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# Effects of Fenugreek Seed Extract on *in vitro* Maturation and Subsequent Development of Sheep Oocytes

**Ibrahim A.H. Barakat[1,2] and Ahmed R. Al-Himaidi[1]**
[1] *Zoology Department, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Kingdom of Saudi Arabia.*
[2]*Cell Biology Department, National Research Center, Dokki, Giza, Egypt.*

**Abstract.-** The present study was conducted to determine the role and optimum concentration of fenugreek seed extract during *in-vitro* maturation on maturation rate and developmental competence of Neaimi sheep oocytes following *in-vitro* fertilization. The Cumulus oocyte complexes (COCs) collected from sheep slaughterhouse ovaries were randomly divided into three groups, and they were matured for 24 hrs in maturation medium containing fenugreek seed extract (0, 1 and 10 μg $ml^{-1}$). Oocytes of a control group were matured in a medium containing 1 μg $ml^{-1}$ estradiol 17β. After maturation, half of oocytes were fixed and stained for evaluation of nuclear maturation. The rest of oocytes were fertilized *in vitro* with fresh semen, then cultured for 9 days for the assessment of the developmental capacity of the oocytes. The results showed that the mean values of oocytes with expanded cumulus cells percentage were not significantly different among all groups ($P<0.05$), but nuclear maturation rate of oocytes matured with 10 μg $ml^{-1}$ fenugreek seed extract was significantly higher than that of the control group. The maturation rate and development to morula and blastocyst stage for oocytes matured at 10 μg $ml^{-1}$ fenugreek seed extract was significantly higher than those matured at 1μg $ml^{-1}$ of fenugreek seed extract and the control group. In conclusion, better maturation and developmental capacity rate to morula and blastocyst stage were obtained by the addition of 10 μg $ml^{-1}$ fenugreek seed extract to maturation medium than addition of 1 μg $ml^{-1}$ estradiol-17β ($P<0.05$).

**Keywords:** Fenugreek seed extract, *in vitro* maturation, sheep oocytes, *in vitro* fertilization, embryo development.

## INTRODUCTION

Sheep are an important livestock that acts as a source of wool, meat and milk to millions around the globe. Sheep are usually seasonal breeders and they do not yield sufficient lambs to meet demand. The *in vitro* embryo production system includes three major steps, namely *in vitro* maturation of the primary oocytes, *in vitro* fertilization of the matured oocytes and *in vitro* culture of presumptive embryos, until transferred or cryopreserved for future use (Cognie *et al.*, 2003; Gandolfi *et al.*, 2005; Zhu *et al.*, 2007). Hence assisted reproduction technologies have been developed over the past few decades to produce high-yielding lambs in large numbers. As with other technologies, *in vitro* embryo production technologies have their share of problems and failures (Camargo *et al.*, 2006) and therefore need to be optimized to produce healthy and viable lamb.

Medicinal plants have been useful in the development of new drugs and continue to play an invaluable role in the drug discovery processes (Amer *et al.*, 2013). Fenugreek (*Trigonella foenum graecum L.*) is an annual plant from the family Papilionaceae–Leguminosae and is extensively cultivated in India, Mediterranean region, North Africa and Yemen (Kassem *et al.*, 2006). Phytoestrogens are natural plant substances which act as endogenous estrogens because of the similarity of their chemical structure. Therefore, they can bind to estrogen receptors of mammalian cells (Cassidy, 2003). In addition, Fenugreek seed contain diosgenin which is used in synthetic estrogen (Billaud, 2001). A steroid sapogenin constituent of fenugreek seed is a precursor of steroid hormones, such as progesterone and anti-inflammatory steroids, such as cortisone (Norton, 1998). Studies show that fenugreek seed have antioxidant properties (Hibasami *et al.*, 2003). Further fenugreek seed polyphenols prevented oxidative hemolysis and lipid peroxidation induced by $H_2O_2$ *in vitro* in human erythorcytes (Kaviarasan *et al.*, 2004). In our previous study (Barakat *et al.*, 2010) addition of fenugreek extract to maturation

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* Corresponding author: ibrahimahb@yahoo.com, ibarakat@ksu.edu.sa
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medium enhanced the quality of less effective medium (CR1aa), as well as optimized the effect of TCM-199 medium for *in vitro* maturation of Egyptian buffalo oocytes.

Moreover, oral administration of fenugreek extract significantly increased the total number and quality of collected mice oocytes as mentioned by Hassan *et al.* (2006).

*In vitro* maturation is one of the essential steps in the *in vitro* fertilization process. Addition of hormones into maturation medium for *in vitro* maturation of oocytes plays an important role in subsequent *in vitro* fertilization and *in vitro* development in goats (Pawshe *et al.*, 1996) and cattle (Saeki *et. al.*, 1991). The addition of steroids (estrogen and progesterone) improves the completion of maturation (Moor *et al.*, 1980) and plays an important role not only in nuclear maturation, but also in cytoplasmic maturation of sheep (Guler *et al.*, 2000), cattle (Ali and Sirard, 2002) and human oocytes (Tesarik and Mendoza, 1997). The presence of steroids in the follicular fluid before and during maturation may play a role in oocyte maturation (Ainsworth *et al.*, 1980). In fact, it has been shown that estradiols as well as other steroids are involved in keeping the oocytes in meiotic arrest (Mingoti *et al.*, 1995). Furthermore, it has been suggested that estradiol is important in the oocyte acquisition of the fertilization competence (Pellicer, 1997). However, the specific role of estrogens in follicular and oocyte maturation as well as ovulation and embryo development seems to be species dependent (Moudgal *et al.*, 1996) and is currently unknown in various species. Pig cumulus oocyte complexes secrete estradiol during cultivation in a steroid free medium, probably as a consequence of the action of gonadotropins (Dode and Graves, 2002). Secretion of estradiol by human and bovine cumulus cells has also been reported (Mingoti *et al.*, 2002).

*In vitro* matured oocytes which were collected from slaughterhouse ovaries may be easily and cheaply obtained and their use may become a useful tool for basic research of the reproductive biology and for application of advanced biotechnologies such as somatic cell cloning and transgenic livestock (Nadi *et al.*, 2002). However, there are some problems encountered with *in vitro* production of sheep embryos such as, still 60% failure of IVM/IVF oocytes reaching the blastocyst stage (Gilchrist and Thompson 2007; Katska-Ksiazkiewicz *et al.*, 2007); the progress in the *in vitro* fertilization techniques, embryo culture procedures and oocytes quality was inadequate over the past years (Cognie *et al.*, 2004; Russo *et al.*, 2007; Morton *et al.*, 2008).

To our knowledge, there are no reports where the fenugreek seed extract has been used as phytoestrogenic agent in the *in vitro* maturation of sheep oocytes and the subsequent development of the matured oocytes to the blastocyst stage following the *in vitro* fertilization. Hence, the aim of the present study was to examine the role and the optimum concentration of fenugreek seed extract during the *in vitro* maturation of Neaimi sheep oocytes and the subsequent IVP.

## MATERIAL AND METHODS

### *Chemicals*

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### *Preparation of fenugreek seed extract*

Fenugreek seed extract was extracted with hot water for once time in the beginning of the experiments. Briefly, fenugreek seed were heated in water (10 g/100 ml) until boiling and evaporate the 75% of the water, then spreading the suspension and leave to dry completely (Maghsoud *et al.*, 2013).

### *Oocytes maturation*

Neaimi sheep ovaries were collected from a local slaughterhouse and transported to the laboratory in saline (30 to 35$^{o}$C) within 1 to 3 hrs. from collection. Ovaries were washed three times with pre-warmed fresh saline (37$^{o}$C), and all visible follicles with a diameter from 2 to 8 mm were aspirated using a 20 gauge needle. After aspiration, only cumulus oocytes complexs (COCs) surrounded by more than 3 layers of cumulus cells were selected for *in vitro* maturation (IVM) (Fig.1). Selected oocytes were washed three times with maturation medium. The basic maturation medium consists of TCM-199 with earle's (Sigma, m4530) supplemented with 10 % Fetal bovine serum (FBS, GIBCO, Grand Island, NY), 40 IU/ml equine chronic gonadotropin (eCG, Folligon, Intervet International BV, Boxmee, Holland), 10 µl penicillin sodium salt and 50µg $ml^{-1}$ gentamycin sulphate. Ten to fifteen selected COCs were transferred into 50 µl droplet of maturation medium supplemented with estradiol 17β or fenugreek seed extract for 24 h. and were cultivated at 38.5°C at 5% $CO_2$ and high humidity.

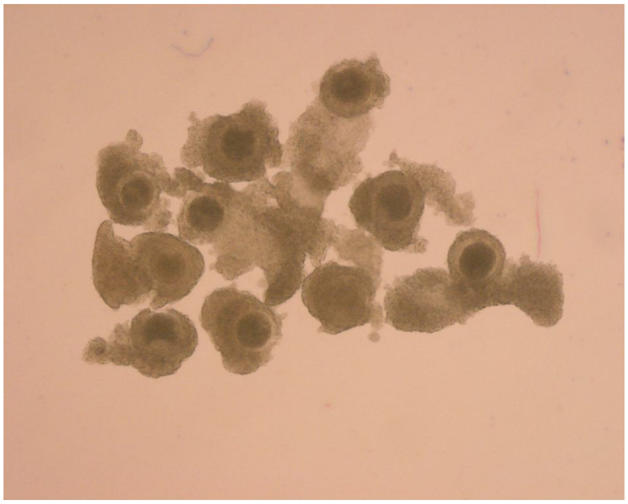

Fig. 1. Sheep oocytes used in *in vitro* production of sheep embryos (200X).

*Experimental design*

To evaluate the role and optimum concentration of fenugreek seed extract on *in vitro* maturation and developmental competence of Neaimi sheep oocytes, *in vitro* fertilization has been followed. Three thousands and two hundred sixty two oocytes were used, 1391 oocytes were used for nuclear maturation assay and 1871 oocytes were used for IVF. Each treatment was repeated five times. According to the culture medium used oocytes were divided into 3 groups: group I (n= 943) – basic maturation medium + 1 µg estradiol 17β/ml media, group II (n= 1143) – basic maturation medium + 1 µg fenugreek seed extract/ml media and group III (n= 1180) – basic maturation medium + 10 µg fenugreek seed extract/ml. Fenugreek seed extract doses were selected depending on the recommended estradiol 17β (1 µg estradiol 17β/ml medium) dose which has been reported used in IVM cultivation medium for most animals (Lv *et al.*, 2010). Group I served as the control group and groups II and III were considered as treatment groups.

*Nuclear maturation assay*

After maturation, cumulus cells were removed from matured oocytes using 0.1% hyaluronidase and mechanical displacement by gentle mouth pipetting using a small-bore glass pipette. The cumulus free oocytes were then fixed in acetic:ethanol (1:3 v/v) for 24–48 h and were stained with 1% aceto-orcein in 45% (v/v) acetic acid (Rao *et al.*, 2002). The nuclear status of the matured oocytes was classified according to Santos *et al.* (2006).

*Preparation of sperm and in vitro fertilization*

For IVF, 35 µL Bracket and Oliphant (B.O.) IVF medium (Bracket and Oliphant, 1975) drops were made in petri dishes. The drops were covered with paraffin oil and equilibrated at 39°C under 5% $CO_2$ in humidified air in the incubator for at least 2 h prior to use. Fresh semen was collected from sheep ram of proven fertility. For swim up, 80 to 100 µl of semen was kept under 1 ml of B.O. medium supplemented with 5 mg BSA/ml and 0.2 mg Heparin/ml in a 15 ml conical Falcon tube at 38.5ºC for up to 45 min. After swim up, the 700 to 800 µl of the supernatant were added to 3 ml of BSA-B.O. medium, centrifuged twice at 1800 rpm for 5 min and the final pellet was re-suspended with BSA-B.O. medium. Insemination was carried out by adding 15 µl of sperm suspension, containing $1.0 \times 10^6$ sperm/ml, into the fertilization drop (10 to 15 oocytes per 45 µl fertilization drop). The gametes were co-incubated with sperm for 18–20 h at 38.5°C, 5% $CO_2$ and high humidified.

*In vitro culture of embryos*

After fertilization, oocytes were denuded by successive pipetting, then 15 to 20 oocytes were transferred into 50 µl droplets of SOF medium (Maraa *et al.*, 2013) supplemented with10% FBS, 0.0027g sodium pyruvate, 100 IU/ml penicillin sodium salt and 50 µg $ml^{-1}$ gentamycin sulphate and were then covered with mineral oil. The fertilized oocytes were cultured for 9 days under 5% $CO_2$, 5%

$O_2$, 90 % $N_2$, 38.5°C with high humidity. The day of IVF was considered as day 0. The final numbers of embryos at various stages (2-, 4-, 8-, 16-, morula, and blastocyst) were recorded at day 9.

*Statistical analysis*

Replicates of experiments were performed on different days with different batches of oocytes and semen. Statistical analyses for all data were carried out using analysis of variance (ANOVA). Statistical differences were considered significantly at $P \leq 0.05$ levels by using Duncan's Multiple Range Test procedure (Duncan, 1955). Results were expressed as mean ± SEM (standard error of mean). Analysis of data was used to compare the maturation and different stages of embryo development *in vitro* in different treatments. All the calculations were performed using the SPSS statistical program.

## RESULTS

*In vitro maturation*

Effects of fenugreek seed extract on cumulus expansion and nuclear maturation of sheep oocytes are presented in Table I. The mean value of oocytes with expanded cumulus cells percentage was not significantly different ($P \leq 0.05$) among all groups (Fig. 2). Regarding nuclear maturation, there were no significant differences in oocytes stages; GV, GVBD, MI, AI, and MII; in group II and group III, group I and group II for GV and M II stages only, and group I and group III for GV only (Fig. 3). The number of oocytes developed to M I and Anaph. I in group II and group III was significantly higher than those in group I, while there was no significant difference between group II and group III. Concerning oocytes with M II stage, it was significant higher in group III compared to group I, but there is no significant difference ($P \leq 0.05$) between group II and group III (Table I).

*In vitro embryo development*

To determine the effect of fenugreek seed extract on the developmental competence of sheep oocytes, we compared between oocytes matured *in vitro* in medium supplemented with or without the extract. The analysis of embryo development stages (Fig. 4) data in Table II showed significant

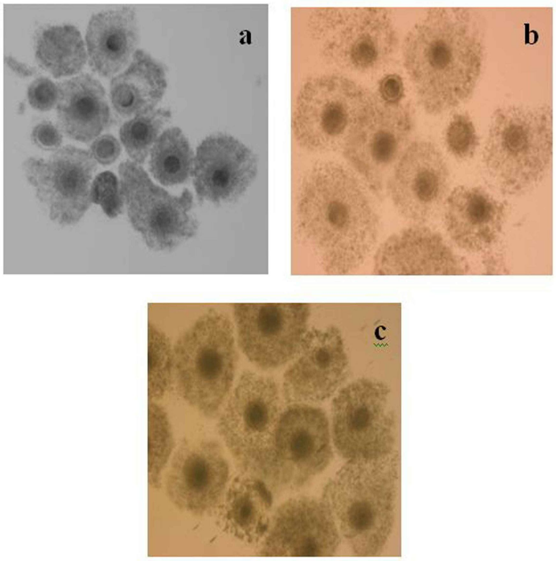

Fig. 2. Expansion of cumulus cells as a result of exposing the sheep oocytes to different concentrations of fenugreek extract. a) Control, b) 10 µg/ml, c) 1 µg/ml (200X).

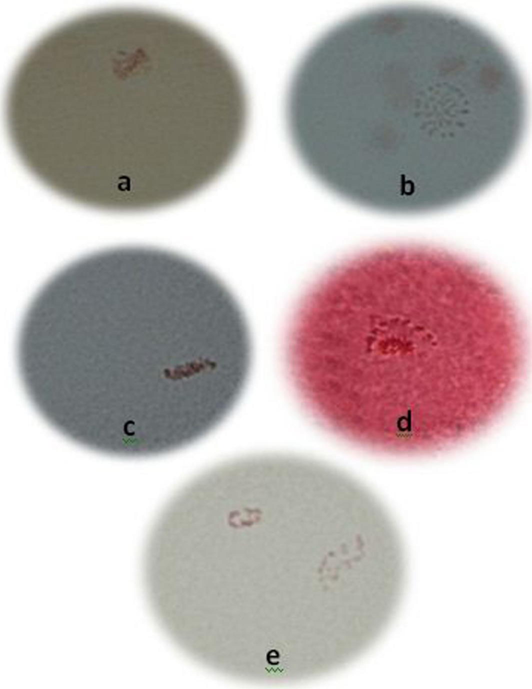

Fig. 3. Stained of Neaimi sheep oocytes with aceto-orcein stain represented nuclear stages during meiosis progression. a) Germinal Vesicle (GV). b) Germinal vesicle break down (GVBD). c) Metaphase I (M I). d) Anaphase. e) Metaphase II (M II) (400X).

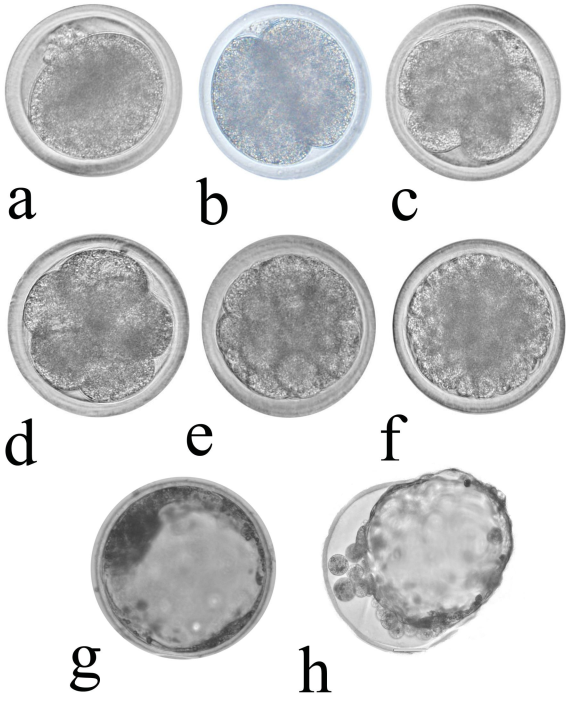

Fig. 4. Neaimi sheep embryos developmental stages; a) Fertilized oocyte, b) 2-cell stage, c) 4 cell stage, d) 8 cell stage, e) 16 cell stage, f) Morula stage, g) Expanded blastocyst, and h) Hatching blastocyst (200X).

differences ($P \leq 0.05$) between group III and group I and group II for degenerated and fragmented embryos, 4-, 16-, and blastocyst stages, while there was no significant differences for 2- and 8- stages. Concerning the morula stage, data analysis showed that the mean value significantly differ between group III and group I, while there is no significant difference between group I and group II. The mean value of oocytes matured *in vitro* in medium supplemented with fenugreek seed extract at 10 µg $ml^{-1}$ and developed to blastocyst stage (28.0±0.7) was significantly higher ($P \leq 0.05$) than those oocytes matured *in vitro* in medium supplemented with 1µg $ml^{-1}$ estradiol 17β (23.8±0.7) and 1 µg $ml^{-1}$ fenugreek seed extract (25.4±0.5) as shown in Table II. Data analysis of embryo development showed that 10 µg $ml^{-1}$ fenugreek seed extract (group III) is better than other treatments (group I and group II).

## DISCUSSION

As far as we know, there are no reports on the effects of addition of fenugreek seed extract into maturation medium used in IVP of sheep oocytes. In this study, addition of fenugreek seed extract to maturation medium of sheep oocytes enhanced the rates of maturation and subsequent development to morula and blastocyst stage after *in vitro* fertilization. So, the discussion will be written from the perspective of the impact of estorogen or estradiol-17β on the maturation of oocytes and embryo growth, where the fenugreek extract has an estrogenic effect (Billaud, 2001; Norton, 1998). Improvement of an IVM system is a major step of *in vitro* embryo production procedures, because it does not only affects the maturation rate of oocytes, but also has a role in subsequent embryonic development (Rizos *et al.*, 2002). Several researchers have studied different aspects of IVM in mammalian oocytes (Rao *et al.*, 2002; Kharche *et al.*, 2005). The maturation media with the selection of protein supplements and hormones play an important role for IVM, subsequent IVF and *in vitro* development (Motlagh *et al.*, 2008). In previous studies reported that the addition of fenugreek extract to maturation medium significantly enhanced the maturation rate and embryo development (Barakat *et al.*, 2010; Choi *et al.*, 2001; Roushandeh *et al.*, 2007) as found in our results. This study showed that the developmental rate of oocytes to morula and blastocyst stage significantly increased when the fenugreek seed extract was added into maturation medium compared to estradiol-17β.

These results may be attributed to the steroid compound in the seed extract, since the addition of steroids to a maturation medium improved the completion of maturation (Moor *et al.*, 1980). Also, *in vivo* oocyte growth and maturation are directly regulated by intra-ovarian factors such as steroids, cytokines and other growth factors acting at key points during the process of follicle development (Campbell and McNeilly, 1996). Among these factors, estradiol (E2) may be of great importance (Zheng *et al.*, 2003).

Our results demonstrated that the addition of fenugreek seed extract to maturation medium

**Table I.** **Effect of fenugreek seed extract on cumulus expansion and nuclear maturation (mean ± SEM) of sheep oocytes.**

| Groups | Traits | Nuclear maturation | | | | | |
|---|---|---|---|---|---|---|---|
| | | Oocytes with expanded cumulus cells (%)$^{\S}$ | GV | GVBD | M I | Anaph. I | M II |
| Estradiol 17β (Control) | Group I | 97.8 ± 0.4 a | 12.6 ± 0.8a | 8.4 ± 1.0a | 8.4 ± 1.1a | 5.2 ± 0.7a | 44.8 ± 6.3a |
| Fenugreek seed extract | 1 µg/ml (Group II) | 98.0 ± 0.3a | 12.2 ± 0.9a | 12.2 ± 0.7b | 11.8 ± 1.1b | 7.8 ± 0.9b | 48.4 ± 2.7 ab |
| | 10 µg/ml (Group III) | 98.0 ± 0.3a | 10.2 ± 0.7a | 13.2 ± 1.3b | 12.4 ± 1.0b | 8.4 ± 0.9b | 62.2 ± 3.8 b |

* Mean values in the same columns with different superscripts (a, b) differ significantly ($p \leq 0.05$).
GV, germinal vesicle; GVBD, germinal vesicle breakdown M-I, metaphase-I; Anaph. I, anaphase I; M-II, metaphase-II.
$^{\S}$ Mean maturation rate % depending on expansion of cumulus cells

**Table II.** **Embryo developments (mean ± SEM) after *in vitro* maturation of sheep oocytes in medium supplemented with fenugreek seed extract.**

| Groups | Traits | Embryo developed to | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Degenerated embryos | Fragmented embryos | 2-cell stage | 4-cell stage | 8-cell stage | 16-cell stage | Morula | Blastocysts |
| Estradiol 17β (Control) | Group I | 12.6±0.5a | 5.6±0.4a | 2.2±0.4a | 1.2±0.4a | 1.8±0.4a | 1.4±0.2a | 60.6±1.8a | 23.8±0.7a |
| Fenugreek seed extract | 1 µg/ml (Group II) | 11.4±0.5b | 19.4±0.8c | 4.2±0.6b | 4.0±0.5c | 3.2±0.4b | 1.6±0.2a | 66.2±1.9ab | 25.4±0.5a |
| | 10 µg/ml (Group III) | 9.0±0.7a | 10.2±0.9b | 3.4±0.5ab | 2.4±0.2b | 2.2±0.4ab | 2.6±0.2b | 71.4±2.0b | 28.0±0.7b |

* Mean values in the same columns with different superscripts (a, b, c) differ significantly ($p \leq 0.05$).

enhance the maturation rate and embryonic development of Neaimi sheep oocytes. This finding suggested that the enhancement may be attributed to the steroid compound in the extract (Billaud, 2001), as well as, the fact that antioxidants present in the extract may enhance the maturation rate and embryonic development (Sur *et al.*, 2001; Hibasami *et al.*, 2003).

## CONCLUSION

According to our results, an alternative natural source of estradiol-17β with fenugreek seed extract at 10 µg/ml concentration seems to improve the *in vitro* maturation rate of Neaimi sheep oocytes and the morula and blastocyst rate. However, additional research is needed to prove the positive effects of using natural sources of extracts in IVP media.

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