

Study the effect of addition alcoholic extract of *Tribulus terrestris* on bovine oocyte maturation in vitro (IVM)

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Summary

The present study included 98 bovine ovary collected from AL-Shulla slaughter house immediately after the animal was slaughtered. Ovaries were preserved in physiological saline at 37 C° and transport to the laboratory within 3-4 hrs. Diameters of the follicles were measured and divided into large (6-10 mm) and small (1-5 mm) follicles. Cumulus-oocyte complexes (COCs) were aspirated from large and small follicles using a 18 G needle connected to a 10 ml syringe COCs surrounded by compact and thick cumulus cell were cultured in an integrated culture media RPMI-1640 , than were divided in to two groups , the first group (control media) using integrated culture media only , the second group alcoholic extract of *Tribulus terrestris* (Tt) plant was added to the culture media RPMI-1640 once a concentration of 50µg/ml , and the other a concentration of 25µ/ml . The results of the current study demonstrated superiority of alcoholic extract of 50µg/ml in the percentage of oocytes maturation which optened or drawn from large and small follicles compared with a concentration of 25µg/ml of the alcoholic extract it was observable the effect of adding alcoholic extract of *T. t.* plant in two concentrations of 25µg/ml and or 50µg/ml on the percentage of mature oocytes compared to control media (culture media RPMI-1640) .The present results indicated that the oocytes retrieved from larger follicles is better than small follicles in maturing oocytes maturation. In conclusion, alcoholic concentration of 50µg/ml preference extracted first and then the concentration of 25µg/ml *T. t.* plant extract that was added to culture media RPMI-1640 , superiority in percentage of the mature oocytes compared to control culture media RPMI-1640 , also the large follicle were better than small follicles in terms of equality suitable for mature oocytes.

Keywords: *Tribulus terrestris* , oocyte , vitro maturation , RPMI-1640.

دراسة تأثير إضافة مستخلص نبات الكطب على إنضاج بيوض الأبقار مختبريا

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الخلاصة

شملت الدراسة الحالية 98 مبيضا ، جمعت من مجزرة الشعلة مباشرة بعد ذبح الحيوان ،حضت المبايض في المحلول أفسلجي بدرجة حرارة 37 م ونقلت إلى المختبر خلال 3-4 ساعات ، تم قياس قطر الجريبات المتواجدة على المبايض وقسمت إلى جريبات كبيرة الحجم (6-10) مليمتر وصفيره الحجم (1-5)مليمتر . سحبت البويضات المحاطة بالركمه المبيضة بطريقه الشفط من الجريبات الكبيرة والصغيرة بواسطة ابره قياس 18 كيج موصولة بسرنجة سعة 10ملمتر . البويضات الملائمة للإنضاج والمحاطة بطبقه ثخينه من خلايا الركمه المبيضة زرعت في الوسط أزرعي المتكامل RPMI- 1640 ، وقد قسمت إلى مجموعتين ، المجموعة الأولى السيطرة (control media) لم يضاف أي ماده للوسط أزرعي المتكامل ، إما المجموعة الثانية فقد أضيف للوسط أزرعي RPMI-1640 المتكامل المستخلص الكحولي لنبات الكطب بتركيز 50 مايكروغرام/مللتر وتركيز 25 مايكروغرام/ مللتر. بعد زرع البويضات أظهرت نتائج الدراسة تفوق المستخلص الكحولي لنبات الكطب بتركيز 50 مايكروغرام / مللتر في النسبة المئوية لإنضاج البويضات المسحوبة من الجريبات الكبيرة والصغيرة مقارنة بتركيز 25 مايكروغرام / مللتر لمستخلص نبات الكطب ، وسجل تأثير أضافه المستخلص الكحولي لنبات الكطب وبالتركيزين 50 ، 25 مايكروغرام / مللتر في النسبة المئوية لإنضاج البويضات مقارنة بالوسط أزرعي (السيطرة) . كما أوضحت نتائج الدراسة الحالية بان البويضات المسحوبة من الجريبات الكبيرة القطر أفضل من الجريبات الصغيرة القطر في الإنضاج والحيوية وتدني النسبة المئوية للبويضات غير الناضجة عند أضافه

المستخلص الكحولي. نستنتج من الدراسة الحالية أفضلية المستخلص الكحولي لنبات الكطب المضاف للوسط الزراعي RPMI-1640 في تفوق النسبة المئوية للإخصاج البويضات مقارنة بالوسط الزراعي (السيطرة)، كما إن الجريبات الكبيرة القطر أفضل من الجريبات أصفيره القطر من حيث نوعية البويضات الملائمة للإخصاج وحيويتها .
الكلمات المفتاحية : مستخلص نبات الكطب , انضاج البيوض , الابقار.

Introduction

The medicinal plants and herbs have been used for many years in the treatment of various diseases in animals and human beings (1). *Tribulus terrestris* (*T.t.*) is natural herb used for treating many diseases like hypertension. It is a member of the Zygophyllaceae family, and an annual herb found in many tropical and moderate areas of the world, including U.S.A and Mexico, the Mediterranean region, and throughout Asia, *T.t.* is also known as Puncture Vine, it contains steroidal saponins, and act as a natural testosterone enhancer(2). *Tribulus terrestris* increases testosterone through increasing luteinizing hormone (LH)(3). There is good confidence that *T.t.* is useful as a sexual enhancement herb(4). In vitro production (IVP) technology including in vitro maturation (IVM), in vitro fertilization (IVF) and embryo culture (IVC) is consider a key technology in the fields of animal reproduction and the biomedical field(5). Oocyte maturation is one of the most important stages for IVP of embryos in domestic animals (6). The aspiration technique, it may be possible to use slaughtered and surgical material as a routine source of viable ova for ova transfer in cattle (7). Slicing and puncture are alternative techniques of oocyte recovery (8). For the success of IVP, several barrier need to be overcome optimizing the culture medium to produce an in vitro environment similar to that of the oviduct and uterus is an important step toward achieving this goal (9). There were no study used the *T.t.* extract plant have been used as to improve in vitro maturation of bovine oocytes, so this study was conducted to detect the effect of herbal extract (*T.t.*) on in vitro bovine oocyte maturation and the effect of follicular sizes on bovine IVM.

Materials and Methods

Tribulus terrestris plant were collected from the gardens of Baghdad University in Al-jadiria during September, which a season of blooming plants and reap the fruits. The plant was identified by Dr. Ali Hussein Al -Muswi (Department of Biology, College Science University of Baghdad) as a *T.t.L* plant, which belongs to the family of Zygophyllaceae. Reaped the fruits of the plant were washed and left to dry in the air for two weeks with the temperature of the laboratory with continuous stirred. Dry fruit were converted into fine powder by an electric grinder. Keeping the powder at 4°C refrigerator until use.

Fifty grams of fruits *Tribulus terrestris* powder were extracted with 500 ml of 70% ethanol under continuous stirring for eight hours at room temperature, the suspension was filtered by Whatman no. 1 filter paper and the filtrate was concentrated using vacuum rotary evaporator (10) The crud extract was stored in dark sterile screw bottle at 4°C until use to prepare the required concentration.

The 98 ovaries of slaughtered cows were collected from the shulla slaughter house, washed with normal saline at a temperature of 38°C, then placed in a container contain 500ml of 0.9% saline solution at 37 -38°C and antibiotic 100 IU/ml penicillin and 100µg/ml of streptomycin . Ovaries were transferred to the laboratory of biotechnology research center, within 3-4 hours. In the Laboratory ovaries repeated washed again with 0.9% saline solution to oocytes withdrawal from the follicles (11).

The diameter of follicles were measure using a vernier and classified during the withdrawn of the oocytes to small and large sized 1-5 and 6-10 mm respectively. Two syringes of 10ml and 18 gauge needle each syringe contained 0.5ml of the culture media RPMI-1640. Were used to withdrawal the oocytes from the small follicles, and large follicles

by aspiration under sterile hood at 38C°. Then discharged the content of syringes in a private test tube with specific measurement of the follicles. Follicular fluid were examined for each test tube under inverted microscope by putting 1ml of content of the test tube in plating dish with a diameter of 10 mm for each test. After that, choose healthy oocytes and appropriate for the maturation which is characterized by being appraised fully compact cumulus cells (COCs) and homogenous cytoplasm while denuded oocytes (DOs) with heterogeneous cytoplasm rejected. Healthy oocytes transferred to the plating dish containing 100µl of culture media, repeated this process 4 times to wash the oocytes and get rid of the debris of the cumulus cell in the follicular fluid. The stage, which precedes incubation, oocytes will be transferred to the plating dish with diameter of 5mm containing 1ml of the culture media RPMI-1640, which will be covered with adequate quantity of mineral oil. In order to prevent temperature changes and the occurrence of cold shock and bacterial contamination. Then incubated plating dish in CO₂ incubator at 38C° and 5% CO₂ 100% humidity in order to prevent drying of the culture media. Incubating oocytes 24hrs for maturation.

Extracted the small- diameter plating dish of 5mm from the incubator to be placed directly under the inverted microscope where the oocytes stripped from cumulus oophorous by aspiration gently and rewind several times to dish using a micro – pipette. After that classified oocytes to mature and immature, then transferred each one of them to the special plating dish containing 100µl of culture media was to differentiate between the mature and immature oocyte according the existence of first polar body in the prevetlin space of mature oocytes(12).

Experimental oocytes were appropriate for laboratory maturation IVM healthy oocytes were divided in to two groups

1-First group (control media):- placed oocytes predisposing maturation in the culture media RPMI-1640 without adding any of the extracts of the *T.t.* plant. Where been confirmed of PH degree by the PH meter and the osmotic pressure through the two group of experiment. After 24hrs of incubation oocyte examination of searching for maturation. Also been microscopic examination of the oocytes, and searching for maturation after 24 hrs of incubation of the two groups of experiment.

2-Tow group (Alcoholic extracts of *T. t.* plant 25µg/ml, 50µg/ml)

Placed oocytes predisposing maturation in the culture media RPMI-1640 with the addition of alcoholic extract of the *T.t.* plant once a concentration 25µg/ml and the other, concentration 50µg/ml calculated doses of alcoholic extract of the plant as follows :-Weight of 1 gm of powder alcoholic extract of *T. t.* plant was dissolved in a container containing 100ml of distilled water and then be nominated by filter with a measurement of 0.45 and 0.22 to get rid of impurities and microbes and bacteria. Closed the container by parafilim-tap and saved in sterile surroundings at 4C°, this is called stock-solution. And with a simple calculation would be estimate the first and second concentration 25µg/ml and 50 µg/ml.

Results and Discussion

The 98 ovaries were divided in to two groups (table 1) the first group, 49 ovaries contain 10 large and 168 small follicle. This finding is consisted with finding and suggestive Diskin *et al.*(13) in lactating dairy and beef cows which negative energy balance or reduced dietary intake, while not affecting the population of small to medium size follicles, affects the size and ovulatory fate of the dominant follicle. While the increasing the number of large follicles 27 table (1) and from 116 follicles which increased were obtained from 20 ovary collected during the spring which, accompanied by improved nutrition and health status of the animals in general and ovarian activity in particular. Diskin 13 mentioned the effects of low levels of energy intake may also compromise the welfare axis, as well as also long-term chronic dietary restriction in cattle causes a gradual reduction in dominant follicle growth rate, maximum

diameter and persistence. More acute dietary restriction causes an immediate (within days) reduction in follicle growth rate and maximum diameter of dominant follicles.

Table 1- Effect of varicose concentration of alcoholic extract of *Tribulus terrestris* on oocytes maturation.

The total number of follicles	Small follicles 1-5 mm				Large follicles 6-10 mm				No. of ovaries	Group
	No. of immature Oocyte	No. of mature Oocyte	No. of oocytes with drawn	The number	No. of immature Oocyte	No. of mature Oocyte	No. of oocytes with drawn	The number		
178	87	37	124	168	5	3	8	10	49	Control media
79	36	18	54	67	6	4	10	12	29	Alcoholic extract 25 µg/ml
116	47	28	75	89	13	10	23	27	20	Alcoholic extract 50 µg/ml

There was a significant increase in the percentage of mature oocytes for large follicles ($p \leq 0.01$) in concentrate of 50µg/ml of alcoholic extract of *T. t.* plant in comparison with the control media (Table 2) .While in concentrate of 25µg/ml there were no significant different in the percentage of mature oocytes in compare between 25µg/ml and the control group. Also tables demonstrates that the percentage of immature oocytes were significantly ($p \leq 0.01$) lower the alcoholic extract 50µg/ml compared with the control media. The present study could be considered as advance research focusing on the benefits of this herb in laboratory maturation of oocytes (IVM). Mustafa and Kulplulu, (14) suggested a higher protein synthesis in oocytes aspiration frame larger follicle (>7mm) were low at small follicle (3-6mm). Follicles >6 mm in diameter yielded significantly more oocytes with many layers of granulosa cells and a higher proportion of in vitro produced blastocysts, while Lonergan, *et al.*, (15) suggesting that larger follicles may contain growth factor enhancing morphological and functional status of the COCs and embryo yields.

Table 3 illustrated that alcoholic extracts of *Tribulus terrestris* in concentration of 50µg/ml were exceeding significantly on oocytes maturity in small follicles (1-5mm) comparative with control media. While concentration of 25µg/ml was insignificant differed in comparison with control media. In addition, the total immature oocytes were significantly lower in both alcoholic extracts 50µg/ml and 25µg/ml in comparison with control media.

Table 2: The effect of alcoholic extract of *tribulus terrestris* on the maturation percentage of large follicles (6-10mm). (M±S.D)

Immature oocytes	Mature oocytes	Parameter
62.5±2.88a	37.5±2.9b	Control media
60± 2.39a	40± 2.31ab	
56.52 ±0.65b	43.48 ± 0.58a	Alcoholic extract 50 µg/ml

Values are means ± S.D.

Different letters (a , b) means significant difference ($P \leq 0.01$) compared between columns groups .

Table 3 the effect of alcoholic extract of *tribulus terrestris* on the maturation percentage of small follicles (1-5mm).(M±S.D)

Immature oocytes	Mature oocytes	Para meter
70.16±1.04 ^a	29.84 ±1.02 ^b	Control media
66.7±1.73 ^a	33.30±1.87 ^{ab}	Alcoholic extract 25 µg/ml
62.67± 1.55 ^b	37.33± 1054 ^a	Alcoholic extract 50 µg/ml

Values are means ± S.D.

Different letters (a , b) means significant difference (P≤ 001) compared between columns groups .

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