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Osteogenic protein 1 does not stimulate a regenerative effect in cultured human degenerated nucleus pulposus tissue.

Short title: OP-1 does not stimulate nucleus pulposus regeneration

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Abstract

Low back pain is a major cause of disability and is heavily associated with intervertebral disc degeneration. Osteogenic protein 1 (OP-1) is a growth factor that has shown potential to regenerate the intervertebral disc in human cells and animal models. However, high doses are required, presumably due to clearance from the tissue. Controlled release may be a solution to this problem. In this study, we developed a pre-clinical, pathophysiological human tissue explant culture model of degenerated nucleus pulposus (NP). The NP explants were cultured for 28 days, and injected with 100 µg OP-1 as a bolus, or injected with sustained release biodegradable microspheres loaded with 16 or 1.6 µg OP-1. After culture, tissue explants were analyzed for biochemical content (water, sulfated glycosaminoglycans (GAGs), hydroxyproline and DNA), histology, cell viability and gene expression (disc matrix anabolic and catabolic markers). Untreated degenerated NP explants lost some of their GAG content when cultured for 4 weeks, but maintained other tissue constituents. Gene expression levels were close to native values. A bolus injection of OP-1 partially restored GAG content to the native level in half of the donors, while the sustained release of OP-1 did not affect NP explants. No effect of treatment was observed on anabolic or catabolic gene expression at day 28. These results demonstrated that the regenerative potential of OP-1 is donor dependent and only at very high doses. This questions the clinical use of OP-1 as a regenerative agent as these high doses may increase the incidence of complications.

Keywords: nucleus pulposus, explant culture, intervertebral disc degeneration, regenerative medicine, osteogenic protein 1, growth factor therapy

1 Introduction

More than 80% of the population suffers from low back pain at least once during their lives (Walker et al., 2004). When it becomes chronic, it has a large socio-economic impact (Dagenais et al., 2008). Current treatments, such as conservative therapy and spinal fusion, have limited success and are not designed to treat the cause of the disease, which is often related to intervertebral disc degeneration (Cheung et al., 2009). When the disc starts to degenerate, there is a shift from an anabolic to a catabolic environment in its core, the nucleus pulposus (NP). The NP cells decrease their production of the main extracellular matrix proteins; aggrecan and type II collagen (Kluba et al., 2005) and increase their production of degrading enzymes (Le Maitre et al., 2004a). Additionally, there is a shift from type II collagen to type I collagen production and these changes in cellular behavior can cause the gradual changes seen in the extracellular matrix with degeneration (Antoniou et al., 1996). This is manifested in loss of proteoglycans (PGs) and water (Roughley, 2004), decrease of disc height and tissue fibrosis, and results in loss of tissue integrity and mechanical function, which may ultimately lead to low back pain (Cheung et al., 2009). Regenerative therapies aim to treat disc degeneration at an early stage to prevent low back pain from occurring and have shown to be promising to stop intervertebral disc degeneration in animal models (Masuda and Lotz, 2010).

Growth factors have potential as regenerative therapeutic agents, as they can be used to stimulate the native cells in a moderate stage of degeneration to increase extracellular matrix production and regenerate the disc (Masuda and Lotz, 2010). Bone morphogenic proteins (BMPs) are a family of growth factors that can stimulate bone and cartilaginous tissue production. Some BMPs are more chondrogenic than others, but BMP-7 (also known as osteogenic protein 1, OP-1) has shown potential to regenerate the disc (Masuda, 2008). OP-1 is effective in stimulating in vitro bovine (Zhang et al., 2004) and rabbit NP cells cultured in alginate (Masuda et al., 2003) to increase aggrecan and collagen type II production. Furthermore, OP-1 was able to stimulate human cells, obtained from early degenerated tissue and cultured in alginate beads, to proliferate and produce extracellular matrix proteins (Imai et al., 2007a). Furthermore, a bolus of 100 µg of OP-1 has also shown potential to increase disc height and PG content in animal models of disc degeneration (Miyamoto et al., 2006; Imai et al., 2007b). Despite the promising results of OP-1 in human cells and in rabbit models of induced disc degeneration, human degenerated disc tissue is more complex and in addition differs greatly from most laboratory animal IVDs. As in vivo research in the human is not feasible, the use of an explant model of early degenerated human tissue would be ideal for studying disc repair.

Another issue is that of equivalent dosages. The amount of OP-1 injected in the rabbit models might not be feasible to use as a regenerative therapy in the clinic when scaled up to a human disc. The large amounts required in animal studies to regenerate the disc are most likely due to the very short in vivo half-life of OP-1 (Larson et al., 2006);

the concentrations used in cell culture are at least two orders of magnitude lower than the amounts injected in the animal models (Imai et al., 2007a). These combined observations indicate the potential of using a sustained release system to deliver OP-1 to degenerated discs, as its effects might be prolonged compared to a bolus and the use of lower therapeutic dosages would reduce the risk of clinical complications.

We have previously cultured early degenerated human NP explants in a close to in vivo state for 1 week using balanced osmotic medium (van Dijk et al., 2014). In the meantime, we observed that an artificial annulus approach that keeps the osmotic pressure that is also present in vivo, is superior to osmotic balance in long-term cultures of bovine explants, as it kept cellular gene expression of extracellular matrix proteins closer to in vivo values (van Dijk et al., 2013). In this study, we cultured early degenerated human tissue (Thompson grade 3) for 4 weeks, using our artificial annulus approach. We then used this NP explant culture model to explore if OP-1 has the potential to regenerate the IVD by stimulating the production of extracellular matrix. Furthermore, we explored whether sustained release biodegradable microspheres loaded with OP-1 were more effective in stimulating extracellular matrix production than a bolus injection of OP-1.

2 Materials and methods

2.1 Culture

Human intervertebral disc tissue was obtained from autopsy subjects within 48 hours post mortem. Collection of intervertebral disc specimens was according to the medical

ethical regulations of the University Medical Center Utrecht (Utrecht, The Netherlands). Five human lumbar discs (L1-5, but exact levels unknown) were harvested from different donors under sterile conditions (Table 1).

Table 1. Overview of the donor discs (all Thompson grade 3)

Donor	Gender	Age	Cause of death
1	Male	73	Myocardial infarction
2	Female	63	Sepsis
3	Female	70	Myocardial infarction
4	Female	67	Influenza pneumonia
5	Male	65	Brain hemorrhage

The discs were opened transversely and the appearance of the NP, annulus fibrosus, endplate and vertebrae were used to determine the grade of degeneration according to the Thompson classification (Thompson et al., 1990). Only Thompson grade 3 discs were used in this study. The NPs were taken out of each disc and cut into 10 pieces of approximately 200 mg. The explants were cultured for 28 days in our artificial annulus system (van Dijk et al., 2013), using a 100 kDa molecular weight cut-off membrane (Figure 1). The culture medium consisted of advanced DMEM (Gibco; Invitrogen, Carlsbad, USA), with 4 mM L-glutamine (Lonza, Basel, Switzerland), 1% penicillin/streptomycin (Lonza), 50 mg/L ascorbic acid (Sigma, Zwijndrecht, the Netherlands), and 10% fetal bovine serum (Gibco).

Figure 1. Artificial annulus explants were added to a deep-well transwell plate with 6 ml of culture medium. As the artificial annulus explants might be buoyant, a stainless steel cylinder was added to a culture insert (left) to keep explants submerged (courtesy of Anthal Smits).

2.2 Osteogenic protein-1

OP-1 (ProSpec Bio, Rehovot, Israel) was dissolved in sterile milliQ water according to the manufacturer's instructions to a solution of 1 mg/ml. OP-1 was supplied lyophilized from a solution containing 1% sucrose, 1.2% mannitol, 20 mM glycine, and 0.05% Tween 20, at pH 4. This solution was dialyzed at 4°C using dialysis cassettes (Pierce; Thermo Fisher Scientific Inc, Rockford, USA) to remove Tween 20, to a 0.2x solution at pH 6.9, which was regularly changed. The dialyzed solution was lyophilized overnight, and stored at -80°C. Before use, the lyophilisate was dissolved in 5x times less water to concentrate the sample to 5 mg/ml in 1% sucrose, 1.2% mannitol, 20 mM glycine, at pH 6.9. OP-1 activity after these steps was confirmed through its capacity to induce ALP production in ATDC5 cells, and compared to OP-1 from other commercial suppliers. Microspheres were produced aseptically (DSM, Geleen, the Netherlands) using a standard water/oil/water emulsion procedure (Sinha and Trehan, 2003). This

procedure is suitable for the encapsulation of both hydrophobic and/or hydrophilic active compounds, varying from small molecules to large proteins, like OP-1. Microspheres were loaded with 3% OP-1 (w/w), with an efficiency of 55%, and stored at -20°C before use.

2.3 Treatment conditions

From every donor, 10 pieces of NP tissue were cultured as follows: 2 explants were immediately harvested as Day 0 controls and 8 explants were placed in the artificial annulus system. Explants were weighed before and after culture to determine the original and final wet weight (ww) of each sample. At day 1, explants were randomly distributed in duplicate in 4 different groups as described in Table 2.

Table 2. Overview of the experimental groups

Group	Injection volume	OP-1	Microspheres	Solution/suspension
Control	0 µl (puncture)	-	-	-
Bolus	20 µl	100 µg	-	Sucrose-mannitol buffer
Microspheres High	20 µl	16 µg	520 µg	PBS
Microspheres Low	20 µl	1.6 µg	52 µg	PBS

Control explants were sham punctured with the needle to control for disrupting the jacket/membrane/tissue system. Bolus explants were injected with 100 µg of OP-1 dissolved in 20 µl of the sucrose-mannitol buffer. Microspheres were suspended in 20 µl phosphate buffered saline (PBS) in two concentrations, loaded with 16 or 1.6 µg OP-1 respectively. The puncture and injections were done with a 26G needle and a 100 µl Hamilton syringe (Da Vinci, Rotterdam, the Netherlands), which was sterilized with 70% ethanol in between explants. All cultures were performed in a humidified incubator at 5% O₂, 5% CO₂ and 37°C. All explants were cultured in 12 deep-well plates (Greiner, Alphen a/d Rijn, the Netherlands) filled with 6 ml of culture medium. Medium was changed two times a week. A 316L stainless steel weight was added to well inserts to prevent floating of explants (Figure 1). At day 0 and 28, explants were cut in 3 pieces. One piece was used for histological and cell viability evaluation, one to determine the biochemical composition and one to assess gene expression levels of the NP tissue.

2.4 Biochemical content

Samples were weighed at the beginning and end of culture, and the percent increase in sample wet weight (ww) was calculated. Subsequently they were stored frozen at -30°C, lyophilized overnight (Freezone 2.5; Labconco) and the dry weight (dw) was measured. The water content was calculated from $ww-dw/ww$. The samples were then processed as described earlier and used to determine their content of: sulfated glycosaminoglycans (sGAG), hydroxyproline (HYP), and DNA, as well as the fixed charge density (FCD) (van Dijk et al., 2013). The amounts of sGAG, HYP, and DNA were

expressed as percentage of the tissue dw. Media samples collected at day 4, 7, 14, 21 and 28 were stored at -30°C and subsequently analyzed for GAG lost to the medium using the chondroitin sulfate reference dissolved in culture medium and normalizing to the original sample wet weight.

2.5 Gene expression levels

At day 0 and 28, samples for gene expression analysis were snap-frozen in liquid N₂ and stored at -80°C. To disrupt the samples a stainless steel 8 mm bead and stainless steel custom-made lid were placed in a 2 mL Eppendorf tube and snap-frozen in liquid N₂. Frozen samples were placed in between bead and lid, and disrupted with a microdismembrator (Sartorius, Goettingen, Germany) for 20 sec at 2000 rpm. This was repeated if necessary with the sample snap-frozen between each cycle until the tissue was pulverized. After disruption, RNA was isolated using TRIzol (Invitrogen) and purified using the Qiagen mini-kit (Qiagen, Venlo, the Netherlands). The quantity and purity of isolated RNA was measured with a spectrophotometer (ND-1000; Isogen, de Meern, the Netherlands). Absence of genomic DNA contamination in the isolated RNA was checked using real time PCR (CFX384, Biorad, Veenendaal, the Netherlands) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. The genes of interest were aggrecan, type I, II and X collagen, Runt-related transcription factor 2 (RUNX2), cyclooxygenase 2 (COX2), ADAM metalloproteinases with thrombospondin type 1 motif 4 and 5 (ADAMTS 4 and 5) and matrix metalloproteinase 13 (MMP13). Primer pairs were developed earlier (van Dijk et al., 2014), except RUNX2 which was obtained from literature (Potier et al., 2007) (Fwd: 5'- GTA CAG CTT TAA GGA TTC CCT CAA TTC-

3', Rev: 5'-TTG CTA ATG CTT CGT GTT TCC A-3', Sigma). The VILO-kit (Invitrogen) was used to reverse-transcribe 77 ng of total RNA. 18S was selected as reference gene from three tested genes (RPL13A, GAPDH and 18S) as the most stable gene throughout our experimental conditions. Gene expression was investigated using real time PCR in duplicate, and expression difference (ΔC_t) was calculated as the difference between the gene of interest and the reference gene 18S. Levels of expression are expressed as $2^{-\Delta C_t}$. When gene expression was not detected, the $2^{-\Delta C_t}$ value was set to 0 to conduct the statistical analysis.

2.6 Histology and cell viability

Samples were covered in Tissue-Tek O.C.T. (Sakura, Alphen a/d Rijn, the Netherlands), snap-frozen in isopentane in liquid N₂, and stored at -30°C. Subsequently, 10 µm thick sections were cut with a cryostat (Microm, Walldorf, Germany) and stored at -30°C. Sections were either fixed with 3.7% formalin, stained with Weigerts Hematoxylin for cell nuclei, Safranin-O for PGs and Fast Green for collagen (Rosenberg, 1971) or stained for lactate dehydrogenase (LDH) to assess cell viability (Stoddart et al., 2006) (fixed with 70% EtOH, permeabilized with 0.1 % Triton and counterstained with propidium iodide). Images were taken with a combined fluorescence and bright-field microscope (Zeiss Observer, Zeiss, Sliedrecht, the Netherlands). Fluorescence and bright field images of the same region of interest were combined using ImageJ software (National Institutes of Health, Bethesda, US) for the LDH stained sections.

2.7 Statistics

Matlab (Mathworks Inc., Natick, USA) was used for statistical analysis. For all biochemical data, one-way analysis of variance was performed, followed by Bonferroni corrected post-hoc independent t-tests. On the gene expression data, Kruskal-Wallis analysis was performed at each time point, followed by Bonferroni corrected Mann-Whitney post-hoc tests. Statistical significance in all cases was assumed for $p < 0.05$.

3 Results

3.1 Histology

Cell viability, assessed with LDH staining, was maintained in all culture groups after 28 days of culture (Figure 2).

Figure 2. Lactate dehydrogenase (LDH) stained sections of fresh and 28-day cultured NP explants: day 0 (A), control (B), bolus (C), microspheres high concentration (D), and microspheres low concentration (E); representative of 5 donors. Dark blue indicates

living cells (active LDH), red indicates cell nuclei (either living or dead). Scale bar = 200 μm .

Figure 3. Safranin-O / Fast Green stained sections of fresh and 28-day cultured NP explants: day 0 (A), control (B), bolus (C), microspheres high concentration (D), and microspheres low concentration (E); representative of 5 donors. Proteoglycans are stained red/orange, collagen is stained green and cell nuclei are stained black. Scale bar = 100 μm .

3.2 Biochemical content

After 28 days of culture, the average water, hydroxyproline and DNA contents of control (sham injected) explants were not significantly different from Day 0 explants (Figure 4A, D, E). The GAG content and the FCD decreased in the control group compared to day 0 (Figure 4B, C). These observed effects were similar for all five donors (Figure 5). There were no significant differences in any of the biochemical parameters between OP-1 treated and sham control explants (Figure 4). The GAG

content of explants treated with a bolus of OP-1 was not different from day 0, but was decreased in all other cultured explants. There were clear differences in the DNA content of the different donors, causing a large variance (Figure 4E). Of the four individual donors treated with a bolus, one donor responded to OP-1 by a strong increase in GAG content compared to sustained release or sham injection; in two donors a response was observed in only one of the duplicates; and in one donor no response was observed (Figure 5). Some GAG was lost from the explants mostly in the first week of culture, and the lost amounts and rates were similar in all culture groups (Figure 6).

Figure 4. Biochemical content of NP explants at day 0 and 28: water content (A), GAG content expressed per dry weight (B), fixed charge density in mEq/g (C), hydroxyproline content expressed per dry weight (D), and DNA content expressed per dry weight (E). Values are means ± standard deviation, n = 5, (4 for bolus). *Different from day 0 at three significance levels; * p < 0.05, ** p < 0.01 and *** p < 0.001.

Figure 5. GAG content of NP explants of the individual donors after 28 days. GAG content expressed per dry weight. Lines are means from duplicates of 1 donor, points are individual explants, n = 5 donors, (4 for bolus).

Figure 6. sGAG content in the explant supernatant expressed per dry weight of the samples. Values are means $\pm$ standard deviation, n = 5, (4 for bolus).

3.3 Gene expression levels

No significant differences were observed for the expression levels of aggrecan, type II collagen, ADAMTS 4 and 5, RUNX2 and COX2 for all cultured groups, compared to day 0 (Figure 7). Expression of MMP13 was significantly upregulated in sham injected and bolus treated groups compared to day 0. Type I and type X collagen expressions were significantly upregulated in all cultured groups compared to day 0. There were no differences in gene expression between any treatment group and the sham injected group.

Figure 7. Gene expression levels at day 0 and 28: aggrecan (A), type II collagen (B), type I collagen (C), type X collagen (D), RUNX2 (E), COX2 (F), MMP 13 (G), ADAMTS 4 (H), and ADAMTS 5 (F). Gene expression levels are relative to 18S expression levels ($2^{-\Delta Ct}$).

Values are means $\pm$ standard deviation, n = 5 (4 for bolus). *Different from day 0 at three significance levels; * p < 0.05, ** p < 0.01 and *** p < 0.001.

4 Discussion

The artificial annulus system to culture human NP tissue in vitro worked sufficiently well to test stimulatory strategies but does require a negative control. After 28 days in this system, there were no changes in cell, collagen, and water content in untreated controls compared to day 0 (Figure 4). The cell viability of the explants was equally preserved (Figure 2). However, GAG content and FCD decreased for all five donors (Figure 4B, C). This was unexpected as the culture system used here was previously demonstrated to preserve the native tissue content of healthy bovine NP explants (van Dijk et al., 2013). This decrease could be due to loss of PGs through the needle puncture. However, as the resulting cut was single, isolated and small, we believe that the use of degenerated tissue mainly contributed to the PG loss. With disc degeneration, PGs become fragmented and less aggregated (Roughley, 2004) and the damaged collagen network allows even more mobility for these smaller PGs. Fragments smaller than 100 kDa (molecular weight cut-off of the membrane surrounding the explants) may then have slowly leached out of the tissue in culture, unlike the healthy tissue with larger aggregated PGs. Finally, as remaining PGs attract water into the tissue, filling the volume left by the leaching GAG, water content may have increased (Figure 4A). This hypothesis is also in agreement with the data for GAG measured in the media, which occurred mostly in the first week of culture (Figure 6). The GAG loss was not visible on Safranin-O / Fast Green stained sections (Figure 3), as

previously observed in another NP explant model (Le Maitre et al., 2004b). Using membranes with a smaller molecular weight cut off could limit the leaching of fragmented PGs, but care should be taken as it may also impede the entrance of growth factors (supplemented via FBS) important for cell survival in the NP tissue.

In terms of cell behavior, gene expression of major disc matrix proteins (type II collagen and aggrecan) was not significantly different in cultured explants compared to native tissue (Figure 7). However, expression of MMP13, type I and type X collagens were upregulated after 28 days in culture. This may result from a statistical artifact in that the expression of these genes was extremely low in day 0 explants. It could also be an indication that the system is not fully stable: as PGs are partially lost and osmotic pressure decreases, the NP cells could respond by remodeling their collagen matrix. Despite unexpected results in terms of gene expression and protein content, the culture system still allowed testing of different therapeutic approaches on one disc sample, and did maintain NP matrix composition and NP cell gene expression close to native values.

To the best of our knowledge, this was the first study that evaluated the regenerative potential of OP-1 in early degenerated human tissue. Although no substantial effect of OP-1 was observed, in bolus form, it did partially restore the GAG content to the Day 0 level (Figure 4), yet there was a high inter donor variability (Figure 5). Previous studies using rabbit models of induced degeneration showed that the OP-1 bolus used in this study (100 µg) could restore PG content and disc height to control levels (Imai et al.,

2007b; Masuda et al., 2006). However, our relative therapeutic dose was one order of magnitude smaller than that of Imai et al., since the weight of a rabbit NP is approximately 25 mg (O'Connell et al., 2007), and the NP explants used in this study were ca. 200 mg. Moreover, though injected in the center of the explant, OP-1 is likely to have diffused through and perhaps out of the tissue through the membrane (100 kDa MWCO) and or the injection hole (OP-1 molecular weight 49 kDa), reducing the exposure time of NP cells to OP-1. Finally, it is also possible that newly synthesized PGs under OP-1 stimulation may have leached out of the tissue. However, no substantial increase was observed in the GAG measured in medium supernatants for bolus injection (Figure 6). This indicates that the stimulatory effect of OP-1 was very small, and the lack of a response could have been due to a dosage and/or exposure time issue. However, clinical usage of this OP-1 amount scaled up to an entire human NP, though, would be of great concern (10 mg to treat a complete NP) given the current knowledge of the clinical complications observed with the use of high doses of BMP-2, as stated in a warning letter from the American Food and Drug Administration (Schultz, 2008), and the cost generated by such a therapeutic approach. In fact, in a recent study using a canine model of spontaneous disc degeneration, OP-1 doses in the order of magnitude used in this study induced ectopic bone formation (Willems et al, 2015).

On the other hand, sustained release from microspheres or hydrogels has the potential to solve the issue of short exposure time of NP cells to OP-1, due to either the leakage of OP-1 out of the system or the short half-life of OP-1. The lack of effect of the sustained release in this study was then probably caused again by a dosage issue. The

highest amount we could deliver with the microspheres was only 16 μg (with 0.52 mg microspheres in 20 μl PBS). It was not possible to inject larger volumes or higher concentrations, as the suspension of microspheres would clog the needle. Hence, a 16 μg dose could be insufficient to trigger a reaction from NP tissue within the time frame of our study. Although 4 weeks were enough to observe small changes of disc height in the rabbit studies (Imai et al., 2007b; Masuda et al., 2006), this period maybe too short when the tissue is bigger (200 mg for our system vs. 25 mg for rabbit studies) or the dosage lower.

Another possibility to explain the discrepancy between human degenerated samples and rabbit NP is the cell composition of these tissues. Mature rabbits used in previous studies retain their notochordal cells (cells involved in disc development) during adulthood (Sowa et al., 2008), whereas human NP tissue as used here only contains chondrocyte-like cells (Hunter et al., 2003); and the notochordal cells might be more sensitive to growth factors. This hypothesis is supported by the observation that cells isolated from a rabbit NP (Masuda et al., 2003) respond 2.5 times stronger to a stimulus of 200 ng/ml OP-1 for 14 days in alginate bead culture, than NP cells isolated from human donors (Imai et al., 2007a). Furthermore, the cell density in discs containing notochordal cells is higher (Liebscher et al., 2011), so both effects might explain the stronger response to OP-1 in the animal models.

The current study again stressed the large donor variations in human patients, in this case there were clear differences in cell density between different explants and

donors. This indicates that even when only Thompson grade 3 discs are treated with growth factor therapy, there might be quite clear differences in response to this treatment. Cell therapy might then be a better option in patients with low cellularity in the disc. This also points out the clear advantage of our explant culture system, which is the use of a matched experimental design: multiple explants can be obtained from the same NP tissue and exposed to different therapeutic treatments, contrary to a whole disc explant system (Junger et al., 2009). This approach is appealing as donor-to-donor variance can be reduced, since the response per donor can be investigated (Figure 5). Furthermore, donors can be divided into responders and non-responders, and differences between these subpopulations can be further studied to identify characteristics that may help to identify patients as potential candidates for growth factor therapy.

5. Conclusion

In conclusion, human early degenerated NP explants were cultured in a simulated in vivo environment for 4 weeks. Stimulation with OP-1, supplied either as a high bolus or sustained lower dosages did not regenerate the explants. Contrary to previous cell culture and animal model work, any regenerative potential of OP-1 on human early degenerated tissue is donor dependent and only at very high concentrations. These very high doses might cause clinical complications, which questions the use of OP-1 as a regenerative therapy.

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