

Hypoxic Preconditioning Effect on the Expression of Intracellular Heat Shock Protein (HSP) 27, HSP 70 and HSP 90 on Cultured Adipocyte-Derived Mesenchymal Stem Cells (AMSCs)

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2 Hypoxic Preconditioning Effect on the Expression of Intracellular Heat Shock Protein (HSP) 27, HSP 70 and HSP 90 on Cultured Adipocyte-Derived Mesenchymal Stem Cells (AMSCs)

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Abstract. New hope in the treatment of myocardial infarction has emerged by transplanting multipotent stem cells into the affected myocardium. Hypoxic preconditioning (HPC) is expected to increase the survival cell potency by inducing upregulation of Heat Shock Protein (HSP) in Adipocyte-derived Mesenchymal Stem Cells (AMSCs). This study aimed to investigate the roles of HPC in AMSCs to observe the in vitro expression of HSP27 as an ATP independent chaperone, and HSP70, HSP90 as an ATP dependent chaperone. This study is a true experimental randomized post-test design study. AMSCs were isolated from adipose tissue and cultured until there were four passages. The samples were then divided into two groups, HPC (O₂ 1%) and normoxia (O₂ 21%). Immunocytofluorescence techniques were used to evaluate the expression of HSP27, HSP70, and HSP90. Data were analyzed using a two paired t-test and Mann Whitney U test. There was a significant increase of HSP27, HSP70, and HSP90 expression in HPC compared to the normoxia group (272,00±92,225 vs 86,19±26,362 p<0.0001; 130,88±45,416 vs 120,75±97,113 p<0.05; 165,75±58,930 vs 96,81±22,578). Hypoxic preconditioning significantly increased the expression of HSP27, HSP70, and HSP90 on cultured AMSCs.

1. Introduction

Myocardial infarction causes cardiomyocyte death and myocardial tissue dysfunction. It may lead to scarring in the myocardium and become heart failure. Recent management of myocardial infarction is still limited to medical and interventional therapy that aims to recover coronary flow and reduce the myocardial burden. The challenge of recent therapy is the inability of the heart to carry out self-regeneration. This statement leads to the development of gene and cell-based therapies for cardiac treatment [1]. Several studies provide hope for myocardial infarction therapy by transplanting the pluripotent stem cells to repair and regenerate myocardium. For several years bone marrow-derived MSCs (BMSCs) are the main source of stem cells for regenerative medicine applications. But several recent studies have shown the same potential role of subcutaneous adipose tissue or adipocyte-derived mesenchyme stem cells (AMSCs) to differentiate into several types of cells and tissues [2].

Survival of the low transplanted stem cell is due to the apoptosis process is the biggest challenge in stem cell therapy. It is presumably because the cells were exposed to a pro-apoptotic

microenvironment after myocardial infarction. Lack of oxygen supply triggering inflammatory factors causes oxidative stress and the presence of cytotoxic processes. It is then followed by failure of the transplanted cell through various pathways, mainly through the mechanism of apoptosis. For this reason, increasing transplanted stem cell resistance against apoptosis is important in achieving successful stem cell therapy [3] [4].

Hypoxic preconditioning (HPC) is a sublethal hypoxic condition. It provokes the internal mechanism of MSCs followed by several cellular processes such as protein expression that can protect these cells from lethal hypoxia and other ischemic conditions. Heat shock protein (HSP) is known to be involved in hypoxia and ischemic conditions. Normally HSP is present in the cells, but its synthesis will rise in conditions of stress, such as hypoxia and ischemia [5].

The role of HSP as a cell protector against apoptosis has been widely developed, but still needs further examination, especially in terms of AMSC application as the basic ingredient of stem cells. Hypoxic preconditioning in AMSCs is expected to increase the survival cell potential by increasing HSP. Therefore, the researchers would like to further review the role of MSCs, especially AMSCs. Technically, AMSCs are given exposure to hypoxic preconditioning with a 1% oxygen concentration to examine the potential of HSP 27, HSP 70, and HSP 90 in vitro.

2. Methods

This was an exploratory laboratory experimental study (in vitro study) with the provision of hypoxic conditions in AMSCs cultures from adipose tissue. This study aims to determine the hypoxic preconditioning effect on the expression of HSP 27, HSP 70, and HSP 90. This was a true experimental post-test study factorial and randomized group design.

This research was conducted through 4 phases, first by isolation and culture of AMSCs from adipose tissue by minimally invasive procedures. Identification of the phenotype of AMSCs from adipose tissue (immunofluorescence and flow cytometry) was conducted by examining the expression of CD 105 +, CD 90+, and CD 45- (cell-surface markers). Hypoxic preconditioning (HPC) AMSCs were in vitro. The last measurement of intracellular HSP 27, HSP 70 and HSP 90 expression is in AMSCs tissue cultures.

2.1 Research phase 1: AMSCs isolation and culture from adipose tissue

2.1.1 AMSCs isolation. The procedure begins with minimal adipose tissue surgery using a standard collected method in a sterile closed tube. Liposuction samples were placed at room temperature after arriving at the laboratory, about 24 hours before use. All procedures were carried out in closed biosafety.

2.1.2 Tissue Washing. A 2 ml tube containing 500 µl of warm PBS and 1% antibiotic solution was added with a small amount of tissue (approximately 150 mg). The sample was then washed with 300 µl PBS warmed with 1% antibiotic solution. The washing stage is repeated several times, usually twice. The tissue is placed on the plate, and tissue fragmentation is carried out.

2.1.3 Tissue Digestion. The volume of fat to be washed was measured (from the last washed) and then prepared with the same warm collagenase enzyme. The washed fat was then poured into a sterile bottle of 600, 1000 or 2000 ml, depending on the estimated volume of washed fat (4 x aspirate tube volume). A mixture of 5 ml of collagenase enzyme was then added. The tube was closed and placed in the tube on the magnetic stirrer or manually placed into the water bath, at 35-38°C prewarmed for 20 ± 5 min. The frequency and amplitude of the magnetic stirrer will be set to prevent separation of the floating tissue from the collagenase fluid. Observation is repeatedly performed after 15 minutes to prevent over-digestion. Digestion can be stopped when the residual quantity of fragments of adipose tissue is estimated at 5% from the initial amount. After that, the digestion was moved to a sterile mouthpiece glass, then left for 5-10 minutes to let the separation begin. Finally, the suspension was filtered on a 100 mm cell filter and collected in a 50 ml centrifuge tube.

2.1.4 Stromal Tissue Separation. The suspension then centrifuges twice with 1800-2000 rpm for 5-6 minutes at room temperature. Then it is immediately shaken in the pellet and mixes the cells. The enzyme liquid is removed, leaving the lipoaspirate at the bottom.

2.1.5 AMSC's culture procedure. First, a plate was prepared with complete α -MEM medium, which contains 20% FBS + anti-mycotic, antibiotics, and nutrients. After the sample was dissolved with three equal volumes and distributed equally into several dishes, each 10 cm culture dish contained of 10 ml of diluted aspirate, then evaluated with a microscopic phase 0 (post isolation). The culture dish was reinserted into the incubator with 5% CO₂ and cultured continuously at 37°C incubator temperature. The cell density was observed every day. It was assumed that AMSCs had achieved the next passage and moved to the new culture dish if the cell is about 80-90% confluent. It was transferred to a culture dish and centrifuged to the cell culture initial process. This is the key action because the cell density > 90% becomes a contact inhibitor and ceases splitting. Each phase in this study, on average, takes 3-4 days. AMSC is grown to phase 4. Every 3-4 days, the medium is replaced until confluence cells are formed. When the cells are at 80-90% confluence, it can be harvested or directly identified for adipocyte characterization. In this study, adipocytes characterization was held when the cell had come to the fourth passage. First of all, the medium was removed from the cell and stored in the sterile media in a sterile tube for next cell culture applications (sterile filtration before applying). Then a volume of 250-500 μ l PBS was added to gain supernatant aspiration and cell suspension in a 100 μ l stromal inoculation of the cell medium and put into a ten well cover plate (Cooke, Germany).

2.2 Research phase 2: AMSCs characterization in the form of phenotype identification of CD90+ CD105+ and CD45- before hypoxia administration

We conducted an examination into the AMSCs phenotype (CD 90+, CD 105+, and CD 45-) that were cultured before being treated with HPC. Examination of phenotype was carried out using Flow cytometry and Immunofluorescence methods.

2.2.1 Flow cytometry: CD90+ and CD 45-. AMSC cells were added to a solution of CD105 and CD44 antigens. Then after a few minutes, the cells were tested in the flowcytometry tool. Results were obtained in the form of images and tables.

2.2.2 Indirect immunofluorescence for CD90+, CD 105+, and CD 45-. Immunophenotype CD 90+, CD 105+, and CD 45- as M-AMSCs markers can be recognized using immunofluorescence indirectly. The identification was done using harvested M-AMSCs cell culture and then inserted into a 15 ml tube and fixed using methanol, after 15 minutes the FITC-labeled reagent was added and then washed with PBS and then poured onto an object glass and examined under a fluorescence microscope.

2.3 Research phase 3: Hypoxia preconditioning on in vitro AMSCs with 1% oxygen concentration for 24 hours

At this stage, AMSCs samples were divided into two treatment groups consist of control Group (P0), Normoxia AMSCs (Oxygen 21%) for 24 hours. And also treatment Group (P1), HPC AMSCs (Oxygen 1%) for 24 hours

2.4 Research phase 4: Measurement of the expression of Intracellular HSP 27, HSP 70 and HSP 90 Levels

Intracellular HSP 27, HSP 70, and HSP 90 levels in this study were analyzed using immunofluorescence and flow cytometry methods.

2.4.1 Immunofluorescence. Expression of HSP 70 (Bioss, USA) and HSP 90 (Santa Cruz Biotechnology) could be examined using indirect immunofluorescence. The identification was made by harvesting AMSCs cell culture. It was then it inserted into a 15 ml tube and fixed using methanol. After

15 minutes the FITC-labeled reagent was added, then washed with PBS and dropped on the object-glass and analyzed under a fluorescence microscope.

2.4.2 Immunocytochemistry Immunocytochemistry in AMSCs cell preparations was intended to see the expression of HSP 27 (eBioscience, San Diego). The cell coloration causes antibody-antigen bonds on the surface or inside cells; then it could be detected by marking with enzymes, isotopes, fluorophore, or colloidal gel. Cells were fixed then localized between cells and examined by electron microscopy or light microscope, to analyze cell morphology using DAB Plus cell staining (Thermo Scientific).

3. Results

3.1 Expression of intracellular HSP 27

HSP 27 is an ATP-independent small Heat Shock Protein (sHSP) that will experience up-regulation in cell stress conditions, such as hypoxia. The presence of HSP 27 expression on the AMSCs surface, that has been exposed to hypoxic preconditions (1% O₂ level for 24 hours), is a sign that these AMSCs have cytoprotective and anti-apoptotic potential thus maintaining cell survival. In this study, qualitatively, HPC stimulation in AMSCs for 24 hours showed stronger expression of HSP 27 compared with normoxia AMSCs on immunocytochemistry examination. It can be seen in cells stained with DAB plus cells (Thermo Scientific) characterized by AMSCs, which has a strong brownish glow (Figure 1).

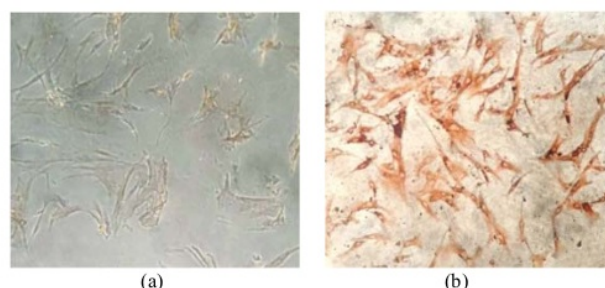


Figure 1. Immunofluorescence examination of the expression of HSP 27 in normoxia (a) and hypoxia (b)

The normality data were tested using the Kolmogorov Smirnov One-Sample Test to evaluate the expression of HSP 27 quantitatively both in the hypoxia and normoxia group and showed the results of a normal distribution. The data were then tested statistically with two paired t-tests, which showed very significant results ($p < 0.05$). The data analysis showed that the average HSP 27 in AMSCs culture with HPC was higher compared to AMSCs O₂ culture 21% (272.00 \pm 92.225 pg/ml vs. 86.19 \pm 26.36 pg/ml) with a p-value < 0.0001 (Table 1)

Table 1. Differentiation test of HSP 27 Expressions in the normoxia and hypoxia groups

	Group	<i>n</i>	Mean \pm SD	<i>p</i>
HSP 27	Normoxia	16	86,19 \pm 26,362	$< 0,0001$
	Hypoxia	16	272,00 \pm 92,225	

3.2 Expression of intracellular HSP 70

The stimulation of hypoxic preconditioning is expected to improve cell survival by inducing cell defense mechanisms so that apoptosis does not occur. HSP 70 is one of the ATP-inducible HSPs that was

stimulated during HPC. With this increase, it is expected to maintain protein folding and inhibit apoptosis through barriers to the apoptosome. These obstacles resulted in inactivation of caspase 9 and caspase three so that apoptosis did not occur.

In this study, qualitatively, HPC stimulation in AMSCs for 24 hours showed higher expression of HSP 70 compared to AMSCs O2 21% on immunofluorescence examinations, characterized by green luminescence of AMSCs (Figure 2).

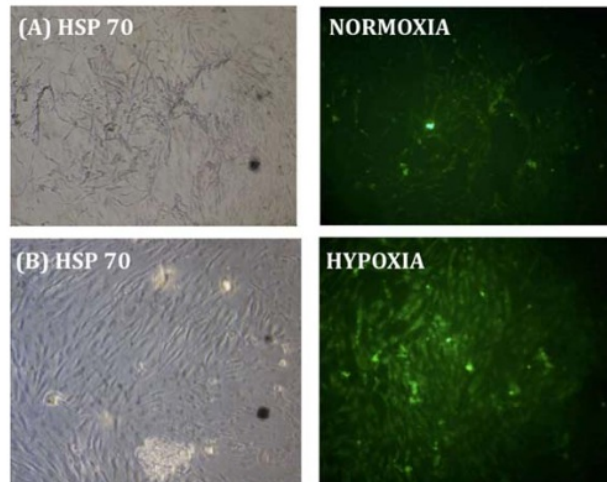


Figure 2. Immunocytochemistry examination of HSP 70 expression in the fourth phase culture. The picture of immunofluorescence in the hypoxia group (B) has a stronger green glow than the normoxia (A) group.

To evaluate the expression of HSP 70 quantitatively in the hypoxia and normoxia group, the normality data were tested using the Kolmogorov Smirnov One-Sample Test and showed abnormal distribution results. The data were then tested statistically with the Mann Whitney-U test, which showed significant results ($p < 0.05$). The results of the data analysis showed that the average HSP 70 in AMSCs culture with HPC was higher than AMSCs normoxia (130.88 ± 45.416 pg/ml vs. 120.75 ± 97.113 pg/ml) with a p-value 0.048 (Table 2).

Table 2. Differentiation test of HSP 70 Expressions in the normoxia and hypoxia groups

	Group	n	Mean \pm SD	p
HSP 70	Normoxia	16	$120,75 \pm 97,113$	0,048
	Hypoxia	16	$130,88 \pm 45,416$	

3.3 Expression of intracellular HSP 90

HSP 90 is ATP-dependent inducible HSP. It can prevent the occurrence of apoptosis by binding to APAF-1. Then it prevents bonding with cytochrome-c and increases cytoprotective eNOS synthesis.

In this study, qualitatively, a stronger HSP 90 expression, showed by green luminescence, was found to be higher in HPC rather than the normoxia group (Figure 3)

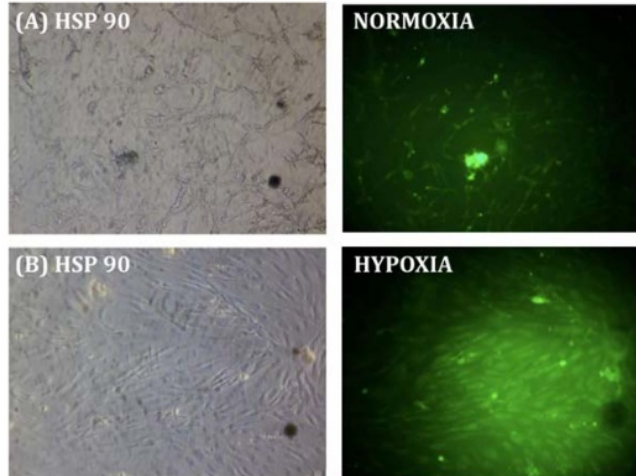


Figure 3. Immunocytochemistry examination of HSP 90 expression in fourth phase culture. The picture of immunofluorescence in the hypoxia group (B) has a stronger green glow than the normoxia (A) group.

The normality data were tested using the Kolmogorov Smirnov One-Sample Test and showed normal distribution results. The data were then quantitatively tested with two paired t-tests, which showed very significant results ($p < 0.05$). The results of the data analysis showed that the average HSP 90 in AMSCs culture with HPC was higher compared to normoxia AMSCs (165.75 ± 58.930 pg/ml vs. 96.81 ± 22.578 pg/ml) with a p -value < 0.0001 (Table 3).

Table 3. Differentiation test of HSP 90 Expressions in the normoxia and hypoxia groups

	Group	<i>n</i>	Mean \pm SD	<i>p</i>
HSP 90	Normoxia	16	96,81 \pm 22,578	$< 0,0001$
	Hypoxia	16	165,75 \pm 58,930	

4. Discussion

Mesenchymal stem cell (MSC) has a promising therapeutic potential with its ability to regenerate and multiply. It also has paracrine effects after being transplanted into the ischemic or infarct heart. Adipose tissue contains large amounts of AMSCs multipotent cells, the important and prerequisite element for stem cells based therapy. AMSCs have many surface antigens (surface markers) in their cells to show the nature or character of the cell. AMSCs cannot be identified using a single surface marker, but several markers are needed for identification of the existing population. Several markers that have generally been reported to have strong positive expressions are CD13, CD29, CD44, CD73, CD90, CD105, CD166, and MHC I. On the other hand, hematopoietic and angiogenic cell markers such as CD31, CD45 and CD133 show low expression in AMSCs. Likewise, MHC II is also not found in ASCs. Levels of expression markers found with sufficient or moderate levels were CD9, CD34, CD49d, CD106, CD146, and STRO-1, which were below 50%. [6] Because MSCs have many surface antigens, the 2006 International Society of Cellular Therapy (ISCT) determined the minimum criteria for characterizing

MSCs, most of which showed positive expressions of CD73, CD90, and CD105, and negative expressions of CD11b or CD14, CD19, CD34, CD45, and HLA-DR.[7] In this study, we used the phenotypic characteristics of CD 90+, CD105 + and CD 45- expressions in post-4 AMSCs cultures. The results explained the characteristics of MSCs in immunofluorescence CD90 + and CD 105+ showed the results of cells that glowed green as markers of characterization of phenotypes of MSCs, besides that CD 90+ flowcytometry examination also proved that these were positively expressing cells. While in immunofluorescence phenotypes examination, CD 45- showed negative expression results and was supported by flow cytometry examination. It reaches the minimum criteria for characterizing MSCs presented by the International Society of Cellular Therapy (ISCT).

However, the use of MSCs on therapeutics is limited to the fact that not all transplanted MSCs survive. It is due to microenvironment conditions in ischemia that induce cell death.[8]. It is reported that only a very small proportion of cells (<10%) will survive 24 hours after transplantation. Various methods were developed to prevent cell apoptosis and increase therapeutic potential, especially in ischemic heart, such as by finding the best stem cell sources based on therapeutic and ethical considerations, genetic modification, the best transplant route, understanding apoptosis inhibition pathways, or increasing cell viability by using preconditioning in vitro. One way to improve cell survival is by giving hypoxic preconditioning. The advantages of hypoxic preconditioning in mobilizing endogenous defense mechanisms have been demonstrated in cell culture.[9][10] The strategy of HPC on stem cell therapy has many advantages in increasing (1) cell survival (cell survival), (2) paracrine activity to construct a supportive environment rich in tropic and angiogenic factors, (3) motility/cell migration, (4) cell proliferation ability, and (5) cell differentiation according to its functional integration.[11] The stimulation of hypoxic preconditioning (HPC) causes a stress response from cells, that stimulate the endogenous defense mechanism, followed by activation of HSF-1 simultaneously with hypoxia shock element will cause upregulation of some inducible heat shock proteins (HSP 27, HSP 70 and HSP 90). Also, stimulation of hypoxic preconditioning (HPC) enhanced HIF-1 α , which has an important role in MSCs, by regulating several genes that contribute to cell adhesion, migration, and paracrine effects.

In our study, there were strong expressions of HSP 27, HSP 70, and HSP 90. It was supported by the results of immunofluorescence tests on the expression of HSP 70 and HSP 90, which showed the presence of green fluorescent cells that were higher in the hypoxia group compared to normoxia. In HSP 27, with the use of DAB Plus cell staining (Thermo Scientific), a light brownish cell glow showed higher expression of HSP 27.

From our study, the examination of HSP 27 expression was higher in AMSCs who were given 1% hypoxia O₂ exposure compared to 21% normoxia O₂ which was 272.00 ± 92.225 vs. 86.19 ± 26.326 with very strong significance ($p < 0.0001$). Additionally, immunocytochemical examination showed HSP 27 expression in AMSCs with normoxia culture becoming stronger after being exposed to hypoxia (Figure 11). It indicates that hypoxia exposure can stimulate greater HSP 27 expression than normoxic conditions. Hypoxic stimulation can increase the expression of small heat shock protein (sHSP), namely HSP 27, which has anti-oxidant, anti-inflammatory, and anti-apoptotic functions. HSP 27 is an ATP-independent chaperone making its expression unlimited by low ATP condition such as hypoxia as shown as the study presented by Whitlock et al., 2008 where ischemic preconditioning (IPC) causes HSP 27 upregulation in the culture of retinal neurons. It is proven that preconditioning can increase the expression of HIF-1 α , which has a protective effect on retinal damage. The mechanism of survival cells by HSP 27 also presented by You et al., 2012 on the excessive expression of HSP 27 in septic rats. The PI3K/Akt activation pathway is thought to be a mechanism of avoidance of cardiac dysfunction in sepsis rats.

Hypoxia can regulate HSP 70 expression, and as previously explained, hypoxia can activate heat shock factor (HSF). Heat shock element (HSE) activate gene transcription and increases HSP expression. In our study, the examination of HSP 70 expression was higher in AMSCs who were given 1% hypoxia O₂ exposure compared to 21% normoxia O₂, i.e., 130.88 ± 45.416 vs. 120.75 ± 97.113 with significant results ($p < 0.05$). Also, immunofluorescence immunocytochemistry showed stronger

HSP 70 expression in AMSCs culture after being given hypoxic exposure. It is characterized by a light greenish glow in the hypoxic group (Figure 12). Similar results were obtained from our study for HSP 90. From the examination of HSP 90 expression, higher results were obtained in AMSCs given HPC exposure than normoxia O₂ 21%, i.e., 165.75 ± 58.930 vs. $96.81 \pm 22,578$ with results significant ($p < 0.0001$). Immunofluorescence immunocytochemistry also showed stronger HSP 90 expression in AMSCs culture after exposure to hypoxia (Figure 13). HSP 70 and HSP 90, ATP-dependent substances, are inducible HSPs. The study by Turman et al., 2012 in the human proximal tubular epithelial cell (PTEC) given hypoxic preconditioning for 12 hours later 24 hours, showed higher expression of HSP 70 and heat shock cognate-70 (HSC70) compared to basal conditions. Northern blot analysis showed HSP 70 > HSP 90 > HSC 70. The same conclusion was explained by Jiang et al., 2013 who used BMSCs from mice given 24 hours of hypoxia in vitro. The result showed up-regulation of HSP 90, where HSP 90 decreases receptor expression BAX and caspase-3 and activate PI3K/Akt and ERK1/2 anti-apoptotic pathways. It also explained that upregulation of HSP 70 and HSP 90 after preconditioning was reported to inhibit the release of second mitochondria-derived activator of caspase (SMAC) to prevent activation of caspase-9 and caspase-3 thereby preventing apoptosis [9].

It is safe to say that HSP 27, HSP 70, and HSP 90 could be expressed in cell stress conditions such as hypoxia so that it is assumed to be able to increase survival cells especially in providing cytoprotective and anti-apoptotic effects. Interestingly, there is a higher expression of HSP 27 compared to HSP 70 and HSP 90 under hypoxic conditions. This is possible because of the ability of HSP 27 as an ATP-independent chaperone. In sum, there are potentials of AMSCs, which still need to be explored further, in the signaling pathway and cell survival ability given by HSP as a basis for regenerative medicine-based therapy.

5. Conclusion

There is a significant difference between the intracellular expressions of HSP 27, HSP 70, and HSP 90 in the group given hypoxic preconditions compared to the control group (normoxia).

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