Original Article

In Vitro Fertilization of Immature Oocytes by Testicular Sperm: Animal Model for Azoospermic Infertile Patients

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Summary:

Background: Men with azoospermia require testicular and epididymal sperm aspiration for intracytoplasmic sperm injection. Women over 37 years of age demonstrate an increase number of immature oocytes after induction of ovulation.

The development of a technique for in vitro maturation (IVM) and fertilization (IVF) of the oocytes using testicular, epididymal and vassal sperm (TS, ES, VS, respectively) will be of therapeutic value for the treatment of azoospermic patients.

Objectives: The goal of the study was to develop an animal model for the treatment of infertile couples with obstructive azoospermia and immature oocytes.

Materials and Methods: Canine ovaries and testes were collected from a local animal clinic. Immature oocytes were retrieved from the ovaries and cultured in modified tissue culture medium (MTCM). The mature oocytes were in vitro fertilized by TS, ES, and VS. The normality of the fertilized oocytes was studied.

Results: Sperm motility index was significantly higher ($P<0.01$) in sperm retrieved from the vas deference compared to ES and TS. The concentration of the sperm was significantly higher ($P<0.05$) in the testes compared to epididymis and vas deference while VS and ES normal morphology were significantly ($p<0.01$) better than TS. Viable oocyte percentage was significantly higher ($P<O.05$) in 72 hours versus 96 hours culture durations. Significant higher IVF was reported in VS compared to other groups ($P < 0.05$) and the SPI was significantly higher after 96 versus 72 hours duration ($P<O.05$).

Conclusion: IVM of the oocytes in MTCM for 72 and 96 h resulted in a significant increase in IVM rate compared to 48,120, and 144 h durations. In vitro viable mature oocytes showed higher IVF rates when fertilized with VS compared to ES and TS. The results showed that canine oocytes are good model for azoospermic patients.

Keywords: IVM, IVF, Testicular Sperm, Animal model, Azoospermia

Introduction:

Many factors may affect the viability of the surgically retrieved spermatozoa and the outcome of intracytoplasmic sperm injection (ICSI) in infertile patients with obstructive and non-obstructive azoospermia. These factors include the site of sperm origin, such as epididymis, testicular or vassal sperm, the developmental stage of the sperm cell in addition to the testicular histology, serum follicular hormone concentration and the age of the male partner (1). Parameters determined by the female partner such as the nuclear and cytoplasmic maturational stages of the oocytes, chromosomal and spindle disorganization of the oocytes, ovarian reserve, luteal phase normality, and age of patients may also have a significant impact on ICSI success (2). Successful fertilization and pregnancy rates can be achieved when epididymal and testicular sperm were retrieved prior to the injection of human chorionic gonadotropin followed by in vitro culture of sperm cells for forty hours before ICSI. The application of ICSI and luteal support therapy found to improve pregnancy rates in infertile women with luteal phase defect. (3-5).

In mammals, in vivo oocytes maturation occurs within the ovarian follicle and the first

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meiotic division takes place prior to ovulation. Oocytes arrested at metaphase I1 are ready to be fertilized within the oviduct. In contrast, canine oocytes are ovulated as immature oocytes and expel the first polar body at 48 to 72 hours after ovulation when oocytes are in the middle part of the oviduct. The canine oocyte is ovulated at the germinal vesicle stage and the maturation of the oocyte occurs in the oviduct and requires up to five days. The extended period of oocyte incubation in the canine oviduct seems to be required for oocyte maturation. Canine oocytes may work as a model for in vitro maturation (IVM) of human oocytes that result from ovulation induction, advance age, inadequacy of ovarian reserve and defective menstrual cycle (6-8).

A normal progression of nuclear and cytoplasmic maturation is essential for the oocytes to undergo optimum and adequate fertilization and embryonic development. Motile canine spermatozoa are found in the lumen of the uterus for four to six days after mating. The oocytes are matured within the oviduct while sperm are present around the eggs even before the initiation of maturation. In most other mammals, sperm are present around cumulus-oocyte complexes in vivo after the oocytes have reached metaphase II (9-10).

The goal of the present study was to develop a model for 1VR.I and IVF of human oocytes by testicular, epididymal and vassal sperm in azoospermic patients using canine oocytes as an animal model. The present study also investigated the effects of cumulus cell removal and culture duration on the nuclear maturation and fertilization of the oocytes when using sperm retrieved from the testes, epididymis and vas deference.

Materials and Methods:

Canine ovaries and testes were collected from a local clinic, placed in warm saline solution and transported to the lab. The oocytes were recovered from the ovaries by mincing them in warm TC-199 medium supplemented with Hanks salts, L-glutamine and HEPES (Gibco, Invitrogen Co.). Canine oocytes with a dark cytoplasm and at least two layers of cumulus cells were cultured in TC-199 medium-199 supplemented with Earle's salts, 2200 mg/ml sodium bicarbonate, 25 mM HEPES, 2 mM sodium pyruvate, 5 ug/ml progesterone, 100 ng/ml epidermal growth factor, 10 IU/ml human chorionic gonadotropin (HCG), 5 ug/ml insulin, 0.05 mM epinephrine, 10% estrus bitch serum, 0.01 mM non-essential amino acids and 20 ug/ml gentamicin. The oocytes were cultured modified tissue culture medium (MTCM) for 72,96,120 or 144 hours (h) at 38.5 C in 5 % C02 in humidified air.

The cumulus cells were removed after a 72 or 96 hour culture period. For the first 48h, the cumulus-oocyte complexes were cultured in the MTCM containing 10 IU/ml HCG and then cultured in the same medium free from HCG. The oocytes were denuded by pipetting and stained with Hoechst 33342 and examined for nuclear maturation. ANOVA was used for statistical analysis of the data. The composition of the MTCM and the technical aspect of IVM and IVF of the oocytes are shown in Table one and two respectively.

Results:

The number of mature oocytes in large size ovaries (> 30 mm) was significantly ($P<0.01$) higher compared to 20-30 mm and <20 mm ovarian sizes (Table 1). The percentage of germinal vesicle oocytes was significantly higher (pcO.05) at 72 hours (h) compared to 120 and 144 h. The percentage of viable oocytes at 72h was significantly higher ($p<0.01$) than 96h, 120h, and 144h (Table 2).

The IVM rate of the oocyte was significantly higher ($P<0.05$) at 72 and 96h compared to 48, 120 and 144h (Figure 1). The removal of cumulus cells at 72h and 96h resulted in 17.9% and 14.8% WM rate respectively (Figure 2). The viability of the cultured oocytes at 72h was significantly better than 96h ($P<0.001$, Table 3). The IVM rate per viable oocytes was 25.6% at 72h and 30.2% at 96h (Table3).

The sperm motility index (SMI = motility percentage X sperm activity grade) was significantly higher in sperm retrieved from the vas deference compared to epididymal and testicular sperm (259 versus 95 and 19.2 respectively, $P<0.05$, Table 4). Normal sperm morphology was significantly higher in vassal sperm (VS) compared to epididymal sperm (ES) and testicular sperm (TS). Abnormal

sperm forms were significantly higher in TS compared to other groups. The concentration of sperm cells in the testes was significantly higher than epididymis and vas deference groups (Figure 3).

The mature oocytes were inseminated by vassal sperm following invitro hyperactivation with HEPES solution supplemented with 3 mg/ml bovine serum albumin. The IVF rates of the oocytes following 72 and 96 hours of maturation in vitro were 48.2% and 40%, respectively (Table 5). Sperm penetration was significantly higher at 96h compared to 72h and the number of sperm heads inside the ooplasm was 3.2 for the 72h group versus 4.8 for the 96h group (P<0.05).

Penetration of immature oocytes at metaphase 1 (M1) by vassal sperm significantly induced resumption of meiosis compared to epididymal or testicular sperm penetration.

Table 1: Composition of modified Tissue Culture Medium-199 (MTCM):

Compound	Quantity
1. Progesterone	5 ug/ml
2. Epidermal Growth Factor	100 ng/ml
3. Human Chorionic Gonadotrophin	10 IU/ml*
4. Insulin	5 ug/ml
5. Epinephrine	0.50 mM
6. Estrus Bitch Serum	10%
7. Non-Essential Amino Acids	0.01 mM
8. Gentamicin	20 ug/ml
9. TC-199 Supplemented with Earle's Salts, 2200 mg/ml Sodium Bicarb, 25 mM HEPES and 2 mM Sodium Pyruvate	100 ml

* The oocyte-cumulus-Complex mass was exposed to the MTCM containing HCG for the first 45 hours of IVM and then culture in same medium free from HCG for another 24-48 h.

Table 2: Technical aspect of IVM of the ovarian follicular oocytes:

1. Used always overnight fresh equilibrated culture medium with 5% CO2 and replace the culture medium every 24 hours.
2. Remove the cumulus cells after 72 hours of culture period.
3. Used unexpired TCM-199.
4. Select large ovaries size > 30 mm and use size > 110 u oocytes for IVM.
5. Expose oocytes to HCG-modified-TCM-199 for the first 48 hours of culture period and then culture is modified TCM-199 without HCG.
6. Aspirate the old culture gently by micropipette and replace with new overnight culture.
7. Watch for humidity, CO2 concentration.
8. Check osmolarity and pH of the MTCM
9. Check sterilization of culture medium.
10. Avoid micromanipulation and exposing the oocytes to light and change in temperature.

Table 3: The effect of ovarian sizes on the in vitro maturation of canine oocytes using modified TC-199 medium supplemented with reproductive hormones and epidermal growth factor

Ovarian Sizes	No. Cultured in Vitro	No. Viable	No. In Vitro Matured (MII)	No. Degenerated
> 30 mm	178	69.1%*	17.9%**	31%*
20-30 mm	88	54.6%	9.1%	45.4%
< 20 mm	135	40.7%***	3.7%***	59.3%***

* P<0.01 significantly different from other groups.
** P<0.05 significantly higher than other groups
*** P<0.01 significantly lower than other groups

Table 4: The effect of in vitro culture period of canine ovarian follicular oocytes on in vitro maturation, viability and germinal vesicles break down rates using modified tissue culture medium- 199 (MTR0506)

Culture Period(hours)	Number Cultured	Number with Polar Body	Percent P.B1	Number Viable	Recent Viable	Number W/GV	Percent GV
48	184	11	6.0	128	69.6	70	38.0
72	126	19	15.1*	85	67.5**	40	31.8***
96	124	21	16.9*	65	52.4	35	28.2
120	121	15	12.4	51	42.1	25	20.7
144	154	14	9.1	54	35.1	24	16.7

* P<0.05 significantly different from 48h, 120h and 144h groups
** P<0.01 significantly different from 96h, 120h and 144h groups
*** P<0.05 significantly different from 120h and 144h groups

Table 5: The effect of the removal of cumulus cells at 72 h and 96 h on in vitro maturation of canine oocytes cultured in modified Medium-199 (MTR0506)

Removal Time of Cumulus cells	Total No. of Cultured Oocytes	IVM/Total Oocytes	Percent Viable Oocytes/Total	IVM/Viable Oocytes
72 Hours	240	43/240	168/240*	43/168
Percentage		17.9	70.0	25.6
96 Hours	236	35/236	116/236	35/116
Percentage		14.8	49.2	30.2
Total	476	78/476	284/476	778/284
Mean %		16.4	59.7	27.5

* P<0.001 significantly different from 96h group
IVM = Mature oocyte at Metaphase-II

Table 6: In vitro sperm hyperactivation following sperm retrieval from vas deferens, epididymis and testes

Sperm Source	Concentration (x million/ml)	Motility % +/- SE	Normal Morph % +/- SE	Activity Grade (0-4) +/- SE	SMI****
Vasal Sperm	18	74 +/- 10.3*	65 +/- 6.8*	3.5 +/- 0.1	259*
Epididymal Sperm	22	38 +/- 7.6	46 +/- 10.5	2.5 +/- 0.2	95**
Testicular Sperm	28***	14.8 +/- 4.1	12 +/- 5.2	1.3 +/- 0.2***	19.2

* P<0.01 significantly different from epididymal and testicular sperm groups.
** P<0.05 significantly different from testicular group
*** P<0.05 significantly different from corresponding groups
**** SMI: Sperm Motility Index = Sperm motility % * Sperm Activity Grade

Table 7: In vitro maturation and fertilization of canine oocytes using vassal spermatozoa

Culture Period	Number Cultured in Vitro	Number Viable	Number Matured Met-I	Number Matured/ Viable oocyte	Number Fertilized	Sperm Penetration Index (SPI)
72h	176	115/176*	27/176	27/115	13/27	3.2
Percentage		65.3	15.3	23.5	48.2	
96h	201	111/201	30/201	30/111	120/30	4.8**
Percentage		55.7	14.9	27.0	40.0	
Total	377	226/377	57/377	57/226	25/57	4.0
Mean %		59.9	15.1	25.2	43.9	

* P<0.05 significantly different from corresponding group
** P<0.05 significantly different from 72h group
SPI = No. of sperm penetrated heads/oocyte

Discussion:

The number of matured oocytes which was retrieved from large ovaries was significantly higher compared to medium and small size ovaries. This may be due to the fact that large ovary contains large oocytes. It was reported in the literature that oocytes with >110 micron in diameter progress to MII while those < 110 micron stay at germinal vesicle stage of development. There is a clear relationship between oocyte diameter and meiotic competence (7). The percentage of viable oocytes at 72h was significantly higher than 96,120, and 144h. This is in good agreement with the observation that oocytes viability in culture decreases with an increase in time of duration (8, 11).

The in vitro maturation rates (IVM) of the oocytes were significantly higher at 72h and 96h versus 48,120, and 144h while the removal of cumulus cells either at 72h or 96h had no significant effects on IVM. Cumulus cells communication with the oocytes at the early stages of development may have significant role in IVM process. It seems that 72-96h culture duration considered being optimal period for IVM of canine oocytes (10-11). The IVM rate per viable oocytes was similar at 72h versus 96h (P<0.05). This indicates that some of the matured oocytes may start to degenerate at 96h of culture duration (12).

The motility index of the vassal sperm was significantly higher compared to epididymal and testicular sperm and this may be due to the fact that vassal sperm are completed their maturation in comparison to other groups (13). The IVF rate at 72h was not significantly different than at 96h. This indicates that the sperm cells may undergo degenerative changes at 96h. On the other hand, an increase in sample size may be advisable since Chi-square test requires large sample size to get significant P value (14). It was reported in the literature that sperm penetration can occur in vitro in immature canine oocytes and that this induces a resumption of meiosis. At 72h, the percentage of oocytes with germinal vesicle decreased significantly and the percentage of oocytes beyond metaphase I increased. At 96h the number of the oocytes with sperm head penetrations increased significantly compared to 72h. This may be due to the aging of the oocytes which allow polyspermic fertilization. Canine sperm can penetrate immature oocytes (15-16).

In conclusion, the data of the present study demonstrated that canine oocytes are adequate model for aged infertile couples with azoospermia.

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