APOTOPSIS, NUTRITION, AND METABOLISM OF TRANSPPLANTED INTERVERTEBRAL DISC CELLS

INTRODUCTION

Apoptosis is a contributing factor to degenerating intervertebral disc (IVD). Disc regeneration has been attempted by transplanting cells into the disc, with some gains in disc height achieved in animal models. Here, we study whether the apoptotic micro-environment affects the transplanted disc cells. Methods: Human annulus fibrosus (AF) and nucleus pulposus (NP) cells were grown in media then starved for 5 days in vitro by not changing the media. Three aspects of apoptotic cell influence on the transplanted cells were tested in a total of 32 samples: 1) the effect of apoptotic cytokines in the media, 2) reduced glucose in the media, and 3) apoptotic cell bodies in the flask. The Trypan Blue, AlamarBlue®, and 1,9-Dimethyl-Methylene Blue assays for sulfated glycosaminoglycan (sGAG) content were performed (n=4). Results: There were significant decreases in cell viability between the control, 25% conditioned media (CM) and starved control group. There were no significant differences in cell number, metabolic activity or sGAG production in cells grown in different conditioned media compared to cells grown in complete media. The cells of the control decreased in viability and number over the 5 days without feeding, then improved dramatically when feeding was resumed. Flasks that received transplanted cells in addition to renewed feeding did not recover as much as the cells in the re-fed group. Conclusions: Cytokines from starved cells negatively impact on the viability of healthy cells. Starving cells that receive new sources of nutrition have even higher viability than transplanted cells. This indicates that altering and improving the nutrient supply problem in the IVD could be a valuable option. Level of Evidence III; Case control study².

KEYWORDS: Apoptosis; Intervertebral disc; Metabolism.

RESUME

Introduction: L'apoptose est un facteur qui contribue à la dégénérescence du disque intervertébral (DIV). La tentative de régénérer un disque a été faite en transplantant des cellules dans le disque, avec des gains de hauteur de disque réalisés dans des modèles animaux. Ici, nous étudions si le micromilieu apoptotique affecte les cellules du disque transplantées. Méthodes: Les cellules humaines de l'anneau fibreux (AF) et du noyau pulmonaire (NP) furent cultivées in vitro au sein de médias privés de nutrition sur une durée de cinq jours, sans alteration des médias. Trois aspects de l'influence des cellules apoptotiques furent testés dans un total de 32 échantillons: 1) l'effet des cytokinés apoptotiques dans le milieu, 2) réduction de la glucose dans le milieu, et 3) corps cellulaires apoptotiques dans le flacon. Les essais avec le bleu de Trypan, AlamarBlue® et 1,9-diméthyl azul de méthyle pour le contenu de glicosaminoglican sulfaté (sGAG) furent effectués (n=4). Résultats: Il y avait des diminutions significatives de la viabilité des cellules entre le contrôle, le milieux conditionnés (MC) à 25% et le groupe contrôle privé de nutrition. Il n'y avait pas de différences significatives dans le nombre de cellules, l'activité métabolique ou la production de sGAG dans les cellules cultivées en différents milieux conditionnés en comparaison avec le milieu complet. Les cellules de contrôle avaient une réduction de la viabilité et de nombre au long de ces cinq jours sans alimentation; à suivre, la viabilité et la production s'aggravèrent. Les fracs qui reçurent des cellules transplantées, en plus de l'alimentation re-nucléée, ne se récupèrent pas tant que les cellules du groupe ré-alimenté. Conclusions: Les cytokinés de cellules privées d'alimentation ont un impact négatif sur la viabilité des cellules saines. Les cellules privées d'alimentation qui reçoivent de nouvelles sources de nutrition ont même plus de viabilité que les cellules transplantées. Cela indique que l'amélioration de la supplémentation de nourriture dans le DIV pourrait être une option valable. Nível de Evidência III; Estudo de caso controle². 

Descritores: Apoptose; Disco intervertebral; Metabolismo.

RESUMEN

Introducción: La apoptosis es un factor que contribuye a la degeneración del disco intervertebral (DIV). La tentativa de regenerar el disco fue por medio de trasplante de células en el disco, con algunos ganhos de altura del disco alcanzados en modelos animales. Aquí estudiamos si el microambiente apoptótico afecta a las células del disco transplantadas. Métodos: Células humanas del anillo fibroso (AF) y del núcleo pulposo (NP) fueron cultivadas in vitro en medio de cultivo privados de nutrición por cinco días, sin alteración de los medios. Tres aspectos de la influencia de células apoptóticas en el medio de cultivo fueron testados en un total de 32 muestras: 1) el efecto de citocinas apoptóticas en el medio, 2) reducción de la glucosa en el medio y 3) los cuerpos celulares apoptóticos en el matraz. Realizaron ensayos con azul de tripano, AlamarBlue® y 1,9-dimetil azul de metileno para el teor de glicosaminoglicano sulfatado (sGAG) (n = 4). Resultados: Constataron-se decréscimos significativos no número de células, atividade metabólica ou produção de sGAG em células cultivadas em diferentes meios condicionados em comparação com o meio completo. As células de controle tiveram redução de viabilidade e de número ao longo dos 5 dias sem alimentação; a seguir, houve melhora substancial ao se retomar a alimentação. Os frascos que receberam células transplantadas, além da alimentação renovada, não se recuperaram tanto quanto as células do grupo realimentado. Conclusões: As citocinas de células famintas tiveram impacto negativo sobre a viabilidade das células saudáveis. As células famintas que recebem novas fontes de nutrição têm viabilidade ainda maior do que as células transplantadas. Isso indica que alterar e melhorar o fornecimento de nutrientes no DIV pode ser uma opção valiosa. Nível de Evidência III; Estudo de caso controle². 

Descritores: Apoptose; Disco intervertebral; Metabolismo.
INTRODUCTION

Back pain affects 70%-85% of the population in developed nations during some point of their lives, and is a symptom of intervertebral disc (IVD) degeneration. The large population suffering from back pain results in significant economic costs due to lost productivity and health care expenses, estimated at $34 billion to the United States alone. Spinal degeneration, including IVD, can take many forms, complicating the understanding of the underlying degenerative mechanisms.

The IVD consists of three distinct anatomical segments: the endplate, the annulus fibrosus (AF), and the nucleus pulposus (NP). The endplate houses the capillaries that brings nutrients into the disc, and connects the rest of the disc with the adjacent vertebral body. The annulus fibrosus, made up of interconnected concentric lamellae, provides structural rigidity for the disc. The NP, a gelatinous structure, helps to evenly distribute compressive force across the disc.

Current treatment options for degenerative discs include conservative medical therapy, chronic pain therapy, transcutaneous minimally invasive disk procedures, and discectomy, whether alone or combined with spinal fusion. While surgical treatment may resolve pain in the short term, mechanical differences in and around fused and healthy spine segments can lead to further degeneration in the adjacent segment discs. Due to mechanical degradation, patients may have to adjust their lifestyle to account for the reduced range of motion. While such restriction is especially difficult for younger patients, even older patients may be severely impacted, leading to loss of ability to walk.

Disc regeneration would be a beneficial treatment for young IVD degeneration patients, who suffer most from adjacent segment wear due to their longer life expectancy. Improving the health of the endogenous cells or introducing new healthy cells into the disc are two possible regeneration strategies. Gruber, in 1998, showed that a high percentage of cells within the IVD undergo apoptosis, with degenerated specimens containing 53.5% viable cells compared to 73.1% in healthy specimens. Therefore, regional interruption or termination of apoptosis are potential regeneration strategies for improving disc health. Another possible treatment may be to introduce healthy cells into the degenerated disc; either by direct injection or incorporation a tissue engineered construct such as a hydrogel. Persoglio et al. attempted to seed hydrogels with NP cells and embryonic stem cells; however, there was no testing to detect how many of the cells were apoptotic or alive, or which originated as MSCs. In a canine animal model, Serigano et al. used TUNEL to quantify the percentage of dead MSCs transplanted. Transplanted cell death was dependent on the quantity of cells introduced to the disc during the 16-week study. Most data is currently from animal IVD models. However, pilot studies are underway in humans in Japan and Spain, with promising initial results.

We investigated whether apoptotic endogenous cells have any influence on introduced healthy, viable cells. This was done by testing multiple factors when applying live cells to a flask containing dead cells in one experiment. In another experimental group, we used multiple levels of cell conditioned media to then investigate the effect of released signaling markers on live healthy cells.

METHODS

Human af and np cell source

Healthy human annulus fibrosus cells (HAFCs) and human nucleus pulposus cells (HNPCs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA), who collected the cells from 24-week fetal tissue. Cells were screened for mycoplasma and tested negative by the supplying company.

Cell culture

T-75 poly-D-lysine (Sigma Aldrich, St. Louis, MO, USA) coated culture flasks were prepared as directed by ScienCell. Briefly, 1.5 µg/mL poly-D-lysine was added to each culture flask and incubated at 37°C for 1 hour. The flasks were then rinsed with sterile 1x PBS prior to storage.

Cells were thawed in a 37°C water bath and expanded for 5 days in 50% Dulbecco’s modified eagles medium (DMEM):50% F12 (Life Technologies, Grand Island, NY, USA) media supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, MO), henceforth called control media. The cells were then passaged into their experimental groups. Each experimental group had a sample size of 4 (n=4) for each cell type, HNPCs and HAFCs.

Conditioned media experiment

Live control cells were grown to confluence fed with control media every two days. (Table 1) The dead control group was not fed over the 5-day period. The media was collected and centrifuged at 200 RCF, and the supernatant was saved. A portion of collected supernatant from the dead control was used as conditioned media for the 12.5% and 25% conditioned media groups, where the media was changed every 2 days.
and absorbance read at 525 nm immediately. A standardized curve of a DMMB reagent at pH 3 was added to 20 µL of each sample heated to >90°C for 10 min to inactivate the proteinase K. 200 µL solution was incubated at 37°C overnight to lyse the cells and then proteinase K (Roche, Branford, CT, USA) in 100 mM Na2HPO4 was removed. The cells were resuspended in 1 mL of 50µg/mL cells were thawed and centrifuged. All but 0.5 mL of the supernatant minutes and resuspended in 1x phosphate buffered saline (Sigma-MO) assay. Cells from the culture were centrifuged at 200g for 4

Experimental design for all groups.

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 10</th>
<th>Day 12</th>
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<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Collected</td>
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<td>Fed with control media</td>
<td>Fed with control media</td>
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<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Fed with 12.5% conditioned media</td>
<td>Fed with 12.5% conditioned media</td>
<td>Collected</td>
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<td>25% Conditioned Media</td>
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<td>Fed with control media</td>
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<td>Fed with 25% conditioned media</td>
<td>Fed with 25% conditioned media</td>
<td>Collected</td>
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<tr>
<td>25% PBS</td>
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<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Fed with media and 25% PBS</td>
<td>Fed with media and 25% PBS</td>
<td>Collected</td>
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<tr>
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<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Fed with media and 25% PBS</td>
<td>No feeding</td>
<td>No feeding</td>
<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Collected</td>
<td></td>
</tr>
<tr>
<td>Live on Dead-Dead</td>
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<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Fed with media and 25% PBS</td>
<td>No feeding</td>
<td>No feeding</td>
<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Collected</td>
<td></td>
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<tr>
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<td>Fed with control media</td>
<td>Fed with control media</td>
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<td>Fed with control media</td>
<td>Fed with control media</td>
<td>Collected</td>
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</table>

25% PBS experiment

To determine the effect of decreased glucose in the CM group as opposed to the presence of apoptotic cytokines, one group was fed with 25% PBS:75% control media.

Feeder layer experiment

Following the steps from the negative control, cells were starved by not changing the culture media following passage. The media was taken off and stored, and new culture media, containing live cells or no cells, were introduced into the flask. The cells were then fed for 5 more days.

Cell viability and population density

Cell viability was quantified following final collection of cells and media for analysis. The cells were centrifuged and then resuspended in 1x PBS. A sample of cells was taken and combined into a 1:1 solution of the PBS and Trypan Blue reagent (Life Technologies, Grand Island, NY, USA). The slides were then analyzed by a Countess II automatic cell counter (Life Technologies, Grand Island, NY, USA) and live/dead percentages, as well as cell densities, were obtained.

Alamar blue assay

Alamar Blue reagent (Life Technologies, Grand Island, NY, USA) was added to the cell culture flask containing media at a 1:10 ratio of AlamarBlue: media and incubated at 37°C for 2 hours. The media was then transferred to a 96 well plate. The absorbance was read at a wavelength of 570 nm with a reference wavelength of 600 nm on a Tecan Infinite 200Pro (Männedorf, Switzerland). The absorbance was normalized by cell count from the Trypan Blue cell viability test.

Dimethylmethylene blue (DMMB) assay

Sulfated glycosaminoglycan (GAG) content was assessed using a 1.9 dimethyl-methylene blue (DMMB) (Sigma-Aldrich, St. Louis, MO) assay. Cells from the culture were centrifuged at 200g for 4 minutes and resuspended in 1x phosphate buffered saline (Sigma-Aldrich, St. Louis, MO) before freezing at -80°C. Before testing, the cells were thawed and centrifuged. All but 0.5 mL of the supernatant was removed. The cells were resuspended in 1 mL of 50µg/mL proteinase K (Roche, Branford, CT, USA) in 100 mM Na2HPO4 adjusted to pH 8.0 using 1N HCl (Fisher, Hampton, NH, USA). This solution was incubated at 37°C overnight to lyse the cells and then heated to >90°C for 10 min to inactivate the proteinase K. 200 µL of a DMMB reagent at pH 3 was added to 20 µL of each sample and absorbance read at 525nm immediately. A standardized curve was prepared using bovine chondroitin-4-sulfate (Sigma-Aldrich, St. Louis, MO) for known concentrations between 0.125-1.0 µg/mL. Final concentrations were adjusted for resuspension volume and cell count from the Trypan Blue cell viability test.

Statistical analysis

Statistical analysis was performed using Statistica software. In all cases, the data were analyzed for normality using the half-normal probability plot. Statistical cutlers were removed prior to ANOVA analysis. A main effects ANOVA was performed for all data in the conditioned media experiment and the 25% PBS vs 25% conditioned media experiment. A 2nd degree factorial ANOVA was used to assess significance between groups in the Feeder Layer Experiment. A post-hoc Fishers multiple comparisons test was used to assess significance between specific groups. The p value for significance was set at <0.05 for all tests.

RESULTS

Conditioned media

Cell viability was highest in the cells grown in control media (98.5%±0.9%) (Figure 1A) and lowest in the dead control group (58.6%±13.2%). There were significant decreases in cell viability between the control group, the 25% conditioned media group (p=0.002), and the dead control group (p=0.000). There was no significant decrease in cell viability between the control group and the 12.5% conditioned media group (p=0.77). Cell type, NP or AF, did not play a significant role in cell viability (p=0.10). (Table 2)

The largest cell population was found in the flasks grown in the 12.5% conditioned media group (1.1x10⁵±6.0x10⁴) (Figure 1B) and the lowest was found in the dead control group (7.1x10⁴±5.8x10⁴); however, there were no statistically significant differences between levels of conditioned media (p=0.42). The HNPCs (1.1x10⁵±5.4x10⁴) had a significantly larger population (p=0.04) than the HAFCs (7.2x10⁴±3.3x10⁴).

Statistical analysis was not significantly dependent on conditioning level (p=0.86) (Figure 2A) or cell type (p=0.75).

Sulfated GAG production (Figure 2B) was not significantly affected by the presence of conditioned media (p=0.59) or cell type (p=0.83).

PBS vs conditioned media

To determine the effect of apoptotic cytokine signaling in the media when compared to the loss of glucose and growth factors that was also present in the 25% conditioned media, media was prepared with 25% PBS. The 25% PBS group had higher cell viability (97.5%±1.3%) (Figure 1C) and cell population (2.7x10⁵±5.7x10⁵) (Figure 1D) than the...
25% conditioned media group’s cell viability (75.9%±18.0%, p=0.003) and cell population (9.9x10^4±2.7x10^4, p=0.000).

Metabolic activity (Figure 2C) was higher in the 25% conditioned media group than the 25% PBS group (p=0.000).

Sulfated GAG production (Figure 2D) was significantly lower (p=0.002) in the 25% PBS group (1.4x10^-7±3.8x10^-8µg/Cell) than in the 25% conditioned media group (5.3x10^-7±2.8x10^-7 µg/Cell).

Feeder layer
Healthy cells were added to a flask of dying cells and given control media to determine whether the presence of apoptotic cell bodies hindered or helped newly supplied cells. Additionally, cells were allowed to die and then given fresh media to determine the ability of the cell populations to recover. Using this system, cells were shown to decrease in viability (Figure 1E) and population (Figure 1F) from the control over the 5 days without feeding, then when cells were re-fed.

Table 2. Statistical significance (p) of independent variables on each dependent variable.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Conditioned media level</th>
<th>Pbs vs conditioned media</th>
<th>Feeder layer</th>
<th>Feeder layer/conditioned media interaction</th>
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<tr>
<td>Cell viability</td>
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<td>0.000*</td>
<td>0.000*</td>
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<tr>
<td>Cell population</td>
<td>0.04*</td>
<td>0.42</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>Cell activity</td>
<td>0.75</td>
<td>0.86</td>
<td>0.67</td>
<td>0.006*</td>
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<tr>
<td>Sulfated GAG production</td>
<td>0.83</td>
<td>0.59</td>
<td>0.002*</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.05).
fed for the following 5 days, their population and viability increased dramatically. In comparison, flasks that received new healthy cells in addition to 5 days of renewed feeding did not recover as much, in viability or population, as the cells in the re-fed only group.

Metabolic activity (Figure 2E) was significantly lower in the re-fed group than in the other 3 groups (p=0.000) and was also significantly higher in the live-on dead group than in the dead control group (p=0.01).

Sulfated GAG production (Figure 2F) was lowest in the re-fed dead control group (9.4x10^-8±2.8x10^-8µg/Cell) and highest in the dead control group (7.5x10^-7±4.9x10^-7µg/Cell).

Discussion

The conditioned media from apoptotic cells caused a decrease in cell viability of healthy disc cells. This effect was not due to a lack of growth factors and glucose, since the 25% PBS group had higher viability than the 25% CM group. Our data suggest that apoptotic cytokines in the media were responsible for decreased viability of the initially healthy cells. Adding live cells to a flask containing dead cells was shown to have significant effect on viability as well with the live on dead group having lower viability than the re-fed dead cells alone.

The metabolic rate of the cells also changed as a result of the environment. Adding the live, viable cells to a flask of dead cells increased the metabolic rates of the cells. Since fresh media was used, the increased metabolic rate was attributed to the apoptotic cells residing at the bottom of the coated flask, and not to the effect of the apoptotic cytokines that accumulated in the media over the 5 days.

Adherence of healthy cells introduced into the flask of dead cells did not appear to be hindered. There was no visual evidence of multiple cell layers, but the high final viability in the live on dead group suggests that dead cells either detached from the surface over time, or were pushed off the lysine flasks as healthy cells invaded the area.

Figure 2. Cell metabolic activity and GAG production for the A-B) condition media experiment, C-D) PBS control experiment, and E-F) and feeder layer experiment.
The dead cells would then have been discarded when the media was changed. In vivo IVD tissue, cellularity is only around 1%\textsuperscript{10} and therefore attachment sites for new cells would not be problematic, even if dead cells were not immediately phagocytosed.

This study had several limitations. First, an in vitro model was chosen for this preliminary experiment to simplify the system and keep the experimental costs low, but it did remove complicating factors that could be important in vivo. Furthermore, this experiment was performed in 2D culture. Although all experiments were complete by passage iteration 4-5, it is possible that some of the cells could have undergone phenotypic changes toward a fibroblastic lineage. Furthermore, we did not perform any cell signaling analysis. From the low viability in the dead control group, we know some of the cells died, but we do not know the state of the remaining cells. For instance, were the surviving cells in both the CM group and the re-fed dead control group senescent, apoptotic, or unaffected? This will be the subject of future work, along with more extensive study of the apoptotic and chondrogenic signaling profiles of those cells.

It has been shown in previous research that evidence of apoptosis has been found in degenerated discs, and that mechanical strain and/or poor transport of nutrients could be to blame for apoptosis and degeneration in human IVDs. The results of this study indicate that introducing live cells to an environment that has apoptotic cytokines can influence the health of the cells. This requires further exploration to determine the level of impact in an in vivo environment, and whether cell therapies could be successful in the long term, regardless of the negative microenvironment. It is also necessary to determine whether these therapies require adjunctive treatments to recover disc nutrition or removal of apoptotic cell debris and signaling factors. The results from the re-fed dead control group appear to indicate that the former might be a valuable treatment to explore, without the addition of transplanted cells. However, other aspects of the health of those re-fed cells would need to be investigated, such as possible senescence and phenotypic changes. Therapeutic developments may be amenable to local or regional spinal therapy strategies. Several teams have sought to address this critical nutrient transport deficit through mechanical and biological means such as spinal traction\textsuperscript{16,17} and enzymatic permeabilization\textsuperscript{18} respectively.

CONCLUSIONS

Cytokines from starved cells negatively impacted the viability of healthy cells. Starving cells that receive new sources of nutrition have even higher viability than transplanted cells. This indicates that treating the nutrient supply problem in the IVD could be a valuable treatment alone, without the need for further biological therapeutics.

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