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Characterization of bovine and canine animal model cartilage endplates and comparison to human cartilage endplate structure, matrix composition, and cell phenotype

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Abstract

There is a need to further explore mechanisms of cartilage endplate (CEP) degeneration, due to its role in the onset and progression of intervertebral disc degeneration and low back pain. Therefore, the goal of this study was to evaluate structure, matrix composition, and cell phenotype between the human and bovine or canine, both clinically relevant animal models currently used to study the intervertebral disc, CEP. This information may be used in addition to other relevant studies, to help determine optimal animal models for use in studying the role of the CEP in intervertebral disc degeneration and back pain. Endplate structure, matrix composition, cell morphology, and gene expression were evaluated using a picrosirius red/alcian blue and hematoxylin and eosin stain, a dimethylmethylene blue assay, and quantitative reverse transcription polymerase chain reaction. The bovine and canine CEPs were thinner with more rounded cells and thicker bony endplates. The canine CEP contained significantly more sulfated glycosaminoglycans. The bovine CEP demonstrated higher expression of ACAN, COL1, and COL2 and lower expression of T, FBLN1, and collagen X (COLX) compared to the human CEP. The canine CEP had higher COL2 and lower COL1, KRT19, MKX, FBLN1, COLX expression compared to human. These similarities and differences between human and bovine or canine CEP are important to consider when evaluating which animal model is most optimal to use in future studies, interpreting research findings using these animal models and assessing translatability to the human condition.

KEYWORDS
animal models, bovine, canine, cartilage endplate, intervertebral disc

1 | INTRODUCTION

Low back pain (LBP) is the leading cause of disability worldwide,[1] is the second most common reason for prescription of opioids[2] and has socio-economic costs of >$100 billion in the United States alone.[3] LBP is highly associated with intervertebral disc (IVD) degeneration,[4] yet current treatment strategies are limited to non-invasive pain management, such as physical therapy and non steroidal anti-inflammatory drugs or highly invasive surgeries, such as lumbar fusions, neither of which target the underlying disease mechanisms-associated IVD degeneration. The IVD consists of the central nucleus pulposus (NP) surrounded circumferentially by the annulus fibrosus (AF). It is enclosed cranially and

caudally by cartilage endplates (CEPs), thin layers of cartilage, which work as an interface between the IVD and adjacent vertebral bodies.[5] The CEP consists mainly of collagen, proteoglycan, and cells resembling articular chondrocytes and is largely thought to resemble articular cartilage (AC). However, unlike AC, the CEP is not directly connected to the bone of the vertebral bodies, but is connected to the disc through the lamellae of the AF. The CEP functions to distribute loads and provide nutrition to the avascular IVD, which relies heavily on the exchange of metabolites through the CEPs for supply of nutrient and waste removal.[5] Vertebral bone marrow lesions, visualized as Modic changes on magnetic resonance imaging,[6,7] and CEP defects[8] are highly prevalent in patients with discogenic LBP, implicating the CEP as a potential source of pain. A recent cross-sectional cohort study of chronic LBP patients found CEP defects as the strongest predictor of pain, even more predictive than IVD degeneration.[8] In addition to being a potential source of pain, CEP degeneration/calcification is associated with the onset and progression of IVD degeneration,[9] and changes in CEP biochemistry can lead to decreased nutrient diffusion from vertebral capillaries across the CEP and a subsequent decrease in NP cell viability.[10] However despite a significant association with IVD degeneration and discogenic LBP, there exists a lack of relevant research models to investigate the mechanisms underlying CEP pathology in the degenerate IVD joint and LBP.

Animal models are extensively used to study IVD degeneration and could provide a relevant tool in studying CEP degeneration/calcification due to decreased subject variability, increased tissue availability, and ability to study disease mechanisms and screen potential therapies in vivo compared to human.[11] However, species specific differences in CEP structure and cell phenotype have been largely neglected to this point when considering animal models of IVD degeneration and back pain, although some distinct structural differences have been previously identified.[12] Past studies have demonstrated that mice, rats, rabbits, goats, and dogs have vertebral growth plates that persist through adulthood, whereas adult humans do not have vertebral growth plates.[12,13] Mice and rats have thicker CEPs with smaller secondary ossification centers and rabbits and goats have thinner CEPs with larger secondary ossification centers, while the human CEP is thicker with both a cartilaginous and bony endplate (BEP).[12] Chondrodystrophic dogs also have both CEPs and BEPs; however, dogs have thicker BEPs and thinner CEPs compared to humans.[13] Bovine and chondrodystrophic canine IVDs are commonly used to study IVD degeneration. They offer clinically relevant models based on similarities in structure and cell composition to the human IVD and are therefore two promising intermediate/large animal models for studying the CEP. Bovine IVDs are commonly used for whole IVD organ and cell culture models in vitro, as they are highly accessible through local abattoirs, have little variability, and are generally healthy.[14,15] Chondrodystrophic dogs have naturally occurring IVD degeneration with degenerative changes and clinical pain symptoms similar to humans, making them of specific interest due to the

TABLE 1 Specimen demographics

Specimen ID	Species	Age	Sex	Level	Thompson grade
Hu8	Human	52 years	Male	L2/L3	Unknown
Hu9	Human	58 years	Female	L4/L5	2-3
Hu10	Human	56 years	Female	L4/L5	Unknown
Hu12	Human	31 years	Female	L1/L2	Unknown
Hu7	Human	56 years	Female	L3/L4	3
Hu15	Human	59 years	Female	L3/L4	3
Hu13	Human	30 years	Female	L2/L3	3
BT12	Bovine	2–3 years	Male	caudal	1–2[a]
BT14	Bovine	2–3 years	Male	caudal	1–2[a]
BT15	Bovine	2–3 years	Male	caudal	1–2[a]
BT16	Bovine	2–3 years	Male	caudal	1–2[a]
BT22	Bovine	2–3 years	Male	caudal	1–2[a]
BT23	Bovine	2–3 years	Male	caudal	1–2[a]
BT24	Bovine	2–3 years	Male	caudal	1–2[a]
K9-7	Canine/hound	Adult-specific age unknown	Female	Lumbar	1–2[a]
K9-8	Canine/hound	Adult-specific age unknown	Female	Lumbar	1–2[a]
K9-9	Canine/hound	Adult-specific age unknown	Female	Lumbar	1–2[a]
K9-16	Canine/beagle	15 months	Female	Lumbar	1–2[a]
K9-17	Canine/beagle	15 months	Female	Lumbar	1–2[a]
K9-18	Canine/beagle	24 months	Male	Lumbar	1–2[a]
K9-19	Canine/beagle	13 months	Male	Lumbar	1–2[a]
K9-20	Canine/beagle	13 months	Male	Lumbar	1–2[a]

Note: Demographics of specimens used in the study including species, age, sex, disc level, and Thompson grade.
[a]Thompson grade equivalent.

ability to study naturally occurring disease progression and potential therapeutic strategies in an actual patient population.[16]

There are currently no studies that have characterized and directly compared the bovine, canine, and human CEP on both the histological and gene level, making it difficult to extrapolate results from these animal models to the human CEP. Direct comparison in structure, matrix composition, and cell phenotype between canine, bovine, and human CEPs will allow for identification of animal models of the CEP most representative of the human CEP and assist in interpretation of research findings utilizing these animal models. Therefore, the goal of this study was to evaluate the structure, matrix composition, and cell phenotype between the human and bovine or canine CEP in order to provide additional information to help determine optimal animal models for studying the role of the CEP in IVD degeneration and discogenic LBP.

2 | MATERIALS AND METHODS

2.1 | Tissue Acquisition

Human IVD tissue was obtained from cadaveric lumbar spines (n = 7) within 36 hours of death (Cooperative Human Tissue Network, Columbus, OH). Canine IVD and AC tissue was obtained from cadaveric lumbar spines and knee/elbow joints of chondrodystrophic dogs (n = 8) within 36 hours of death per IACUC protocol number 2018A00000131. Bovine IVD (n = 7) and AC (n = 6) tissue was obtained from cadaveric caudal spines and metacarpophalangeal (MCP) joints from the local abattoir (Table 1 Demographics). AC tissue was scraped from all condyles of bovine MCP (1 joint/animal) and canine knees/elbows (2 knees and 2 elbows/animal) for cell isolation and tissue analysis. Individual IVD motion segments (vertebral body-IVD-vertebral body) were removed from each spine. Images of IVDs were taken, blinded, and used for identification of Thompson grade by two individual expert spine researchers. Images for Hu8, Hu10, and Hu12 were not available and therefore Thompson grades are unknown. Sagittal whole sections were cut from each bovine, canine, and human disc and fixed in 10% neutral buffered formalin (NBF) (22-050-104, Thermo Fisher Scientific, MA) for additional processing. The remaining IVD tissue from human, bovine, and canine was dissected from the vertebral bodies for isolation of cells and analysis of NP and AF tissue. The vertebral bodies of human, bovine, and canine disc specimens were scraped to remove NP or AF tissue and expose the CEPs. CEP tissue was carefully dissected from the vertebral bodies to ensure shiny, translucent cartilage was removed for cell isolation. Extreme care was taken in separating the three tissue types to decrease potential for cross-contamination.

2.2 | Cell isolation

Cells were isolated from human (n = 7) CEP, bovine (n = 6) CEP, NP, AF, and AC; and canine (n = 6) CEP, NP, AF, and (n = 4) AC tissue using 0.2% protease (from *Streptomyces griseus*-Type XIV:P5147, Sigma–Aldrich, MO) for 1 hour at 37°C, followed by digestion in 0.012% collagenase II (CEP, NP, and AC) (from *Clostridium histolyticum*-17 101 015, Thermo Fisher Scientific, MA) or 0.012% collagenase I (AF) (from *C. histolyticum*-C2674-100MG, Millipore Sigma, MA) for 12 hours at 37°C as previously described.[17] Cells were expanded in standard culture media (Dulbecco's Modified Eagle Medium (4.5 g/L glucose-11 995 115, Thermo Fisher Scientific, MA), 10% fetal bovine serum (FBS:10437028, Gibco, MD), 1% penicillin/streptomycin (15 140 122, Gibco, MD), 50 μg/mL ascorbic acid (97061-072, VWR, PA), 0.5% Amphotericin B (15 290 018, Gibco, MD)) in standard culture conditions (5% CO_2, 20.7% O_2, 37°C) until 80% confluent. Cells were frozen at passage 1 (p1) at 0.5×10^6 to 1×10^6 cells per cryogenic vial in 1 mL freezing solution (10% dimethyl sulfoxide [D2438-5X, Sigma–Aldrich, MO] in FBS).

2.3 | Gene expression

To isolate RNA from human CEP cells (n = 7), bovine CEP, NP, AF, and AC cells (n = 6); and canine CEP, NP, AF (n = 6), and AC cells (n = 4), cryogenic vials were thawed and centrifuged at 400*g* for 5 minutes. Freezing solution was removed from each cell pellet and RNA was isolated using the TRIzol Plus RNA Purification Kit (12 183 555, Thermo Fisher Scientific, MA). RNA content was quantified using Nanodrop 2000. Samples

TABLE 2 Gene expression primers

Gene	Abbreviation	Marker	Human Primer	Bovine Primer	Canine Primer
Aggrecan	ACAN	Matrix	Hs00153936_m1	Bt03212186_m1	Cf02674826_m1
Collagen-1	COL1	Matrix	Hs00164004_m1	Bt03225322_m1	Cf01076765_m1
Collagen-2	COL2	Matrix	Hs00264051_m1	Bt03251861_m1	Cf02622867_m1
Brachyury	T	NP	Hs00610080_m1	Bt04313980_m1	Cf02624791_m1
Keratin-19	KRT19	NP	Hs00761767_s1	Bt03219428_m1	Cf03986270_s1
Mohawk	MKX	AF	Hs00543190_m1	Bt04292311_m1	Custom
Fibulin-1	FBLN1	AC	Hs00972609_m1	Bt00972620_m1	Cf02642766_m1
Collagen-X	COLX	Possible CEP	Hs00166657_m1	Bt03215582_m1	Hs00166657_m1

Note: Human, bovine and canine matrix and gene markers, Taqman primers and associated catalog numbers.
Abbreviations: AC, articular cartilage; AF, annulus fibrosus; CEP, cartilage endplate; NP, nucleus pulposus.

were converted to complementary deoxyribose nucleic acid (cDNA) using Maxima H Minus cDNA Synthesis Master Mix (M1662, Thermo Fisher Scientific, MA) and diluted to a final concentration of 15 ng/reaction. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was completed in duplicate using Taqman Master Mix and primers

(Table 2). The canine MKX primer was custom designed using the Custom Taqman Assay Design Tool. Seven canine MKX transcript variants (https://www.ncbi.nlm.nih.gov/gene; Gene ID: 487079) were evaluated using the basic local alignment search (BLAST-https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the aligned sequence used to develop the
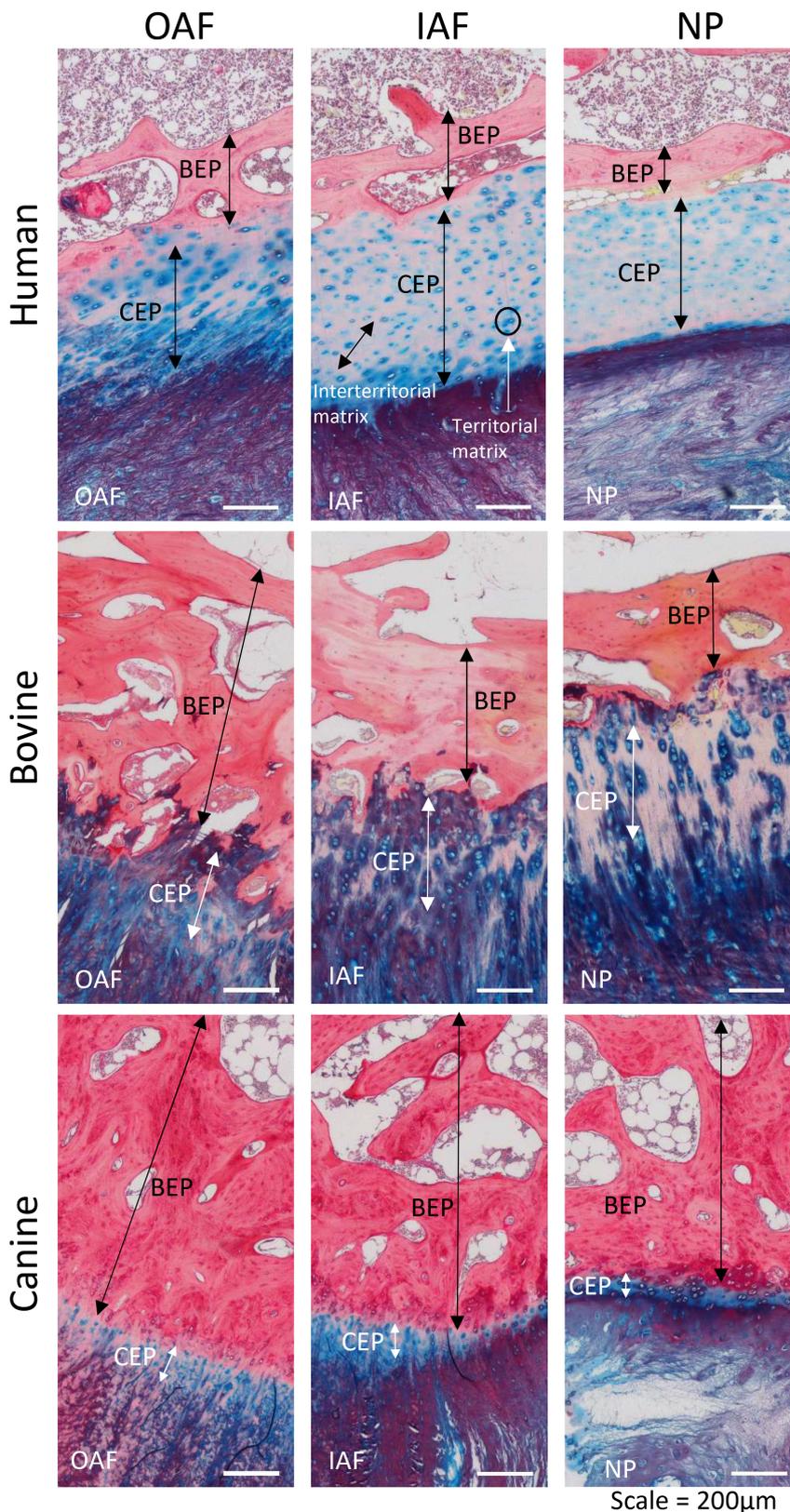
FIGURE 1 Endplate structure and matrix composition. Representative images of picrosirius red/alcian blue stain of OAF, IAF, and NP-EP interfaces in sagittal sections of human, bovine, and canine intervertebral discs. BEP, bony EP; CEP, cartilage EP; EP, endplate; IAF, inner annulus fibrosus; OAF, outer annulus fibrosus; NP, nucleus pulposus

Scale = 200μm

custom primer (Table S1). BLAST was also used to determine human and canine collagen-X (COLX) sequence homology due to lack of available COLX Taqman primer for canine. Homology was 92%, therefore the human COLX Taqman primer was utilized for canine samples. Gene expression was determined using the ΔΔCT method relative to the housekeeping gene 18s.[17] Gene expression for bovine and canine NP, AF, and AC were normalized to bovine and canine CEP, respectively. Gene expression for bovine and canine CEP were normalized to human CEP.

2.4 | Histology

Sagittal whole disc sections (n = 3 human, n = 3 bovine, and n = 3 canine) were fixed in NBF for >24 hours and then decalcified in 14% ethylenediaminetetraacetic acid solution until bone was soft to touch. After decalcification, tissue was dehydrated in an ascending series of ethanol (70%, 95%, 100%) for 5 minutes each, embedded in paraffin, cut into 4 μm sections and heat fixed to histology slides. Cell morphology was evaluated using a hematoxylin and eosin (H&E) as previously described.[18] To determine matrix composition, the sections were deparaffinized and stained using a picrosirius red (collagen)/alcian blue (proteoglycan) (PR/AB) dual stain as previously described.[14] Stitched whole disc ×4 images were taken of stained PR/AB sections using a Nikon TiE Inverted Microscope with a high-resolution DS-Fi2 color camera (#MQA11020) to evaluate CEP structure and matrix composition. CEP and BEP thickness and IVD height, including the CEP and BEP, were measured along the outer AF (OAF), inner AF (IAF) and NP-CEP interfaces of each disc using the length tool in the NIS Elements AR software. CEP and BEP thickness were normalized to total disc height at respective regions and presented as percent of disc height. ×20 and ×40 images of CEP were taken of H&E stained sections to evaluate cell morphology.
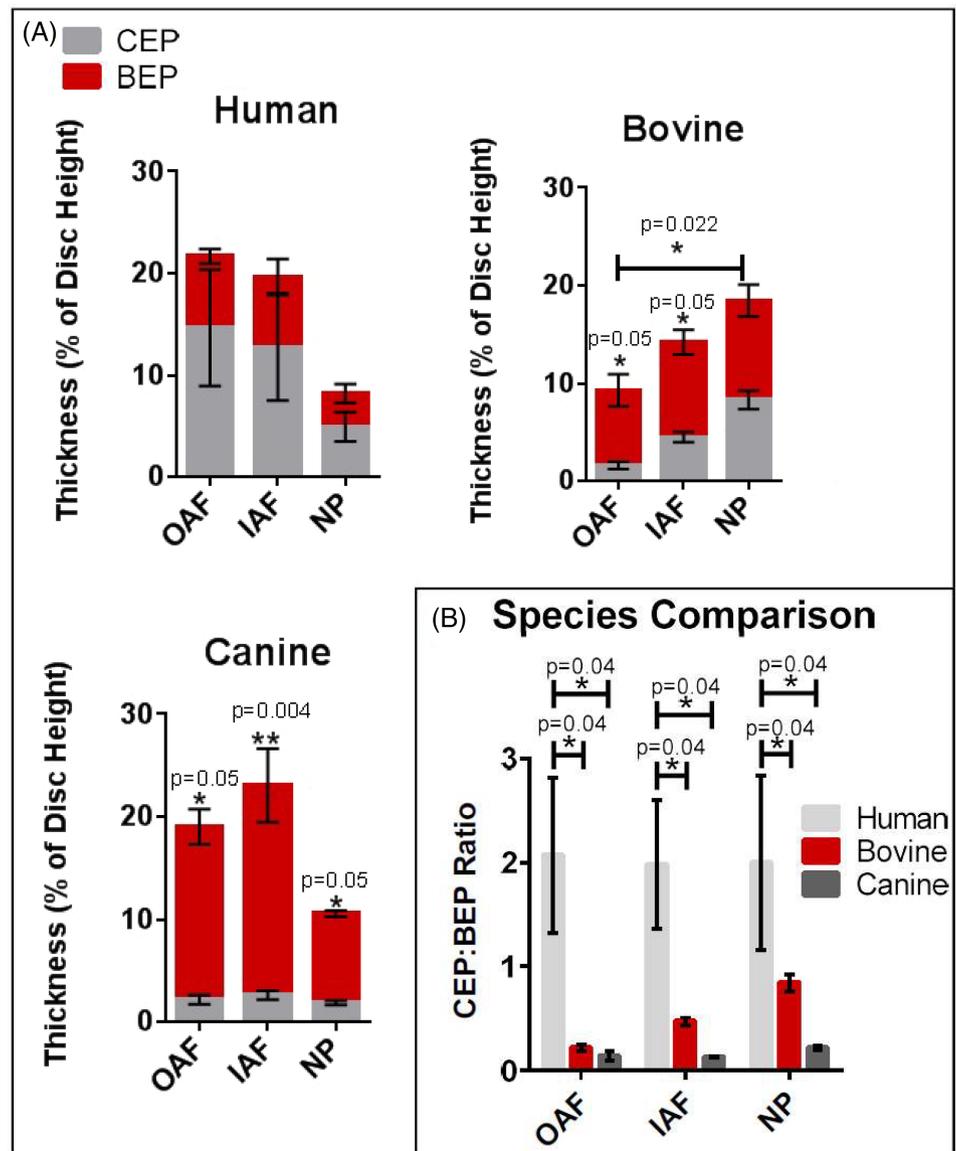

FIGURE 2 Cartilage and bony endplate thickness. Quantified cartilage and bony endplate thicknesses presented as percent of disc height (A) and cartilage endplate to bony endplate ratios (B) in human (n = 3), bovine (n = 3), and canine (n = 3) intervertebral discs. Error bars are shown as SEM. * indicates significance $P < .05$. BEP, bony endplate; CEP, cartilage endplate; IAF, inner annulus fibrosus; OAF, outer annulus fibrosus; NP, nucleus pulposus

(A)

Species Comparison s-GAG Content

(B)
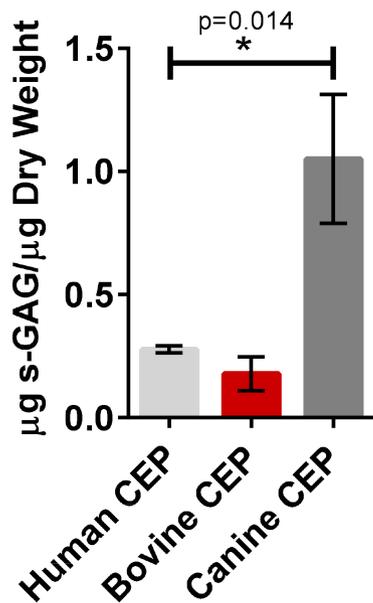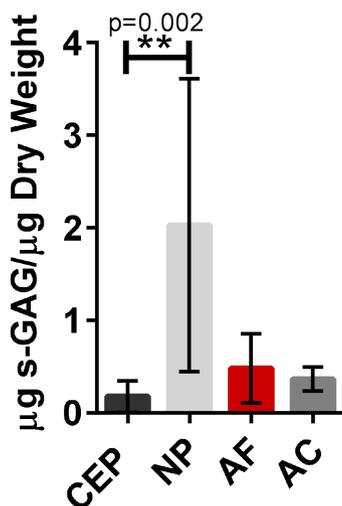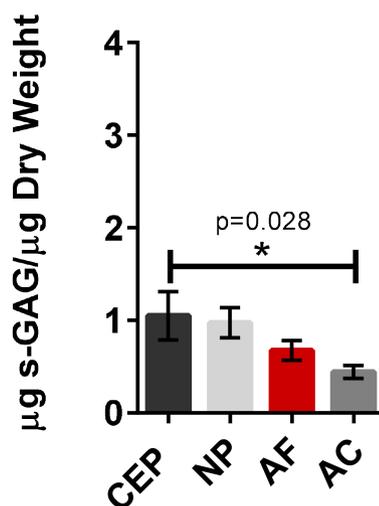
Bovine s-GAG Content

Canine s-GAG Content

FIGURE 3 Glycosaminoglycan content. Quantified sulfated glycosaminoglycan (s-GAG) content in the human (n = 6), bovine (n = 6), and canine (n = 6) CEP (A) and in the bovine and canine CEP, NP, AF (n = 6 bovine, n = 6 canine) and AC (n = 6 bovine, n = 4 canine) (B). Glycosaminoglycan content was normalized to dry weight of tissue. Error bars are shown as SEM. * indicates significance $P < .05$. AC, articular cartilage; AF, annulus fibrosus; CEP, cartilage endplate; NP, nucleus pulposus

2.5 | Quantification of glycosaminoglycan

Human CEP (n = 6) and bovine and canine CEP, NP, AF (n = 6 bovine and canine), and AC (n = 6 bovine and n = 4 canine) were lyophilized, weighed to obtain dry weight (DW) and digested in 1 mL of proteinase K at 60°C overnight. Samples were centrifuged at 10000*g* for 10 minutes at room temperature and supernatant was removed. Remaining tissue was relyophilized and weighed to obtain DW of nondigested tissue. Supernatant was vortexed and used for dimethylmethylene blue (DMMB) assay to assess sulfated

glycosaminoglycan (s-GAG) content.[19] S-GAG concentration was normalized to DW of digested tissue.

2.6 | Statistics

Due to a sample size of n = 3 to -7, the assumption of normally distributed data cannot be made. Therefore, nonparametric statistical tests were utilized to determine significant differences with α = 0.05 and $P < .05$ considered significant. For gene expression and s-GAG

content data comparing the human CEP and bovine or canine CEP, or comparing the bovine or canine CEP and AC, a Mann-Whitney test was performed. Mann-Whitney tests were also performed to determine differences in BEP and CEP thickness and a nonparametric Kruskal-Wallis with a post-hoc Dunn's test was used to evaluate differences in CEP and BEP thickness between OAF, IAF and NP regions. Since CEP, NP, and AF cells were isolated from the same bovine or canine IVD, a paired Wilcoxon test was performed to determine gene expression between CEP and NP, and CEP and AF cells.

3 | RESULTS

3.1 | Structure

Structural differences in endplate (EP-combined CEP and BEP) were observed between human and bovine or canine IVDs (n = 3) and along the IVD-EP interface in bovine in PR/AB stained motion segments (Figure 1). Human EPs consisted of both a CEP and a BEP. The CEP appeared thicker than the BEP but when quantified, no significant differences in CEP and BEP thickness or differences in CEP or BEP thickness along the IVD-EP interface were observed (Figures 1 and 2A). The human CEP:BEP ratio was significantly higher at each EP-IVD interface compared to the bovine and canine CEP:BEP ratios (Figure 2B). Bovine EPs had a significantly thinner CEP compared to BEP at the OAF-EP and IAF-EP interfaces, but not at the NP-EP interface. Bovine CEP thickness at the NP-EP interface was significantly greater compared to the OAF-EP interface (Figure 2A). Fibers of the bovine NP and AF appeared interdigitated with the CEP (Figure 1). Canine EPs consisted of a significantly thicker BEP at all IVD-EP interfaces. No significant differences in canine CEP or BEP thickness along the IVD-EP interface were observed (Figure 2A).

3.2 | Matrix composition

A PR/AB stain and DMMB assay were used to qualitatively and quantitatively evaluate matrix composition in the bovine and canine CEPs compared to human. Qualitatively, the human CEP had slightly more proteoglycan at the AF insertion point, but otherwise consisted mainly of a collagen rich extraterritorial matrix and proteoglycan rich territorial matrix (Figure 1). The bovine CEP appeared to have regional differences in matrix composition. From the thinner CEP at the OAF-EP interface to the thicker CEP at the NP-EP interface, there was a gradual transition from proteoglycan rich interterritorial and territorial matrix to a proteoglycan rich territorial and collagen rich interterritorial matrix. The CEP at the NP-EP region more closely resembled the matrix composition of the human CEP. The opposite was observed in the canine CEP, where the CEP at the OAF-EP interface had a collagen rich interterritorial and a proteoglycan rich territorial matrix and the CEP at the NP-EP interface had a proteoglycan rich territorial and interterritorial matrix. In the canine CEP, the CEP at the

OAF-EP interface more closely resembled the human CEP (Figure 1). The DMMB assay quantifying s-GAG content in the human, bovine, and canine CEP revealed a significantly higher s-GAG content in the canine CEP compared to the human CEP, and no significant differences in s-GAG content between the human and bovine CEP (Figure 3A). To characterize the bovine and canine CEP further, DMMB was also performed on NP, AF, and AC tissue from both
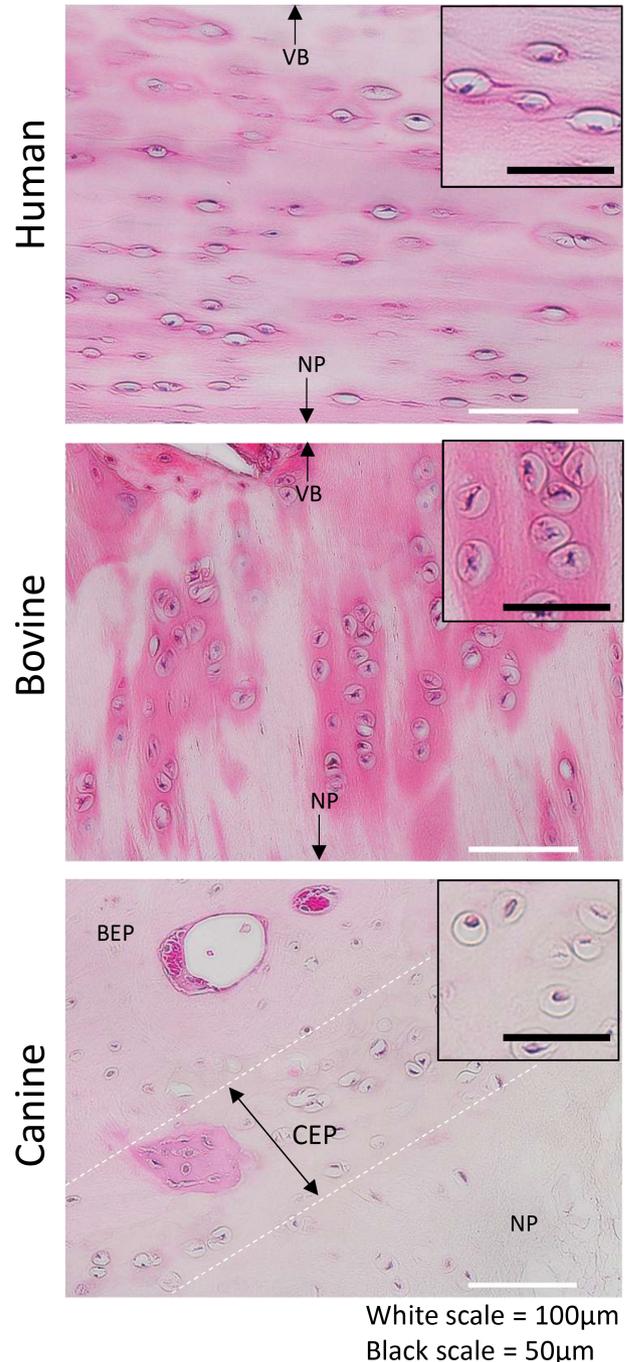

White scale = 100μm
Black scale = 50μm

FIGURE 4 Cell morphology. Representative images of cell morphology in the human, bovine, and canine CEP at both ×20 and ×40 magnification. BEP, bony endplate; CEP, cartilage endplate; NP, nucleus pulposus, VB, vertebral body
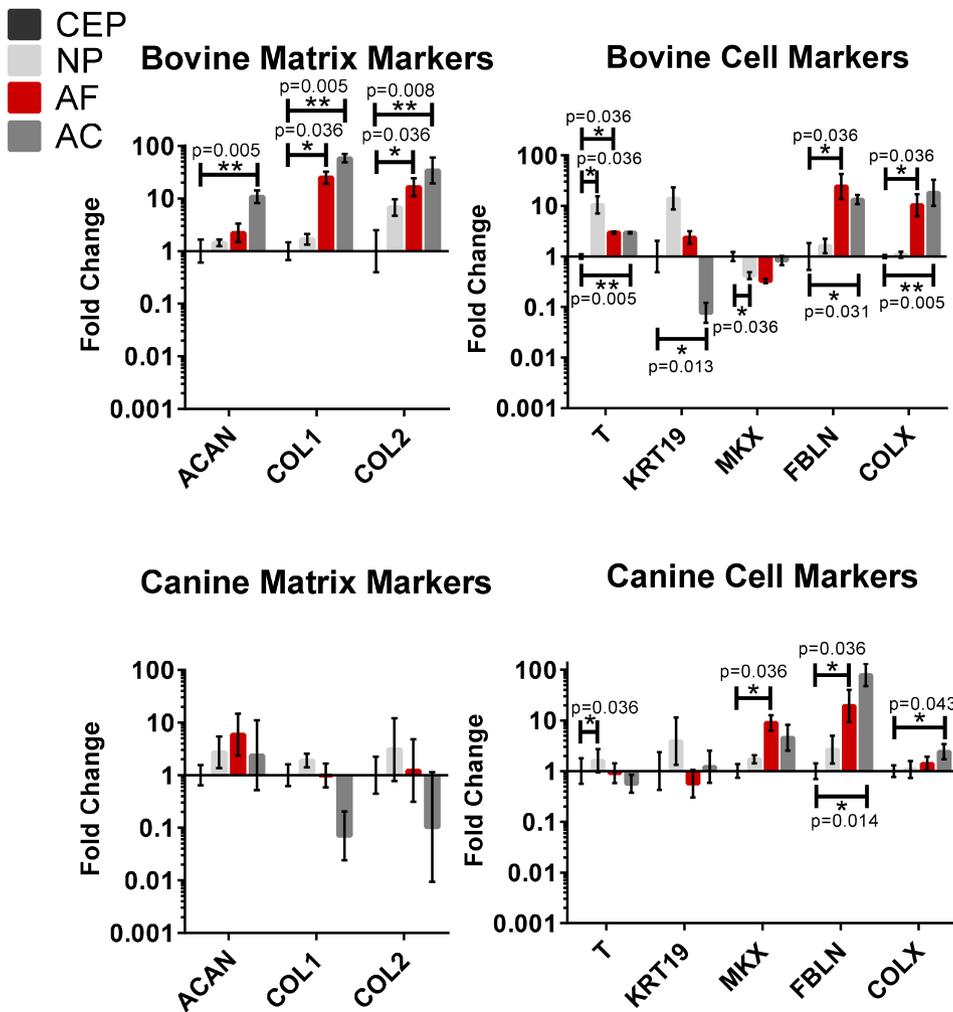
FIGURE 5 Validation of CEP isolation protocol and further characterization of bovine and canine cartilage endplate. Evaluation of matrix and intervertebral disc cell marker expression in the bovine and canine CEP to validate successful isolation of cartilage endplate cells without NP or AF cell cross-contamination and to further characterize the bovine and canine cartilage endplate compared to NP, AF, and AC. (n = 6 bovine NP, AF, and AC; n = 6 canine NP, AF; n = 4 canine AC). Error bars are shown as SEM. * indicates significance $P < 0.05$. AC, articular cartilage; AF, annulus fibrosus; CEP, cartilage endplate; NP, nucleus pulposus

species. There was significantly more s-GAG in the bovine NP compared to the bovine CEP and significantly less s-GAG in the canine AC compared to the canine CEP (Figure 3B). S-GAG was normalized to dry weight of tissue.

3.3 | Cell morphology and gene expression

Cells of the human CEP were elongated and were aligned parallel to the IVD and mainly appeared as singled, nonclustered cells. Bovine cells appeared rounded and stacked in columns perpendicular to the IVD. Canine cells appeared rounded but did not seem to be directionally organized. They appeared in both small clusters and as single cells (Figure 4). To characterize and compare the CEP cell phenotype further, bovine and canine CEP, NP, AF, and AC cells were evaluated for IVD joint matrix and IVD/AC cell marker gene expression (Figure 5). In addition, these comparisons would also ensure there was no cross-contamination during dissection and isolation, validating our previous method of CEP isolation for human cells.[20,21] There was significantly higher COL1, COL2, and ACAN, and COL1 and COL2 expression in the bovine AF and AC compared

to the CEP, respectively. There was significantly higher T expression and lower MKX expression in the bovine NP compared to the CEP. There was significantly higher T, FBLN1, and COLX expression in the bovine AF and AC compared to the CEP. There was significantly lower KRT19 expression in the bovine AC compared to the CEP. There were no significant difference in matrix marker gene expression between canine CEP and NP, AF or AC cells. There was significantly higher T expression in the canine NP compared to CEP. There was significantly lower MKX and FBLN1 expression in canine AF compared to CEP and significantly less FBLN1 and COLX expression in the canine AC compared to CEP (Figure 5). Matrix and cell marker gene expression were also evaluated between human and bovine or canine CEP (Figure 6). There was significantly more ACAN and COL2 and less COL1 expression in the bovine CEP compared to the human CEP. There was significantly higher COL2 and lower COL1 expression in the canine CEP compared to the human CEP. There was significantly less T, FBLN1, and COLX expression in the bovine CEP compared to the human CEP and significantly less KRT19, MKX, FBLN1, and COLX expression in the canine CEP compared to the human CEP (Figure 6).
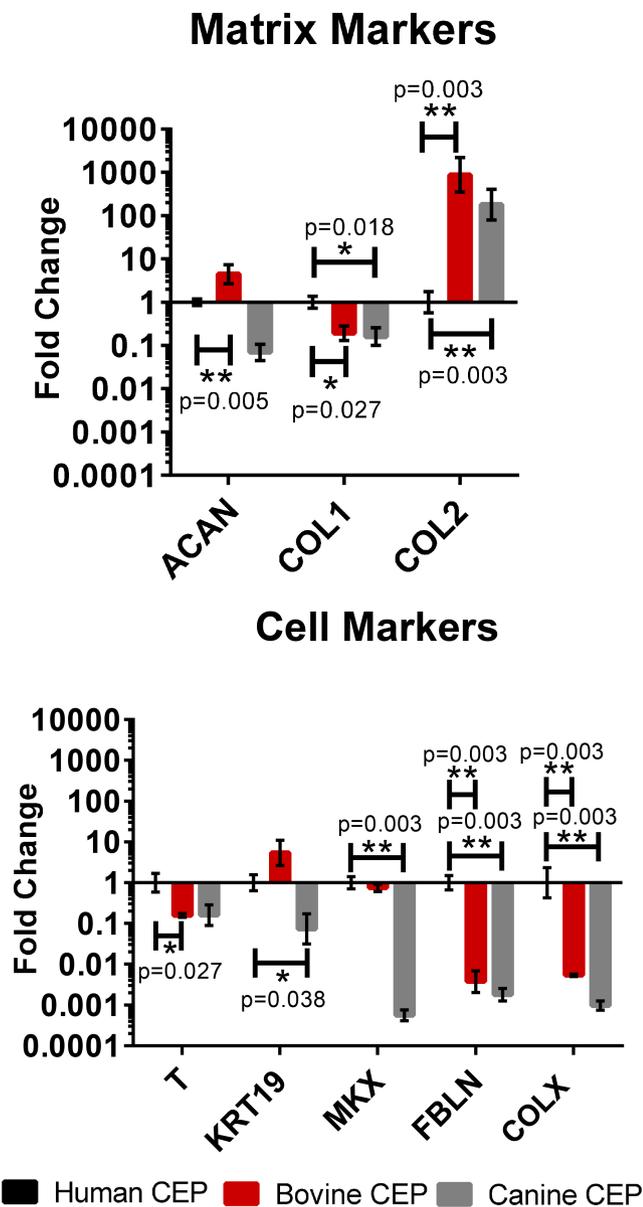
Matrix Markers

Cell Markers

■ Human CEP ■ Bovine CEP ■ Canine CEP

FIGURE 6 Cell and matrix markers. Evaluation of matrix and intervertebral cell marker expression in bovine (n = 6) and canine (n = 6) cartilage endplate cells compared to human cartilage endplate cells (n = 7). Error bars are shown as SEM. * indicates statistical significance $P < .05$, ** indicates statistical significance $P < .01$. CEP, cartilage endplate

4 | DISCUSSION

Developing a better understanding of degenerative mechanisms in the CEP can help elucidate the role of the CEP in IVD joint degeneration and discogenic LBP and inform potential treatment strategies that target it. Animal models are valuable tools for studying the mechanisms of CEP degeneration due to limited variability between research animals, increased tissue availability, and ability to study disease mechanisms and test potential therapies in vivo. Therefore, the aim of this study was to evaluate and compare CEP structure/matrix composition and gene expression between bovine and canine animal models

and human to provide an additional data set to potentially help determine which animal model is optimal for use in studies which aim to elucidate the role of CEP degeneration in IVD joint degeneration and back pain. Both bovine and canine CEPs demonstrated similarities and differences from the human CEP which may be important to take into consideration when determining which animal model is most appropriate for a specific study. Additionally, the results of this study can help interpret the relevancy of findings from bovine or canine studies to the human CEP.

Regional differences in matrix composition along the IVD-EP interface appeared similar between the human and bovine CEP with the OAF-EP region containing more proteoglycan compared to the NP-EP region. There were no significant differences in s-GAG content between the human CEP and bovine CEP, further supporting these findings. In addition, s-GAG content in the human and bovine CEP were similar to past findings.[22-24] Interestingly, Wu et al found higher s-GAG content in the bovine[23] and human[22] NP-CEP region compared to the AF-CEP region, whereas our PR/AB stain revealed more intense blue staining in the AF-CEP region compared to the NP-CEP region. Since alcian blue stains for both s-GAGs and nonsulfated GAGs (ns-GAGs) and DMMB only detects s-GAGs, this alcian blue staining of greater intensity may indicate more ns-GAGs in the NP-CEP region compared to the AF-CEP region in both human and bovine. Additionally, there was significantly more s-GAG in the canine CEP compared to human CEP. Regional differences in alcian blue (proteoglycan) staining were observed in the canine CEP with more intense staining in the NP-CEP region compared to the AF-CEP region, opposite of the staining pattern observed in the human CEP. It is unclear why this is, though it may likely be attributed to differences in disc size, shape, and loading patterns between canine and human. Mechanical properties may differ based on matrix composition, which is important to consider when conducting studies evaluating mechanical properties or loading patters in the CEP/IVD using bovine or canine discs.

Both similarities and differences in cell morphology and gene expression between the human and bovine or canine CEP and between canine and bovine CEP and NP, AF, or AC were also observed. Bovine CEP cells had significantly higher expression of matrix markers ACAN, COL1, and COL2 than human CEP cells which may suggest more extracellular matrix remodeling in the bovine CEP. Canine CEP cells had lower COL1 and higher COL2 matrix gene expression compared to human CEP cells. These differences may be attributed to the fact that bovine and canine discs were isolated from younger subjects (2-3 year old bovine and 1-2 year old canine) where the age of bovine subjects were approximately equivalent to 18 to 22 year old humans and canine subjects approximately equivalent to 14 to 24 year old humans. With age and degeneration, there is a shift from healthy collagen-II to more fibrotic collagen-I matrix content in the human CEP[25] which may account for the lower COL2 and higher COL1 expression in the human CEP compared to canine.

NP markers T and KRT19,[26] AF marker MKX,[27] AC marker FBLN1,[28] and potential CEP marker COLX[20,21] were examined in bovine and canine CEP cells compared to human. We have previously

evaluated expression of T, KRT19, MKX, and FBLN1 in human CEP cells and found human CEP cells express T and KRT19, MKX, or FBLN1 at equal or greater levels than human NP, AF, or AC, respectively. Furthermore, we previously demonstrated significantly higher COLX expression in the human CEP compared to NP on the protein level.[20,21] There was lower expression of T, FBLN1, and COLX in bovine CEP cells and lower expression of KRT19, MKX, FBLN1, and COLX in canine CEP cells compared to human CEP cells. FBLN1 was identified by Minogue et al as an AC marker in bovine tissue[29] and our results indicate similar findings where FBLN1 is significantly higher in the bovine AC compared to the bovine CEP. While T and FBLN may be suitable markers to distinguish NP or AC and CEP cells, respectively, in bovine and canine tissue, our data demonstrate the human CEP expresses higher levels of T and FBLN and these markers may not be suitable to distinguish human NP or AC and CEP cell phenotypes. While no significant differences in COLX gene expression between human CEP and NP, AF, or AC cells were previously identified, our current study demonstrates higher expression of COLX in bovine and canine AC cells compared to bovine and canine CEP cells and lower expression of COLX in bovine and canine CEP cells compared to human CEP cells. Although COLX is expressed in the human CEP at all ages, its expression is known to increase with age.[30] Therefore, the significantly higher COLX gene expression in human compared to bovine or canine may be an artifact of differences in age between the species. Further research is necessary to determine whether, COLX may be an appropriate bovine or canine CEP cell marker. While both bovine and canine CEP cells exhibited differences in gene expression, less differences were observed between human and bovine CEP cells compared to human and canine CEP cells, suggesting that bovine are likely more phenotypically similar to human CEP cells on the gene level.

Canine and bovine CEPs consist of rounded, chondrocyte-like cells and the human CEP consists of cells elongated parallel to the disc in the direction of collagen fibers, more similar to fibroblast-like cells of the human AF. The differences in cell morphology between human and bovine or canine could imply differences in EP loading and matrix organization patterns in the EP. The human CEP cells lie along collagen fibers parallel to the disc, suggesting the human CEP is experiencing tensile loading, likely in response to hydrostatic pressure in the center of the disc.[5] Bovine cells appear stacked in columns similar to chondrocytes undergoing endochondral ossification during long bone development and canine cells do not have any apparent organizational or directional patterning. When evaluating CEP degeneration on the cellular level using bovine or canine cells, it is important to take into consideration these cell specific differences when interpreting results.

Differences in CEP thickness observed between human and canine or bovine may have implications for use in studying CEP transport or mechanical properties. For example, human CEPs with poor diffusive properties have higher amounts of matrix constituents, such as collagen, aggrecan, and mineral,[10] and treating the CEP with MMP-8 reduces these matrix constituents and increases diffusivity.[31] Diffusivity of the CEP is essential for proper IVD nutrition and waste removal. Transport properties of thinner CEPs may be better than thicker CEPs due to a smaller distance for metabolites to travel. The bovine and canine CEP:BEP ratio was significantly less than the human CEP:BEP ratio, suggesting the bovine and canine CEP is thinner with a thicker BEP compared to human. In the human CEP, diffusivity has been found to decrease with increase in compressive strain, inhibiting the transport of solutes[22] and these affects are largely magnified in the human CEP relative to the bovine and canine CEP given the increased CEP:BEP ratio/CEP thickness and should be considered when selecting an animal model to assess the role of CEP diffusivity in IVD degeneration. Increased irregularities in the human CEP have been identified as a limitation when compared to the canine model,[16] a limitation that may be extended to the bovine model based on CEP thickness. The bovine CEP had similar matrix composition as assessed by DMMB and PR/AB stains, and a thicker CEP in the NP region compared to canine, suggesting that the bovine model may be a more suitable animal model for studying transport or mechanical properties of the CEP.

There are many important considerations when selecting an appropriate animal model. Molecular similarities between bovine and human CEP, such as proteoglycan concentration and distribution, and gene expression suggest this may be an appropriate model for studies exploring the structural and mechanical properties of the IVD. Previous studies have identified that bovine caudal discs experience similar compressive strain to that of the human lumbar,[32] further supporting bovine IVD as a strong model for investigating IVD biomechanics. However, the canine model has merit as a disease model due to the occurrence of spontaneous degeneration and age-related loss of aggrecan and notochordal cells, similar to human.[11,16] This phenomenon is not observed in the non-chondrodystrophic dog breeds but is also seen in ovine models, making the sheep model a candidate for further characterization.[33] Additionally, the goat has been used as an effective translational model to evaluate the impact of disc nutrition and CEP permeability on the etiology of IVD degeneration.[34]

This study provides valuable insight into species specific differences between the human, bovine and canine CEP on the structural, tissue, and cell level; however, there are some limitations which are important to note. For example, a majority of human tissue was acquired from female autopsy samples, whereas bovine tissue was acquired from males and canine tissue an even mix between males and females. Additionally, age-related differences between human, bovine, and canine may be a confounding factor, as bovine and canine tissue was from a younger population compared to human cadaveric samples. It should be noted, however, that while age could not be accounted for in the human IVD samples, only healthy-mildly degenerate samples, tissue grades were selected. Furthermore, while canine tissue was obtained from purpose bred chondrodystrophic dogs, tissue from both large and small hounds was used and may account for variability in gene expression. A low sample size also likely contributed to variability in the data. Increasing sample size of all three species would be beneficial to account for variability and improve robustness of statistical analysis. Additionally, cells used in gene expression analysis were expanded in monolayer through one passage to allow enough cells to be isolated for qRT-PCR. Cells were frozen in dimethyl

sulfoxide which may have affected mRNA expression and should be considered as a limitation. While extreme care was taken when separating CEP, AF, and NP tissue during dissection and clear differences in marker expression were observed at the gene level between cells suggesting isolation of distinct cell populations, cross-contamination could still be a small concern. To assess COLX expression in canine CEP and IVD cells we used a human primer and probe set due to lack of availability of a COLX canine primer as the gene sequence of human and canine demonstrates 92% homology; however, this could be considered a potential limitation. Regional variation in CEP matrix composition was observed but region was not taken into consideration during cell or tissue isolation to ensure an adequate amount of tissue for DMMB/cell isolation was obtained. Based on current findings, further exploration of regional differences in the CEP from different species may be of interest. Evaluation and comparison of the CEPs of additional species used for IVD research for example, rodent, rabbit, goat, sheep, or non-chondrodystrophic dogs may also prove useful; however, for the current study we focused on bovine because of its similarities to the structure and cell composition of the healthy human IVD[35] and the chondrodystrophic dog given its utility as a model for IVD degeneration and back pain.[16]

5 | CONCLUSIONS

In conclusion, the human, bovine, and canine CEPs demonstrate significant differences in structure/matrix composition and gene expression compared to human which are important for researchers to take into consideration when choosing the most suitable animal model for their specific research studies and when interpreting findings from CEP studies utilizing these species.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

All authors meet the following criteria: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and drafting the work or revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors listed have read and approved the final submitted manuscript.

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SUPPORTING INFORMATION