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# Efficient isolation and identification of primary endothelial cells from bovine aorta by collagenase P

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## Abstract

**Introduction:** Endothelial cells are widely used among researchers for investigation of cardiovascular diseases, particularly atherosclerosis. Since aortic endothelial cells are able to be cultured in high passages, these cells are suitable for physiological and pathological studies of blood vessels.
**Objectives:** The aim of this study was employing a digestion method to isolate the endothelial cells from bovine aorta by utilizing collagenase P and, establishing and characterizing isolated primary endothelial cells (IPECs) cultures.
**Materials and Methods:** IPECs were isolated from fresh bovine aorta via enzymatic digestion method using collagenase P. Cell morphology and its functions were assessed by measuring the gene expression of endothelial nitric-oxide synthase (eNOS) and endothelin-1. In order to validate them as IPECs, they were compared with bovine aortic endothelial cells (BAECs) and vascular smooth muscle cells (VSMCs). Effects of synthetic endothelin-1 (100 nm) were assessed on the phosphorylation of Smad2 transcription factor via western blotting in IPECs for periods of one to four hours.
**Results:** In this study, the morphology of IPECs from bovine aorta was identical to that of the BAECs. The gene expressions of endothelin-1 and eNOS were higher than those of BAECs and VSMCs. In addition, synthetic endothelin-1 resulted in the time-dependent boost of phosphorylation of carboxy-terminal Smad2 in the IPECs for periods of two and four hours.
**Conclusion:** The results of this study confirm the efficacy of the enzymatic digestion method in isolating a large number of endothelial cells with morphological and functional characteristics.

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## Introduction

The endothelium layer covers the inner surface of *vascular walls*; in addition to being a barrier between plasma and vascular walls, this layer has remarkable roles in response to physiological and pathological stimuli (1). It is noteworthy to mention that endothelial dysfunction happens when disruption of vascular homeostasis regulation occurred. This is commonly scrutinized in patients with coronary artery disorders such as atherosclerosis, diabetes, and high blood pressure, as well as in smokers (2, 3). Therefore, in vitro culture of vascular endothelial cells is a significant tool for studying vascular physiology and disease pathology. It has been proven that endothelial cells, depending on the vascular bed from which they are derived, have diverseness of function and phenotype (4). Particularly, bovine aortic endothelial cells serve as a comprehensive model in studying all the aspects of cardiovascular function including signaling responses to bioactive compounds (5,6). The human embryonic cord endothelial cells retain the features of endothelial cells only in low passages, whereas endothelial cells of the cystic aorta also maintain their endothelial cell properties in high passages (7). A few studies have evaluated isolated primary endothelial cells (IPECs) based on their functional characteristics; therefore, developing appropriate identification methods would greatly assist in isolating and confirming endothelial cells. Numerous genes such as angiopoietin-2, Von Willebrand factor, endothelial nitric oxide synthase (eNOS), and Pecam-1 (CD31) have been recognized as being endothelial cell-limited gene expression profiles (8). It has been determined that eNOS

**Key point**
Collagenase digestion is an efficient technique for isolation of bovine aorta endothelial cells. The genes expression of endothelial nitric-oxide synthase (eNOS) and ET-1 is comparable with the endothelial-specific markers. The function of isolated endothelial cells is similar to the endothelial cell line.

gene expression is restricted to the vascular endothelium (9). In other words, the eNOS transcripts are mostly located in the vascular endothelium. Endothelin-1 (ET-1) is predominantly secreted by endothelial cells, leading to amplified vasoconstriction and arterial blood pressure (10). Studying the enhancer/promoter region of the human ET-1 gene has demonstrated that the expression of ET-1 gene is adjusted in a cell type-dependent manner (11). Studies in vascular biology have shown that the expression of ET-1 and its receptors increase in the human and animal models of atherosclerotic lesions (12).

## Objectives

In this study, the endothelial cells were isolated from bovine aorta by utilizing collagenase P. In order to confirm the IPECs as endothelial cells, the expression of endothelial cell-limited genes, namely eNOS and ET-1, were compared in IPECs, and in bovine aortic endothelial cells and vascular smooth muscle cells (VSMCs) as non-endothelial cells. Subsequently, the IPECs (isolated by enzymatic digestion) were evaluated based on their functional characteristics and the effects of synthetic endothelin-1 were investigated on phospho-Smad2C in these cells.

## Materials and Methods

### *Preparation and isolation of isolated primary endothelial cells*

Fresh adult bovine aortas were obtained from a slaughterhouse in Ahvaz, Iran. The connective and adipose tissues were removed from thoracic aorta. Then, they were washed three times with cold sterile PBS with pH 7.4, and the aortas were filled with 10 mL DMEM-high Glucose (Life Technologies, NY, USA) supplemented with 0.5 mg/mL collagenase P (Sigma–Aldrich, USA), 50 µg/mL gentamicin, and 50 µg/mL amphotericin B. Then, all of their branches and their two ends were fastened tightly and they were incubated at 37°C for 20 minutes in an incubator. The digestion fluid obtained from aorta was poured into a 15-mL tube and in order to detach the loosened endothelial cells, the entire surface of the interior of aorta lumen was gently scraped; then, the contents were poured into the same 15-ml tube. In the next step, all the collected fluid was centrifuged at 300×g for 5 minutes and the cell pellet was re-suspended in 5 mL DMEM medium.

### *Cell survival after isolation*

To evaluate cell survival and the number of cells in the collected outflow, the IPECs were stained with 0.4% trypan blue using Neubauer's chamber. Cell survival rate was obtained using the following formula:

$$\%\ Survival = \frac{Number\ of\ non-colored\ cells}{Total\ number\ of\ cells} \times 100$$

### *Cells cultivation*

The IPECs were plated at $15 \times 10^4$ cells in the T25 flasks with DMEM-Low Glucose medium enriched with 10% FBS, streptomycin (50 µg/mL), and penicillin (50 units), and they were incubated at 37°C with humidity of around 95% and $CO_2$ level of 5 percent. After 24 hours of growth, the culture medium was replaced with a new medium. Afterwards, the cells were examined morphologically by light microscopy for 5 days a week.

### *Quantitative real-time PCR*

When the confluence of the three types of cells (IPECs, VSMCs, and BAECs) reached to about 80%, total RNA was extracted by total RNA Extraction Mini kits (Favorgen). Subsequently, NanoDrop (Thermo Scientific) was used to measure the concentration and purity of the extracted RNA, and cDNA synthesis was carried out using PrimeScript™ RT Reagent Kit (TaKaRa, Japan). The eNOS and ET-1 genes expression were measured by quantitative polymerase chain reaction (qPCR) using specific primers (Table 1) and SYBR Premix Ex Taq II (Tli Plus) (TaKaRa, Japan). Moreover, GAPDH gene was used as a loading control.

### *Western blot*

The IPECs were cultivated in DMEM-Low Glucose medium (Life Technologies, NY, USA), including 10% FBS (Gibco, USA) and 1% antibiotic (Penicillin-Streptomycin; Invitrogen, Iran). The cells were incubated at 37°C and 5% CO2. IPECs were seeded $5\times10^5$ cells/50 mm petri dish. When the cell density reached about 80%, serum starvation of cells was performed for 18 hours, and finally, the cells were exposed to synthetic endothelin-1 (100 nm) for different periods of 1, 2, and 4 hours.

After treatment, cell lysates were prepared with RIPA buffer and concentration of the proteins was measured by BCA assay (Protein assay Quantification kit, Parstous Biotechnology, Iran). Sixty micrograms of the whole proteins was separated on 10% SDS-PAGE, and then PVDF membrane (Roch Diagnostics, Germany) was used for transferring the proteins. PVDF membranes were blocked with 3% skim milk solution for one hour at room temperature and then covered with phospho-Smad2 (Ser465/467) antibody (Cell Signaling Technology, USA). GAPDH antibody was obtained from Cell Signaling

**Table 1.** Sequence of primer used in this study

| Primer | Forward | Reverse |
|---|---|---|
| ET-1 | TCTGGACATCATCTGGGTCA | CTTGGCAAAAATTCCAGCAT |
| eNOS | CATTGAGAGCAAAGGGCTGC | GTACGTAGGTCTTGGGGCTG |
| GAPDH | CAAGTTCAACGGCACAGTCA | CATACTCAGCACCAGCATCACC |

Technology, USA, which was used to detect GAPDH protein as internal control. Secondary antibody was conjugated with HRP that was obtained from Sigma-Aldrich. The bands were detected using the enhanced chemiluminescence ECL detection kit (ECL Clarity kit; Bio-Rad, Hercules, CA, USA) and Bio-Rad ChemiDoc XRS+.

### *Ethical considerations*

This research was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (#ethical code; IR.AJUMS.REC.1396.104). The research also followed the tenets of the Declaration of Helsinki. This study is part of the PhD thesis of Faezeh Seif at this university.

### *Statistical analysis*

All statistical evaluations were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Results were reported as mean ± standard deviation (SD) and significant differences were measured by one-way analysis of variance (ANOVA) followed by post hoc Tukey test to detect individual differences.

## Results

### *IPECs morphology*

Phase-contrast microscopy was used to evaluate the morphology of IPECs. More than 95% of the cells displayed typical cobblestone morphology (Figure 1).

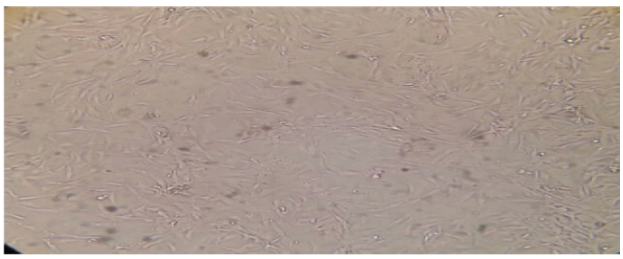

**Figure 1.** Morphology of isolated primary endothelial cells (IPECs) from bovine aorta under phase contrast microscope.

### *mRNA expression of eNOS*

Endothelial NOS is mostly produced in vascular endothelial cells, particularly in the endothelial layer of aorta. The eNOS mRNA expression was assessed by quantitative PCR in IPECs, BAECs, and VSMCs. The data demonstrated that the expression of eNOS mRNA in IPECs ($P<0.001$) and BAECs ($P<0.01$) was significantly higher than its value in VSMCs (Figure 2).

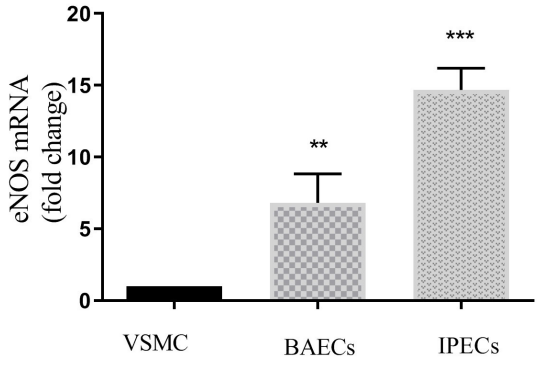


**Figure 2**. eNOS mRNA expression in IPECs, BAECs and VSMC. eNOS mRNA expression level were quantified by qRT-PCR and normalized to GAPDH mRNA level. The result shows the mean ± SD of three independent experiments (**$P<0.01$, ***$P<0.001$).

### *mRNA expression of ET-1*

ET-1, a potent vasoconstrictor peptide, is expressed and released by endothelial cells. In this study, the amounts of ET-1 mRNA expression in IPECs and BAECs were evaluated in comparison with VSMCs. The results showed that the expression of ET-1 mRNA in IPECs ($P<0.01$) and BAECs ($P<0.05$) was significantly higher than its value in VSMCs (Figure 3).

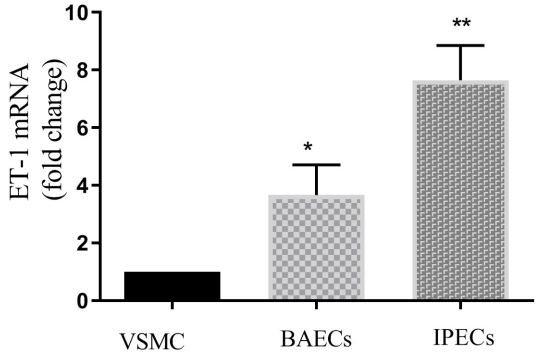


**Figure 3**. ET-1 expression in IPECs, BAECs and VSMC. The ET-1 mRNA expression level were quantified by qRT-PCR and normalized to GAPDH mRNA level. The result shows the mean ± SD of three independent experiments (*$P<0.05$, **$P<0.01$).

### *Phospho-Smad 2 (Ser465/467) induced in IPECs*

G protein-coupled receptors (GPCRs) agonists such as thrombin and endothelin-1 can activate TGF-β receptor and increase the phosphorylation of Smad2 carboxy region in BAECs and VSMCs (13,14). To investigate the ability of IPECs to respond properly to environmental stimuli, similar to what was observed in BAECs, phospho-Smad2C was measured in endothelin-1-treated IPECs by Western blotting. In this work, it has been demonstrated that synthetic endothelin-1 results in phosphorylation of Smad2C (Ser465/467) in IPECs in periods of two ($P<0.01$) and four hours ($P<0.01$; Figure 4).

## Discussion

In this study, an efficient technique was used for detaching IPECs from bovine aorta by collagenase digestion,

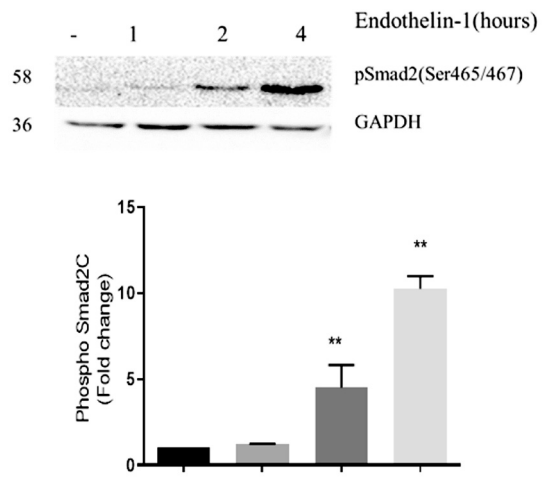


**Figure 4**. Synthetic endothelin-1 induced phospho-Smad2C. IPECs were pre-incubated with synthetic endothelin-1 (100 nM) for 1, 2 and 4 h. ** $P < 0.01$ vs untreated. Results are the mean ± SD from triplicate experiments.

while these detached cells, maintained the shape and characteristics of endothelial cells. In addition, the differences in the gene expression were revealed in a cell type-specific manner. There are various procedures for isolation of primary endothelial cells such as collagenase digestion, Matrigel matrix enriched with endothelial growth supplement, collagen gel bead, and lumen digestion (15-17). In this research, an enzymatic digestion method was used for utilizing collagenase P to isolate endothelial cells from bovine aorta. The main advantage of applying this method is easy detachment of a large number of pure primary endothelial cells from large vessels. The common shape of the isolated cells (spindle-shaped) was similar to the shape of other endothelial cells taken from the microvascular bed (18). Primary cells have several features that are very similar to the tissue from which they originate. On the other hand, immortalized cell lines can maintain their phenotype after multiple passages lasting for several months. However, several studies have demonstrated the occurrence of phenotypic and functional changes in the cultured endothelial cells after subsequent passaging (18). Lidington et al have shown that gene expression of adhesion molecules such as E-selectin and VCAM widely vary among endothelial and primary HUVEC cells. This variation is probably due to the potential of the cells to have phenotypic and genotypic changes during consecutive passages (4). In the current work, the specificity of IPECs was assessed through comparing the endothelial cell-specific genes expression including ET-1 and eNOS in IPECs, BAECs, and VSMCs. Our results revealed that the eNOS expression in the IPECs is higher than its expression in BAECs and VSMCs. Yvonne et al have shown that the methylation pattern within eNOS promoter in endothelial cells is completely different compared to that of other cell types. This diversity in pattern of methylation results in differences in the gene expression on an endothelial cell-specific fashion (19). One of the endothelial-specific genes is ET that has different isoforms (ET-1, ET2 and ET3) and is expressed in different tissues and cells, whereas ET-1 has the highest expression among endothelial cells (20). Moreover, it was discovered that the ET-1 expression was higher in IPECs in comparison with BAECs and VSMCs. This is also in agreement with the results of Harrison et al study, in which they extracted ET-1 from primary bovine endothelial cells using the sucrose density gradients (21). This research follows a previously-conducted study, in which it was determined that GPCR agonists such as thrombin induce phospho-Smad2C (14). Therefore, in this paper, the function of IPECs was investigated via measuring the phospho-Smad2C induced by synthetic endothelin-1. The phospho-Smad2C increased by synthetic endothelin-1 in IPECs was similar to the increase observed in BAECs.

## Conclusion

The data presented here show that IPECs isolated by the enzymatic digestion method are morphologically similar to BAECs. It was observed that the genes expression of eNOS and ET-1 is comparable with the endothelial-specific markers such as CD31/PECAM and Von Willebrand factor. Additionally, the function these cells have is similar to that of the endothelial cell line.

## Limitations of the study

The major limitation of our study is a limited number of genes were examined. We suggest further genes will be investigated in the future.

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### Authors' contribution

Study concept and design, HBR; acquisition of data: FS; statistical analyses; FS; drafting of the manuscript: FS, AK; analytic review of the pamphlet for important intellectual content: All authors read and signed the latest version.

### Conflicts of interest

Writers state they do not have any conflict of interest.

### Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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