ISSN 2307-8073 MRVSA Publisher House
Fundamental Novelty of research

Mirror of Research in Veterinary Sciences and Animals (MRVSA)
Journal homepage: http://mrvsa.com/
DOI: 10.22428/ MRVSA. 01.01.017

Original Article

Impact of xenotransplantation of sheep ovarian cortex and follicular fluid- enriched SMART medium on the morphology of recovered of sheep oocyte

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ARTICLE INFO

Received: 28.12.2016
Revised: 28 .01. 2017
Accepted: 29.01. 2017
Publish online: 01.02.2017

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Abstract

Ovarian follicles and cortical tissue can survive viable after ovariectomy and use successfully for xenotransplantation or in vitro maturation. This study designed to assess in vitro maturation (IVM) of sheep oocytes recovered from immature follicles using simple medium for assisted reproductive techniques (SMART) medium enriched with follicular fluid (FF) using xenotransplanted ovarian tissue cortex inside the body of female mice injected with or without different hormonal stimulation protocols. In this study, follicular fluid (FF) aspirated from randomly sheep ovarian follicles. Seventy-five healthy and mature female mice were used for transplantation of sheep ovarian tissue (OT) on the inner side of the peritoneum. Later on, these female mice were classified into two groups. Group A: control (without medication). Group B: hormonal programs (hormonal stimulation). The last experimental group was Group C: the direct examination of sheep ovarian cortex group. The sheep ovarian cortex was recovered from female mouse then oocytes were collected by slicing for assessment and classified into three groups. Group1: oocytes incubated within SMART medium alone (control group). Group 2: oocytes incubated within SMART medium enriched with 5% FF, and Group 3: oocytes incubated within SMART medium enriched with 10% FF. The normal oocytes morphology was assessed post-xenotransplantation and parameters were statistically analyzed. No significant (P> 0.05) difference of normal oocytes morphology was seen between treated group (GB) and control group (GA). However, significant (P= 0.043) difference was observed in the percentage of normal oocyte morphology of recovered oocytes for the group (GC) in compare to (GA). Meanwhile, there was highly significant (P< 0.001) difference between GB and GC groups. Addition 5% FF to SMART medium of GB revealed significant (P= 0.044) difference in compare to the GA. Moreover, highly significant (P< 0.001) difference was observed in GC in compare to GA and GB compared to GC. Meanwhile, addition 10% FF to SMART medium of G3 revealed significant (P< 0.05) difference in the percentages of the normal and abnormal morphology of recovered oocytes. In conclusion, this study approved that normal oocyte morphology and maturation was valuable when using ovarian tissue grafts. In addition, the combination of SMART medium with 10% FF was revealed the best oocyte morphology and maturation.

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To cite this article: Muhammad Baqir M.R. Fakhrildin; Nadia J. Sudani Al-Behadili ; Mohammad Oda Selman. (2017). Impact of xenotransplantation of sheep ovarian cortex and follicular fluid-enriched SMART medium on the morphology of recovered of sheep oocyte. MRVSA. 6 (1), 9-18.

Key words: Ovarian tissue transplantation, in vitro maturation, xenotransplantation.

Introduction

Xenotransplantation is an approach in which human ovarian tissue is transplanted into immune-deficient patient (National Center for Health Statistics, 2010). It has been explored as a method to eliminate the risk of cancer transmission and recurrence and as a possible application for women with hormone-sensitive malignancies. Moreover, follicular development can be easily monitored and the tissue is readily accessible for oocyte retrieval. For now, xenotransplantation remains a scientific challenge at the bench and will not be translated into applications until the safety issues can be resolved (Seli &Tangir, 2005). The development of technologies to grow and mature oocytes from ovarian tissue holds many attractions for assisted conception, clinical practice, human and animal production technology and research. However, despite much research attention, it has proved difficult to grow follicles from early stages to maturity in vitro (Picton *et al.*,2003). Ovarian tissue transplant (OTT) is the technologically advanced and successful of all the reproductive tissue transplants (RTTs), with a number of human experiments showing great promise. It is considered experimental, and all major reproductive societies recommend that protocols for human experimentation be regulated by research standards (Blake, 2013). In general, OTT would hold potential for a wide variety of individuals with ovarian factor infertility, most especially women undergoing cancer treatment who may be able to bank ovarian tissue prior to undergoing chemotherapy or radiotherapy (Mahajan, 2015). The technique of OTT could permit a woman to conceive naturally with her own eggs provides the pregnancy chances at the right time in patients or those who want to postpone childbearing for social or financial reasons. However, OTT present even newer issues than hand and face transplants because they are intended not only for life improvement but the creation of life as well (Hosseini *et al.*,2014). The full potential of this tissue to restore fertility could be achieved by the development of in vitro systems that support oocyte development from the most immature stages to final maturation. The techniques of in vitro maturation (IVM) are being developed in human (Telfer & Zelinski, 2013). However, IVM refers to the maturation of immature oocytes in culture after their recovery from small antral follicles at the stage prior to selection and dominance. It has been proposed as an alternative to conventional in vitro fertilization (IVF), since it reduces the primary adverse effects caused by controlled ovarian stimulation (Chang *et al.*,2014), and it is for woman who is not able to undergo hormone treatments because of her type of cancer (Woodruff, 2010). Thus, IVM becomes an efficient means of obtaining healthy birth because of the short time necessary to reach oocyte retrieval. Furthermore, IVM is a promising technique

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for fertility preservation (Grondahl,2008). Review of literature revealed paucity publication regarding the applications of xenotransplantation of ovarian cortex and follicular fluid- enriched SMART medium. Therefore, this study was designed to assess the in vitro maturation (IVM) for sheep oocytes recovered from immature follicles and its application as xenotransplanted ovarian tissue cortex inside body of female mice injected with or without different hormonal stimulation protocols.

Materials and Methods

Animals (Sheep and Mice)

This study was approved from research and animal ethical committee in the college. Seventy-five healthy, non-pregnant and mature female mice were received from the animal house unit / High Institute for infertility diagnosis and assisted reproductive technologies ((HIIDART), were used in this study. In addition, the ovaries were collected randomly from ewes which were slaughtered in Al-Shualla local abattoir. All collected ovaries were transferred immediately within one hour to the HIIDART/AL-Nahrain University laboratory. This study was conducted during the period from September 2015 to April 2016.

Collection of Sheep Ovaries

The ewes ovaries were collected from a local abattoir (AL-Shullah) at Baghdad. Both ovaries collected from each animal immediately after slaughtering and kept in sterile normal saline solution (0.85% NaCl) supplemented with antibiotics (100µg/ml streptomycin, 100IU/ml penicillin, and 100µg/ml metronidazole). Later on, samples were placed into thermos at 30-35 °C. The ovaries were transported to the HIIDART laboratory within less than 1 hour. At the laboratory, the ovaries were washed three times with warmed (30 °C) normal saline supplemented with antibiotics (100IU/ml penicillin and 100µg/ml streptomycin) to insure the removal of blood clotting and reduce the contamination on the ovarian surfaces (Amitabh *et al.*,2015).

Aspiration and Preparation of Follicular Fluid

The follicular fluid (FF) from the visible ovarian antral follicles on the ovarian surface, was aspirated using disposable syringe containing SMART medium (0.5ml) and heparin 0.01. The FF transferred to the test tube and centrifuge at 2600 rpm/8 min. at 15ºC. The aspirated upper layer of FF kept in the water bath at 56 ^{0}C for 30min and then frozen at -20 °C until use (Salha *et al.*,1998).

Preparation of Ovarian Cortex Fragments

Each ovary divided into two halves, then cortex and medulla were separated. Then, the cortex of each ovary cut into small fragment (2X2 mm in area and 1mm in

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thickness) (Richards & Pangas, 2010). The fragments transferred into Petri dish containing 0.5mL of SMART medium to xenotransplantation (Faddy,2000).

Xenotransplantation of Ovarian Cortex

The female mice anesthetized using inhaled Ether, and a small slit made in the abdomen and peritoneum from one side of the body, the sheep ovarian cortex fragments transplanted to the inner side of the peritoneum with the abdominal wall and closed by 1 or 2 stitches. Each mouse was administered Prisolone (Prednisolone) (0.178µg) orally daily for immunological inhibition. The sheep ovarian cortex was taken out from mice and washed three times in SMART medium, and then oocytes were collected by slicing ovarian cortex fragment (Järvelä *et al.*,2002).

Ovulation Induction Programs (OIP)

-Group A: control / female mice with no medication.
-Group B: hormonal programs / female mice under hormonal stimulation protocols. Day1: FSH 15 IU. Day2: FSH 15 IU. Day3: FSH 15 IU. Day4: FSH 10 IU + HCG 10 IU. Day5: HCG 10 IU.
- Group C: direct examination of sheep ovarian cortex.
The IVM group, oocytes classified into three groups:
-Group 1: oocytes with SMART medium alone (control group).
-Group 2: oocytes with SMART medium enriched with 5% FF.
-Group 3: oocytes with SMART medium enriched with 10% FF.

Assessment of Ova Morphology after IVM

All obtained oocytes were washed three times in SMART medium containing 20% HSA, then, about 5-10 oocytes per droplet (1ml) from culture SMART medium supplied with HCG (5 IU/ml), PMSG (10 IU/ml) and Estradiol (1µg). Each 5-10 oocytes per droplet (1ml) was supplied with 5% FF and another with 10% FF, then cultured within four well petri dish, covered by liquid paraffin and incubated for about 24hr in CO2 incubator (5% CO2) at 38.5^{o}C with high humidity (95%). The oocytes were examined under an inverted-phase microscope to assessment of ova morphology (Lebbe& Woodruff, 2013). The percentage of IVM was recorded based on the following equation: IVM% = mature oocytes / cultured oocytes *100

Statistical Analysis

The data were statistically analyzed using statistical package for social sciences (SPSS version-21(by application the number and percentage of Chi-square analysis test), a (p) value of < 0.05 considered statistically significant (Moran & Carter, 2013).

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Results

Effect of Xenotransplantation Techniques on Morphology of Recovered Sheep Oocytes of Control Group (G1)

The percentage of recover normal morphology sheep oocytes using ovarian xenotransplantation techniques revealed no significant (P> 0.05) difference of normal sheep oocytes morphology between treated group (GB) and control group (GA). A significant difference (P= 0.043) observed in the percentage of normal morphology of oocytes that recovered from group (GC) sheep when compared to control group (GA). However, there is highly significant (P< 0.001) between group (GB) and group (GC) as shown in Table (1).

Effect of Xenotransplantation Techniques on Morphology of Recovered Sheep Oocytes of SMART medium Enriched with 5% FF, (G2)

A significant difference appeared in the percentages of recovered normal sheep oocytes morphology for the group (G2), addition 5% FF to SMART medium of the hormonal stimulated group (GB) when compared to control group (GA). Meanwhile, a highly significant (P< 0.001) difference recorded in the group (GC) in comparison to the control group (GA) and group (GB) when compared to group (GC), (Table 2).

Effect of Xenotransplantation Techniques on Morphology of Recovered Sheep Oocytes of SMART medium Enriched with 10% FF, (G3)

The percentages of normal and abnormal morphology of recovered sheep oocytes when adding 10% FF to SMART medium of group (G3) using ovarian xenotransplantation techniques (Table .3). Both treated (GB) and (GC) groups showed the significant differences (P= 0.049) and (P= 0.047) respectively, in comparison to control group (GA). Moreover, a significant difference (P= 0.035) also seen in group (GB) in comparison to group (GC).

Table 1: Comparison among the percentages of normal sheep oocytes morphology for control group, hormonal stimulated group and direct examination of sheep ovarian cortex group following addition of SMART medium

Xenotransplantation G 1 (Control group)	Morphology of recovered oocytes					
	Normal		Abnormal		Total	P-value
	No.	%	No.	%		
G A (Control group)	26	68.4	12	31.6	38	-
G B (Hormonal stimulated group)	29	70.7	12	29.3	41	>0.05 NS
G C (Direct examination of sheep ovarian cortex group)	20	47.6	22	52.4	42	=0.043 S <0.001 HS

GA&GB: *P*>0.05 Non Significant; GA&GC: *P*= 0.043 Significant; GB&GC: *P*<0.001 High Significant

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Table 2: Comparison of normal sheep oocytes morphology percentage between control group, hormonal stimulated group and direct examination of sheep ovarian cortex group following addition of SMART medium enriched with 5%FF.

Xenotransplantation G 2 SMART medium enriched with 5% FF	Morphology of recovered oocytes					
	Normal		Abnormal		Total	P-value
	No.	%	No.	%		
G A Control group	29	74.4	10	25.6	39	-
G B Hormonal stimulated group	32	71.1	13	28.9	45	=0.044 S
G C Direct examination of sheep ovarian cortex group	19	46.3	22	53.7	41	<0.001 HS
						<0.001 HS

GA&GB: P=0.044 Significant; GA&GC: P<0.001 High Significant; GB&GC: P<0.001 High Significant

Table 3: Comparison of normal sheep oocyte morphology percentage between control group, hormonal stimulated group and direct examination of sheep ovarian cortex group following addition of SMART medium enriched with10% FF

Xenotransplantation G 3 SMART medium enriched with 10% FF	Morphology of recovered oocytes					
	Normal		Abnormal		Total	P-value
	No.	%	No.	%		
G A Control group	24	58.5	17	41.5	41	-
G B Hormonal stimulated group	26	56.5	20	43.5	46	=0.049 S
G C Direct examination of sheep ovarian cortex group	19	43.2	25	56.8	44	=0.047 S
						=0.035 S

GA&GB: P=0.049 Significant; GA&GC: P=0.047 Significant; GB&GC: P=0.035 Significant

Discussion

Oocyte morphology can be considered as a marker for predicting embryo quality and implantation potential, in addition, to use the standard morphological embryo scoring (Cauffman, 2015). Results of the present work showed different percentages of normal oocyte morphology when using different protocol and techniques post-xenotransplantation.

Non-significant (P >0.05) difference observed between (GB) (hormonal stimulated group) in compare to GA (control group). However, high significant (P >0.001) difference seen in GC (direct examination of sheep ovarian cortex group) in compare

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with (GB). These results might be due to many factors that impact the normal morphology of recovered oocytes. Temperature and pH are the most crucial factors which should be managed during the procedure of oocyte retrieval. In fact, oocytes are extremely sensitive to changes in temperature, especially declines (Wang, 2001). On the other side, pH alternations during oocyte handling may have the adverse aftermath, because the cellular metabolism and cytoskeletal dynamics are highly dependent on pH (Cauffman, 2015). Meanwhile, the best percentage observed in the hormonal stimulated group (GB) and revealed a significant difference ($P<0.05$). Besides, a significant ($P<0.05$) difference noticed in the percentage of normal oocyte morphology of (GB) group when 10% of FF added to the SMART medium. The impact of follicular fluid (FF) on medium provides an essential microenvironment for the development of oocytes. It is feasible to anticipate that few biochemical characteristics of the FF enclosing the oocyte may act a challenging role in determining oocyte quality and the subsequent potential to achieve fertilization and embryo development (Revelli, 2009). A previous study warns that the differences in the composition of the medium can alter the percentage of oocytes completing maturation. Moreover, the follicular fluid combined with hormones appeared to give better conditions for pig oocyte maturation in vitro (IVM) (Margot and Charles, 2001). However, SMART medium owns all the necessary ingredients for activation and growth of oocytes and sperm. Fakhrildin and Aljuaifri, (2015) also mentioned these observations. They concluded that the vitrification technique with cryoprotectants EG and PrOH supplemented with sucrose used SMART medium recorded improvement in the ability of oocytes for IVF and early embryonic development post-xenotransplantation (Fakhrildin and Aljuaifri, 2015).

In this study, a high percentage of normal morphology of recovered oocytes was noticed in the (G2) (hormonal stimulated group), these female mice injected gonadotropins for five days. The results of the current study are in agreement with work of Mendoza *et al.,* (2002), who cited that adding LH to the medium is seen to be constantly higher in follicles containing oocytes and lead to successful IVF attempts with embryos (Mendoza *et al.*,2002). However, OTT in mice approved the results of previous work carried out by Weissman and his colleagues (Weissman *et al.*,1999). The intrafollicular concentrations of FSH and LH are affected by their circulating levels. High concentrations of FSH, hCG and LH have been reported to promote normal oocyte maturation and related to large fertilization chance of eggs (Armstrong *et al.*,1991). In contrast, it is mentioned that the elevation level of FSH produces high numbers of abnormal and atretic oocytes (Falcone *et al.*,2004). The results of the present work also revealed that the levels of hCG hormones, FSH and LH was identified as the physiologic stimulants of oocyte maturation during the final stages of development (Armstrong *et al.*,1991). It appears that higher levels of gonadotropins would improve these processes and lead to normal oocytes and better embryos. Meanwhile, Fakhrildin *et al.*,(2006) reported that low dose of hCG could support oocyte maturation. In conclusion, this study approved that ovarian tissue grafts offered valuable normal oocyte morphology and maturation. Moreover, the best

oocyte morphology and maturation was observed in the combination of SMART medium with 10% FF. The authors recommend further future studies on the in vitro maturation and fertilization of retrieved ova and embryo transfer.

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