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Migration of Human Wharton's Jelly Stem Cells Seeded Onto the Chorioallantoic Membrane Into the Chick Liver

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Abstract

Background: Developing chicken embryo culture techniques provides methods for manipulating chicken embryos. In this study, the cell-based approach investigated human Wharton's jelly stem cells (HWJSCs) proliferation and migration into internal embryonic chick tissue.

Methods: Wharton's jelly cells were cultured on an acellular dermal matrix (ADM) and seeded on the chorioallantoic membrane (CAM) of a 4-day-old chicken embryo. Using an RT-PCR assay with the COLA-I human gene, molecular analysis was performed on chicken liver tissue after hatching to confirm the presence of these cells.

Results: The chicken liver tissue from Wharton's jelly stem cells/ADM sample tested positive for the human COLA-I gene. The COLA-I gene sequence analysis shows this sequence belongs to the COLA-I gene in humans. The cells cultured on CAM can migrate and proliferate in the liver of a chicken embryo.

Conclusion: These findings suggest that human cells cultured on the CAM can appear in the liver of chickens. However, further investigations are necessary to confirm this finding.

Keywords: Chimeric animal, Human Wharton's jelly stem cell, Chorioallantoic membrane, Acellular dermal matrix, Chicken embryo

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Introduction

Producing human cells, tissues, and whole organs in animals is possible. The animal-generated human organs or cells are introduced as human-animal chimeras. Several benefits of animals producing human organs include: (I) animals can be used for the preparation of cells or organs before transplantation; (II) the generation of human organs in the animal body is natural and biological; (III) the organ derived from the patient's stem cells reduces the risk of immune rejection (1). Various methods can be used to produce transgenic and chimeric animals: direct DNA microinjection into the pronuclear (2), injection of in vitro modified embryonic stem cells into the blastocyst (3), transfer of transfected nuclei into enucleated oocytes for cloning (4), and viral vector transfer (5). However, the bird's transgenic technology has been hampered due to the ovum birds' unique reproductive system and yolk-laden structure. Direct DNA microinjection into the male pronucleus cannot be done because bird fertilization involves multiple sperm, and it takes place in the infundibulum of the reproductive system. Also, identifying the male pronucleus and the return of the ovum to the oviduct is difficult (6). The general methods for producing transgenic and chimeric birds include viral transfection and embryonic cells that have been genetically modified and placed directly into the recipient embryo (7). The viral transfection systems have limitations, such as inserting endogenous genes and distributing them (8), limiting the vector genome size to below 8 to 10 kb (9), and Lentiviral vectors are influenced by positional effects (10). The isolation of primordial germ cells (PGCs) from donor embryos and the transfer of these cells into recipient embryos is one of the methods for generating chicken chimeras. Chojnacka-Puchta et al used blood

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primordial germ cells (bPGCs) transfected with the pEGFP-N1 plasmid to produce chicken chimeras. The cells were subsequently injected into the dorsal aorta of the host embryo. Molecular analysis showed that 16.7% of the embryos were transgenic hens (11).

The chorioallantoic membrane (CAM) of chick embryos is widely used as a host for organ culture. Histologically, the membrane consists of an outer layer of ectoderm originating from the ectoderm, the lower layer of the epidermis forming from the endoderm, and a stromal layer of mesoderm. Blood and lymph vessels are located in the stromal layer (12). The growth and development of the CAM of chickens is similar to the growth of this membrane in humans (12-14). It is also noteworthy that the CAM of chickens has significant molecular and functional similarities to the lymphatic system in mammals. This membrane acts as the respiratory organ of the chick embryo and naturally attracts the most attention in the field of angiogenesis (15). This membrane shields the extra-embryonic respiratory vessels, transporting sodium and chloride ions from the alveolar sac and calcium from the eggshell to the embryonic vessels. It also forms part of the alveolar sac that expels waste products. In fact, on days 11-12 of the chick embryonic development period, the CAM contains a dense volume of angiogenic capillary network, and the response of this membrane to angiogenesis is almost rapid (16,17). Cost-effectiveness, ease of surgical procedures, visibility, availability, and the rapid growth of the CAM during embryonic development are some factors that make it suitable for studying many biological systems. Chick embryos are used in animal specimens because they can be used for transgenic studies by viral vectors as a screening system in pharmacology to investigate the toxicity and causes of cancer. Bird eggs have been used as a proven biotechnological resource for vaccine production and have recently been used as a potential bioreactor to produce therapeutic proteins (18,19). Nonetheless, the efficacy of transgenic and chimeric approaches in poultry is quite limited. In this study, we assessed cell-based methods for producing chimeric birds. We used the CAM as a substrate for stem cell growth and migration to the chicken's internal tissues. The Wharton's jelly stem cells loaded on the acellular dermal matrix (ADM) will be implanted on the CAM. Finally, molecular analyses will be performed to trace the presence of Wharton's jelly stem cells in internal organs, including the liver.

Methods

Figure 1 shows the study's workflow.

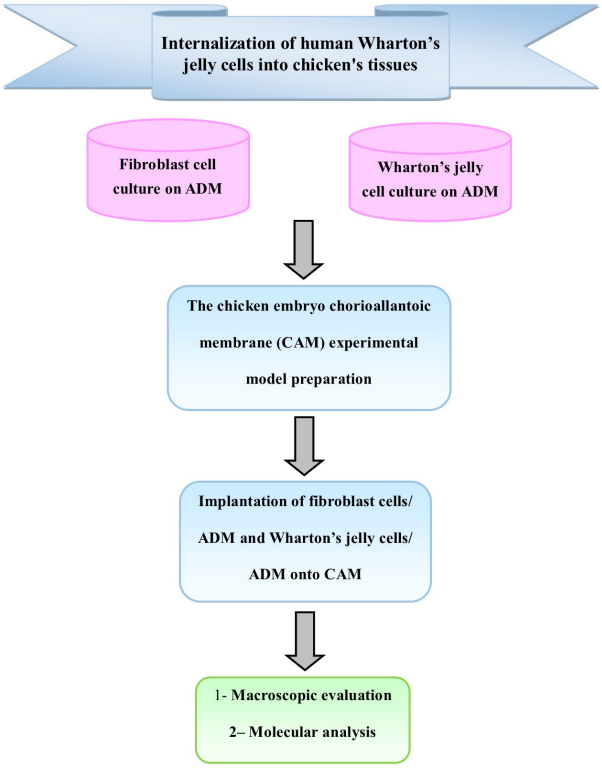

Figure 1. A graphical overview of the workflow used in this study

Cell culture

The human umbilical cords were obtained from chosen cesarean sections performed in the 36th and 37th weeks of pregnancy. Informed written consent was acquired from all participants to gather umbilical cords for research purposes. The 3-cm tissue sections were rinsed with sterile phosphate-buffered saline (PBS), and the opened tissue segments were positioned in a sterile dish, which contained an enzymatic cocktail solution (collagenase type I: 2 mg/mL, collagenase type IV: 2 mg/mL, and hyaluronidase: 100 IU) (Sigma, MO, United States). Finally, the Petri dish was kept in an incubator at 37 °C with 5% CO_2. The suspension of cells was centrifuged for 10 minutes at 2000 rpm. Cell pellets were cultivated in tissue culture flasks containing the human Wharton's jelly stem cells' (HWJSCs') culture medium, which was made up of 90% Dulbecco's modified eagle's medium (DMEM) enriched with 10% fetal bovine serum (FBS), 1% insulin–transferrin–selenium, 1% non-essential amino acids, and 1% penicillin/streptomycin antibiotic mixture. They were incubated in a 5% CO_2 incubator at 37 °C (20).

Characterization by flow cytometry

Flow cytometry was conducted using the 3rd passage of HWJSCs to express mesenchymal and hematopoietic surface markers. CD105 and CD90 (Dako) were considered for the assessment of mesenchymal markers, and CD34 (Dako) was considered to evaluate hematopoietic surface markers.

Growth kinetics

A total of 4×10^4 HWJSCs from passage three were placed in 24-well culture plates and maintained in an incubator

with 5% CO_2 and high humidity at 37 °C. The media was replaced every three days, and cell viability along with population doubling time (PDT) were assessed over seven days using 0.4% trypan blue solution (Biowest), and a Neubauer hemocytometer. Cells were cryopreserved at a concentration of 1×10^6 cells/mL using 10% dimethyl sulfoxide (DMSO; MP Bio) (V/V), 50% fetal bovine serum (V/V), and 40% DMEM.

Acellular dermal matrix preparation

The human skin samples were prepared under sterile conditions in the Department of Plastic Surgery at Shiraz University of Medical Sciences, Shiraz. The samples were stored in a PBS solution containing penicillin and streptomycin. The samples were washed twice with 0.5% Triton X-100 and 10 mmol/L EDTA (Sigma). The mixture of 1M NaCl, 0.5% Triton X-100, and 10 mol/L EDTA was employed to eliminate epidermal tissue in a shaking incubator at 37 °C for 24 hours. For the decellularization process, the tissue was immersed in a solution containing 0.5% sodium dodecyl sulfate (SDS), along with 10 mmol/L HEPES and 10 mmol/L EDTA solutions (Sigma) for one hour at 37 °C inside an incubator (21).

The chicken embryo chorioallantoic experimental membrane model preparation

Ten fertile chicken eggs (Ross 308) weighing an average of 51.3 ± 0.7 g were chosen. Fertilized chicken eggs were set at 37.5 °C and 60% relative humidity inside an incubator (Easy-bator 2, Eskandari Industrial Group, Urmia, Iran). After four days of incubation, the shells were sterilized with a 70% ethanol solution, and a hole was made in the top of the eggshell by a high-speed drill. Prepared eggs were used for cell implantation (22).

Implantation of HWJSCs onto the CAM

The HWJSCs implantation with and without scaffold was performed on day four of eggs. The specimens of 10×7 mm were adapted in a 24-well plate, and HWJSCs were seeded separately at a density of 6×104 cells/well on the specimens. The cells were carried on an ADM scaffold for three days before implantation on a CAM. The cells with and without scaffold were placed on the upper surface of the CAM (22).

Macroscopic evaluation

On day 18 of the embryonic period, the eggshell was opened, and embryos were evaluated using the stereomicroscope. The liver samples were harvested and used for molecular analysis.

Primer design

Primers used for real-time PCR of the COLA-I gene were designed using Gene Runner version 5.1 (Informer Technologies Inc., Spain). Finally, primers were validated by NCBI-BLAST. Table 1 shows primer information.

RNA extraction

Molecular analysis was done on liver tissue to confirm the migration of HWJSCs. From each group, one sample was selected for molecular analysis. Total RNA was isolated from the liver tissue. In this research, 1 mg of liver tissue was homogenized, and 1 mL of cold QIAzol lysis reagent (Cat. No. 79306, Qiagen, USA) was incorporated into the tissue. The mixture was vortexed and incubated for 5 minutes at room temperature. 0.2 mL of chloroform (CAS 67-66-3, Merck, Germany) was added and, after inverting, was centrifuged for 15 minutes at 14000 RPM at 4 °C. The supernatant was transferred into a micro-tube. The total RNA was precipitated with 1 mL of absolute cold ethanol (CAS 64-17-5, Merck, Germany) and 2 µL of glycogen (20 mg/mL, Cat. No. G024, ABM Inc., Canada). Finally, the RNA pellet was washed with 75% ethanol, and after being centrifuged and air-dried, the pellet dissolved in 100 µL of DEPC-treated water (Cat. No. CH8141, Cinnagen, Iran).

cDNA synthesis and real-time PCR

After the extraction of total RNA, cDNA synthesis and qPCR were done using the RevertAid first-strand cDNA synthesis kit (Cat. No. K1622, Thermo Scientific, USA) and SYBR-Green PCR Kit (Cat. no. K0223; Thermo Fisher Scientific, Inc.) following the guidelines provided by the manufacturer (23).

PCR and sequencing

After qPCR, the general PCR was performed on two samples with a Col-I specific primer with the following conditions: 95 °C for 5 minutes (primary denaturation), 95 °C for 45 seconds (denaturation), 59 °C for 45 seconds (annealing), 72°C for 30 seconds (extension), and 72 °C for 5 minutes (final extension) in 40 cycle. Finally, the samples were loaded on a 2% agarose gel with a 100-bp ladder, and sequencing was performed for positive samples. The results of sequencing were analyzed with chromas software (v. 2.5.1) and NCBI-BLAST.

Results

Cell characterization

HWJSCs at passage 3 adhered to the culture plates and appeared spindle-shaped (Figures 2A and 2B). These cells showed positive expression for CD90 and CD105, which are mesenchymal markers, while they displayed negative expression for CD34, a hematopoietic marker (Figure 2C).

Growth kinetics

HWJSCs at 3rd passage for seven days showed a PDT of 40.1 hours. Cell proliferation increased until day 6, and then, a decrease was visible (Figure 3).

Table 1. Primers information

Gene	Sequence (5′-3′)	Size (bp)
COLA-I	Forward: GAGGGCCAAGACGAAGACATC	140
	Reverse: CAGATCACGTCATCGCACAAC	

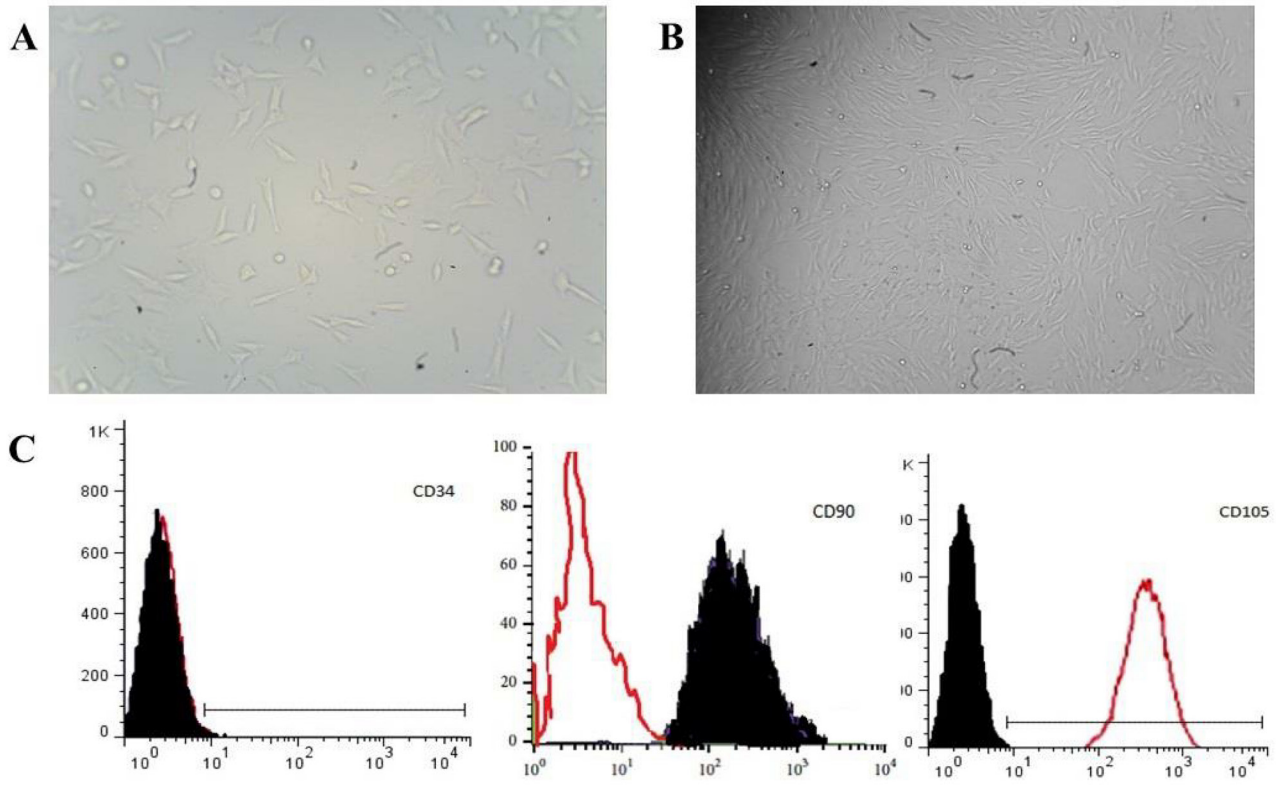

Figure 2. A) HWJSCs in passage 1, B) HWJSCs in passage 3, C) Flow cytometry of HWJSCs, indicating positive mesenchymal markers for CD105 and CD90 and negative expression of hematopoietic markers for CD34

HWJSCs culture on ADM

HWJSCs cultured on ADM formed a dense layer of cells with normal morphology (Figure 4).

Macroscopic evaluation

After 18 days of the implantation of HWJSCs/ADM onto CAM, the macroscopic evaluation in HWJSCs/ADM group showed 7 (70%) embryos with average growth and 3 (30%) death embryos.

Real-time PCR, PCR, and gel electrophoresis

The real-time PCR was done with COLA-I gene-specific primers for one sample from each group. The sample of HWJSCs/ADM was positive for the COLA-I gene; the PCR was done on a sample that was positive for the COLA-I gene with specific primers. The detection of the PCR product showed a 140-bond for the COLA-I gene on the agarose gel at 2% (Figure 5). Finally, PCR products were sequenced.

Sequencing analysis

The sequencing analysis of the PCR product obtained with COLA-I gene-specific primers on liver tissue (HWJSCs/ADM sample) was done with Chromas software (Figure 6A). The analysis of the COLA-I gene sequence by BLAST-NCBI shows this sequence belongs to the COLA-I gene in humans with 100% identity (Figure 6B).

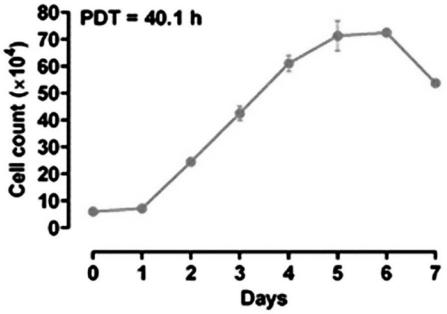

Figure 3. Growth kinetics of HWJSCs. PDT = population doubling time

Discussion

Organ transplantation is the most promising cure for patients suffering from organ failure. However, donor organ storage and a substantial risk of rejection pose a global problem that has limited the development of organ transplantation. Stem cell technology is promising for regenerative medicine and tissue engineering development. Organs generated from the patient's cells are suitable for end-stage organ failure patients because immune rejection and immunosuppressive agents reduce the risks of infection and tumorigenesis in the transplantation of autologous cells. Animal fetuses can be a biological incubator for human organogenesis (1).

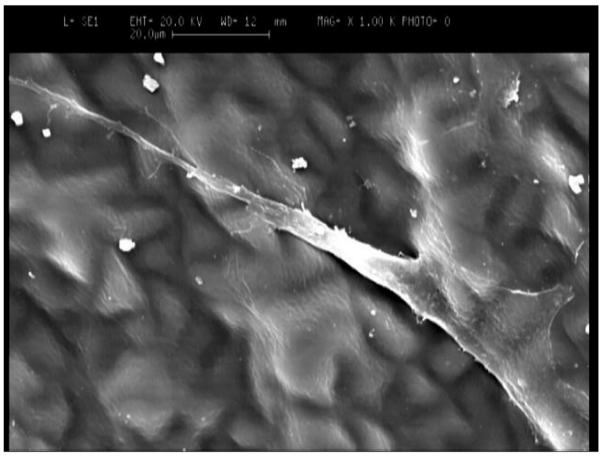

Figure 4. Wharton's jelly Mesenchymal stem cells culture on ADM after 72 h

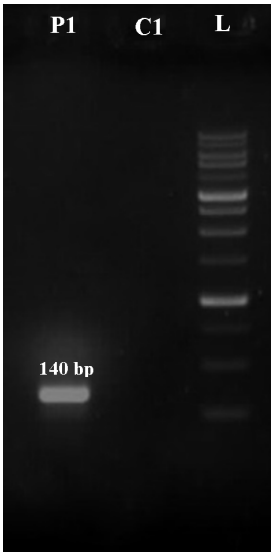

Figure 5. Amplification of the COLA-I gene with a product size of 140 bp seen in P1.
C1: negative control, L: 100 bp DNA ladder

When an adult chicken organ is implanted into CAM, it can stimulate the growth of the homolog organ (24). Ovarian tissue culture on CAM showed that this tissue can connect to CAM and follicles and stromal cells proliferate, and neovascularization has also been reported (25). The CAM can provide nutrients and growth factors for proliferating cells. Maeda and Noda have shown that the transplantation of bone onto CAM inhibits perichondrium-derived suppressive signals against the growth of epiphyseal cartilage. Therefore, CAM or other chicken cells can be an appropriate substitute for cartilage development (26). The cells were derived from adult human kidneys and implanted into CAM. After a week, cells were arranged into tubular formations (27). Mozdziak et al produced germline chimeric chickens by injecting FACS-sorted gonadal PGCs into the bloodstream of stage 17 embryos or the sub-germinal cavity of stage X embryos (28). Jiang et al used modified PGCs by lentiviral vectors to generate transgenic chickens (29). This study investigated the culturing and proliferation of HWJSCs associated with ADM on chick embryonic CAM and their migration to the internal tissues of chickens. The CAM is full of blood vessels suitable for various studies, including evaluating drug delivery, studying the transport of cells or chemicals to a tumor, and studying angiogenesis and metastasis (12). After the implantation of cells on ADM, this structure facilitates revascularization and provides a scaffold for donor cells (30). The acellular matrix can serve as a framework for cell proliferation, blood vessel formation, and differentiation. On the other hand, this matrix can be integrated into the CAM without being rejected. Numerous research studies indicate that acellular matrix transplants derived from the brain, aorta, femur, esophagus, and diaphragm can trigger a robust angiogenesis response (31-35).

In this study, it was observed that chick embryo CAM could provide a suitable platform for the proliferation of HWJSCs and their migration to the internal tissues of the chicken. The CAM model is widely used to study angiogenesis and vascular biology. The CAM displays a densely vascular structure on the inner surface of the shell (16). Angiogenesis is an essential process for the study of cell migration. HWJSCs are migrated to the liver using an extensive angiogenesis network in CAM. The expression of the COLA-I reference gene in the chicken liver and sequencing analysis showed that this gene was related to humans. Hen et al described the transfer of genes into chickens utilizing a lentiviral vector obtained from the feline immunodeficiency virus (FIV) within the CAM. They detected the expression of the yellow fluorescent protein (YFP) gene in the liver and spleen of chicks two days later (36). Naito et al isolated PGCs from embryonic blood and transferred a construct containing exogenous DNA (lacZ gene) into the cells using lipofectamine. The transfected cells were subsequently implanted into partially sterilized host embryos. Three days after PGC injection, lacZ gene expression was observed in the gonads of the chimeric embryos (37). Park et al transferred piggyBac transposition into PGCs, showing the expression of GFP in 52.2% of the chicken offspring (38).

Conclusion

In this study, HWJSCs were seeded on an ADM scaffold and cultured on a CAM. Molecular studies performed on chicken liver tissue showed that HWJSCs could migrate to the liver tissue through the vast network of vessels in the CAM. Our results indicate that human cells grown on the CAM can be present in the chicken's livers. However, further investigations are necessary to confirm this finding.

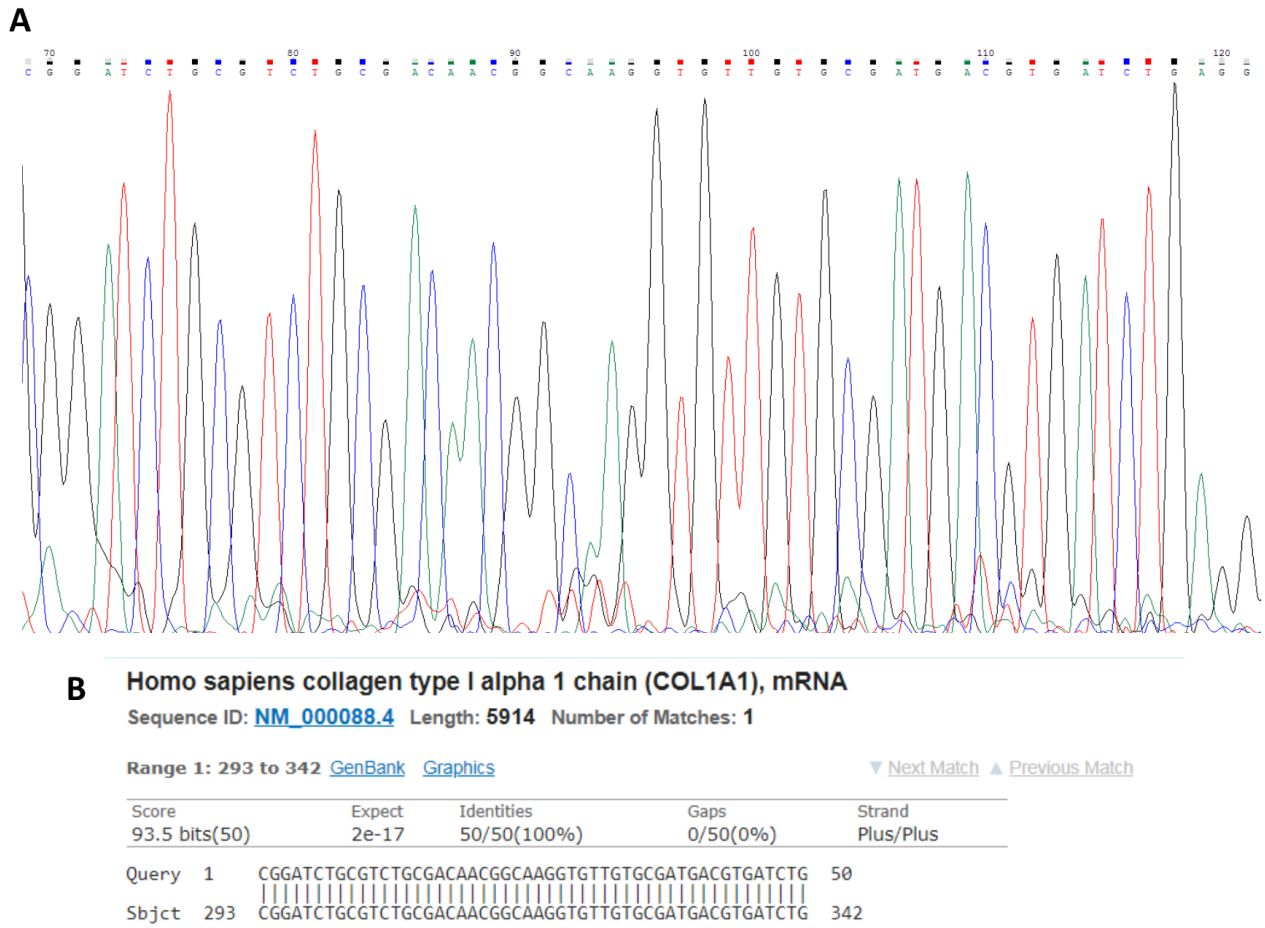

Figure 6. (A) The results of sequencing of COLA-I gene. (**B**) The results of BLAST-NCBI of sequenced COLA-I gene with the human genome

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Authors' Contribution
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Funding acquisition: Amin Derakhshanfar.
Investigation: Javad Moayedi, Seyedeh-Sara Hashemi.
Methodology: Javad Moayedi, Iman Jamhiri.
Project administration: Amin Derakhshanfar.
Resources: Amin Derakhshanfar.
Software: Zeinab Dehghan, Seyedeh-Sara Hashemi.
Supervision: Zeinab Dehghan.
Validation: Zeinab Dehghan, Javad Moayedi.
Visualization: Zeinab Dehghan, Javad Moayedi.
Writing–original draft: Zeinab Dehghan, Javad Moayedi.
Writing–review & editing: Amin Derakhshanfar, Javad Moayedi, Seyedeh-Sara Hashemi, Iman Jamhiri, Zeinab Dehghan.

Competing Interests
The authors declare that there is no conflict of interests.

Ethical Approval
The research protocol was approved by the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (Ethical code: IR.SUMS.REC.1396.S111). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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