Study the Effect of Undifferentiated Mesenchymal Stem Cells on Experimental Model of Peripheral Ischemia

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Abstract

Background: The adult vasculature possesses remodeling attempts to increase blood supply to better serve tissue metabolic demand. Better understanding of the mechanisms of vascular remodeling could lead to more effective treatments for these conditions.

Objectives: We aimed to study the effect of human wharton jelly derived-mesenchymal stem cells (hMSCs) on angiogenesis in a rat model of unilateral peripheral vascular disease (unilateral femoral artery ligation).

Methods: The work divided into 2 parts in vitro one for cells isolation and characterization followed by second in vivo part for cells transplantation in surgical induced peripheral ischemia. Animals were grouped; control sham-operated rats (n=12), ischemia group (n=12) subjected to surgical left femoral artery ligation with no further intervention, and a third ischemic groups (n=12) subjected to surgical left femoral artery ligation followed by 24 hr transplantation with undifferentiated hMSCs (IM injection of 5x 10^6). In vitro, hMSCs was isolated from human wharton jelly. The cells characterization was assessed morphologically in culture. Ischemic group transplanted with undifferentiated MSCs group showed an improvement in blood vessels formation, detected by high significant level of SDF-1, HIF-1α and CXCR4 genes expression and histopathological angiogenesis compared to ischemic group without further transplantation.

Conclusion: MSCs transplantation in peripheral vascular diseases may improve the angiogenesis in ischemic rat model.

Key Words: Rat ischemia – hMSCs – hypoxia inducible factor-1α – Chemokine receptor 4 – Stromal derived factor-1 – Angiogenesis.

Introduction

IN the Western world, peripheral vascular disease (PVD) has a high prevalence with high morbidity and mortality. In a large percentage of these patients, lower limb amputation is still required [1].

Several types of stem cells have been used for therapeutic neovascularization, including the mesenchymal stem cells (MSCs), which have attracted a great attention. These cells mediate their therapeutic effects by homing to and integrating into injured tissues, differentiating into endothelial cells, and/or producing paracrine growth factors [2].

MSCs are primitive cells originating from the mesodermal germ layer and were classically described to give rise to connective tissues, skeletal muscle cells, and cells of the vascular system. These cells were able to differentiate into adipocytes, chondrocytes, osteocytes, and myoblasts, both in vitro and in vivo. In addition, it has also been demonstrated that MSCs are capable of differentiating into cardiomyocytes, neurons, and astrocytes in vitro and in vivo [3,4-8]. Isolation of MSCs has been reported from several tissues, including adipose tissue, liver, muscle, amniotic fluid, placenta, umbilical cord blood, and dental pulp [3,8-10].

Wharton's jelly (WJ) is the connective tissue which is gelatinous in nature present between the amniotic epithelium and the umbilical vessels providing protection within the umbilical cord [11]. The MSCs presented in Wharton's jelly are often regarded as umbilical cord matrix (UCM) cells and their occurrence is believed to be trapped during their migration from the aortic–gonadotropin-mesonephric region to the fetal liver through
the umbilical cord in the early embryogenesis period \[12\], additionally due to their dependence on the source of oxygen and nutrients, they would most likely be located closest to the vasculature. Several types of stem cells have been used for therapeutic angiogenesis and neovascularization, including the human wharton jelly-derived mesenchymal stem cells (hMSCs), which have attracted a great attention from investigators because of their plasticity and availability \[13\]. These cells mediate the therapeutic effects by homing to and integrating into injured tissues, differentiating into endothelial cells, and/or producing paracrine growth factors. However, recent studies have shown that patients with PAD are often coincident with cardiovascular risk factors, such as aging, diabetes mellitus, which reduce the availability of progenitor cells \[14-16\]. Therefore, cell therapy and neovascularization technology application for patients with PAD and cardiovascular diseases are needed. So, the aim of the present work was to investigate whether human wharton jelly-derived mesenchymal stem cells (hMSCs) delivery may give better prognosis in a rat model of unilateral peripheral vascular disease (unilateral femoral artery ligation).

**Material and Methods**

This work was performed at the Unit of Biochemistry and Molecular Biology at The Medical Biochemistry Department, Faculty of Medicine, Cairo University, and Cairo, Egypt From December 2013 – January 2015. The work was in collaboration with Gynecology and Pathology Departments, Faculty of Medicine, Cairo University. Ethical committee approval was taken from our Faculty of Medicine, Cairo University.

**This work was done in 2 parts:**

**In Vitro:** Twenty samples of Wharton jelly for isolation and culture of MSCs.

**In Vivo:** Experimental study (surgical induced ischemia model) performed on thirty six rats.

**Inclusion criteria:**
1. Cord blood of full term labor.
2. Age: from 25-40 years.
3. Not complaining of chronic disease: Ischemia or vascular occlusion disease.

**Exclusion criteria:**
1. Cord blood of abortion or fetal death.
2. Patients having chronic disease as diabetes mellitus and hypertension.
3. Ischemic patients.

**In vitro study:**

**Isolation, expansion and characterization of different types of stem cells:**

Human umbilical cord (hUC) specimens were obtained using protocols approved by the ethical committee of Faculty of Medicine, Cairo University. hUC samples were obtained by collaboration with the Labor and Delivery nursing staff.

**Procedure:**

1. **UC samples collection and processing:**

   After obtaining patient’s Own informed consent, 20 fresh UC samples of women with healthy pregnancies were retrieved at the end of gestation during caesarean deliveries from the Department of Obstetrics and Gynecology.

2. **Culture of human umbilical cord mesenchymal stem cells:**

   hUC Wharton jelly was harvested from term deliveries at the time of birth with the mother's consent. hUCMSC was isolated by 3mg collagenase II enzyme (IgG, C.histoliticum. US Biological life science) digestion and maintained in 2% fetal bovine serum and 1x Pen/Strep (Invitrogen, CA). Cells were incubated at 37°C in an incubator with 5% CO\(_2\) at saturating humidity until cells will reach 70%-80% confluency, cultures were washed twice with phosphate buffer saline (PBS) and cells were trypsinized with 0.25% trypsin for 5 minutes at 37°C. After centrifugation, cell pellets were resuspended with serum-supplemented medium and incubated in 50cm\(^2\) culture flask (GIBCO/BRL). The resulting cultures were referred to as first-passage cultures \[17\].

3. **Characterization of mesenchymal stem cells:**

   Characterization of wharton jelly mesenchymal stem cells were assessed by their morphology, adherence, and their power to transdifferentiate into osteocytes. The differentiation was achieved by adding 1-1000 nM dexamethasone (Invitrogen), 0.25 mM ascorbic acid (Invitrogen), and 1-10mM beta-glycerophosphate (Sigma Aldrich) to the medium. Differentiation of MSCs into osteoblasts was achieved through morphological changes and confirmed by Alzarin red staining of differentiated osteoblasts.

**In Vivo part:**

**Experimental animals:**

Thirty six male white Albino rats belonging to local strain weighing between (150-170) gm from the Animal House of Faculty of Medicine, Cairo University were included in this study. The animals
were housed in wire mesh cages at room temperature with 12:12h light-dark cycles and maintained on standard chow and tap water. Veterinary care was provided by Animal House Unit of Cairo University. The animals were randomly divided into 3 groups as following:

- **1st** group: (n=12) control group (sham-operated rats) (–ve control) (No ischemia).
- **2nd** group: (n=12) ischemia was induced in them (+ve control) by surgical ligation of left femoral artery without further intervention.
- **3rd** group: (n=12) ischemia was induced by the same manner and they received undifferentiated MSCs (5x10^6 cells) after 24 hours post ischemia induction [18]. Animals were scarified after 8 weeks.

**Surgical left femoral artery ligation:**

Rats were anaesthetized with pentobarbital (50mg/kg, intraperitoneally) and unilateral left limb ischemia was created. Ventral longitudinal midline incision was performed and the left femoral artery was carefully dissected free, separated from around the tissues and ligated by a nylon suture, the incision was closed with sterile silk sutures, the wound was sterilized and I.M. tetramycin injections and skin ointment were used to prevent sepsis. The rats were allowed to recover with free access to food and water for 8 weeks. Sham-operated animals (control group) underwent identical surgical treatment, including isolation of left femoral artery; however artery ligation was not performed.

**Ischemia was assessed by pathological detection of muscle infarction.**

**After scarification of all groups:**

A- Quantitative real time PCR was done to detect CXCR-4, SDF-1 and HIF1 α:

Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOneTM, USA). The qPCR assay with the primer sets were optimized at the annealing temperature. All cDNA including previously prepared samples (for CXCR-4, SDF-1 and HIF1 α), internal control (for GAPDH gene expression as housekeeping gene), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture), were in duplicate. Primers sequence of all assessed genes were illustrated in Table (1).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence: 5′-3′</th>
<th>GBAN#</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR-4</td>
<td>F: 5′-AAAGCTAGCGGTGATCCTCA-3′  R: 5′-CACCATTTCAAGCCTTTGTTT-3′</td>
<td>NM_009911.3</td>
</tr>
<tr>
<td>SDF-1</td>
<td>F: 5′-AAAGCTAGCGGTGATCCTCA-3′  R: 5′-TTACTTGTAAAAAGCTTTTTC-3′</td>
<td>L12029.1</td>
</tr>
<tr>
<td>HIF1 α</td>
<td>F: 5′-AAGTCTAGGGATGCAGCAC-3′  R: 5′-CAAGATCCACAGCTTAG-3′</td>
<td>NM_024359.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F:CTCTACTGGCGCTGCAAGGCT  R:GTCCACCACTGCAGTGTTG</td>
<td>NT009759.16</td>
</tr>
</tbody>
</table>

#GBAN: Gene bank accession number.

B- Histo-pathological assessment of different groups of muscle tissues. Muscle tissues were collected for assessment of angiogenesis. The tissue section was washed with PBS and fixed overnight in 40g/l paraformaldehyde at 4 °C. Serials
sections of the dissected muscle tissues were stained with hematoxylin and eosin (HE) and were examined histopathologically.

Statistical analysis:

Statistical analyses were done using Statistical Package for Social Studies (SPSS) Software, version 11 for Windows. The clinical scores were shown as the mean ± standard deviation. Data were analyzed statistically by Friedman’s test and the Wilcoxon signed-rank test. p-values of less than 0.05 were accepted as significant.

Results

This study was conducted at Medical Biochemistry Department, Cairo University. The study was done on thirty six rats and was divided into two parts, in vivo study and in vitro study.

A- In vitro study:

Wharton jelly derived MSCs were isolated, propagated and characterized by morphology and ability of differentiation into other tissues like adipocytes.

B- In vivo study:

1- Induction of ischemia by ligation of the left femoral artery.

2- Animal scarification and quantitative RT-PCR analysis for the expression of CXCR4, HIF 1 α and SDF 1.

3- Histopathological examination of ischemic gastrocnemious muscle.

A- In vitro results:

1- MSCs Isolation, propagation and identification:

MSCs were identified by their morphological fibroblast spindle shape as shown in Fig. (3).

![Fig. (3): Spindle shaped MSCs after two weeks in culture with 80-90% confluence.](image)

2- MSCs characterization by ability of differentiation into osteocytes:

![Fig. (4): Characterization of MSCs.](image)

B- In vivo results:

After animal scarification, quantitative RT-PCR genes expression of HIF 1, CXCR-4 and SDF-1 were assessed and histopathological examination of gastrocnemious muscle of the studied rats.

<table>
<thead>
<tr>
<th></th>
<th>Control (8 weeks)</th>
<th>Ischaemia (8 weeks)</th>
<th>Ischaemia + undifferentiated MSCs (8 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4</td>
<td>.196±.076</td>
<td>.398±.055*</td>
<td>.204±.062#</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.

*: Statistically significant compared to corresponding value in control group (p<0.05).

#: Statistically significant compared to corresponding value in ischemia only group (p<0.05).

The quantitative RT-PCR of CXCR4 gene after 8 weeks of induced ischemia treated with undifferentiated MSCs was statistically significant compared to the corresponding value in the untreated group.

<table>
<thead>
<tr>
<th></th>
<th>Control group (8 weeks)</th>
<th>Ischemia only (8 weeks)</th>
<th>Ischemia + undifferentiated MSCs (8 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF1</td>
<td>.149±.073</td>
<td>.203±.078</td>
<td>.694±.140*</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.

*: Statistically significant compared to corresponding value in control group (p<0.05).

#: Statistically significant compared to corresponding value in ischemia only group (p<0.05).
The quantitative RT-PCR of SDF 1 gene after 2 months of induced ischemia treated with undifferentiated MSCs was statistically significant compared to corresponding value in control group and was also statistically significant compared to the untreated group.

Table (4): Quantitative RT-PCR of HIF 1 \( \alpha \) gene after 8 weeks of induced ischemia among the studied groups treated with undifferentiated MSCs and those without treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control group (8 weeks)</th>
<th>Ischemia only (8 weeks)</th>
<th>Ischemia + undifferentiated MSCs (8 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1( \alpha )</td>
<td>.223±.091</td>
<td>1.082±.294*</td>
<td>.431±.138#</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.
*: Statistically significant compared to corresponding value in control group (\( p<0.05 \)).
#: Statistically significant compared to corresponding value in ischemia only group (\( p<0.05 \)).

The quantitative RT-PCR of SDF 1 gene after 8 weeks of induced ischemia treated with undifferentiated MSCs was statistically significant compared to corresponding value in the untreated group.

**Histopathological examination:**

**A- Normal healthy tissue:**

Striated muscles were demonstrated in control/placebo group as normal muscle cells with preserved nuclei and cytoplasm. No inflammatory cells or oedema and normal vasculature were detected as illustrated in (Fig. 5).

**B- Ischemic tissues:**

- Architect: Focal areas of muscle necrosis.
- Myocytes: Necrosis and atrophic dark myocytes nearby and vacuolar degenerative changes
- Inflammation: Reduced signs of inflammation
- Interstitium: Edema hemorrhage & large dilated congested vessels with rupture.
- Damage Necrosis, degeneration & atrophy score 4.
- Regeneration minimal focal.

**C- Ischemic tissue injected with undifferentiated MSCs:**

- Damage Necrosis score 2.
- Mild healing and regeneration.
- Neovascularization.

Fig. (5): Normal striated musculature (stained with MT with X100 magnification).

Fig. (6): Hematoxylin and eosin staining (A, B &C) showed ischemic muscle tissue with large area necrosis, PNLs & mononuclear infiltrate around necrotic muscle inflammation (arrow) X100 (A), X200 (B) & X400 (C).
Study the Effect of Undifferentiated Mesenchymal Stem Cells

Discussion

The present study was designed to examine the therapeutic effects of human wharton jelly derived MSCs in the treatment of ischemic hindlimb. Overall, this study demonstrates more pronounced angiogenic response, and improved muscle performance of ischemic muscle following human wharton jelly derived MSCs transplantation. Wharton jelly derived MSCs have been identified as a potential new therapeutic option to induce therapeutic angiogenesis. The main advantage of using wharton jelly derived MSCs in treating ischemic disease is that MSCs can be isolated by ex vivo non invasive expansion before implantation. Under specialized culture conditions, wharton jelly-derived MSCs have the capacity to differentiate into cells such as bone, cartilage, adipocytes and endothelial cells [19]. The present study shows that transplantation of hMSCs to ischemic hindlimb significantly increases CXCR-4, SDF-1 and HIF1 α genes expression, and improves muscle tissue angiogenesis. In our experiment, the fusiform isolated and propagated cells were characterized by their ability to differentiate into osteocytes. Capacity for migration towards an injured region is an important characteristic of MSCs. When transplanted into ischemic hindlimb muscle after left femoral artery ligation, MSCs survived and migrated to the ischemic site, where they restored damaged ischemic cells. The transcription factor hypoxia-inducible factor-1 (HIF-1) is a central regulator that expresses in response to hypoxia which mediates systemic homeostatic responses to low levels of oxygen [20]. Under hypoxic conditions HIF-1 α accumulates and activates the transcription of downstream target genes encoding multiple angiogenic growth factors and cytokines of potential importance in wound healing [21]. In this study, we have observed that HIF-1 α increased in the hindlimb of the rat model following surgical induced femoral artery ischemic ligation. MSCs migration may involve various chemokines, cytokines, and integrins. Among the chemokines and their corresponding receptors, the SDF-1α/CXCR4 axis is the most extensively studied system [22-24]. SDF-1 α, a member of the CXC subfamily, is widely expressed in many organs. The ischemia microenvironment in the injured tissues can up-regulate its expression. We have observed increased SDF-1 α expression in the current study. Ceradini et al., (2004) indicated that SDF-1 α gene expression is regulated by HIF-1, resulting in selective in vivo expression of SDF-1 α in ischemic tissue in direct proportion to reduced oxygen tension. In addition, HIF-1-induced SDF-1 α expression increases the adhesion, migration and homing of circulating CXCR4-positive progenitor cells to ischemic tissue [28]. We propose that HIF-1-induced SDF-1 α expression also increases the homing of injected MSCs to the injured muscle tissue. In the present study, we have found that SDF-1 α increased significantly in the hypoxic ischemic rat. The results suggested that SDF-1 α might be an important factor in the microenvironment of the muscle and trigger the migration of MSCs. Translation of these data to similar significant clinical improvements in tissue function, such as increases in walking distance, would signify a
substantial advance in the treatment of peripheral vascular disease. In this study, we showed that successfully isolated and cultured hMSCs, when transplanted into the ischemic hindlimb, led to quantitative high significant changes in CXCR-4, SDF-1 and HIF1α in muscle tissue at the molecular level compared to control non ischemic and to ischemic none-injected rats. These observations suggest that transplantation of hMSCs can change the ischemic microenvironment and provide an organization and formation of new blood collaterals. At the histopathological levels, the ischemic muscle tissue showed eosinophilic necrosis, loss of cell nuclei, fibrous tissue, edema and collection of inflammatory cells compared to control non-ischemic muscle which showed intact nuclei and normal striated muscle bundles. Ischemic transplanted muscle with hMSCs showed definite angiogenesis, neovascularization and collateral formation. Lim et al., [26] proved that administration of human umbilical cord MSCs potentially improved ischemic stroke and may be a clinically feasible means of providing less invasive and repeatable transplantation therapy. Li et al. [27] reported that MSCs express factors that support cell survival and avoid apoptosis thereby preserving cells which would otherwise be destroyed. The authors also reported that, hindlimb muscle cells underwent severe ischemic apoptosis after artery occlusion, however the apoptosis of cells in ischemic muscle regions were significantly reduced after simvastatin and bone marrow-derived MSCs combined treatment. In conclusion, human wharton jelly-derived mesenchymal stem cells (hMSCs) transplantation may be a helpful tool for better treatment and prognosis for ischemic stroke and peripheral arterial disease (PAD). This makes hMSCs potent candidates for the clinical application of MSC-based therapies.

Conclusion:

MSCs transplantation in peripheral vascular diseases may improve the angiogenesis in ischemic rat model.

References

18- J.C. ZHANG, G.F. ZHENG, L. WU, et al.: Bone marrow mesenchymal stem cells overexpressing human basic


تملخص العربي

يتمثل قصور الشرايين الحاد واحد من أصعب التحديات التي يواجهها أطباء أمراض الأوعية الدموية. ويعرف قصور الشرايين الحاد بالانخفاض المفاجئ في ضغط الدم الذي يسبقه خطراً محتلاً على بقاء الطرف (يتحلل في حدوث أم تقصف، وقحة بالطرف، و/ أو الغريتنا) في المرضى الذين يضمنون خلال أسبعين من الحدود الحادة.

تكمل هذه المشكلة، تعرّض الرسمي أن الجهاز الدموي، وهو عامل كيميائي يُتميز بكفاءة عالية لخلايا T، الخلايا الحرة، و ما قبل الخلايا الأصلية، والخلايا الأصلية المتكونة لدى SDF-1، والخلايا الجذعية، والخلايا الأصلية المتكونة لدى (CXC) chemokine، طريق النقل بواسطة CA و/ أو الهجرة الخلية، وتصنيع الأوعية الدموية بشكل طبيعي بالانتحار، باستقبال التحذيرات.

والهدف من هذا العمل هو دراسة قدرة الخلايا الجذعية الوسيطة المستمدة من وارتون جيلي على تصنيع الأوعية الدموية في حيوانات التجربة.

ويتم هذا العمل من جزيئين:

- في المختبر: يتكون من عشرين عينة من وارتون جيلي لعزل الخلايا الجذعية الوسيطة.
- في الحيوانات: دراسة تجريبية (نموذج قصور الشرايين تم إحداثه جراحياً) أجريت على ستة وثلاثين فأرًا. تم حقن الفئران التي أصيبت بقصور الشرايين بخلايا جذعية وسليطة.

- تم تقسيم الدراسة إلى 3 مجموعات:
  - مجموعة 1 (العدد 16 فأرًا) المجموعة الضابطة (لا تعاني من قصور الشرايين).
  - مجموعة 2 (العدد 16 فأرًا) تم إحداث قصور الشرايين من خلال الرهين الجراحية لدى الفئران دون مزيد من التجربة.
  - مجموعة 3 (العدد 16 فأرًا) تم إحداث قصور الشرايين من خلال نفس الطريقة واثارة الخلايا الجذعية الوسيطة غير متحورة (6x10^6 خلية).

- بعد 24 ساعة من حدوث قصور الشرايين، وشملت الدراسة تقييم ما يلي:
- الكشف عن جينات 4 PCR في الأنسجة الدموية التي تم تشريحها بواسطة تسلاسل البلمرة الكمي.
- الكشف عن النسخة الجينية HIF1α، SDF-1، CXCR-4 في الأنسجة المرضية التي تم تشريحها بواسطة تسلاسل البلمرة الكمي.

ولقد لاحظنا اختلافاً في مستويات جينات HIF1α و SDF-1، والإنزيمات التي لم يتم حقنها والدراسات التي حققت بخلايا غير متحورة.

وتشمل نتائج الدراسة في الحالات التي تم إحداثها من خلال الربغ الجراحية لدى الفئران، وتشمل قصور الشرايين المحايدة نمو وتطور الخلايا الجذعية الوسيطة، وظيرت النتائج اقتنا، والتحدي البؤري، والانتقاص، والخلاصة: أثبتت هذه الدراسة نجاح الخلايا الجذعية الوسيطة في سلسلة الأوعية الدموية في حيوانات التجربة المصاب بقصور الشرايين.