0.25 M sucrose and re-homogenized at 4ºC.[14] The suspension was centrifuged at 800 x g for 5 min, at 4ºC, and the supernatant stored at -70ºC for the measurement of 3β-HSD activity.

Determination of ovarian 20α-HSD activity

Determination of 20α-HSD activity was based on the procedure of Bunce et al.[15] The incubation mixture for assaying this enzyme, in both test and control tubes, contained the following ingredients: 60 µmol Tris-HC1 buffer (pH 8.0); 100 µl of freshly extracted ovarian supernatant; and 0.625 µmol $NADP^+$ (nicotinamide adenine dinucleotide phosphate; in 100 µl Tris buffer) in a final volume of 0.85 ml. The tubes were incubated for 3 min at 37ºC. Then 50 µl of 95% ethanol was added to the control tubes and the spectrophotometer was set to zero absorbance at 340 nm using these tubes. To initiate the reaction in the test tubes, 0.08 µmol of 20α-hydroxy progesterone in 50 µl 95% ethanol was added and the increase of absorbance at 340 nm was followed for 6 min, where the reaction was found to be linear with time. Enzyme activity measurements were performed in duplicate. One milliunit (mU) of the enzyme corresponds to the amount of the enzyme producing one nmol of NADPH/min under assay conditions. Protein concentration in the 105,000 x g supernatant was measured by the procedure of Bradford,[16] using bovine serum albumin as a standard. Enzyme specific activities were expressed in mU/mg protein.

Determination of ovarian 3β-HSD activity

The activity of ovarian 3β-HSD was measured on the 800 x g supernatant using the procedures of Kawano et al.[14] In brief, the incubation mixture for the assay of this enzyme in both test and control tubes contained the following ingredients in a final volume of 0.8 ml: 40 µmol glycine-NaOH buffer (pH 9.4); 0.9 mg bovine serum albumin; 0.5 µmol Na2-NAD (nicotinamide adenine dinucleotide); and 100µl of the 800 x g ovarian supernatant. The tubes were pre-incubated at 37ºC for 3 min. After adding 50 µl of 95% ethanol to control tubes, the spectrophotometer was set to zero absorbance at 340 nm. To initiate the enzymatic reaction in the test tubes, 50 µl of a solution containing 0.1 mg pregnenolone (Sigma Chemical Company, USA) in 95% ethanol was added and the increase in absorbance at 340 nm was recorded for ten min.

The reaction was linear with time for approximately 2 min. Enzyme activity measurements on each pair of ovaries were performed in duplicate. Based on the linear part of the activity vs. time curve, enzyme activity/ml of the ovarian extract was calculated .One milliunit (mU) of the enzyme corresponds to the amount of the enzyme producing 1 nmol of NADH/min under assay conditions. Protein was measured and enzyme specific activities were expressed in mU/mg protein. Serum progesterone was measured using the radioimmunoassay kit (Orion Diagnostica, Espoo, Finland).

Statistical analyses

Data are presented as Mean±SEM. Paired Student's t-test was used to determine the differences between the mean values of both groups and $p<0.05$ was considered statistically significant.

Results

Administration of WCF from day-8 to day-15 of pregnancy decreased serum concentration of progesterone by 30%, the number of live embryos by 67% and the specific activity of ovarian 3β-HSD by 47% in late pregnancy (Table 1). However, it did not have any effect on the specific activity of ovarian 20α-HSD and the total number of embryos.

Discussion

It has been shown that increases in the concentration of progesterone in sera of normal

Table 1: Effects of aqueous extract of WCF on the concentration of serum progesterone (PRG; ng/ml), its metabolizing enzymes (20α-HSD and 3β-HSD; mU/mg protein) and the number of total (TOTAL) and live (LIVE) embryos of pregnant rats.

Groups (n=5)	PRG	20α-HSD	3β-HSD	Total	Live
Control	50.0 ± 4.4	11.3±1.5	51.5 ± 5.7	12.0 ± 0.4	12.0 ± 0.4
Experiment	34.6 ± 6.3*	11.1±1.5	27.3 ± 4.0*	10.6 ± 0.8	4.0 ± 0.6**

Values are significantly different from control at *= $p<0.05$ or **=$p<0.01$.

pregnant rats are evident between day-1 and 4 and also between day-10 and -15 of pregnancy.[3] The increase between day-10 and -15 was more pronounced and reached to 160 ng/ml. A similar increase in serum estradiol concentration of pregnant rats is reported by Bridges between day-10 and -18 of pregnancy that reached to the level of 80 pg/ml on day-18.[3] A high level of these hormones together with an appropriate ratio of the two is essential at this period for maintaining pregnancy. We had previously noted that the ip injections of the aqueous extract of WCF had no effect on the number of implantation sites, but significantly reduced the number of pups born to these animals.[6] In the present investigation we found that ip injection of this extract to pregnant rats, before and during the time of progesterone and estradiol surge, significantly decreased the activity of ovarian 3β-HSD and the concentration of serum progesterone, which concomitantly reduced of the number of live embryos in the last stages of pregnancy (Table 1). It is shown that the aqueous extract of WCF contains steroidal compounds with estrogen antagonistic properties.[6,7,10-13] These compounds may lower the activity of ovarian 3β-HSD, whose activity is under the control of estradiol.[2] Therefore, the inhibition of ovarian 3β-HSD has lowered synthesis and or secretion of progesterone. Maintenance of pregnancy is very sensitive to the ratio of progesterone to estradiol,[3] and the level of plasma progesterone has decreased significantly (Table 1), hence reducing the number of live embryos and eventually leading to a significant reduction of the number of pups born.

Conclusion

Aqueous extract of *Physalis alkekengi* fruits, which contains cyclosteroids, probably acts as a 3β-HSD inhibitor similar to synthetic steroidal compounds such as trilostane,[1] onapristone,[4] and RU486,[5] in decreasing progesterone production. Further work on the isolation of such cyclosteroids and the study of the effects of the pure compounds on ovarian 3β-HSD is needed.

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