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Detection of Proteomic Biomarker in Fibroblast Cell Derived Nuclear Transfer Fertilization Compared to Natural Fertilization

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ABSTRACT

Background: One of the major problems in the treatment of infertility and in vitro fertilization (IVF), as well as for conducting tissue engineering in the case of using cell donor is reproductive failure or transplant rejection by the recipient's immune system. Nuclear transfer technology may be a more accurate means for preventing from rejection of transplanted cells. The aim of this study is to investigate the possible changes in the protein profile of somatic cells obtained from nuclear transfer fertilization method compared with the same for normal fertilization via studying the protein profiles of both cells and detection of the protein expression (major histocompatibility complex (MHC)) and by means of proteomics; so that accessing new therapeutic strategies for cell therapy and tissue engineering may be facilitated.

Methodology: This experimental study was conducted using simulated goat fibroblast cells (experimental group) compared with goat fibroblast cells obtained from natural fertilization (control group). After culturing the cells, the level of protein in cell suspension and MHC biomarker expression in both groups were studied and compared using ELISA and western blotting methods.

Results: The data from ELISA technique and the gels obtained from one-dimension electrophoresis showed decrease in the amount of protein in the experimental group (E) compared with the control group (C group). However, such decrease was statistically insignificant ($P \leq 0.05$). It also showed that MHC protein, which has been considered as a biochemical marker recently, was expressed in both groups, and the cells from both control and experimental groups were identical in terms of structure and function of the immune system.

Conclusion: Probably, the same immune response from fibroblast cells obtained from nuclear transfer technique and fibroblast cells derived from natural fertilization indicates that fertilization method has no impact on the production of studied fibroblast cells. Thus, it is conjectured that this can be a good prognosis for researchers in order for using nuclear transfer method to conduct research and tissue engineering.

KEYWORDS: Nuclear transfer, Fibroblasts, MHC, Proteomics, Stem cells.

INTRODUCTION

Transplant rejection by the recipient's immune system is one of important treatment problems in the area of fertility, in vitro fertilization (IVF), as well as for conducting tissue engineering. Moreover, many patients with specific genetic diseases, diabetes, as well as neurological, cardiovascular, and gland disorders who cannot recover despite huge costs. Nuclear transfer technology with cloning therapeutic purposes may be a more accurate tool for preventing the rejection of transplanted cell transplantation and conducting tissue engineering, cloning of stem cells specialized for replacing apotosed cells of nervous system (in Alzheimer's, Parkinson's diseases, etc), cloning of necessary proteins and genetic modification of an individual or a generation (4, 5 and 6). Fertilization using nuclear transfer technique is among reproduction methods. As the most common applied technology in cloning animals, nuclear transfer technology was first introduced for animals by Wilmut et al (1, 2 and 3). Review and identification of protein profile of somatic cells obtained from transfer nuclear transfer method provide use with important information regarding possible differences occurred in cellular protein profiles during nuclear transfer. It is also a valuable tool for studying the interactions within the nucleus and cytoplasm as well as the apoptosis process (1, 7 and 2).

Several studies have been performed on the affecting factors on SCNT, abortion and fetal mortality due to nuclear transfer procedure, including a study of protein expressions during the evolution. Neil et al conducted a comparative assessment and showed that the major proteins of cells derived from IVF, SCNT and

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parthenogenetic methods are different in such a way that certain proteins show normal expression, some show decrease in expression (annexin 1 and 2), while other show increase. The researchers found in their study that decreased expression of this protein might reduce the efficiency of nuclear transfer methods and the resulting low reproduction of viable embryos in SCNT technique (1, 2). Several other studies showed that the expression of certain proteins, including serotonin, SHP2, caspases and annexins are different in nuclear transfer method compared with parthenogenetic and in vivo technique, so that defects in the expression of annexins and serotonin causes problems in the implantation process. Another study found that in the absence of serotonin, fetal development to the blastocyst stage was nearly impossible and produced the embryo will be unable to implantation (7 and 8).

Takeda et al in a comparative study on the protein profiles of mitochondria of the liver somatic cells obtained from nuclear transfer method performed in pigs concluded that the 2 protein expression changes in volumetric ratio of one-to-two compared with that of the control group (9). In a similar study on the mitochondria of the liver somatic cells resulting from nuclear transfer technique in cattle, it was found that the protein profile of the somatic cells obtained from nuclear transfer method is 2.1 times greater than that of the protein in the control group (9).

Recently, some researchers also isolated, identified and measured fetal protein profile and obtained new proteomic findings (10, 11, 12 and 13). One of the secreted biomarkers is called HLA class I histocompatibility antigen, alpha chain G precursor [Homo sapiens] = MHC: major histocompatibility complex. MHC is the name of HLA-G alternative (13).

HLA-G is a non-classical antigen (antigen class Ib) and has a limited polymorphism but has 7 isoforms; four of the isoforms are membrane-bound and three others are in solution. As far as it was known, the implantation process requires that embryo many not be rejected by the maternal immune system (11, 12 and 14). The expression of the protein has been confirmed by fetus and cancerous adult somatic cells in human being and other mammals (15 and 16).

Both soluble and membrane-bound isoforms are immunosuppressive. Moreover, adjusting T helper cells (T_h cells) at the contact point of embryo's cells plays role in the tolerance of the embryo by the maternal immune system and the subsequent placentation (15, 16 and 17). NK cells are the major cells during the luteal phase of endometrium that have receptors for expressed HLA-G by cells of interstitial trophoblast cells at the contact point of mother-fetus, where the endocytosis of HLA-G by the receptors of the cells stimulates proinflammatory /proangiogenic specific mediators and cytokines which contribute to implantation and placentation (79). The presence of this protein in trophoblast cells was first reported in 1990 (18), while its presence was reported in embryo culture medium in 1999 (19 and 20).

In sHLA-G positive embryos, pregnancy improved 3-fold compared with the sHLA-G negative embryos (21), moreover, the abortion rate in sHLA-G negative embryos increases (79). Meanwhile, sHLA-G expression is significantly correlated with the strength of implantation and clinical pregnancy rates (12).

New research shows that the protein is found in somatic cells of colorectal, ovarian, breast, milk ducts, melanoma, thyroid cancer etc and is deemed a new tumor marker (21, 22, 23, 24, 25 and 26). For embryo, this protein is considered as a vital marker, while it is a probable marker for somatic cells. Given that the previous researches have been focused on tracing the protein in embryo culture medium and cancerous tissues, no report has been provided on the existence or possible changes of the protein in cell lines resulting from nuclear transfer compared with cell lines obtained from normal fertilization. Therefore, in order to obtain more complete information in this regard and to achieve the main objective of this study, the expression levels of the protein (MHC) were assessed in order to study the protein profiles of cell lines in both experimental and control group as a biomarker.

Having information on possible changes in the protein profiles of cloned fibroblast cells will help us in interpretation and finding the causes of underdevelopment of the fetus and its abortion, and also the leading cause of abnormalities after birth and deteriorated health in this period, as well as in finding solutions to the problems of fertilization by means of nuclear transfer methods (27, 28 and 29).

It is hoped that with the discovery of the main cause of abortion and underdevelopment of the fetus, abnormalities after birth and as well as unknown cloning problems and overcoming it in the case of the reports being positive, this technique can be used for research and treatment purposes.

MATERIALS AND METHODS

Materials:

Standared medium consisted of RPMI 1640 containing of 25 mm glucose,4mm glutamine supplemented by 12% fetal bovine serum (FBS), FBS was obtained from GIBCO-BRL …… the penicillin, streptomycin. Trypsin– EDTA solutions were supplied by GIBCO-BRL (Grand Island, NY, USA).
MTT with formula ,,,,,were purchased from SIGMA chemical Co. All other reagents were obtained from SIGMA Chemical Co.

Cell line:

One and a half years old cloned goat fibroblast cell line (Cd1529), as experimental groups, and same aged goat donor fibroblast cell line (cd 1530),as control groups, was purchased from Research Institute of Royan(www.royaninstitue.org/cmsfa), Tehran, Iran. These cell lines was maintained as stock cells in cryotubes at temperature 800c .

Defreeze and sample preparation:

The cryotubes containing fibroblast cell lines was defreezed by heating in a water bath at +37°C, then all of the cryotube contents was carefully transferred to a sterile centrifuge falcons with 10 mL of a fresh medium, and centrifuged. The obtained cell plates cells was again re-suspended in a fresh medium and transferred to a 75-cm² cell culture flask with RPMI containing 12% fetal bovine serum (Life Technologies, Inc.), 100 U/ml penicillin, 100 g/ml streptomycin (Life Technologies, Inc.), and 0.25g/ml fungi- zone (amphotericin B) (Life Technologies, Inc.), and cultured in a humidified incubator at 95% air, 5% CO2 at 37°C. The medium was changed after 24 h. When the cultured cells were multiplied to 90%–95% of confluence (within 9 to 15 days), the cells was enzymatically detached by application of 0.05% trypsin at 37°C for 3 minutes. The suspended fibroblasts was centrifuged at 1500 rpm for 5 minutes, and the supernatant were removed. Cells was re-suspended in 2 mL of culture medium, and then they were equally divided as 0.5 ml per flask with 9,5 mL culture medium, 6 flasks for each groups, totally 12 flasks. 3 flask from each groups was prepared for the study of the viability and proliferation rate during 2, 4, 6 and 8 days after incubation, and 3 other flask was prepared for study of protein materials in third passage. These were true for the control cell lines as described bellow.

Briafly at the end of experiments we had 12 groups which named them by the numbers 1-12 including;

The flasks 1to 3 containing 1529 CD cell line named as experimental groups and the flasks 4-6 including 1530cd as control groups were designed for protein content assay at third passage.

The flasks 7 to 9 including 1529cd cell line named as experimental groups and the flasks 10-12 including 1530cd as control groups designed for proliferation assay in days 2,4,6 and 8.

The viability assay of cloned and donor fibroblast cell lines - MTT Assay test:

The flasks of 7-9, containing donor cell line with the codes 1530CD, and the flask 10-12, containing cloned cell line with code 1529 CD, was designed for estimation of cells proliferative ability by MTT Assay test. For estimation of cell proliferation rates, the growth curve was designed as bellow.

Growth curve:

the cells with concentrations of 5 × 103, 10× 103 ,20 × 103 and 40× 103 in 200 µl per well for each groups were grown overnight in 96-well plates.

After drawing of standard grow curve, the cells with concentrations of 200000 in 200 µl per well were grown for 2, 4, 6 and 8 days in 96-well plates. After end of incubation periods, medium was replaced with fresh complete medium (200 µl).Here PBS (40 µl) containing MTT (5 mg/mL) was added to each wells. In the absence of light, samples was incubated for 4 hours, followed by the removal of the medium. Precipitates were re-suspended in 100 µL DMSO. Absorbance was measured on a plate reader at 570 nm. Each experiment was performed in triplicate for each groups and the obtained data was presented by OD value.

Proteomics analysis:

Determination of protein concentration in donor and cloned cell lines by Bradford's method:

Here the flasks 1-6 was designed for protein contents assays.

5 × 106 cells from each flask was grown overnight in 96-well plates. Cells were re-suspended in 100 µL chilled cell lyses' buffer and incubated on ice for 10 minutes. After incubation, cells was centrifuged for 1 minute in a microcentrifuge (10,000g). The supernatant (cytosol extraction) was transferred to a fresh tube and left on ice. The concentration and density of total proteins of cytosol extraction was determined by Bradford's method, with Coomassie blue G250 as the reagent and bovine serum albumin as the standard protein (Sigma Chemical Co., St Louis, MO, USA). All samples were analyzed in triplicate. The lyophilized samples were reconstituted by adding 50 µL of Milli-Q water (Milli-Q plus Ultrapure water system, Millipore Corp., MA, USA) and homogenized with a mechanical agitator (Vortex AP-56, Phoenix, São Paulo, Brazil). Subsequently, 5 µL was withdrawn, and 1 mL of the Bradford solution was added, rehomogenized, and left to rest for 10 min. Absorption was measured at 595 nm in a spectrophotometer (Ultraspec 1100, Pharmacia, Cambridge, UK).

SDS-PAGE ANALYSIS:

SDS-PAGE was performed on a minivertical electrophoresis system (Bio-Rad Mini-PROTEAN 3 Cell; Bio-Rad Laboratories, Hercules, CA). Each sample with an equal amount of total obtained proteins was separated on a 12% acrylamide resolving gel (0.1% SDS, 1.5 M Tris-HCl, pH 8.8) with a 5% acrylamide stacking gel (0.1% SDS, 0.5 M Tris-HCl, pH 6.8). Electrophoresis was performed in electrode buffer (0.1%

SDS, 0.25 M glycine, 0.025 M Tris-HCl, pH 8.3) at 60 V for 10 minutes, and then at 120 V for 120-150 minutes. Each experiment was repeated three times in different gels and running buffers. The results was presented according to the instructive for the analysis of protein gels (SDS-PAGE) protocol (experimental biosciences).

Staining and Image Analysis:

Gels were stained with Coomassie blue R-250 in accordance with the protocol. Briefly, after seperating the gel from the glass they were fixed in 40% (v/v) methanol and 10% (v/v) acetic acid for 1 hour and then stained in a staining solution (0.025% Coomassie blue R-250 in 10% acetic acid) heated to 80–90°C. The gels were destained in 10% acetic acid. All gel images were recorded immediately after de-staining to minimize any possibility of fading. Images were prepared for examine the lane profiles. The method introduced in the bellow was used for this examination:

1- Determination of relative mobility for standard and samples proteins: Relative mobility was determined by measuring the distance from the top of the gel (separating gel) to the middle of the day front or arbitrary reference point, measuring from the top of the gel to the middle of the band, and then dividing the second measurement by the first. This is named Rf which is always between 0-1.
2- Estimating of apparent molecular mass for each band: After calculating the relative mobility for each bands of interest, the standard curve was drawn for protein standard markers, and then according to the standard curve, the apparent molecular mass was estimated for each bands of interest. Here the resolution in the appropriate region of a gel and thickness of the band of interest was calculated and reported.
3- Estimating of intensity of for each band: The intensity of protein bands in SCNT line groups was obtained using a densitometer and compared to the donor cell line protein bands.

RESULTS

In the gel obtained from one-dimension electrophoresis for proteins of cell lines in the experimental group, the protein spots were detected and were arranged by molecular weight in comparison with markers (Figure 1).

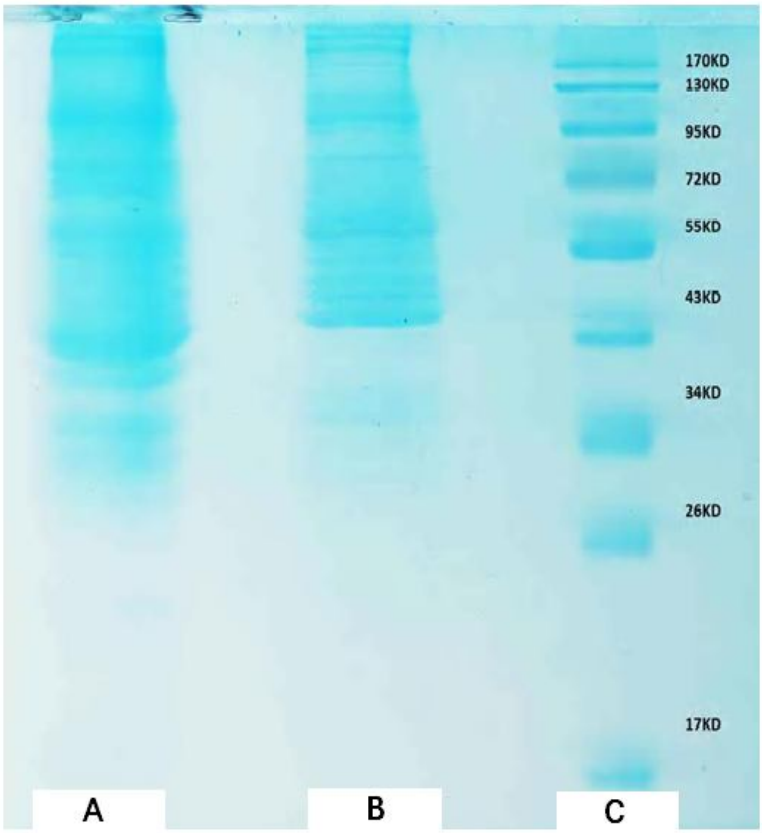

Figure 1: The proteomic model of the protein derived from fibroblast cells with similar concentration on 12.5% electrophoresis gel using SDS-Page method

A-Sample N.1529 (32 micro + 8 sample buffer 2 me) B - Sample N.1530 (32 micro + 8 sample buffer 2 me) C: Marker: SM0671 (Fermantaze)

This figure shows decrease of concentration and size of derived proteins from sample cloned fibroblasts compared with fibroblasts derived from normal fertilization, although this difference was not significant.

Assessment of MHC biomarker expression level (sHLA-G) by western blotting and ELISA techniques showed that the protein was not expressed in both experimental group (S: 1530) and control group (S: 1529) based on the indicator used in these methods (sHLA-G Ab (A)) (Figure 2).

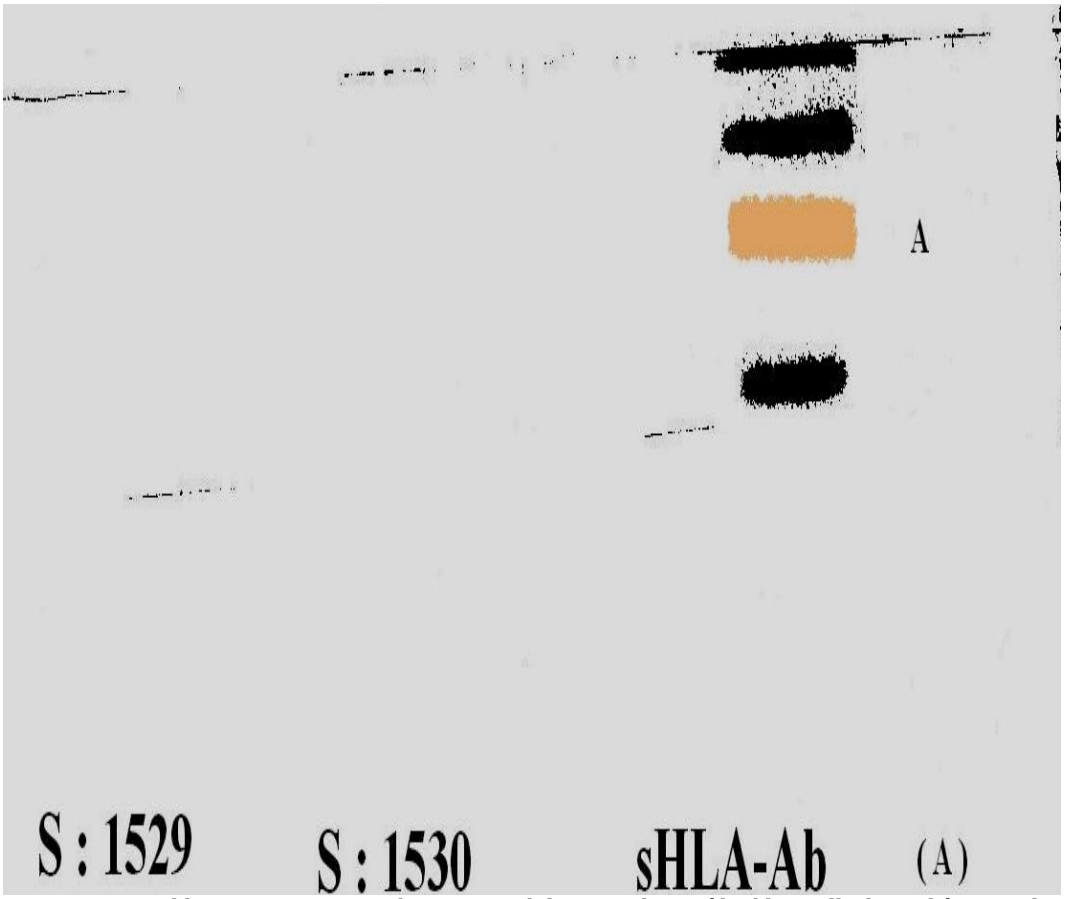

Figure 2: Immunoblotting image. Major histocompatibility complex in fibroblast cells derived from nuclear transfer fertilization method (S: 1530) and fibroblast cells derived from natural fertilization method (S: 1530), compared with sHLA-G Ab. (A)

Mean density of the extracted proteins in the experimental group was less than that in the control group (45.1 unit/ml vs. 55.07 unit/ml); however, this difference was not statistically significant.

Rate of cell growth and cell proliferation after melting stage for the sample compared with the control sample showed that both samples have the same normal growth rate.

DISCUSSION

The current study has been conducted using a proteomics and in line with the studies conducted by other researchers. In this study, the proteins in cell lines derived from fertilization by nuclear transfer method are compared with the proteins found in cell lines derived from fertilization due to normal method. Many researchers have used proteomics to achieve significant developments in the field of molecular medicine and for improving early diagnosis and developing treatment protocols. The main hypothesis of this study is that knowledge of possible changes in protein profiles of lipid droplets, excreted vesicles and Golgia system in cloned animal cells can be the basis for us to interpret and find the causes of underdevelopment of the fetus and its abortion, and also the leading cause of abnormalities after birth and deteriorated health in this period, as well as in finding solutions to the problems of fertilization by means of nuclear transfer methods. They are also helpful in determining that, scientifically, whether this is a useful method or not (30, 31 and 32). Many

researchers believe that one of the important factor in the decline of fertility via in vitro method compared with other methods may be related to morphological changes and abnormal changes ultrastructure of the cells, as well as changes in the expression of different proteins in embryos at the time of pre-hatching or after it. Among other factors that can influence the formative power for each donor's cell in the process of SCNT is the nature of the tissue (27, 33 and 34), defects in the expression of the gene or lack of certain protein expression in cells of the donor or the recipient (31). These reports confirm our findings regarding clinically performed changes in the protein profiles with of the experimental group and the control group.

Researchers believe that the fetus or newborn produced using in vivo fertilization method is different from in vitro fetus in terms of infrastructure and protein profiles. For example, during the embryos produced by in vitro method morula are more dense with more lipid droplets, reduced relative volume of mitochondria, increased density of vacuoles, increased nucleus to cytoplasm ratio and also decreased or increased expression of specific proteins (e.g. annexin) (35). According to this view, the findings of this study are confirmed regarding the detection of quantitative changes in the protein profiles. Based on the finding of this study, the reduced expression of a number of proteins in the protein profiles of the cells in the experimental group could be a sign of the beginning of a series of cellular changes, and this change may be due to a change in the method of reproduction; although the difference of such change compared with that of the control group is not statistically significant. It is possible that these changes in long-term cause the cloned animal to undergo a series of changes related to the cellular proteome. However, to demonstrate this, it is necessary that quality control tests to be performed. Decreased expression of proteins in cell lines of the experimental group strengthens the hypothesis that those proteins without expression will target in some critical systems of the cloned animal in long-term and may poses a series of specific diseases to the animal such as respiratory disease, like dystrophy disorder of the respiratory tract. According to many reports, this the same disease that causes mortality or morbidity of the cloned animals (1, 3, 5). Several studies have been performed on the affecting factors on abortion and fetal mortality due to nuclear transfer procedure. Neil et al conducted, for example conducted a study on the expression of a number of proteins during evolution. This comparative assessment showed that the major proteins of cells derived from IVF, SCNT and parthenogenetic methods are different in such a way that certain proteins show normal expression, some show decrease in expression (annexin 1 and 2), while other show increase. The researchers found in their study that decreased expression of this protein might reduce the efficiency of nuclear transfer methods and the resulting low reproduction of viable embryos in SCNT technique (5, 3, 1 and 18). Several other studies showed that the expression of certain proteins, including serotonin, SHP2, caspases and annexins are different in nuclear transfer method compared with parthenogenetic and in vivo technique, so that defects in the expression of annexins and serotonin causes problems in the implantation process. Another study found that in the absence of serotonin, fetal development to the blastocyst stage was nearly impossible and produced the embryo will be unable to implantation (7 and 8). Takeda et al in a comparative study on the protein profiles of mitochondria of the liver somatic cells obtained from nuclear transfer method performed in pigs concluded that the 2 protein expression changes in volumetric ratio of one-to-two compared with that of the control group (36). In a similar study on the mitochondria of the liver somatic cells resulting from nuclear transfer technique in cattle, it was found that the protein profile of the somatic cells obtained from nuclear transfer method is 2.1 times greater than that of the protein in the control group (37). Such reports confirm the results of this study regarding the changes in protein profiles in the experimental group compared with the control group. Although this difference is not statistically significant.

MHC or HLA-G was another protein as a quality control indicator for embryo and somatic cells in this study. As reported by the researchers, HLA-G is a non-classical antigen (antigen class Ib) and has a limited polymorphism but has 7 isoforms; four of the isoforms are membrane-bound and three others are in solution. As far as it was known, the implantation process requires that embryo many not be rejected by the maternal immune system. The expression of the protein has been confirmed in human being and other mammals as a biomarker and in some somatic cells as a tumor marker (9, 22, 23, 24, 25, 26 and 38). The presence of this protein in trophoblast cells as a biomarker was first reported in 1990 by Ellis (18), while its presence was reported in embryo culture medium by Menicucci in 1999 (39). Drik et al examined the level of abortion in selected embryos based on GES and sHLA-G expression after transfer in 2010. They showed that abortion rates reduced after implantation of the sHLA-G positive embryos between 6 weeks and 12 weeks. Therefore, SHLA-G perpetuates implantation and increases subsequent pregnancy rate. So sHLA-G can be used as a marker for predicting pregnancy outcomes (12 and 40). However, a number of researcher believes that this protein is as a diagnostic marker (tumor marker) for stomach, esophagus, colon, rectum, lung, ovary, breast, bladder and thyroid cancers. In another study, the protein expression has been reported in milk ducts. The increase of this protein was also confirmed in melanoma patients. (22, 43 and 26) Some researches show that the existence of the protein in embryo culture medium is a biomarker and in some somatic cells as a tumor marker and is of great importance as an indicator of the quality control. The results of this study indicate that lack of protein expression in the cell lines of both groups. This finding may reflect the precise duplication of genome and molecular structural adjustment and cellular adaptation during development of the cloned fetus.

Le Gal et al (1999) showed that the protein cause the maternal immune system to be suppressed, increase the tolerance of the fetus by the mother and consequently improves the implantation and pregnancy rates (42 and 41). This report seems to confirm the results of this research, because the absence of this tumor marker suppressor and cell destruction factor in the cells of the experimental group means lack of expression of cancerous genes in the studied somatic cells. That is why these cells can be regarded relatively healthy. The results can offer great optimism for the researchers in this field since the lack of this tumor marker indicate the healthy structure of the cell derived from nuclear transfer. So it can be perceived as a new finding. If this cell were among the cells, which were changed into tumor, then the tumor marker would be present in it. Therefore, lack of this marker indicates the healthy gene structure and safety of the cell and this is the major finding of this study. The findings also seem to confirm the use of somatic cell derived from cloning method for research and clinical purposes.

Accurate identification and study the structure of other vital proteins in cell lines of the experimental group, particularly the proteins involved in apoptosis and cytoskeleton structure are recommended for future research. Identification of the proteins as an indicator may be helpful for research purposes, tissue and cell engineering, and clinical purposes including early diagnosis, follow-up improvement or progression of the disease or the treatment, and may help physicians along with other clinical and pathological findings.

Conclusion

Given the findings and their role in research and clinical affairs, the following hypotheses are derived:

1. The almost identical protein pattern in fibroblast cell lines of the newborn produced by nuclear transfer method compared with fibroblast cell lines derived from normal fertilization may be promising for success of cloning with therapeutic purposes.
2. No expression of tumor marker (sHLA -G) in cell lines of the newborn produced by nuclear transfer method compared with fibroblast cell lines derived from normal fertilization may reflect the stability and health of the safe structure of the cells.
3. It seems that cloning using somatic cells for the reproduction of endangered animals or species with high economic and values can be successful.

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The authors declare that they have no conflicts of interest in the research.

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