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EFFECT OF SPERM- OOCYTE INCUBATION TIME ON IN VITRO FERTILIZATION AND EMBRYO DEVELOPMENT OF BUFFALO OOCYTES MATURED INVITRO

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ABSTRACT

This experiment was planned to investigate the effect of sperm- oocyte incubation time on the fertilization rate and subsequent developmental competence of buffalo oocytes matured in vitro. In vitro-matured buffalo oocytes collected from abattoir-derived ovaries divided into three groups, each group fertilized by frozen thawed semen in F-TALP separately for (4-6h., 14-16h, 21-24h.). Following sperm exposure for different periods of times, the presumptive zygotes were co-cultured in SOF. Sperm-oocyte incubation for 21-24h yielded optimum penetration (62.76%) and fertilization (44.57%) rates than those of 14-16h (56.14 and42.22percentage) and 4-6 h (56.29 and 40.52%). The group of 21-24h achieved maximum cleavage (38.48%), morula (14.15%), and blastocyst (10.18%) rates among the other two groups. Those data concluded that 21-24h sperm-oocyte incubation is ideal for optimizing fertilization rates of buffalo oocytes.

INTRODUCTION

In vivo, the ovulated bovine oocyte is surrounding by a small number of free motil sperm at the situation of fertilization **(First andParrish, 1987)**. In comparison, the bovine oocyte in the IVF medium is encircled by huge numbers of spermatozoa 10,000 to 200,000:1 **(Lambert et al., 1986)** and incubated for different time. Different laboratories use different incubation times with different results .This experiment was performed to study the effect of sperm exposure time on optimization the rate of fertilization and subsequent development of in vitro matured (IVM) buffalo oocytes.

MATERIAL AND METHOD

The present study was conducted in Theriogenology Department, Faculty of Veterinary Medicine, Mansoura University, Egypt in association with the Department of Artificial Insemination and Embryo Transfer, Animal Reproduction Research Institute (ARRI), Al-Haram, Giza

Chemicals

All the chemicals used in this study were purchased from Sigma Chemical co. (St. Louis, MO, USA).

1. Oocyte collection and in vitro maturation

Buffalo Ovaries were collected from Bahtem slaughterhouse into Saline at 35 ºC, transported to the laboratory within 2 h. cumulus oocyte complexes were aspirated from 2 to 8 mm follicles using an 18-gauge needle attached to 10 ml syringe. COCs with an evenly granulated cytoplasm and a compact cumulus cell layer were selected. Selected oocytes were washed three times in sterile PBS. For IVM, COCs were cultured in petri dishes (20) oocytes per drop for 24 h in TCM-199 supplemented with 10% FBS, 0.005 AU/mL FSH , and 1 mg/mL 17b-estradiol at 38^{oC} in a humidified atmosphere of 5% CO2.

2. Semen preparation and invitro culture

Motile sperm were selected using a swim-up technique **(Mehmood et al., 2009)**. After 24h of IVM, invitro-matured buffalo oocytes divided into three groups, each group incubated With 10^6 spermatozoa/mL in 75µl drop of F-TALP for (4-6h., 14-16h, 21-24h.) consequently. The presumptive zygotes of each group (5 zygote/50 µl droplets) were cultured in embryo development medium at 38 ºC and 5% CO2 in air **(Sharma et al., 2010)**. Developmental competence of embryos was assessed separately for each group every 48h and the culture medium were replaced with fresh medium**.**

Evaluation the early buffalo embryo

Assessment Embryo Quality

1.Morphological and morphometric parameters according to (Stringfellow andSeidel, 1998).

2. Assessment mitochondrial function of in vitro produced buffalo embryo:

Mitochondrial functions and cell proliferation was quantified by colorimetric analysis based on the metabolic cleavage of the tetrazolium salt MTT. Embryos were incubated with 0.25 mg/ml MTT in the culture media at 37 °C for 3 h. Absorbance was measured at 575 nm after using a spectrophotemeter **(Green andLeeuwenburgh, 2002).**

3. Total cell number assessment

Embryos were washed twice in PBS containing 1 mg/ml Polyvinylpyrrolidone (PVP). Then embryos were fixed in 100 µl paraformaldehyde solution [4% (w/v) in PBS, pH 7.4] for 1 h at room temperature. Washing of embryos 3 times in 100 µl drop PBS/ PVP then transferred to 50 µl of Hoechst 33342 dye for 10 minutes, then washed twice in PBS/ PVP **(Critser andFirst, 1986)**. Embryos were mounted into a clean slide then covered by coverslip and then were examined under the fluorescent microscope with a UV filter. Nuclei will appear blue that is the base of counting

RESULTS

1. Effect of sperm- oocyte incubation time on in vitro fertilization of buffalo oocyte:

Application of **21-24h** sperm-oocyte incubation yielded optimum penetration (62.76%) and fertilization (44.57%) rates than those of 14-16h (56.14 and42.22%) and 4-6 h (56.29 and 40.52%) as shown in **table**1.

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Table 1: Effect of sperm- oocyte incubation time on in vitro fertilization of buffalo oocyte

Treatment	No. of oocyte	Penetration rate	Fertilization rate
4-6h	38	21 (56.29±8.30)[a]	15 (40.52±7.58) [a]
14-16h	42	23 (56.14±9.44)[a]	17 (42.22±13.53)[a]
21-24h	39	24 (62.76±14.14)[a]	17 (44.57±11.43)[a]
Over all mean		58.39±5.56	42.44±5.58

Significant different at (P< 0.05)

2. Effect of sperm- oocyte incubation time on in vitro embryo development of buffalo oocytes:

The group of 21-24h achieved maximum cleavage (38.48%), morula (14.15%), and blastocyst (10.18%) rates among the other two groups **(table 2).**

Table 2: Effect of sperm- oocyte incubation time on in vitro embryo development of buffalo oocyte

Treatment	No. of oocyte	Cleavage rate	Morula	Blastocyst
4-6h	48	15 (31.48±4.27)[a]	5 (10.74±2.59) [a]	2(4.07±2.06)[a]
14-16h	46	17(36.95±5.79)[a]	6(13.19±3.97)[a]	3 (6.67±3.85)[a]
21-24h	49	19(38.48±6.06)[a]	7 (14.15±3.94)[a]	5 (10.18±1.93) [a]
Over all mean		35.64±2.91	12.96±1.58	6.97±1.64

Significant difference at (P< 0.05)

3-Effect of sperm- oocyte incubation time on total cell number and mitochondrial function of in vitro produced buffalo embryo

The **21-24h group** had the best effect on the quality of invitro produced embryo among the other two groups with reflected improvement in total cell number (**64.67**) and mitochondrial function (0.249) as shown in table **3, 4**

Table 3: Effect of sperm- oocyte incubation time on total cell number of in vitro produced buffalo embryo

Treatment	Total cell number
4-6h	47.67±3.85[b]
14-16h	46.67±4.34[b]
21-24h	64.67±6.12 [a]
Over all mean	53.00.6±3.80

Values with different superscripts in the same column are significantly different at (P< 0.05)

Table 4: Effect of sperm- oocyte incubation time on mitochondrial function of in vitro produced buffalo embryo

Treatment	Mitochondrial function
4-6h	0.214±0.013[a]
14-16h	0.218±0.012[a]
21-24h	0.249±0.025 [a]
Over all mean	0.227±0.011

Values with different superscripts in the same column are significantly different at (P< 0.05)

DISCUSSION

In the existing study 21-24h sperm-oocyte incubation yielded optimum penetration and fertilization rates than those of 14-16h and 4-6 h **table 1**. Also this group of 21-24h achieved maximum cleavage, morula, and blastocyst rates among the other two groups **table 2**. These results are in coordination with **(Rehman et al., 1994)** who concluded that 24 h period of gametes co-incubation produced the maximum fertilization rates; compared to 4–12h.These findings also matching **with (Kochhar et al., 2003; Ward et al., 2002)** who concluded that minimizing co-incubation time to 8 h has achieved a significant decrease of oocyte cleavage. In several laboratories, 18–20 h is ideal fertilization period for bovine oocytes **(Gordon, 2003; Khurana and Niemann, 2000; Tanghe et al., 2003)**. Other established that16 h co-incubation time is suitable for optimizing the blastocyst income in buffalo **(Gasparrini et al., 2008).** On the other hand some authors concluded that protracted oocyte and sperm co-incubation period during IVF negatively effects on following embryo development. In addition to an extreme creation of ROS, the polyspermy rate is also exceed with prolonged incubation period **(Gomez andDiez, 2000)**. It is clear that the present study showing significant improvement in mitochondrial function and total cell count and this can explained as: Regarding oocytes and embryos, mitochondria are essential for adequate reproduction and also for developmental competence **(Cummins, 2004).** In oocytes, mitochondria are structurally immature, after fertilization and during early development; mitochondria mature showing different levels of activity, at different maturation phases **(Bavister and Squirrell, 2000).** The cell number may be a valid indicator of quality and viability of in vitro-developed pre-implantation embryos **(Totey et al., 1996).**

CONCLUSION

Those data concluded that **21-24h** sperm-oocyte incubation is ideal for optimizing fertilization rates and embryo quality of buffalo oocytes through improving of mitochondrial function and total cell count.

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الملخص العربي

تاثير استخدام اوقات مختلفه لتحضين البويضات مع الحيوانات المنويه على الاخصاب المعملى والنمو الجنينى لبويضات الجاموس الناضجه معمليا

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تهدف الدراسه الحاليه الى محاوله تحسين الاخصاب المعملى لبويضات الجاموس عن طريق دراسه تاثير اوقات مختلفه للاخصاب على كلا من معدلات الاخصاب ومعدلات التطور والنمو الجنينى و جوده الاجنه الناتجه لبويضات الجاموس.

بصفه عامه يتم جمع المبايض بعد الذبح مباشره من المجزر المحلى .يتم نقل المبايض الى المعمل فى ترمس حرارى يحتوى على محلول ملحى متعادل. فى المعمل يتم سحب البويضات من جريبات (follicles)على سطح المبيض يتراوح قطرها من٢ -٨مم .بعد ذلك يتم غسل البويضات عده مرات ب PBSثم فحصها تحت الميكروسكوب للاختيار. البويضات ذات الجوده العاليه يتم انضاجها عن طريق تحضينها فى وسط الانضاج فى الحضانه عند٣٨درجه مئويه و٥%ثانى اكسيد الكربون لمده ٢٤ساعه.

للاخصاب يتم تقسيم البويضات الناضجه الى ثلاث مجموعات كل مجموعه يتم اخصابها فى وسط ملائم وتحضينها مع الحيوانات المنويه لاوقات مختلفه (٤- ٦و١٤-١٦و٢١-٢٤ساعه). . يتم زراعه الزيجوت الناتج فى وسط مناسب للنمو الجنينى (SOF) ثم تحضينه تحت نفس الظروف السابقه حيث يتم تغيير الوسط وفحص الاجنه كل ٤٨ساعه.

اوضحت هذه التجربه ان فتره التحضين لمده ٢١-٢٤وقت مناسب لتحضين البويضات والحيوانات المنويه للحصول على معدلات مناسبه من الاخصاب والنمو الجنينى بالمقارنهبين فترات التحضين ٤-٦ و١٤-١٦ساعه على التوالى.

من السابق يتضح ان ٢١-٢٤ساعه للاخصاب وقت مناسب للحصول على اعلى معدلات الاخصاب ومعدلات التطور والنمو الجنينى لبويضات الجاموس الناضجه معمليا.