Original Article

Isolation of granulosa cells from follicular fluid; applications in biomedical and molecular biology experiments

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Abstract

Background: Recently, a lot of research has been conducted to investigate the molecular mechanisms of the low quality of oocytes with granulosa cells (GCs). GCs are one of the major cell types found in follicular fluid and purification of these cells from the follicular fluid is very important for further studies. Although, there are different techniques of purification, a method for separation of highly-pure and minimally-damaged cells is necessary. In this paper, we presented a novel method for high purification of GCs with a large quantity and high purity.

Materials and Methods: Follicular fluid was collected from patients who referred for *in vitro* fertilization and GCs in follicular fluid were extracted by Ficoll, Percoll and Red blood cell lysing buffer (RLB) methods. Then purity of extracted GCs was assessed by flow cytometry and morphological properties of GCs were observed by differential interference contrast microscopy. The purity of deoxyribonucleic acid and ribonucleic acid extracts was examined by NanoDrop 1000, pre-restriction fragment length polymorphism and electrophoresis techniques. Quality and quantity of extracting GCs were affected during the cell separation procedures.

Results: Our results showed that each of purification method can affect quality and quantity of extracted cells.

Conclusion: RLB method for extraction of GCs was shown to be a convenient procedure in comparison with Ficoll and Percoll methods.

Key Words: Ficoll, follicular fluid, granulosa cell, percoll, red blood cell lysing buffer

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INTRODUCTION

Follicular fluid harbors cumulus cells and mural granulosa cells (GCs). In the antral follicle, cumulus cells surround the oocyte and mural GCs which make the follicular wall. Therefore, there are three major cell types in the ovarian follicle including; oocyte, GCs and theca cells.[1] The oocyte is in the center of the follicle and is surrounded by GCs, the theca cells are in the external layer of the follicle, separated from GCs by a basement membrane.[2] Studies have shown that these cells and oocytes have a bi-directional communication with each other, which is essential for follicular differentiation processes including oocyte development, ovulation, fertilization and subsequent

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implantation.[3] Quality and quantity of GCs can be affected by oocyte quality.[4] Simultaneous with oocyte maturation, some of the genes are expressed in GCs and their expression levels are different in the various physiological conditions and the embryo quality depends on the final maturation of the follicle.[5] GCs secret a wide variety of hormone and growth factors that may affect oocyte maturation; therefore, the expression evaluation of these factors or genes in GCs can predict follicular health. Furthermore, the biochemical and molecular assessment of GCs may generate additional information that is necessary for understanding of successful oocyte fertilization.[6]

In *in vitro* studies, GCs are often taken from patients undergoing *in vitro* fertilization (IVF). These cells are limited in the number and half-life because they are under stimulation with supraphysiological doses of follicular stimulation hormone and human chorionic gonadotropin during assisted reproductive technique.[7] During puncture, after taking oocytes, GCs, which are abundant in follicular fluid, are usually discarded. Both free cells and aggregated GCs are present in this fluid. Red blood cells and white cells are also rich in these samples.[8] Since, follicular fluid contains a heterogeneous population of cell types; purification of human GCs for *in vitro* studies is inevitable. For further studies, separating GCs from follicular fluid with high quality and sufficient number is essential.

In recent years, researchers have used different methods to extract GCs, each having their own advantages and disadvantages.[9,10] Furthermore, the choice of method affects the quality and quantity of nucleic acids as well as GC survival. The purpose of our study was to find a suitable method for the extraction of GCs from follicular fluid that it can provide highly pure ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) for use in a range of molecular studies and cause less damage to GCs.

MATERIALS AND METHODS

Materials

For cell isolation Percoll solution was purchased Sigma-Aldrich (Dorset - UK) and Ficoll-Paque solution was supplied by Amershan Pharmacia Biotech (Uppsala - Sweden). For making Red blood cell lysing buffer (RLB) solution, Tris-HCl was obtained from Sinagen (Tehran - Iran) and $MgCl_2$, NaCl was purchased from Merk, (Darmstadt - Germany). CD45-Fluorescence Isothiocyanate (FITC) was purchased, Sigma-Aldrich, (Dorset - UK). RNA extraction was made by RNAX-Plus solution that was supplied by Cinnaolon, (Tehran - Iran). DNA digestion was made by EcoRI that was purchased from Fermentase (Germany).

Purification of GC from the follicular fluid

Subjects were selected from Alzahra Hospital of Tabriz, Iran who had been admitted for IVF. The Ethics Committee of the Hospital approved this study and subjects signed consent form before participation. Among subjects of oocyte donors, patients who had an average age less than 30 years were selected. During the puncture, after removing the oocytes, follicular fluids were collected separately from individuals. Each sample was poured at a sterile tube and was tested in less than an hour.

These samples were initially divided into three groups. In the first group, GCs were extracted from follicular fluid using a 50% Percoll (Dorset - UK) gradient.

In the first group, aspirated follicular fluid was centrifuged at 1000 g for 3 min at 21°C, then 4 ml of phosphate buffer saline (PBS) was added to the pellet and it was slowly layered on a 50% Percoll gradient and centrifuged at 400 g for 30 min at 21°C. The cells, which were at the interface of Percoll and serum were removed by using a Pasteur pipette and washed a few times with PBS.[11] Samples were taken for cell count and viability testing by trypan blue, RNA and DNA extraction.

In the second group, after retrieval of oocytes, follicular fluids that contained GCs and heterogeneous cells were centrifuged at 1000 g for 3 min at 21°C. Then, 4 ml of PBS was added to pellets and diluted solution was layered carefully on 6 ml of Ficoll-Paque (Uppsala-Sweden).[12] The samples were centrifuged at 400 g for 30 min at 21°C. The cells that were in the interface were removed and taken for cell count, viability testing, RNA and DNA extraction.

In the third group, the follicular fluid without oocytes, was pooled and transferred to 50 ml of sterile polypropylene centrifuged tube. The tube was centrifuged at 1000 g for 2 min at 21°C and then 20 ml RLB was added to the pellets. RLB solution contained 2 M Tris-HCL (Tehran-Iran) with pH 7.6 and 1 M $MgCl_2$ (Germany) and 3 M NaCl (Darmstadt-Germany).[13] The diluted solution was kept at room temperature for 2-5 min and occasionally the tube was agitated gently and centrifuged at 300 g for 3 min at 21°C. Then, the pellet in each tube was washed with PBS and used for counting, viability testing and RNA-DNA extraction. Table 1 presents the view granulosa cells that isolated from folicles in the three groups.

Flow cytometry method

Aggregated GCs were washed, resuspended in 1 ml PBS with 1% BSA. The clumps of GCs were mechanically dissociated by gently pipetting the

sample several times with Pasteur pipettes with different diameters. The obtained cell suspensions were selected against CD45, a surface marker specific for leukocytes. To perform this, 5 µl of CD45-FITC, Sigma-Aldrich was added into falcon tubes and 100 µl of the cell suspension of 1×10^6/ml were added and gently mixed. Then, the sample incubated for 30 min in the dark at room temperature. Cells were washed twice with PBS and centrifuged at 300 g for 5 min. The sample was fixed by 200 µl of 0.5% formaldehyde and stored in the dark until analysis.

RNA and DNA extraction

Total RNA of purified cells was extracted by RNAX – Plus solution (Tehran-Iran) according to the manufacturer's instructions. The quantity of RNA was measured by NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE, USA).

The DNA extraction process was carried by cetyl trimethyl ammonium bromide lysing procedure.[14] The quality of extracting DNA was evaluated by restriction digestions and agarose gel electrophoresis.

DNA digestion with pre-restriction fragment length polymorphism (RFLP) method

For further evaluation of the quality and purity of the samples, extracting DNA was digested by pre-RFLP method. For each digestion, in a total volume of 50 µl, 5 µg (10 µl) of genomic DNA, 5 µl of ×10 fast digest buffer and 5 µl of restriction enzyme EcoRI (Germany) were added to a sterile tube and incubated at 37°C for 6 h.

RESULTS

Morphological study

The aim of this study in these series of experiments was finding the most reliable method for the extraction of GCs that increase the amount and purity of extracted cells, causes less damage to cells and has no effect on cell survival, on its function. Besides it should provide highly pure RNA and DNA for use in a range of molecular studies.

GCs are relatively big cell with higher lipid granulity and compared with GCs, the leukocytes are smaller, lighter and less granulity.[15] Figure 1 showed image of GCs, which have been extracted with the mentioned purification methods. In this Figure, 1a and b showed extracted cells by the Ficoll method. As it is clear, single primarily GCs and few debris or deformed GCs were observed in this method. In some cases, crystals of sucrose have been shown [Figure 1a]. Figure 1c and d showed GC extraction by Percoll method. The quantity of leukocyte and derbies decreased, but single and relatively aggregated GCs have been observed in this method. Finally, Figure 1e and f showed GCs extracted by RLB method. As it is shown, there were huge bundles of aggregated GCs and the number of leukocytes and erythrocytes carcasses was negligible.

Table 1: The number of retrieved oocyte and extracted granulosa cells and their viability % in Ficoll, Percoll and RLB methods

Methods	No. of retrieved oocytes	No. of granulosa cells (cells/ml)	Viability %
Ficoll method	8 (6-10)	$0.7\times10^6\pm0.2\times10^6$*	65-70
Percoll method	9 (7-12)	$1.3\times10^6\pm0.3\times10^6$*	75-80
RLB method	9 (7-11)	$1.7\times10^6\pm0.5\times10^6$*	75-80

*Data are mean±SD; No significant difference was found between groups by a $P<0.05$; RLB: Red blood cell lysing buffer

Flow cytometry study

The flow cytometric distribution pattern of GCs showed in Figure 2. To determine whether the GCs exist in any of the suspension and are major population of cells, flow cytometry analysis was performed and during flow cytometric analysis, GCs were identified as CD45 negative cells. A minimum of 10,000 cells was counted in each experiment and the percentage of CD45 positive cells was determined in each population. The flow cytometric distribution pattern of extracted cells suspension with Ficoll method showed that 45-55% of the population were CD45 positive cells while 95-98% isolated GCs were pure as assessed by flow cytometry

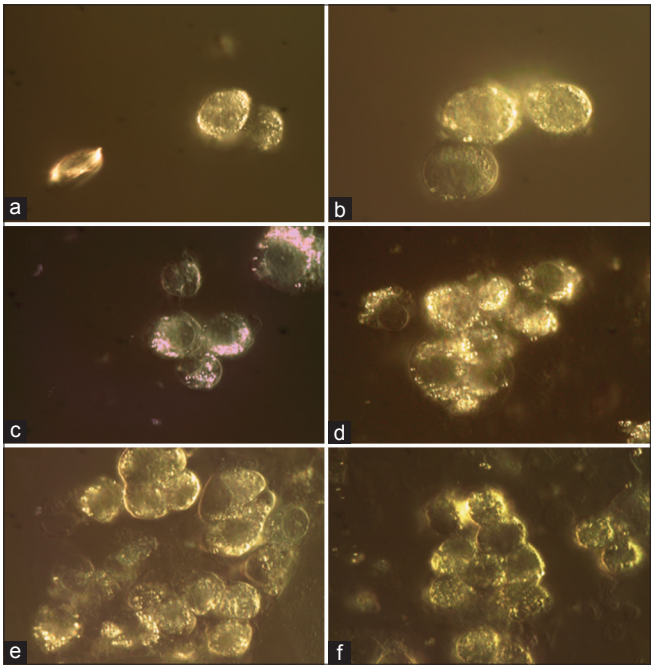

Figure 1: The images of extracted granulosa cells (GCs) with the mentioned methods (a and b) demonstrates images had been taken for extracted GCs with Ficoll method. (a) The Ficoll crystals and presence of free GCs with deformed cells in the sample and (b) the presence of GCs with lipid granules in their cytoplasm and mononuclear cells without lipid granules. (c and d) by Percoll method, the existence of relatively aggregated GCs with high lipid granulity, (e and f) using red blood cell lysing buffer method, which showed huge bundles of aggregated GCs

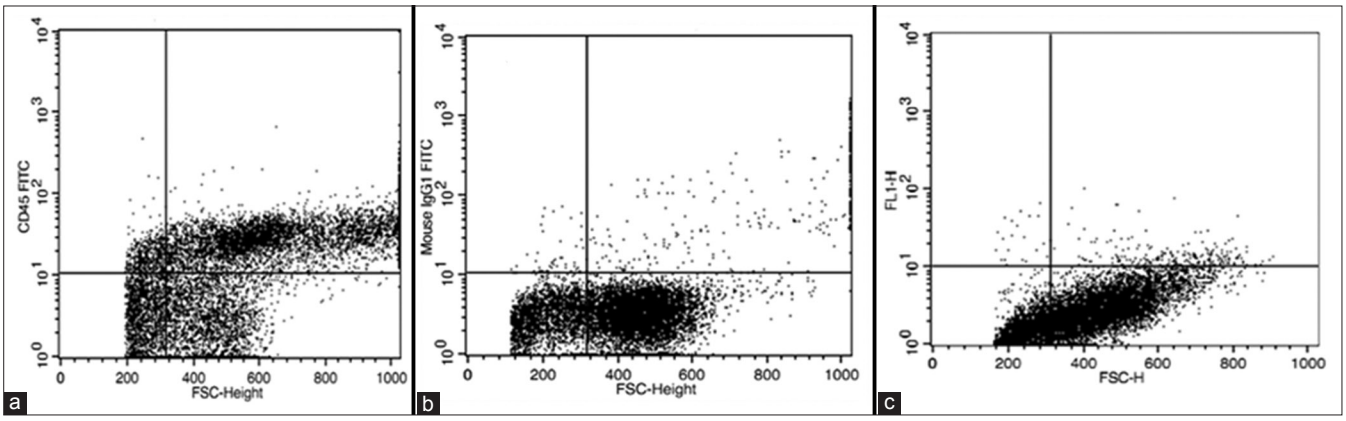

Figure 2: The flow cytometric distribution pattern of extracted granulosa cells (GCs) from (a) Ficoll, (b) Percoll and (c) red blood cell lysing buffer method. GCs identified as CD45 negative cells and leukocytes as CD45 positive cells

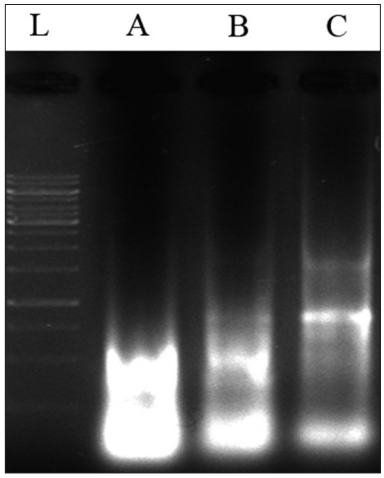

Figure 3: Distribution pattern of ribonucleic acid (RNA) on agarose gel electrophoresis from extracted granulosa cells (GCs). (a) Extracted RNA from GC that separated by Ficoll method and showed distribution pattern of RNA degradation, (b) Extracted RNA from isolated GCs by Percoll method and (c) The bands of RNA from extracted GCs by red blood cell lysing buffer method in the presence of Fermentase 1 Kb Deoxyribonucleic acid ladder

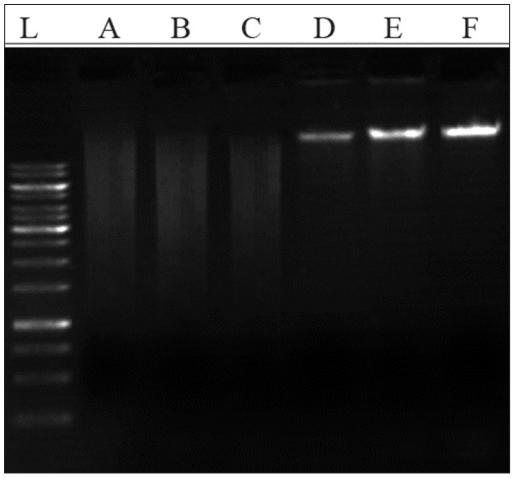

Figure 4: Distribution pattern of extracted deoxyribonucleic acid (DNA) from granulosa cells (GCs) isolated by two common protocols and the presented protocol and pre-restriction fragment length polymorphism results: L) Fermentase 1 kb DNA Ladder; lines A-C, the digestion of extracted genomic DNA by restriction enzyme EcoRI. (a) Extracted DNA of GCs by Ficoll method, (b) Extracted DNA of GCs by Percoll method and (c) Extracted DNA of GCs by Red blood cell lysing buffer (RLB) method. Lines D-F showed distribution pattern of extracted DNA from isolated GCs (d) Ficoll, (e) Percoll and (f) RLB procedures

analysis in Percoll and RLB methods. We classified fluorescen intensity during flow cytometric; <10% negatively, 10-40% low expression, 40-70% moderate expression and > 70 high expression.[16]

RNA and DNA extraction study

Figures 3 and 4 showed extracted RNA, DNA and pre-RFLP results on 1% agarose gel electrophoresis. Figure 3 showed RNA samples from GCs that extracted by Ficoll [Figure 3a], Percoll [Figure 3b] and RLB methods [Figure 3c]. Extracted RNA from GCs that were purified by Ficoll procedure has been degraded. However, RNA samples from the GCs were extracted using the Percoll and RLB methods showed better quality and less damage. Figure 4 showed extracted DNA distribution pattern and pre-RFLP results from each of the mentioned methods of GC extraction. The quality and quantity of extracted DNA were relatively acceptable because DNA digestion using restriction enzyme EcoRI was complete.

DISCUSSION

As mentioned in previous section, GCs have a major role in the maintenance of ovarian function and oocyte quality. Human follicular fluid can be a source of GCs for molecular and *in vitro* specific studies. Moreover, purification of GCs from red and white blood cells is essential.[17]

The previous studies have shown that morphological properties and cell survival is affected by cell

purification. A rapid and simple procedure is necessary to maintain RNA and DNA integrity that have been extracted from GCs.[18]

Percoll and Ficoll purification methods are used to remove red blood cells from GCs. Based on the evidence, although sample contamination with red blood cells may have little effect on extracted nucleic acid from GCs, during purification of GCs, vitality and viability of cells are changed which in turn, may affect the amount, purity and integrity of extracted RNA and DNA.[3,19,20]

Overall, Ficoll-Paque products have the advantages such as being sterile and provide ready-to-use density gradients for isolating mononuclear cells. These products are especially mononuclear cells present in the original blood sample. Ficoll-Paque media products have Ficoll PM400 and sodium diatrizoate with calcium disodium ethyenediamine tetra-acetic acid. Ficoll PM400 is a polymer of sucrose with high molecular weight and epichlorohydrin which is readily soluble.[21]

According to the results [Figure 1], there is the possibility that sucrose crystals form and cause damage to the GCs. On the other hand, Ficoll method is a general method for isolating single cell, lymphocyte cells and GCs in follicular fluid and this finding is consistent with our flow cytometry results. However, this method cannot separate the huge bundle intact GCs [Figure 1]. Furthermore, in this method the number of deformed and ruptured cells was higher than the other methods.

In most studies, Percoll method is used for GC extraction, which separate both single and bundle GCs.[22] Based on our results, it is less likely to cause cellular damage and this method is more suitable for GC extraction; however, the extraction time is slightly longer than other methods.

RLB method can be used to separate GCs, which are integrated and in this procedure, because of the short duration of extraction process, less damage is exerted to cells. This method is also less expensive. Therefore, extraction using the Percoll solution compared to RLB method is complicated, very expensive and time-consuming.

Studies indicate the fluorescence-activated cell sorting (FACS) is a gold standard technique for isolation of cell population; however, FACS may decrease viability rate.[23] Our flow cyometry data showed that Percoll and RLB methods are suitable for GC extraction.

Other studies indicate that the purity of extracted cells affects the degree of purity and quality of extracted DNA and RNA,[19] which is in consistent with the results arisen from our study [Table 2].

The spectrophotometric analysis of absorbance in 260/280 and 260/230 nm with NanoDrop 1000 showed that the extracted DNA and RNA from isolated GCs with RLB and Percoll methods were free from proteins and polyphenolic or polysaccarid compound contamination. These results were probably due to the high quality of the isolated cells and absence of any additional chemical contamination associated with isolated cells.

Furthermore, the extracted DNA and RNA distribution on agarose gel electrophoresis demonstrate that although purity and quality of DNA are almost intact after the cell extraction process, the quality and purity of RNA may be affected through the protocol. Moreover, the results of enzymatic digestion indicated fairly high quality of DNA.

Based on our results, the novel RLB extraction method is able to provide high number, high purity and viability for extracted GCs. Besides, DNA and RNA with high quality are needed for subsequent use in experiments such as cell culture, real-time (RT) polymerase chain reaction (PCR), RT-PCR, and array analyses.

CONCLUSION

In conclusion, many confounding factors such as duration time of extraction and environment factors, which may have important roles on the purity and quality of GCs.

According to our results, RLB method can be considered as a the most suitable procedure for GCs extraction because not only it cause less cell damage, but also is a rapid and cost-effective way in comparison to Ficoll and Percoll methods. Furthermore, RLB method can be an appropriate technique for extraction of DNA and

Table 2: The quality and quantity of RNA and DNA extracted from granulosa cells with three methods; Ficoll, Percoll and RLB

Concentrations	GC extraction by Ficoll method	GC extraction by Percoll method	GC extraction by RLB method
RNA concentration (ng/μl)	1706.4	1741.6	1944.4
260/280	1.97	1.98	1.99
260/230	1.77	1.96	2.09
DNA concentration (ng/μl)	1992.5	2700	3100
260/280	1.78	1.84	1.9
260/230	1.58	1.92	1.9

RNA: Ribonucleic acid, DNA: Deoxyribonucleic acid, RLB: Red blood cell lysing buffer, GC: Granulosa cell

RNA from the GC with high quality and quantity that can be used for molecular techniques.

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