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Keratin Biomaterials Attenuate Hypoxia-Mediated Cell Toxicity

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Abstract

Myocardial infarction (MI) or heart attack is a leading cause of death in the United States. MI occurs when a coronary artery becomes occluded, leading to localized ischemia, which results in heart muscle cell death and propels the heart into an arrhythmic state. In this state, the heart cannot adequately deliver oxygen to tissues within the body. This can lead to death and/or longer-term congestive heart failure (CHF). Unfortunately, individuals that survive MI are plagued by problems associated with CHF due to damage sustained to the heart muscle caused by the original MI injury – which never fully heals. Regenerative medicine research focuses on improving long-term outcomes of MI survivors through several avenues of research 1) by providing healthy cells to the damaged region of the heart for regeneration and repair and 2) by rescuing pre-apoptotic cells from death. To this end, studies have shown that a biomaterial made from human hair, keratin, can be used as a carrier for cell delivery, but may also play a dual role in promoting cardiac regeneration after MI. In the current study, the feasibility of using keratin to attenuate *in vitro*-modeled MI cell death was investigated. Three objectives were completed. 1) An *in vitro* model of MI was developed to expose human umbilical vein endothelial cells (HUVECs) to low oxygen (hypoxia) using Anaerobic Gas Pouches. 2) HUVEC toxicity to hypoxia was measured to identify the duration of low oxygen exposure required to kill ~50% of cells. 3) The ability of keratin to rescue HUVECs from hypoxia-mediated cell toxicity was measured. The results showed that low oxygen conditions that mimic MI-induced hypoxia could be achieved using the method developed (1.43% dissolved oxygen). Cell toxicity to low oxygen was observed as a function of hypoxic exposure. Finally, HUVECs treated with keratin and exposed to long-term hypoxia (72hrs) proliferated in response, indicating that keratin provides a protective effect to cells in hypoxia. These data provide preliminary evidence to support the further study of keratin as a biomaterial treatment for cells exposed to hypoxia.

Keywords: Hypoxia, Keratin, Myocardial Infarction, Regenerative Medicine, Apoptosis, HUVECs

1. Introduction

Naturally-derived biomaterials are increasingly utilized in regenerative medicine and tissue engineering (RM/TE) studies as therapeutics due to their biocompatibility[7]. Therapeutic biomaterials can be used alone or in combination with cells and provide a promising avenue of research for tissue regeneration and replacement. Although cell-based therapeutics are commonly researched in RM/TE studies, the clinical application of cells is limited by cell source, viability, *ex vivo* expansion potential, and the use of non-FDA approved cell growth supplements[6]. Therefore, studies aimed at using biomaterials alone to promote tissue regeneration may be easier to translate to the clinic than those that require cells[3]. Characteristics of an effective biomaterial include biocompatibility, nontoxic degradation products, degradation rates that allow for tissue repair while maintaining structural support, and a structure that physically supports the tissue prior to and after repair[19]. Critically important is that the introduced material should not induce a

host immune response[3]. To this end, naturally derived biomaterials *that lack cellular material*, but contain protein(s) commonly found in the human body are less likely to instigate an immune response[20]. Keratin is one such material that is found in abundance in human tissues. Specifically, cytokeratins are proteins found in nearly every cell in the body and provide structural support to individual cells[18]. Although cytokeratins are abundant, isolation of these proteins for biomaterial use would be tedious and difficult to perform without also isolating cellular material. Hair and fingernail keratin, however, is similar structurally and much more readily available and easily obtainable than cytokeratin.

Hair-derived keratin is particularly appealing as a biomaterial due to its abundance, noninvasive methods used to obtain the material (hair trimmings are commonly used), and biocompatibility[5,8,13]. Keratin from hair contains proteins that when processed can be used to form a 3D structural framework[13,17]. Present in human-hair derived keratin are various protein residues and motifs that provide a substrate for cell attachment[4]. This framework of proteins and various motifs provides structural and functional support to cells. In fact, recent evidence suggests that human-hair derived keratin may be capable of attenuating cell death related to injury[11,15,16]. Of particular interest is that keratin has been shown to mitigate damage to cardiac tissue and promote regrowth after myocardial infarction *in vivo*[15]. This work suggests that keratin biomaterials may have the potential to be used as a treatment that could be applied directly to damaged heart tissue initiating repair, without the need for cells.

Occlusion of an artery within the heart leads to cardiac cell exposure to hypoxia, which is responsible for localized cell death that leads to arrhythmia and subsequent myocardial infarction (heart attack)[12]. Hypoxia or oxygen deprivation inhibits physiological function and initiates programmed cell death pathways (apoptosis)[10]. Apoptosis is initiated in cardiomyocytes by the binding of one of the two death ligands to receptors on the cell's surface[9]. These death ligands, Fas ligand and tumor necrosis factor-alpha, activate membrane receptors and initiate the caspase cascade by activating caspase-8 and other downstream caspases[9]. Caspase activation leads to a cascade of protein and cell structure breakdown within the cell, ultimately leading to death and degradation of the cardiomyocyte.

The study performed by Shen et al.[15] suggests that keratin biomaterials can promote cardiac repair following myocardial infarction in an *in vivo* rodent model. However, the complexity of *in vivo* models makes it hard to discern keratin's exact effect on cell viability. To this end, we aimed to test whether the introduction of keratin -- as a liquid supplement to cells in culture -- prevents cells from undergoing programmed cell death when exposed to a hypoxic environment. We completed three objectives to test this aim. 1) An *in vitro* model of MI was developed to expose human umbilical vein endothelial cells (HUVECs) to hypoxia using Anaerobic Gas Pouches. 2) HUVEC toxicity to hypoxia was measured to identify the duration of low oxygen exposure required to kill ~50% of cells. 3) The ability of keratin to rescue HUVECs from hypoxia-mediated cell death was measured. Cell toxicity to hypoxia with and without keratin was measured using a WST-1 cell toxicity assay. Based on previous studies suggesting that keratin provides protective effects to cells in hypoxia and in high heat conditions[11,15], we hypothesized that keratin treated cells exposed to hypoxia would exhibit decreased cell death versus untreated cells. Confirmation of this hypothesis is the first step towards further investigation of keratin as a natural biomaterial that provides protection from programmed cell death –thereby attenuating apoptosis and aiding in tissue regeneration and repair following myocardial injury.

2. Methods & Materials

2.1 Cell Culture

HUVECs were purchased from the American Type Culture Collection (ATCC) and were used in all experiments presented here. This cell type (HUVECs) was selected due to its ability to respond and proliferate in low oxygen conditions –providing a cell type that could be used to develop/test extreme model conditions for our studies. Subsequent studies will utilize more physiologically relevant cell types such as cardiomyocytes and cardiac stem cells. The cells were maintained in culture at 37°C, in 5% carbon dioxide, within a humidified atmosphere. The cells were cultured in Vascular Cell Basal Medium supplemented with an Endothelial Cell Growth Kit (ATCC). Medium was changed every 2-3 days to maintain cell viability. Cells at passage 4 were used for all experiments.

2.2 Creation of Hypoxic Growth Conditions Using Anaerobic Bags

The first objective of this study was to create an *in vitro* hypoxia model system that could be used to expose cells to conditions similar to myocardial infarction. To this end, we identified a low cost alternative to expensive hypoxia cell culture chambers –BD's Anaerobic GasPaks. To ensure that cells were exposed to low oxygen conditions, we

determined the rate of oxygen depletion in the medium as a function of time within the bags. Briefly, we placed 5mL of prewarmed medium into culture dishes that were placed in the GasPaks (per manufacturer's instructions) for varying lengths of time (3 replicates per time point, ranging from the initial ambient air 0 min to 48 hrs). Samples were placed in the cell culture incubator to replicate cell growth conditions. The dissolved oxygen content of samples at various time points was measured in parts per million (ppm) using CHEMetrics Dissolved Oxygen Kits following the manufacturer's instructions (Indigo Carmine 1-12ppm/ Rhodazine D 0-1ppm). Data were averaged and plotted vs. time to determine the rate of oxygen sequestration as well as the extent of hypoxia achieved by the bags.

2.3 Cell Viability in Response to Hypoxia

The second objective of this study was to determine the effect of hypoxia on cell viability in order to select the appropriate duration of exposure to hypoxia that elicited measurable toxicity, but not to the extent that all of cells underwent programmed cell death. Briefly, cells were seeded at 30,000 cells/cm^2 into 96-well dishes. We seeded three hypoxia time points (placed in Anaerobic GasPaks after seeding) and corresponding normoxia controls (allowed to remain at ambient air within the incubator) (time points: 24 hrs, 48 hrs, & 72 hrs; 12 wells per time point). All dishes were placed back in the incubator for the designated time. Cell toxicity was measured by WST-1 assay (G-BioSciences according to their instructions) after the designated incubation period. Absorbance of the assay product for each replicate was measured as the area under the curve between 420-480nm on a SpectraMax 190 microplate reader. Cell number was determined as a function of cellular dehydrogenase activity. Numbers were compared as a percentage of the same time point normoxia control.

2.4 Keratin Treatment of Hypoxia-Exposed Cells

The final objective of this study was to determine whether hypoxia-mediated cell toxicity could be attenuated by introduction of hair-derived liquid keratin into the medium. To this end, cells were seeded at 30,000 cells/cm^2 into 96-well dishes and either placed in normoxia or hypoxia. In addition to hypoxia-exposed cells, another group was exposed to hypoxia *and* treated with 0.5mg/mL of liquid keratin (lyophilized keratin provided as a gift from Dr. Mark Van Dyke at Virginia Tech). All hypoxia samples were placed in anaerobic bags for 72 hours. Cell toxicity was measured using the WST-1 assay as described above.

2.5 Statistical Analyses

All replicate data were averaged and presented as means +/- standard deviations. Student's T Tests were used to compare individual means and analyses of variance (ANOVA's) with post hoc analyses were used to compare multiple means. A $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 Dissolved Oxygen Content

Dissolved oxygen in the medium showed rapid decline as a function of time in the bags (Figure 1). Ambient dissolved oxygen was approximately 10 ppm, but showed an almost immediate decline upon placement within the Anaerobic GasPak. A rapid decline in oxygen was observed between 10-20 minutes of exposure to the bags, reaching a minimum oxygen content by 2 hours exposure (~0.2 ppm). The average dissolved oxygen content of the medium between 6 hours and 72 hours was 0.43 ppm (long-term data not shown).

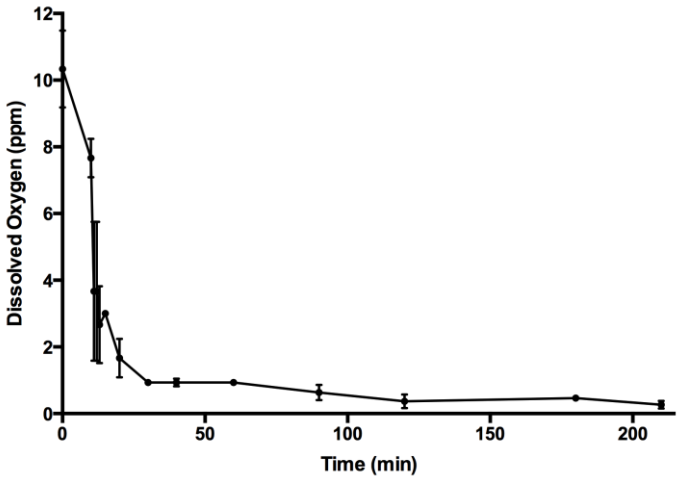

Figure 1. Dissolved oxygen decreases rapidly within the medium upon placement in Anaerobic GasPaks. A minimum is reached after ~2 hours of placement within the bags.

3.2 Cell Toxicity to Hypoxia and Response to Keratin

Cell toxicity was determined by WST-1 assay. This particular assay measures toxicity as a function of cell numbers – determined by cellular dehydrogenase activity. Cells exposed to 24, 48, and 72 hours of hypoxia (via Anaerobic GasPaks) displayed varying levels of toxicity (Figure 2). Exposure of HUVECs to increasing amounts of hypoxia suggested that the cells initially proliferate (24 hour exposure) prior to declining in numbers at 48 and 72 hrs (compared to normoxia same time point controls). Specifically, after 24 hours of exposure to hypoxia, cells increased by 132% compared to the normoxia control ($p \leq 0.001$). Cells then began to decrease in number at later time points. At 48 hours of exposure, cells were decreased to 70% of the normoxia control ($p=0.04$) and at 72 hours of exposure, cells decreased by 66% ($p \leq 0.001$). Based on these results, we selected 72 hours as our exposure time point to test the effect of keratin treatment on hypoxia-mediated cell toxicity.

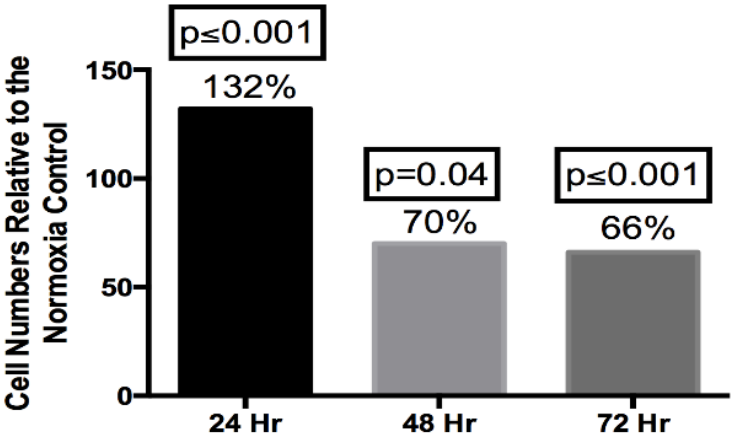

Figure 2. Cell numbers (expressed as a percentage of the normoxia control) indicate that hypoxia duration has altering effects on cell proliferation.

The 24-hour hypoxia-exposed group showed a 132% increase in cell numbers versus the same time point normoxia group. The 48-hour hypoxia-exposed group contained 70% of the cells observed in the same time point normoxia control. The 72 hour hypoxia-exposed group further decreased in cell numbers to 66% of the normoxia same time point control. Cell numbers were determined as a direct correlation to absorbance reading of the WST-1 assay product, measured as the area under the curve between 420-480nm.

Keratin treatment of cells was tested at 72 hours of hypoxia exposure. Cells proliferated and displayed reduced toxicity in response to keratin treatment (compared to the hypoxia-exposed cells). However, the difference in treatments was not shown to be statistically significant, possibly due to the large standard deviation (p=0.06).

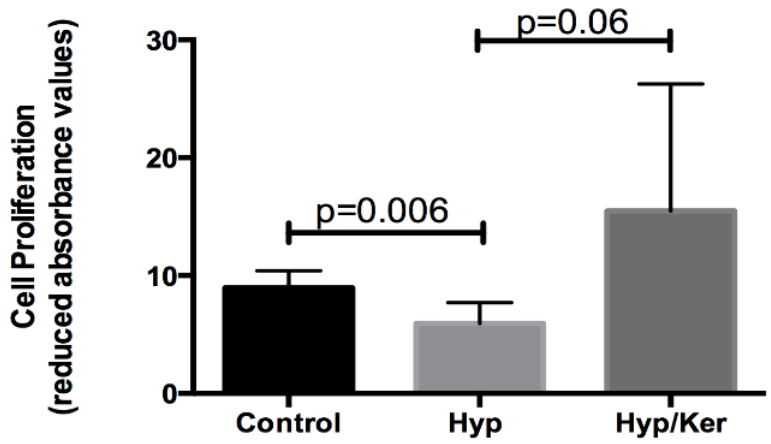

Figure 3. Keratin treated cells (0.5mg/mL) exposed to 72hrs of hypoxia display high proliferation compared to the normoxia and hypoxia groups.

*Reduced absorbance values = area under the curve for absorbance measurements taken between 420-480nm.

4. Discussion

Our results indicate that dissolved oxygen within the medium can be reduced to a low of 0.2 ppm, with an average long-term low of 0.43 ppm. Input of these measurements into PreSen's Precision Sensing Oxygen Unit Calculation tool at a temperature of 37°C and atmospheric pressure of 942.1 hPa (corresponding to the altitude at which measurements were recorded) revealed that these measurements correlate to an oxygen concentration of 0.67% and 1.4%, respectively. These values are consistent with hypoxic states within the heart that are sufficient to induce apoptosis during myocardial infarction[2]. We were able to move forward into testing the cells for toxicity from the hypoxia model to determine cellular death

There was also a significant reduction in the amount of cells following prolonged hypoxia exposure as compared to the normoxia controls determined by the results. A cell toxicity model was successfully achieved by exposing HUVECs to the hypoxic atmosphere created using Anaerobic GasPaks. However, the observed toxicity differed slightly from what we expected to see. Specifically, an initial, significant rise in cell numbers (132%, $p \leq 0.001$) was observed in the 24-hour group compared to the same time point normoxia control. This result suggests that shorter durations of hypoxia may increase HUVEC proliferation. This observation is potentially supported by the cell's physiological function as embryonic sensors/responders to oxygen conditions[14]. HUVECs may proliferate in response to low oxygen in order to generate blood vessel cells in the developing embryo/infant to establish and maintain appropriate oxygen levels[14].

After the observed initial spike in proliferation, HUVECs then responded to prolonged hypoxia (48 & 72 hrs) with significantly increased toxicity (48 hrs= 70% decrease in cell numbers, p=0.04; 72 hrs = 66% decrease in cell numbers, $p \leq 0.001$). The HUVECs did display a significant reduction in cell numbers after the trial, this displayed a result was not what was expected. The expectancy was a very pronounced reduction at the 72 hr time point than what was observed. A possible reason for this discrepancy may again be attributed to the native function of HUVECs. This particular type of cell reside naturally in human umbilical veins and respond to low oxygen conditions by initiating the formation of blood vessels[14]. Therefore, the cells chosen for the model system naturally proliferate in deprived oxygen conditions – more so than would be expected of cardiac cells. A recent study found that HUVECs can be successfully cultured at oxygen levels as low as 1%[1], indicating that our experiments may not lower the oxygen to the

extent required to induce significant cell death. However, our results do suggest that the hypoxic environment created still results in cell toxicity, significantly different than that of cells exposed to normoxia for the same time period -- particularly at the 72-hour time point. For this reason, we selected this time point for determination of keratin's effect on hypoxia-mediated cell toxicity.

Keratin treatment of cells exposed to 72 hours of hypoxia resulted in increased cell proliferation compared to those not treated with keratin but exposed to hypoxia. Although not achieving statistical significance, this corresponds with previous studies that suggest that keratin may enhance cell survival under apoptosis-inducing conditions[11,15]. Determined by the observation of experimental method, the significance value is likely explained by technique error, rather than experimental variation. by the introduction of bubbles into the WST-1 assay plate while pipetting –a factor that the manufacturer suggests can obscure the data and which was observed and noted during the experiment. This could alter the data in such a way as to give a more accurate standard deviation when statistically analyzed.

Keratin is a naturally occurring biomaterial that may be used as an effective scaffolding due to its durable and flexible structure[15]. Applying the material to a damaged heart after a myocardial infarction may provide a means of support to the wounded tissue to assist the essential repair mechanisms in functioning more easily[15]. In summary, we created a method of exposing cells *in vitro* to hypoxic conditions and confirmed that these conditions are toxic to cells. We also tentatively showed that keratin may attenuate hypoxia-mediated cell toxicity. However, due to variation observed in our experimental results, future experiments will first confirm keratin's effect on cell proliferation. More importantly, future studies will utilize the method developed here to test keratin's effect on cells that are more applicable to myocardial infarction. These studies will provide support for the use of keratin as a naturally-derived biomaterial to treat myocardial infarction.

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6. References Cited

1. Abaci, H. E., Truitt, R., Luong, E., Drazer, G. and Gerecht, S. 2010. Adaptation to oxygen deprived in cultures of human pluripotent stem cells, endothelial progenitor cells, and umbilical vein endothelial cells. *Cell Physiology*, 298, 1527-1537.
2. Angelos, M. G., Kutala, V. K., Torres, C. A., He, G., Stoner, J. D., Mohammad, M., Kuppusamy, P. 2006. Hypoxic reperfusion of the ischemic heart and oxygen radical generation. *Am J Physiol Heart Circ Physiol*, 290, H341-7.
3. Browne, S. and Pandit, A. 2015. Biomaterial-mediated modification of the local inflammatory environment. *Front Bioeng Biotechnol*, 15, 67.
4. Burnett, L. R., Rahmany, M. B., Richter, J. R., Aboushwareb, T. A., Eberli, D., Ward, C. L., Orlando, G., Hantgan, R. R., Van Dyke, M. E. 2013. Hemostatic properties and the role of cell receptor recognition in human hair keratin protein hydrogels. *Biomaterials*, 11, 2632-40.
5. Dias, G. J., Mahoney, P., Swain, M., Kelly, R. J., Smith, R. A., Ali, M. A. 2010. Keratin-hydroxyapatite composites: biocompatibility, osseointegration, and physical properties in an ovine model. *J Biomed Mater Res A*, 95,1084-95.
6. Doppler, S. A., Deutsch, M. A., Lange, R., Krane, M. 2013. Cardiac regeneration: current therapies-future concepts. *J Thorac Dis*, 5, 683-97.
7. Dunn, D.A., Hodge, A.J. and Lipke, E.A. 2014. Biomimetic materials design for cardiac tissue regeneration. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 6, 15-39.
8. Ito, H., Miyamoto, T., Inagaki, H., Noishiki. 1982. Biocompatibility of denatured keratins from wool. *Kobunshi Ronbunshu*, 39, 249-56.
9. Kim, N. H. and Kang, P. M. 2010. Apoptosis in cardiovascular disease: mechanism and clinical implications. *Korean Circ J*, 40, 299-305.

10. Lenihan, C. R. and Taylor, C. T. 2013. The impact of hypoxia on cell death pathways. *Biochem Soc Trans*, 41, 657-663.
11. Poranki, D., Whitener, W., Howse, S., Mesen, T., Howse, E., Burnell, J., Greenguaz-Roberts, O., Molnar, J., Van Dyke, M. E. 2013. Evaluation of skin regeneration after burns in vivo and rescue of cells after thermal stress in vitro following treatment with a keratin biomaterial. *J Biomater Appl*, 29, 26-35.
12. Rota, M., Leri, A. and Anversa, P. 2014. Human heart failure: is cell therapy a valid option? *Biochem Pharmacol*, 88,129-38.
13. Rouse, J. G. and Van Dyke, M. E. 2010. A review of keratin-based biomaterials for biomedical applications. *Materials*, 3, 999-1014.
14. Sen, S., Rao, R. and Chaudhuri, G. 2013. Endothelial cell function in utero-placental circulation physiology and pathophysiology. *Curr Vasc Pharmacol*, 11, 730-6.
15. Shen, D., Wang, X., Zhang, L., Zhao, X., Li, J., Cheng, K. and Zhang, J. 2011. The amelioration of cardiac dysfunction after myocardial infarction by the injection of keratin biomaterials derived from human hair. *Biomater*, 32, 9290-9299.
16. Sierpinski, P., Garrett, J., Ma, J., Apel, P., Klorig, D., Smith, T., Koman, L. A., Atala, A., Van Dyke, M. E. 2008. The use of keratin biomaterials derived from human hair for the promotion of rapid regeneration of peripheral nerves. *Biomaterials*, 1, 118-28.
17. Srinivasan, B., Kumar, R., Shanmugam, K., Sivagnam, U. T., Reddy, N. P., Sehgal, P. K. 2010. Porous keratin scaffold-promising biomaterial for tissue engineering and drug delivery. *J Biomed Mater Res B Appl Biomater*, 92, 5-12.
18. Toivola, D.M., Boor, P., Alam, C. and Strnad, P. 2015. Keratins in health and disease. *Curr Opin Cell Biol*, 32, 73-81.
19. Wang, J., Yang, W., Xie, H., Song, Y., Li, Y., Wang, L. 2014. Ischemic stroke and repair: current trends in research and tissue engineering treatments. *Regen Med Res*, 3, 3.
20. Wong, M.L. and Griffiths, L., G. 2014. Immunogenicity in xenogeneic scaffold generation: antigen removal vs. decellularization. *Acta Biomater*, 10, 1806-16.