INTRODUCTION:
Articular cartilage and intervertebral disc degeneration are extremely common ailments in the general population. Novel biologic treatments are being developed including the use of growth factors and gene therapies. To facilitate these efforts, it is essential to identify inexpensive and readily available biofactors involved in the maintenance of joint and disc homeostasis. In our study, we study the effect of neuropeptide factors on in vitro bovine articular cartilage and intervertebral disc cell proliferation and function.

In soft tissue injury, a diminished healing response in neuropathic tissues suggests an important regulatory role for factors derived from the peripheral nervous system (PNS). Connective tissues without peripheral innervation (e.g. cartilage) heal poorly. Growing anatomic and physiologic evidence suggests that the peripheral nervous system (PNS) is important in the proliferative and reparative processes in injured tissue. In the PNS, neurogenic factors, such as neuropeptides (NPs), are chemical agents that mediate these functions.

Recent studies demonstrated that local delivery of specific NPs can reverse the functional deficits of ruptured medial collateral ligaments (MCLs) in normal and neuropathic rat models. These results intensified our interest in the effects of neuropeptides on other connective tissues. Based on these studies, we hypothesized that these neuropeptides will influence the healing of cartilaginous tissue as well. The aim of this study is to measure the influence of peripheral neuropeptides on articular and intervertebral disc cell growth and function.

METHODS:
Bovine calf spines and femoral condyles were obtained from the slaughterhouse and freshly transported to our laboratory. Nucleus pulposus tissue was carefully dissected, avoiding annular and endplate cartilage tissues, from each disc. Articular cartilage from femoral condyles was dissected in the same manner. Tissues were incubated in Dulbecco’s modified Eagle’s medium supplemented with fetal calf serum, antibiotics, and amino acids. Cells were harvested by overnight digestion with hyaluronidase and collagenase, followed by filtration. Cells were plated and incubated in the presence of each neuropeptide or media only (control). At various time points in growth, cells were collected for proliferation and glycosaminoglycan production assays. Neuropeptide concentrations used in previous ligamentous healing studies were used.

Well established colorimetric and luminescent assays (CellTiter 96® AQueous Non-radioactive Cell Proliferation and CellTiter-Glo® Luminescent Cell Viability Assays, Promega) were used to quantify the effect of each neuropeptide on cell proliferation. In addition, corresponding cell cultures during growth were digested with papain to release glycosaminoglycans (GAGs). Dimethyl methylene blue (DMMB) colorimetric assays were used to quantify the amount of GAGs in each culture.

Multiple samples for test and control groups were performed simultaneously. Statistical analyses using one way analysis of variance (ANOVA) with large (n=16 for GAG assays; n=24 for proliferation assays) degrees of freedom were performed to establish the significance of differences between test groups and control groups for each day of growth in culture.

RESULTS:
We initially performed in vitro experiments with bovine articular cartilage chondrocytes. In culture, CGRP, NPY, SP, and VIP stimulate proliferation of these chondrocytes compared to controls (Figure 1). As expected, GAG production is also affected (data not shown). As chondrocyte cells are in a proliferative phase, GAG production initially decreases as cell metabolism is dedicated to proliferation. Confluent cell cultures have not yet been included in our experiments.

We then examined the effect of neuropeptides on intervertebral disc cell proliferation in order to further elucidate the pathophysiology of degenerative disc disease. Bovine nucleus pulposus cells were incubated with the same neuropeptides. Interestingly, CGRP, NPY, and SP stimulate disc cell proliferation compared to controls throughout growth (days 1, 5, and 8; see legend Figure 2), while VIP ultimately had a negative influence on disc cell viability. These results are statistically significant (p<0.05, n=24). Production of GAG follows a similar pattern described above with articular chondrocytes.

![Figure 1: Effects of Neuropeptides on Bovine Articular Chondrocyte Proliferation at Subconfluence](image)

![Figure 2: Effect of Neuropeptides on Bovine Nucleus Pulposus Cell Proliferation during Growth to Subconfluence](image)

DISCUSSION:
Results of this preliminary study support our hypothesis that NPs affect articular chondrocytes and intervertebral disc cells. Our data demonstrate the influence of neuropeptides on proliferation and GAG expression. These results not only identify agents of potential therapeutic value, they also help elucidate the pathophysiology of degenerative joint and disc disease and the emerging role of the peripheral nervous system in soft tissue healing and homeostasis. These preliminary results suggest that future studies should characterize the functional implications of neuropeptides on cartilaginous tissues, including GAG, aggregan, and collagen production and, ultimately, tissue maintenance in vivo.

Neuropeptides possess several advantages as an approach to therapy over growth factors such as OP-1. Since they are small peptides, they are less expensive as they are easily synthesized and easier to integrate into systems of targeted drug delivery. Importantly, they have been show to have broad positive effects, including modulation of the inflammatory response, promotion of angiogenesis and neurogenesis, and stimulation of cell proliferation, on soft tissue healing. Thus neuropeptides advantageously orchestrate effects over a broader spectrum of soft tissue healing than targeted molecules.

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