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MATHEMATICAL MODEL OF BONE MORPHOGENETIC PROTEINS' INFLUENCE ON INTERVERTEBRAL DISC CELL PROLIFERATION *IN VITRO*

Bardonova L.A.[1,2,3], Belykh E.G.[1,2,3], Byvaltsev V.A.[1,2,4], Theodore N.[3,5], Preul M.C.[1,3], Giers M.B.[1,3,*]

[1]Irkutsk Scientific Center of Surgery and Traumatology, Irkutsk, Russia
[2] Irkutsk State Medical University, Ministry of Health, Irkutsk, Russia
[3] Department of Neurosurgery Research, Barrow Neurological Institute, Phoenix, Arizona, USA
[4] Railway Clinical Hospital on the station Irkutsk-Passazhirskiy of Russian Railways Ltd., Irkutsk, Russia
[5] Department of Neurosurgery, Johns Hopkins Hospital, Baltimore, Maryland, USA

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Abstract

Bone morphogenetic proteins change the cellular expression of extracellular matrix components. In the early stages of intervertebral disc degeneration this can be the basis for biological treatments intending to regenerate the structure and function of the intervertebral disc. The success of this therapy depends on the presence of a sufficient number of viable cells.

The study was aimed to investigate the proliferation of intervertebral disc cells under the influence of bone morphogenetic proteins.

Healthy human annulus fibrosus and nucleus pulposus cells were cultured in control medium (1), and medium supplemented with bone morphogenetic protein-2 (2), bone morphogenetic protein-7 (3) and bone morphogenetic protein-14 (4). Daily cell counts were collected from phase contrast micrographs using the FIJI program. On the 4th day, cells were fixed and stained with phalloidin for F-actin and DAPI for nuclear DNA, and imaged using a laser confocal microscope. The growth rate for cells in each condition were calculated by mathematically modeling the cells in their exponential growth phase.

Growth rates of annulus fibrosus and nucleus pulposus cells were not significantly affected by the presence of bone morphogenetic proteins. The growth rate was the same for both cell types. Cells were spindle shaped with long protrusions and cell morphology did not appear qualitatively different between cell types.

Bone morphogenetic proteins' potential as regenerative therapies are supported by their influence of extracellular matrix synthesis rather than their stimulation of cell proliferation. The changes in intervertebral disc cells' nutrient consumption under the influence of growth factors requires further investigation.

Keywords: *intervertebral disc, degeneration, bone morphogenetic protein, cell proliferation.*

Introduction

Degeneration of the intervertebral disc is a complex process involving: changes in intervertebral disc nutrition, decreased cell density and viability, qualitative and quantitative changes in extracellular matrix, as well as changes in disc biomechanics [*Evans C, 2006*]. Which processes are primary and which are secondary remains unclear. Moreover, genetic factors play an important role in the development of disc pathology [*Virtanen I et al., 2007; Martirosyan N et al., 2016*]. The complexity of the pathophysiological mechanisms of intervertebral disc degeneration complicate the search for potential therapeutic targets, including biological treatment approaches.

Address for Correspondence:
Morgan B. Giers
Department of Neurosurgery Research
Barrow Neurological Institute
350 W Thomas Rd, Phoenix, AZ 85013, USA
Tel.: (+1 602-406-3181)
E-mail: Morgan.Giers@gmail.com

Conservative treatment of intervertebral disc degeneration is aimed at acute pain relief and its effects are temporary in the majority of cases. Surgical intervention is required at later stages of degeneration. Surgery is invasive and can lead to adjacent segment degeneration due to changed biomechanics [*Maldonado C et al., 2011*]. Biological approaches for intervertebral disc regeneration, such as the application of growth factors, gene therapy, cell therapy and tissue engineering are promising therapeutic [*Kepler C et al., 2011*]. Therapeutic outcomes may differ depending on the method and the degree of intervertebral disc degeneration. In research of mild intervertebral disc degeneration growth factor injection therapy is a popular biological strategy for regeneration. In particular, the transforming growth factor β superfamily consists of a number of proteins believed to be associated with the synthesis of proteoglycans and collagen, playing an important role extracellular matrix accumulation. Bone morphogenetic proteins -2, -7, and -14 are members of the transforming growth factor β superfamily [*Than K et al., 2012; Belykh E et al., 2015*]. In mild intervertebral disc degeneration growth factors stimulate expression of extracellular matrix by viable cells, which can repair the structure and function of the intervertebral disc [*Masuda K, An H, 2006; Moriguchi Y et al., 2016*]. The success of this therapy depends on the presence of a sufficient number of viable cells [*Vasiliadis E et al., 2014*]. In this paper, we investigate the dynamics of cell proliferation under the influence of different bone morphogenetic proteins.

Material and Methods

Healthy human annulus fibrosus and nucleus pulposus cells were cultured in the control medium (1), and medium supplemented with bone morphogenetic protein-2 (2), bone morphogenetic protein-7 (3), and bone morphogenetic protein-14 (4). Daily cell counts were collected from phase contrast micrographs using the FIJI program. On the 4th day, cells were fixed and stained with phalloidin for F-actin and DAPI for nuclear DNA, and imaged using a laser confocal microscope Zeiss LSM 7 Duo (Carl Zeiss, Germany).

The design of the experiment. Healthy human annulus fibrosus cells and nucleus pulposus cells were thawed and cultured in monolayer in 25 cm^2 flasks incubated at 37°C and 5% CO2 until 85-90% confluent. During this time all cells received the control medium consisting of 50:50 Dulbecco's Modified Eagle Medium: Ham's F-12 (ThermoFisher) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. Once confluence was achieved cells were passaged into 35 *mm* Petri dishes with a 1.5 *mm* coverslip glass bottom 10 *mm* in diameter. Annulus fibrosus and nucleus pulposus cells were grown in: 1) control medium, 2) or medium supplemented with 100 *ng/mL* bone morphogenetic protein-2, 3) bone morphogenetic protein-7, or 4) bone morphogenetic protein-14 (Bone Morphogenetic Proteins Human Recombinant, ProSpec, USA). Medium was changed every two days. Culture was terminated on the 4th day.

Estimation of cell count and cell morphology: Daily assessment of live cell count and morphology was performed under the inverted microscope using a green phase contrast filter Axio (Carl Zeiss, Germany). Digital images were obtained by photographing (Canon camera) 6 fields of view in each culture dish at 240× magnification. Photography was performed every 24 hours for 4 days. Quantitative digital image analysis was performed on the jpegs with FIJI software (NIH, USA) using a plug-in for cell [*Schindelin J et al., 2012*].

Confocal microscopy: After 4 days of culturing the cells were fixed in 4% paraformaldehyde, stained with Alexa Fluor 633 phalloidin for actin and DAPI for nuclear DNA, with consequent slide investigation on a laser confocal microscope Zeiss LSM 7 Duo (Carl Zeiss, Germany).

Statistical analysis was performed using Microsoft Excel and Statistica 9.0 software. The cell counts were normalized to plating density. A multivariate ANOVA was performed for all normalized cell count data. P-values <0.05 were considered as significant. Post-hoc t-test was performed for specific comparison within significant data sets. All data were fit to the following cell growth model,

$$P=P_0e^{rt} \quad (1),$$

where p is normalized population, p_0 is the initial normalized population, r is the growth rate coefficient, and t is time. In this study p_0 is always equal to 1 because of the normalization of our data. Cell growth rates and Pearson correlation coefficients are reported.

Results

The initial plating density of the annulus fibrosus cells (Fig. 1) was significantly higher than the plating density of the nucleus pulposus cells (p=0.01) (Fig. 1A-B). To account for this difference in comparing cell population growth all data was normalized to the plating density (Fig. 1C-D). Normalized population densities were compared in a multivariate ANOVA, which showed that bone morphogenetic protein type or presence did not have a significant effect on cell proliferation (p=0.17). The nucleus pulposus cells showed significant growth every day for all 4 days (p=0.01 to p=0.04). The annulus fibrosus cells showed significant growth in the first 2 days (p>0.01) and then reached confluence (Fig. 2). When confluent the cells did not have significant increases in population (p=0.38). These cells were considered to be under the influence of contact inhibited growth and no longer in the exponential growth phase. Therefore, population growth rate was calculated using only the data from the first 2 days in the annulus fibrosus cells, but was calculated using the data from all 4 days in the nucleus pulposus cells. The growth rate was calculated by fitting normalized cell counts to equation 1. This generated growth rate coefficients for all cell populations (Fig. 3). The Pearson's coefficients showed strong correlations in all samples, with the lowest correlation being R2=0.68 (Table 1). It was found with this method that there was no difference in growth rate between the two cell types (p=0.80) and bone morphogenetic protein also did not affect growth rate (p=0.51). The cell population on day 4 was much larger in the annulus fibrosus cells (231±9) than in the nucleus pulposus cells (159±4) (p=0.05) even though both populations were considered confluent (Fig. 2). This could indicate a difference in cell size between annulus fibrosus and nucleus pulposus cells.

When performing histochemical staining for the actin cytoskeleton, annulus fibrosus and nucleus pulposus cells showed similar morphological forms. At full confluence annulus fibrosus and nucleus pulposus cells tended to be spindle-shaped with cell groups orientated longitudinally or radially towards the centers of increased cell density. Non-confluent nucleus pulposus and annulus fi-

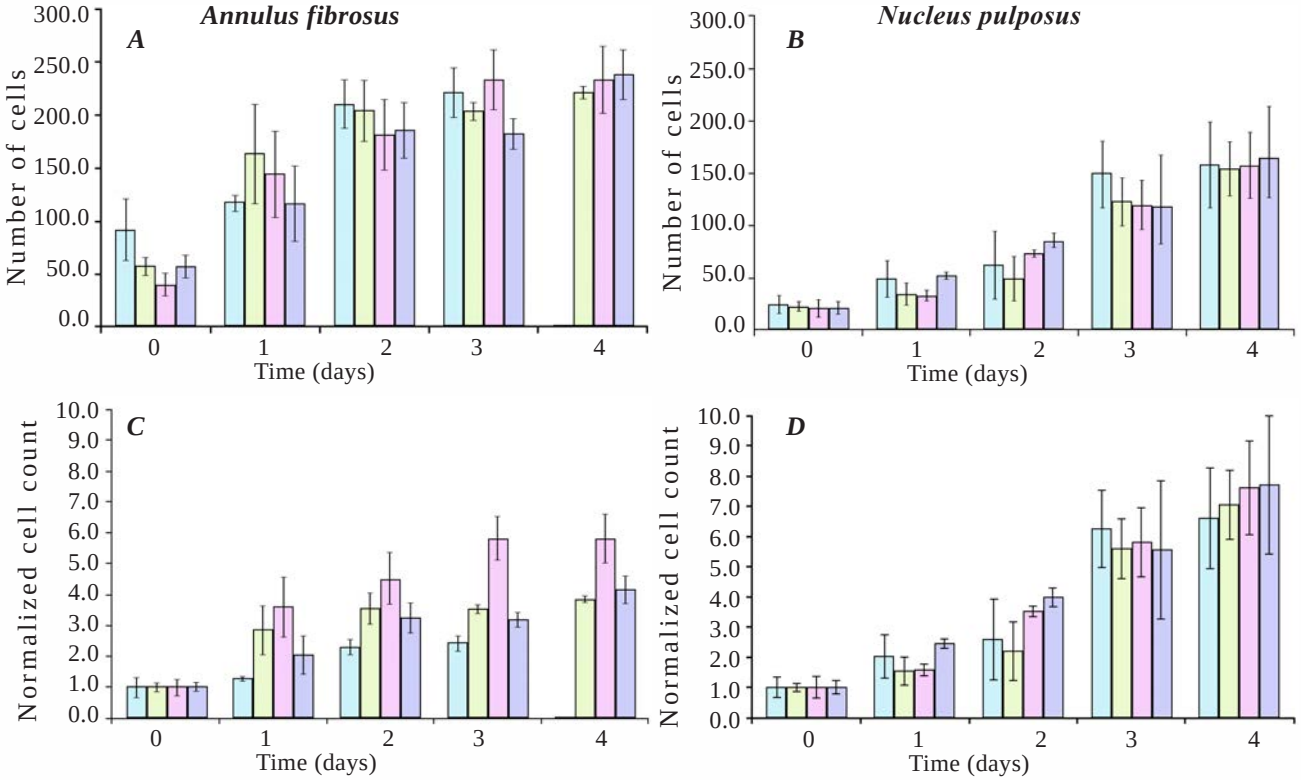


Figure 1. *Raw number of annulus fibrosus (**A**) and nucleus pulposus (**B**) cells counted in each flask (mean value ± standard deviation). Normalized number of annulus fibrosus (**C**) and nucleus pulposus (**D**) cells counted in Petri dish (mean value ± standard deviation)*
Note: *Control ( ), bone morphogenetic protein-2 ( ), bone morphogenetic protein-7 ( ), bone morphogenetic protein-14 ( )*

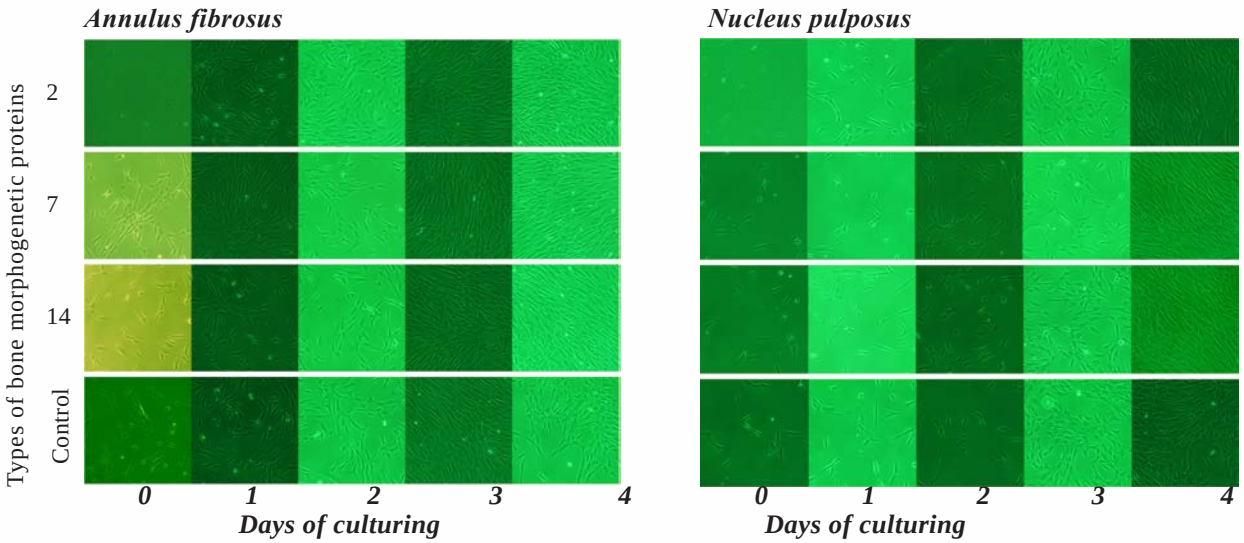


FIGURE 2. Microphotographs of live cells cultured under the influence of different bone morphogenetic proteins at different time points (0,1,2,3,4 days of culturing). Images were taken with phase-contrast microscopy with a green filter at 240x magnification.

brosus cells were spindle-shaped, stellate with 3-4 processes, or rarely fusiform. Flattened cells with flattened cytoplasm were also seen. Some of the nucleus pulposus and annulus fibrosus cells formed long thin processes on days 1-3, reaching 250 µm length. Actin cytoskeleton was well defined. The filaments were located evenly throughout the cell area following the protrusions. Closer to the edges of the cells, the actin filaments formed intense granules, indicating active assembly of actin during the formation of protrusions. The cell nuclei were round and oval in shape, 15 to 30 µm in diameter. No obvious difference in size was noted between the two cell types.

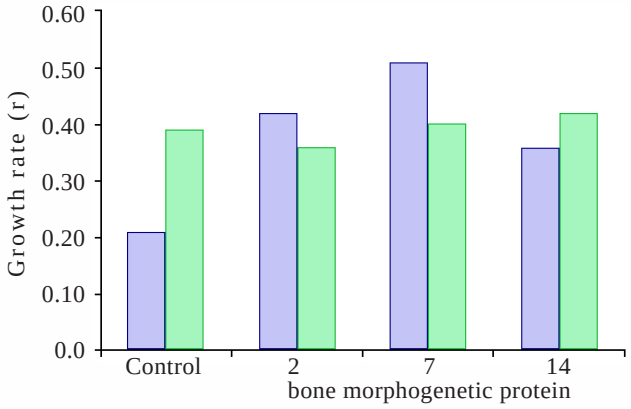


FIGURE 3. Estimation of growth rates of intervertebral disc cells under the influence of bone morphogenetic proteins. These values were obtained by mathematical modeling of exponential growth rate of cells in each Petri dish

Note: *Annulus fibrosus ( ), nucleus pulposus ( )*

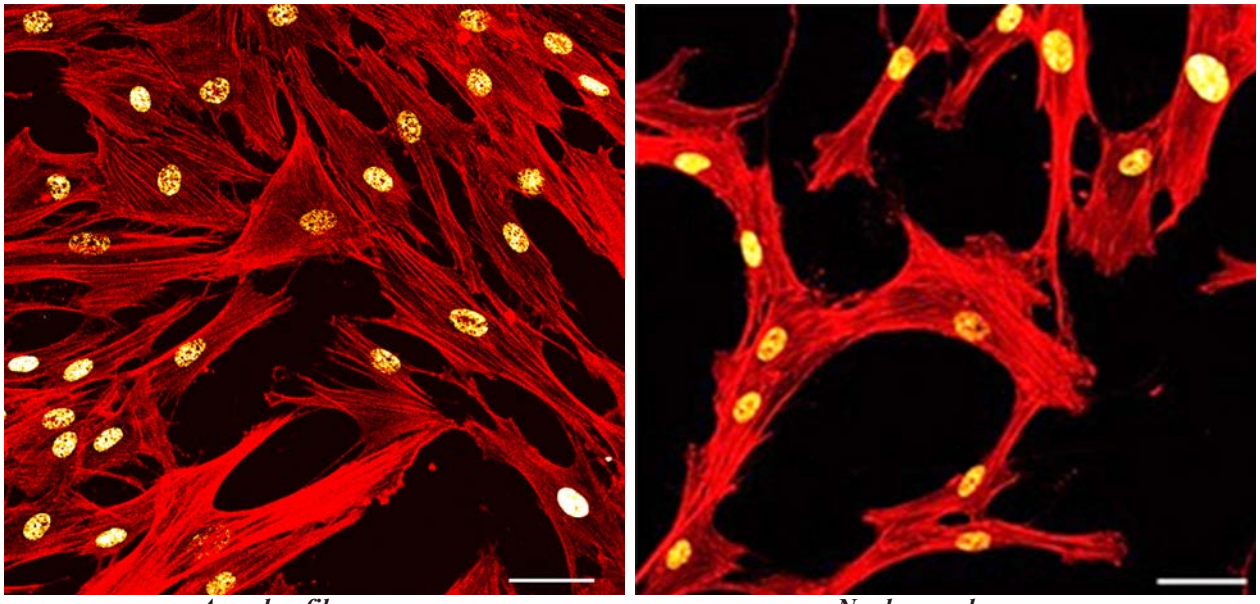
DISCUSSION

In the present study we evaluated proliferation of nucleus pulposus and annulus fibrosus cells in vitro in media supplemented with bone morphogenetic protein-2, -7 and -14 compared with a control. Studies have shown that bone morphogenetic protein-2 stimulates the production of extracellular matrix, in particular proteoglycan synthesis [*Kim H et al., 2009*]. Bone morphogenetic protein-7 is also known as "osteogenic protein 1" or OP-1 ™ (rhBMP-7, Stryker Biotech, Massachusetts, USA) and like

TABLE

Estimation of growth rates of intervertebral disc cells under the influence of bone morphogenetic proteins and Pearson correlation coefficients

Groups		Growth rate (r)		Pearson Coefficient (R2)	
		Annulus fibrosus	Nucleus pulposus	Annulus fibrosus	Nucleus pulposus
Control		0.21	0.39	0.68	0.84
Bone morphogenetic protein-	2	0.42	0.36	0.76	0.86
	7	0.51	0.4	0.75	0.89
	14	0.36	0.42	0.8	0.92
Mean value		0.38	0.39	0.75	0.88
Standard deviation		0.13	0.03	0.05	0.04



Annulus fibrosus ***Nucleus pulposus***

Figure 4. *Cells were fixed on the 4th day of cultivation and stained for actin (Phalloidin-Alexa 633, pseudocolored in red) and nuclei (DAPI, pseudocolored in yellow). Images were taken using laser scanning microscopy (scale 50 µm).*

bone morphogenetic protein-2 is used to promote osteosynthesis during spinal fusion and other orthopedic procedures on bone [*Lo K et al., 2012*]. Bone morphogenetic protein-7 has also shown potential to stimulate the production of the intervertebral disc extracellular matrix components [*Imai Y et al., 2007*]. Bone morphogenetic protein-14, otherwise known as GDF-5, is another member of the transforming growth factor β superfamily, and has a stimulating effect on the intervertebral disc cells, increasing the synthesis of proteoglycans and expression of type II collagen [*Li X et al., 2004*].

This study showed bone morphogenetic protein had no effect on cell proliferation in either cell type. Furthermore, there were no differences in growth rate between the two cell types. One limitation of this study is the uneven initial plating density. We tried to account for differences in the initial cell density by only modeling cells in their exponential growth phase. Confluent cells may experience contact inhibition of proliferation and therefore decrease the difference in the cell count. Due to the limited nutrient supply in the intervertebral disc regenerative therapies should focus on influencing protein production, but not necessarily increasing cell density, which would increase the nutrient demand. Here we have shown that bone morphogenetic proteins -2, -7, -14 do not increase cell proliferation. Changes in the intervertebral disc cell protein production and nutrient requirements under the influence of growth factors and its contribution to the overall regeneration requires further investigation.

Conclusion

In this in vitro study the nucleus pulposus and annulus fibrosus cells did not significantly change their proliferative activity when cultured with bone morphogenetic proteins -2, -7, -14. Bone morphogenetic proteins' potential as regenerative therapies are supported by their influence of extracellular matrix synthesis rather than their stimulation of cell proliferation. The changes in intervertebral disc cells' nutrient consumption under the influence of growth factors requires further investigation.

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