Original Article

IL-10 AND TGF-β1 EVALUATION IN 3D HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS CULTURE AND HYPOXIC CONDITION

AVALIAÇÃO DE IL-10 E TGF- β 1 SOB CONDIÇÕES DE HIPÓXIA EM CULTIVO CELULAR 3D DE CÉLULAS-TRONCO MESENQUIMAIS DE TECIDO-ADIPOSO HUMANO

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ABSTRACT: Mesenchymal stem cells (MSC) are multipotent cells derived from layer mesoderm and that have potential for self-renewal and cellular differentiation. These cells can be extracted from various tissues, being the main sources the bone marrow (BM) and adipose tissue (AT). Therefore, human Adipose-derived Mesenchymal Stem Cells (AdMSCs) are potentially able to differentiate in several cell types such as neurons, adipocytes and osteoblasts. The objective of this work was to quantify levels of the cytokines TGF-β1 and IL-10 in the conditioned medium (CM) of AdMSCs cultivated in 2D and 3D culture after the induction of hypoxia by Cobalt chloride chemistry (CoCl2). When the AdMSCs reached 80% of confluence, the cells were transferred to the 24 plates wells, where they were treated with $CoCl_2$ in 2D and 3D culture. Quantification assay was made using human TGF-β1 and IL-10 kits. The analysis was done through the sandwich ELISA assay. The IL-10 and TGF-β1 production have increased when the AdMSCs were in three-dimensional culture and under hypoxic conditions, indicating that supplies of oxygen associated to the 3D culture influenced significantly the production of these cytokines. This can be a potent and low-cost strategy to improve Adipose-derived Stem Cells conditioned medium when it comes to the release of IL-10 and TGF-β cytokines.

KEYWORDS: Stem cell. 3D culture. Cytokines.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stem cells derived from the mesoderm layer with self-renewal and differentiation potential (CAO et al., 2015). MSCs can be easily isolated and expanded *in vitro* (LINERO et al., 2014) due to its high capacity of proliferation and adhesion to plastic. Thus, human Adipose-derived Mesenchymal Stem Cells (AdMSCs) are able to differentiate into a variety of cell lineages such as neuron cells, osteocytes and adipocytes besides its ability of self-renewing. Furthermore, the use of AdMSCs has been taken into account for its regenerative features (RITTER et al., 2015), even though its ability of differentiation into bone cells has been discussed (LIAO e CHIEN, 2014) and still need further investigation within the scientific community

Remarkably, some *in vitro* studies have shown cell culture provides a defined platform for investigating cell and tissue physiology outside the organism. Traditionally, it has been achieved by culturing single cells populations in poli-dimensional substrate constructs, being the bi-dimensional (2D) substrates the most common (TIBBITT et al., 2009). Ingber and his colleges (2001) inferred that the way through which a cell distends itself in a 2D cell culture construct, determines its lower growth rating and apoptosis (CHEN et al., 1997; SINGHVI et al., 1994). Thus, to develop an appropriate cell culture technique Adipose-derived stem cells cultivation *in vitro* remains a challenge. Recently, studies demonstrated that tridimensional cell cultures contain several advantages over traditional assays. For instance, 3D cell cultures appear to maintain native cells characteristics, facilitating cell-cell communication and cell-extracellular matrix (CEM) interactions (CUKIEMAN et al., 2001; ABBOTT, 2003). Cells in tridimensional models also present variable

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surface for molecules exposition, which in the traditional assay could not happen due to cell distribution on monolayer cultures (TSENG et al., 2015).

Moreover, some researchers have reported a higher chondrogenesis in embryonic 3D cell cultures when compared with bi-dimensional models (TIBBITT, 2009), which shows the importance of studying and considering different approaches in cell culture regarding to its paracrine activity and microenvironment composition. Additionally, the AdMSCs mediated paracrine effects are influenced by bioactive factors secretion. These factors such as cytokines can be found in the conditioned medium (CM) (LINERO et al., 2014), which is the medium where a cell culture is expanded. Usually, CM contains high amounts of growth factors and tissue regenerating agents that are secreted by stem cells. Interestingly, among various cytokines secreted by AdMSCs, TGF-β1 (Tumoral Growth Factor beta 1) and interleukine-10 (IL-10) play very important roles (PAWITAN, 2014).

Transforming growth factor beta (TGF-β) is a key element regulating osteochondral differentiation and it is prevalent during fracture repair. Likewise, the influence of TGFβ is important because it leads to the induction of early markers of osteogenesis as collagen type 1 and Sp7 and inhibits indicators of the final steps of osteogenic differentiation shown by suppression of alkaline phosphatase (ALP) (GLUECK et al., 2015). Several researchers have concentrated their efforts in modulation of the paracrine environment and it's utility to improve cell and tissue therapeutic approaches (BARANIAK; MCDEVITT, 2010).

In addition, various articles have pointed out that MSCs are considered producers of high levels of IL-10 (BISHOPRIC, 2015). This cytokine is capable of lower the levels of pro-inflammatory cytokines and chemokines such as IL-1, IL-16 and TNF-α. Zhang et al. (2014) demonstrated that it can also downregulate the synthesis of nitricoxide, gelatinase, and collagenase. Besides, a specific neutralization of IL-10 may results in upregulating the synthesis of IL-1 and TNF-α. Therefore, IL-10 has been considered as an important regulator of bone homeostasis, in homeostatic and inflammatory conditions.

A relevant approach that has been discussed to enhance outcomes on cell communication is induced hypoxic condition. Under hypoxia, the hypoxia inducible factor alpha (HIFα) is stabilized and it regulates several genes believed to be involved in angiogenesis or transport of oxygen. Cell proliferation and viability in hypoxic conditions might differ depending on cell types that one decides to work with (WU; YOTNDA, 2011). Hopefully, previous treatment of *in vitro* cell cultures with low oxygen levels (<5% O_2) can increase paracrine activity of AdMSCs by inducing HIFα which binds to the hypoxia response elements in various target genes, including several angiogenic growth factors (BARANIAK; DEVITT, 2010).

Studies have shown that CM from AdMSCs could be more effective, in terms of its composition, under hypoxia than when cultivated under normal oxygen concentration, i.e. normoxia (GNECCHI et al., 2005; KAWAK e MACH, 2005). Consequently, many believe that during hypoxia there is a boost in expression and releasing of paracrine mediators into the cell medium (LIM et al., 2011). Thus, it is meaningful to evaluate the influence of hypoxia and its benefits on AdMSC CM when cultivated using 3D and 2D structures, which in turn could be extremely useful for cellular therapy involving regenerative medicine.

The present study aimed to quantify levels of TGF-β1 and IL-10 in human Adipose-derived mesenchymal Stem Cells conditioned medium when the cells are cultivated under hypoxia and normoxia conditions using both, 2D and 3D cell culture methods.

MATERIAL AND METHODS

Culture of human adipose-derived stem cells using 2D and 3D techniques.

Human adipose tissue samples were obtained by liposuction with informed consent from patients in accordance with the Research and Ethics Committee of the Federal University of Uberlandia, CEP/UFU (CAAE: 15373113.8.0000.5152) following the allowance of all donor patients. Firstly, 120 mL of fresh adipose tissue were collected and transferred into two squirts (60 mL each) which were cooled for an hour to split remaining blood material out of the target tissue. Once isolated, the target tissue was then washed for three times with Phosphate Buffer Solution (PBS). Afterwards, two aliquots of 2 mL were placed into a small culture flask and then completed with Fetal Bovine Serum (FBS). Next, incubation time was performed under 37°C in humid atmosphere with 5% CO_2. After 24 hours, BFS was replaced by *Dulbecco's modified Eagle's medium* (DMEM) supplemented with 10% of FBS and the culture was incubated as previously described. The medium was renewed 3 times a week until reach confluence.

Then, cells were counted in a Neubauer chamber and added to a 24-well-plate where half of it received treatment with hypoxic-medium prepared previously. The hypoxic condition was achieved by making up a 100mM final solution of chemical hypoxic inductor, Cobalt Chloride hexahydrate ($CoCl_2 \cdot 6H_2O$, MM = 237. 9) in DMEM medium supplemented with 10% of FBS. To the remaining wells were added only low glucose DMEM medium plus 10% FBS. 2D-cell culture technique was performed containing 1.5 x 10^4 cells/well. The supernatant was collected in the intervals 0h, 20h, 30h and 55h.

Subsequently, for the 3D assay, a second 24 well plate was treated with hypoxic (12 wells) and conventional (12 wells) media in the exactly same way performed in the 2D approach. Additionally, tridimensional structures were obtained using a kit (Bio-Assember- n3D Biosciences Inc.). After confluence, the cells were incubated with 200µL of magnetic nanoparticles (Nanoshuttle™-PL, Nano 3D Bioscience, Inc.) for 24 hours. Then, the cells were collected using trypsin reagent (Invitrogen®) and then they were resuspended into two falcon tubes, one containing hypoxic medium and the other with the conventional one (DMEM + 10%BFS). The suspension was, then, transferred to a low-adherence-24-well plate (1.5 x 10^4cells/well) in which 12 wells had the cells + hypoxic media and the remaining 12 wells had cells + low glucose DMEM with 10% FBS. Lastly, a magnetic drive was placed under the plate aggregating cells at the bottom of the well within a few hours to form spheroids. The supernatants were collected within the following intervals of 0h, 20h, 30h 55h.

MTT cell viability Assay

AdMSCs were plated out, their incubation lasted for 24 hours under 37°C, and 5% of CO_2 until adherence to the plastic. Once this happened, four different concentrations of the hypoxic media were added into the wells for a final volume of 200µL/well. One more time, the plate was incubated for 24 hours and the media was removed, and a washing step was performed using PBS 1X. To the MTT assay, 180 µL of fresh media and 20 µL of PBS-diluted MTT were mixed and added to each well where it was left still for 4 hours. A SDS solution was used for solubilizing the formed dimethyl formamide crystals. A 570-nm wavelength was performed with an overnight incubation.

Cytokines analysis by ELISA®

A sandwich ELISA assay was made using BD OptEIA™ Set Human TGF-β1 kit and the kit BD OptEIA™ Set Human IL-10 from Bioscience ®. Both experiments followed all the producer instructions.

The quantification of IL-10 was achieved through the same method used for TGF-β1 detection although the standard concentrations varied for each cytokine investigated.

Statistical analysis

The results were analyzed by the Prism 5.0 program using two-way ANOVA and Bonferroni post-test. *P*-values <0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

MTT cell viability Assay

The outcomes for $CoCl_2$ cytotoxic analysis over AdMSCs has been shown in the Figure 1 below. A positive control was obtained by adding diluted Triton (1%) solution to the cells while the negative control contained simply AdMSCs in fresh conventional DMEM media.

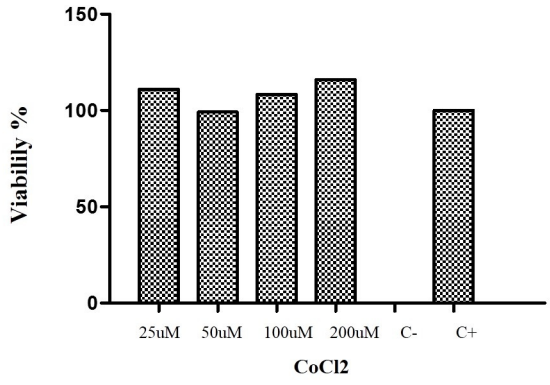

Figure 1. MTT AdMSCs viability assay

The results showed that the Chloride Cobalt did not provide cell death in any of the concentrations tested in this study (25μM - 200μM). Therefore, we can infer that the selected concentration of 100 μM of $CoCl_2$ for inducing hypoxia in the cellular environment was a safe choice.

Quantification of IL-10 in AdMSCs conditioned medium in 2D and 3D assays by ELISA®

The levels of IL-10 in the AdMSC conditioned medium from monolayer culture was not statistically significant in any of the periods set in the experiment in comparison with the controls (Figure 2). However, we could observe a slight trend of the IL-10 to raise after 30 hours of incubation in both, 2D and 3D assays.

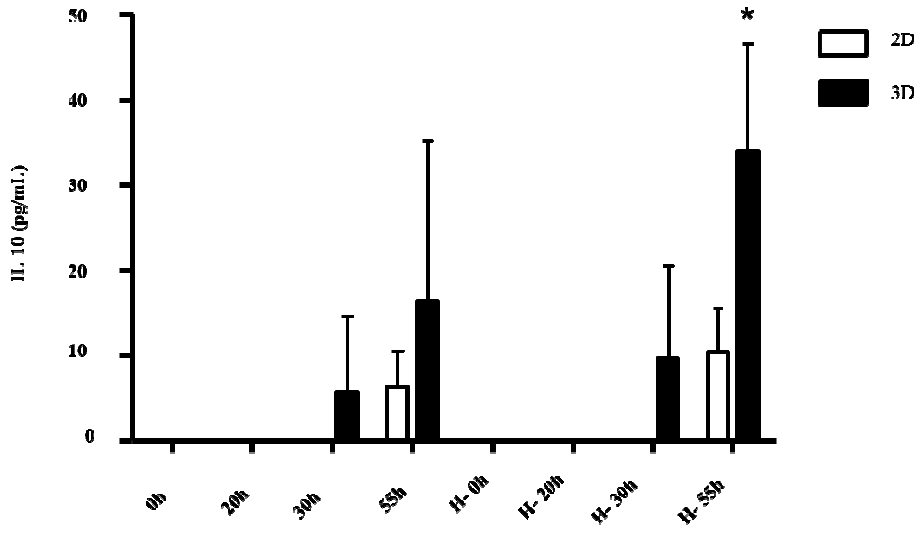

Figure 2. Detection of IL-10 in the AdMSCs CM in 2D and 3D assays. H= hipoxya.* $P < 0.05$

Indeed, some researchers have reported that IL-10 is a late-expression cytokine and it has been highly detected only after 24 hours of incubation (MALEFYT et al., 1991).

Regarding to the 3D technique, the levels of IL-10 measured in the hypoxic group have raised significantly at the time 55h in comparison with control samples ($P < 0.05$) (Figure 2). Thus, the amount of IL-10 released was higher in hypoxic environment (Figure 2) than in control samples, being detected in the last analysed period following a late releasing behaviour of this cytokine. Similarly Xi et al (2016) showed that hypoxia increased IL-10 secretion from ADSCs.

Over all, IL-10 was particularly present after 30h of the studying range in cells treated with Chloride Cobalt. There are several studies investigating the effects caused by lower oxygen offering on AdMSCS in both long and short-term. They argue that hypoxia might affects the cells by promoting a set of alterations along the time of incubation. This may be related to characteristics such as viability, proliferation and secretion of growth factors, interleukins and chemokines of AdMSCs (BURAVKOVA, 2014). Therefore, the present work findings agree with literature data so far.

Furthermore, observed outcomes from this study suggest that 3D cultures, due its disposition, favour paracrine actions by simulating *in vivo* environment more efficiently (CUKIEMAN et al., 2001; ABBOT, 2003) than monolayers approaches. Notably, when comparing both 2D and 3D cell culture methods, it comes out that the supernatant from 3D-hypoxic group at 55h of incubation was statistically significant ($P < 0.001$) (Figure 2). This means that although it is possible to note a statistical parameter in the 3D-hypoxic group, aslight increasing during all experiment could also be observed.

To sum up, considering the late expression behaviour of IL-10 in CM, our findings suggest a potential for tridimensional AdMSC cultures when combined with hypoxia condition in order to enhance the expression of IL-10 in its conditioned medium.

Quantification of TGF-β1 in AdMSCs conditioned medium in 2D and 3D assays by ELISA®

Comparing between hypoxia and normoxia conditions in the 2D assays, the results did not show in the conditioned medium of AdMSCs great levels of TGF-β1 in both hypoxia and normoxia circumstances (Figure 3), corroborating with Rajangan et al (2016) that showed great levels of TGF-β (12000pg/ml) in 3D human adipose-derived stem cell culture. However, comparing between 2D and 3D assays, it is possible to assume the TGF-β1 levels raises constantly in 2D structures under hypoxic conditions whereas the tridimensional approach showed a significant rising between 20h and 30h of incubation on hypoxia and a trend to decline after 30h of incubation under normal oxygen concentration (Figure 3).

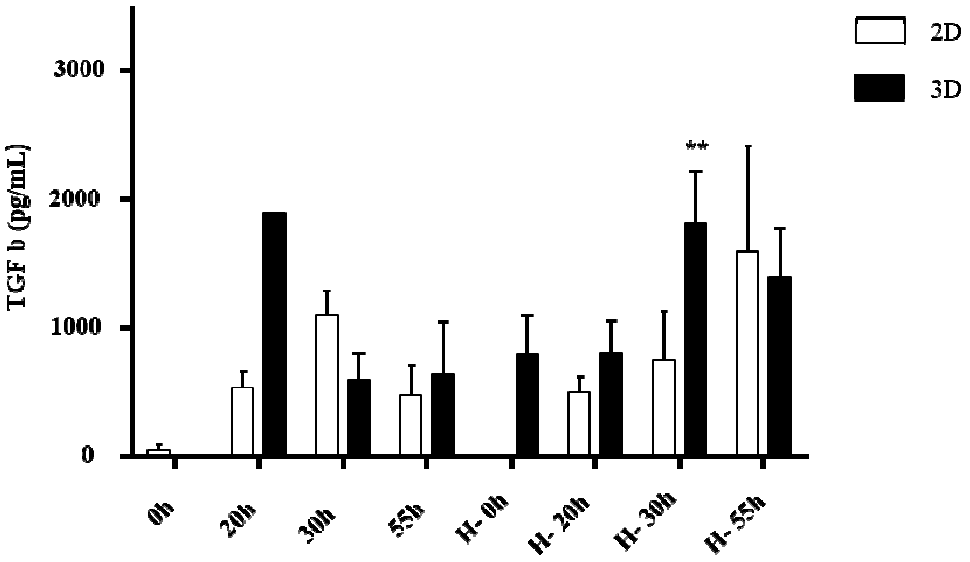

Figure 3. Quantification of TGF-β1 from AdMSCs cultivated in both, 2D and 3D, under normoxia e hypoxia environments.

The graph shows a notable raising on levels of TGF-β1 under hypoxia in comparison with normoxia situation. Lan et al (2015) stated that, in fact, a hypoxic preconditioning leads to a higher releasing of paracrine agents by MSCs such as protective proteins including TGF-β1.

In contrast, the levels of TGF-β1 under normoxia remained relatively steady after 20 hours of incubation (Figure3). Numerous researchers have reported a more appropriated environment for cell culture in 3D assays by demonstrating better *in vivo* features than 2D approaches (TSENG et al., 2015; CUKIEMAN et al., 2001; ABBOTT, 2003).

Thus, comparing both, 2D and 3D cell culture methods we can suppose, not only about IL-10, but also about TGF-β1 secretion, that the tridimensional method was more advantageous, which means it appeared to influence the cells so that the releasing of both TGF- β1 and IL-10 by the AdMSCs was higher. To confirm this, the statistic analyse method, two-way ANOVA were used to present the effect of 2D and 3D culture separately and the interaction between then. The results has shown significant parameters between both techniques suggesting better results by using 3D approach.

In spite of that, it is not plausible to affirm that only the strategy of cell culture was key factor for the observed results, once under normoxia the cells in tridimensional culture did not present higher amounts of both cytokines on its supernatant being mostly stable along all periods of incubation. For this reason, we consider hypoxia induction when associated with 3D cell culture method constitute a more effective approach in order to maximize paracrine activity of AdMSCs by enhancing the release of anti-inflammatory interleukins such as IL-10 and TGF- β1.

CONCLUSION

Chemical-induced-hypoxia by Chloride Cobalt hexahydrate combined with cell culture techniques that promote cells to form tridimensional structures can be a potent and low-cost strategy to improve Adipose-derived Stem Cells conditioned medium when it comes to releasing of IL-10 and TGF- β1 cytokines.

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RESUMO: Células-tronco mesenquimais (CTMs) são células multipontes derivadas da camada mesoderma e que possuem potenciais de auto-renovação e diferenciação celular. Estas células podem ser extraídas de diversos tecidos, sendo as principais fontes a medula óssea (MO) e o tecido adiposo (TA). Assim, CTM-TA são potencialmente capazes de se diferenciarem em diversos tipos celulares como adipócitos, neurônios e osteoblastos através de ações parácrinas do microambiente de cultivo. Este trabalho teve como objetivo dosar os níveis das citocinas TGF-β e IL-10 no meio condicionado de CTM-TA cultivadas em 2D e 3D após a indução de hipóxia química por Cloreto de Cobalto ($CoCl_2$). As CTM-TA foram cultivadas até atingirem a confluência de 80% e em seguida foram transferidas para placas de 24 poços, onde foram tratadas com meio indutor de hipóxia em cultivos realizados em 2D e 3D. A quantificação foi realizada utilizando os kits TGF-β1 e IL10 humanos. Nas análises foi utilizado o ensaio ELISA sanduíche. Os resultados mostraram que a produção de citocinas IL10 e TGF-β aumentaram quando o cultivo celular foi tridimensional em condições de hipóxia, indicando que os fornecimentos de oxigênio associado ao cultivo em 3D influenciaram de maneira significativa na produção de tais citocinas. Esta pode ser uma estratégia potente e de baixo custo para aumentar a liberação TGF-β e IL-10 no meio condicionado de CTM-TA**.**

PALAVRAS CHAVES: Células-tronco. Cultura 3D. Citocinas.

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