

Introduction

The non-human primates (NHP) have several similarities with human. Due to this fact, NHP have been considered as the suitable models for the biomedical researches. The primary purpose of using NHP was studying human reproductive physiology.

The field of *in vitro* embryo production (IVEP), a branch of assisted reproductive techniques (ART), has pervasive applications such as enhancing the reproductive effectiveness of livestock, and improving the laboratory animals' productivity for use as human-disease models and vaccine/pharmaceutical assurance quality assays (Sankai, 2000).

Due to the existent similarities between NHP and humans, these animal models have been noted as an important model for the studying of human infectious diseases, and vaccines and pharmaceutical products quality control tests. For the aforementioned reasons, NHP are substantial for the investigations on neurology, reproductive physiology, metabolism, and endocrinology. On the other hand, since NHP have a long life span, they would be considered as a suitable model for the studies on longevity. To outstretch and preserve these invaluable animal research resources, it is pivotal for us to establish techniques for the *in vitro* manipulation of gametes, and *in vitro* embryo production (Curnow and Hayes, 2019).

The grivet monkey (*Chlorocebus aethiops*), which is native to Africa is a member of the family *Chlorocebus*. The grivets are mostly herbivorous monkeys. They have specific phenotype characteristics as follows: black faces (Figure 1A), grey body hair color with long white tufts of hair along the sides of its face (Figure 1A), light blue scrotum (Figure 1B), and their body length ranging from 40-45 cm for females, to about 50 cm (20 inch) for males (Figure 1C). The non-human primates especially grivets are useful models in understanding the genetic and social behaviors of humans, developmental biology, human physiology, etc. They have been recognized with human-like characteristics, such as hypertension, anxiety, responses to the pharmaceutical products and vaccines. Therefore, grivets have been considered as interesting lab animals and play a key role in the several aspects of biomedical researches (Boatman and Bavister, 1984; Sankai, 2000).

For nearly five decades, the Razi Vaccine and Serum Research Institute (RVSRI) has produced Oral Polio Vaccine (OPV) against polio virus. All the quality assurance tests of the OPV have been conducted on the grivet monkeys before releasing the vaccines to the Ministry of Health, Islamic Republic of Iran. Due to the importance of this animal model for the RVSRI Polio Vaccine and the high expenses of purchasing these animals, the *in vitro* embryo production in NHP grivet has gained a great attention in Iran.

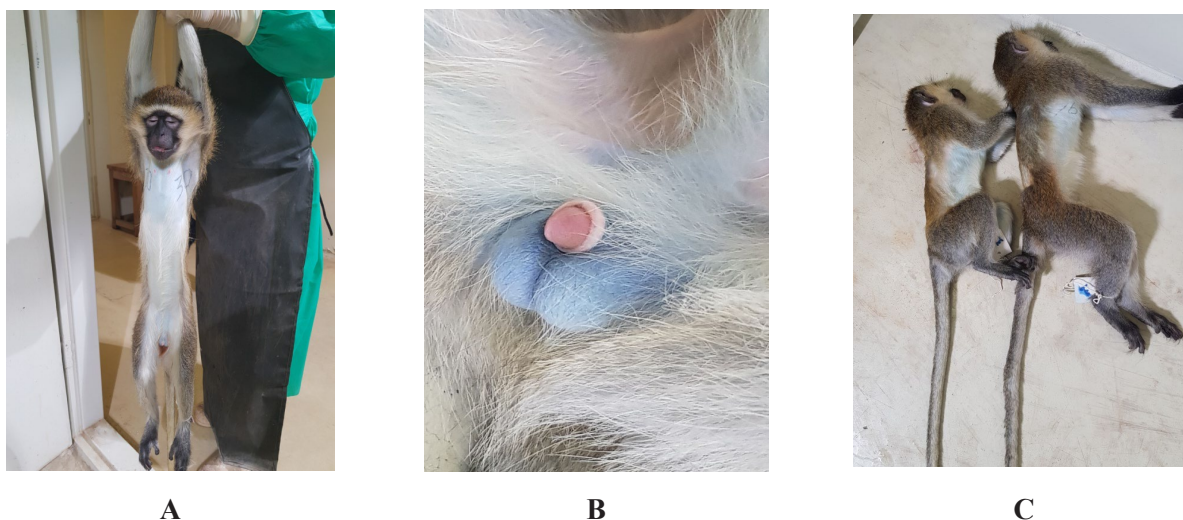


Figure 1. A) General Morphological features of Grivet, B) Grivet male genital system morphology, C) Grivet Adults male and female

Therefore, this study was designed to establish a detailed procedure for the *in vitro* maturation (IVM) of germinal-vesicle stage oocytes, *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC) of grivet monkey.

Materials and Methods

Chemicals

All the chemicals were purchased from Sigma Aldrich Co. unless otherwise stated.

Ethics and Experimental Conditions

According to the Animal Ethics Committee guidelines, all the efforts were made to minimize discomfort and pain for the animals (2-18-18-001-980182). The monkeys were anesthetized by the injection of ketamine hydrochloride (Vetanarcol 15 mg/kg intramuscular; König S.A., Avellaneda, Argentina) and xylazine hydrochloride (Kensol 1 mg/kg intramuscular; König S.A.), followed by intramuscular injection of 20 mg/kg of pentobarbital (König S.A., Avellaneda, Argentina). All the surgical and dissection procedure was performed using the Bovie machine as previously described (Dadashpour Davachi, 2019).

Ten adult male and 4 adult female grivet monkeys were used to obtain the reproductive organs after the proper anesthesia and before performing the heart infusion for the CNS dissection as the final step for the OPV vaccine test.

Ovary and Oocyte Recovery

The ovaries were recovered following the anesthesia of the females. After removal, the ovaries were transported to the laboratory in less than 2 h at 37°C in a container filled with phosphate buffered saline (PBS), supplemented with penicillin-streptomycin 100 µg/mL (Gibco, Grand Island, NY USA). Two different oocyte recovery methods were used in this study: 1) the visible ovarian follicles were aspirated using an aspiration pump (MEDAP Sekretsauger P7040, Tilburg, NL) fitted with a disposable vacuum line and set at flow rates of 10 mL H₂O/min with a disposable 25 gauge needle attached (Dadashpour Davachi *et al.*, 2016), and 2) the oocyte recovery via centrifugation (ORC), in which the ovarian surface was scratched several times with scalpel blade and then ovaries were placed in a modified falcon tube (MFT's) as described before (Dadashpour Davachi *et*

al., 2012b). In brief, the MFT's containing scratched ovaries were filled with 3 mL of a pre-incubated oocyte washing medium and centrifuged at 750 × g for 3 min.

Oocyte *in vitro* Maturation

The basic maturation medium contained the following ingredients: TCM199 supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.2 mM sodium pyruvate, 5 mg/mL gentamicin, 10 mg/mL follicle stimulating hormone (FSH), 15 mg/mL human chorionic gonadotropin (hCG), and 1 mg/mL estradiol. All the recovered oocytes were examined under the light microscope. The COCs with compact and cumulus cell layers and homogenous oocyte cytoplasm were selected for IVM (Dadashpour Davachi *et al.*, 2012a). Each 10 COCs were cultured in a drop of IVM medium (50 µL), covered with mineral oil for 48 h, at 38.5°C in a 5% CO₂ and 95% humidified incubator.

Sperm Collection

Several sperm recovery methods have been applied for the collection of non-human primate and mammalian spermatozoa (Didarkhah *et al.*, 2020; Seifi-Jamadi *et al.*, 2017). Of these, collecting spermatozoa from ejaculated semen and epididymis are the two most generally used methods (Cho and Honjo, 1973; Seifi-Jamadi *et al.*, 2017; Kamrani *et al.*, 2021). In this study, collecting the spermatozoa from epididymis was the preferred method. Following the anesthesia, the testes were removed, placed in a cool box at 5°C, and immediately transported to the laboratory. At the laboratory, the testicles were evaluated morphologically and the testicles with normal appearance were selected for further processing. For the sperm recovery, the epididymis (Figure 2) were dissected, the tails were sliced several times in the sperm washing medium (Quinn's Sperm Washing Medium, Cooper Surgical, Denmark), and incubated for 15 min in an atmosphere of 95% humidity and 5% CO₂. After the incubation time, the active and live spermatozoa swam out to the sperm washing medium. Then, the sperm suspension was placed on the top of a Percoll gradient (45% over 90%) in a disposable 50 ml Falcon tube and then centrifuged at 1000×g for 5 min. After the centrifugation, the motile spermatozoa sedimented at the bottom of the Falcon tube formed a thin white

plate. This plate was gently aspirated and diluted to $1 \times 10^6/\text{mL}$. Prior to IVF, the sperm capacitation procedure was done by incubation of the spermatozoa for 4 h in capacitation medium which contained caffeine and dibutyryl cyclic AMP (Boatman and Bavister, 1984). At this point the spermatozoa were ready for fertilization of the matured oocytes.



Figure 2. Removed testicle with fat pad, connective tissues, and epididymis

In vitro Fertilization and in vitro Culture of Embryos

The fertilization medium used in this study was Universal IVF Medium (Cooper Surgical, Denmark). This newly developed IVF medium is suitable for the fertilization and culture until 2-8-cell stage. At least 15 min prior to insemination, the oocytes were transferred to the fertilization drops. At the time of insemination, a portion ($10 \mu\text{L}$) of the pre-incubated spermatozoa was introduced into $90 \mu\text{L}$ of fertilization medium containing about 20 matured oocytes (Heydari *et al.*, 2021). The co-incubation of gametes was carried out for 48 h at 37°C , 5% CO_2 and 5% O_2 . After co-incubation, the spermatozoa attached to the zona pellucida (ZP) were freed from oocytes by gentle pipetting and the 4-8-cells embryos were transferred to Sydney IVF Blastocyst Medium (Cooper Surgical, Denmark) as the IVC medium for the next 6-8 days after insemination. Throughout the 8-day period, all the embryos were placed in an incubator. At day 7 after insemination, the rate of embryo development to the blastocyst stage was recorded (Schmidt and Golos, 2019).

Statistical Analysis

Data are presented as mean \pm SEM and all the percentages were modeled according to the binomial model of variables and arcsine transformation to achieve normal distribution. The variables in all the experiments were analyzed by one-way ANOVA. When the ANOVAs revealed a significant effect, the values were compared by the Tukey post-hoc test. $P\text{-value} < 0.05$ was taken to denote statistical significance (Masoudi and Dadashpour Davachi, 2021).

Limitation of the Study

Based on the ethical concerns, live colony management, and the limited availability of the female grivet monkeys; we could only use 4 females in our study.

Results

The rate of oocyte recovery and IVM success rate are tabulated in [Table 1](#). A total of 64 grivet oocytes were recovered by means of both recovery methods. The recorded data on the oocyte recovery rate showed significant increase ($P < 0.05$) in the oocyte recovery rate using the ORC technique (9.8 ± 0.41) compared to the oocyte recovery rate via aspiration (4.45 ± 0.32). In this study, the COCs with the expanded cumulus were considered as the oocytes reaching to the metaphase II (MII) stage. However, the oocytes with no expanded cumulus cells were not discarded but inseminated in a separate IVF medium for the probable successful insemination. The results of the IVM showed significantly higher rate of successive maturation for the recovered oocytes via ORC (90.90%) compared to the aspiration technique (80.00%) ([Table 1](#)).

The results of *in vitro* fertilization showed that MII oocytes from ORC had significantly higher potential for the successful IVF compared to those oocytes recovered by the aspiration technique ([Table 2](#)). The data showed that the rate of cleavage was similar in both groups of MII oocytes (71%). The rate of successful development to the blastocyst stage was significantly higher for the cleaved embryos in the ORC group (43%) compared to the cleaved embryo on the aspiration group (33.33%) ([Table 2](#)). On the other hand, as expected, none of the oocytes in either groups, ORC or aspiration, which had not shown some degrees of cumulus expansion, could pass the IVF procedure successfully.

Table 1. The mean (\pm SE) COCs recovery rate and MII oocyte (%)

Oocyte recovery technique	Ovary No.	Oocyte recovery rate	No. of cultured oocytes	MIH oocyte% (No.)
ORC	4	9.81 \pm 0.41 ^a	44	90.90 (40)
Aspiration	4	4.45 \pm 0.32 ^b	20	80.00 (16)

Different superscripts (a, b) in the same column differ significantly ($P < 0.05$).

Table 2. Embryo development rate

Oocyte recovery technique	MIH oocytes* No.	Cleaved Embryo No. (%)	Blastocyst No. (%)	Oocyte number with non-expanded cumulus	Cleave Embryo No. (%)
ORC	40	28 (71)	12 (43.00)	4	0 (0)
Aspiration	16	12 (71)	4 (33.33)	4	0 (0)

*Oocytes with fully expanded cumulus cells

Discussion

Several different studies have been conducted previously on the field of reproductive biology and biotechnology in different mammals (Abaspour Aporvari *et al.*, 2018; Boatman and Bavister, 1984; Cho and Honjo, 1973; Curnow and Hayes, 2019; Sankai, 2000; Schmidt and Golos, 2019; Tello *et al.*, 2020). However, there are few published works about different aspects of non-human primates' reproductive physiology. This is the first report describing the detailed procedure of *in vitro* embryo production in grivet monkey.

The oocyte recovery method has been defined as one the most important steps in the IVEP from the slaughtered materials or dead animals (Wani and Skidmore, 2010; Wani *et al.*, 2000). The reported data of the current research showed differences in the oocyte recovery rate between the ORC technique and the aspiration technique. It could be explained as follows: the aspiration technique has a great limitation in terms of detecting small- and medium-size follicles. Therefore, when we use this technique for the oocyte recovery from the slaughtered or dead animals' materials only the visible follicles are used for the oocyte recovery. Thanks to the centrifugation force used in the ORC technique, it seems that the follicles embedded deep within the cortex can be utilized in this retrieving method. Due to this fact the

betterment in the oocyte recovery rate when applying the ORC technique can be explained. These findings which mentioned that the oocytes recovery method have the great influence on the successful rate of the oocytes recovery method were in agreement with previously published works in different mammals (Dadashpour Davachi *et al.*, 2016; Davachi *et al.*, 2014; Sankai, 2000; Schmidt and Golos, 2019; Wani, 2002; Wani and Skidmore, 2010; Wani *et al.*, 2000; Widayastuti *et al.*, 2017).

The results of IVM (Table 1) shows that oocytes recovered with ORC were more competent for IVEP compared to the recovered oocytes using aspiration device. As previously investigated by several research teams, it has been approved that the intact cumulus oocytes complex with the compact cumulus layers is of great importance in terms of oocyte competence (Crocomo *et al.*, 2013; Dadashpour Davachi *et al.*, 2012a; Lin *et al.*, 2018; Lin *et al.*, 2016; Shahedi *et al.*, 2013). In fact, the gap junctions in this situation remain healthy; therefore, better communication between oocyte and the surrounding cumulus cells is established.

The data from the IVF section of this study showed that the cleavage rate was similar for both ORC and aspiration techniques. This may be due to the importance of sperm quality for the attachment

and penetration of spermatozoa to the MII oocytes. Actually, in this step of IVEP the most important thing for the success of IVEP would be the sperm quality. The oocytes competence does not play an important role compared to the sperm quality. These findings were in contradictory with the previous findings in ovine (Dadashpour Davachi *et al.*, 2016). However, it seems that the method of oocyte recovery and the oocyte competence have a pivotal role in the rate of embryo development to the blastocyst stage (Dadashpour Davachi *et al.*, 2016). Indeed, better nursing and previous communication of oocyte with the cumulus cells during the *in vitro* maturation procedure led to some unknown events, which consequently improve the competence of the presumptive zygotes to reach the final steps of *in vitro* embryo development.

Conclusion

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تولید برون تنی رویان میمون Girvet در شرایط آزمایشگاهی

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زمینه مطالعه: میمون گریوت (*Chlorocebus aethiops*)، که یک پستاندار غیرانسانی (NHP) به شمار می‌رود، به دلیل شباهت‌هایش با انسان، نقش قابل توجهی به‌عنوان یک مدل حیوانی در مطالعات سیستم‌های بیولوژیکی را عهده‌دار است.

هدف: این مطالعه به‌منظور انجام بلوغ آزمایشگاهی (IVM) تخمک‌های مرحله ژرمنال-وزیکول، لقاح آزمایشگاهی (IVF) و کشت جنین آزمایشگاهی (IVC) میمون گریوت طراحی شده است.

روش کار: ۱۰ میمون نر بالغ و ۴ میمون زن بالغ پس از بیهوشی مناسب برای به دست آوردن اندام‌های تناسلی مورد استفاده قرار گرفتند. فولیکول‌های تخمدان با روش اسپیراسیون یا با استفاده از بازیابی تخمک با سانتریفیوژ (ORC) اسپیره شدند. برای بازیابی اسپرم، اپیدیدیم از بیضه جدا شد و دم اپیدیدیم در محیط شستشوی اسپرم خرد شد و به مدت ۱۵ دقیقه انکوبه شد. در زمان تلقیح، بخشی از اسپرم انکوبه شده (۱۰ میکرولیتر) به ۹۰ میکرولیتر محیط لقاح حاوی حدود ۲۰ تخمک بالغ وارد شد.

نتایج: داده‌های ثبت‌شده در مورد نرخ بازیابی اووسیت و نرخ بلوغ برون تنی اووسیت‌ها به ترتیب نشان دهنده افزایش قابل توجهی در نرخ اووسیت‌های بازیابی شده و موفقیت در حصول بلوغ برون تنی از طریق تکنیک (۹۰/۹۰٪، $9/8 \pm 0/41$) در مقایسه با اووسیت‌های بازیابی شده با استفاده از روش اسپیراسیون (۴۵/۳۲٪، $4/0 \pm 45/32$) است. همچنین نتایج نشان داد که میزان موفقیت در انجام لقاح برون تنی و رسیدن به مرحله کلیواژ در هر دو گروه اووسیت‌های بازیافت‌شده از طریق ORC و اسپیراسیون مشابه بود (۷۱٪). در حالی که نرخ رشد جنین تا مرحله بلاستوسیست در گروه ORC (۴۳٪) در مقایسه با گروه اسپیراسیون (۳۳/۳۳٪) به میزان قابل توجهی بالاتر بود.

نتیجه‌گیری نهایی: بررسی نتایج این مطالعه مشخص نمود که تکنیک بازیابی تخمک از نظر تاثیر بر کیفیت اووسیت‌ها در تولید جنین آزمایشگاهی در NHP اهمیت زیادی دارد. همچنین مشخص شد که با استفاده از محیط‌های کشت بلوغ، لقاح و کشت برون تنی رویان که به‌صورت تجاری برای مباحث درمانی در انسان استفاده می‌شود می‌توان از این محیط‌های کشت استفاده نمود.

واژه‌های کلیدی: بازیافت اسپرم، مجموعه کمولوس-اووسیت، پرایم‌های غیر انسان، لقاح برون تنی، بلوغ برون تنی

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