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Expression of adipokines and adipocytokines by epidural adipose tissue in cauda equina syndrome in dogs

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Abstract

Background: Compression of epidural adipose tissue (EAT) within the scope of cauda equina syndrome (CES) could lead to an enhanced expression of inflammatory mediators, possibly contributing to pain amplification in dogs.

Objectives: To analyze expression of inflammatory adipo(-cyto)kines within the EAT of dogs with CES.

Animals: Client-owned dogs: 15 dogs with CES and 9 dogs euthanized for unrelated medical reasons (controls).

Methods: Prospective, experimental study. Epidural adipose tissue and subcutaneous adipose tissue were collected during dorsal laminectomy and used for real-time quantitative polymerase chain reaction. Tissue explants were cultured for measurements of inflammation-induced release of cytokines.

Results: Results show a CES-associated upregulation of the cytokines tumor necrosis factor alpha (TNFα: mean ± SD: 18.88 ± 11.87, 95% CI: 10.90-26.86 vs 9.66 ± 5.22, 95% CI: 5.29-14.02, *: $P = .04$) and interleukin- (IL-) 10 (20.1 ± 9.15, 95% CI: 14.82-25.39 vs 11.52 ± 6.82, 95% CI: 5.82-17.22, *: $P = .03$), whereas the expression of the adipokine leptin was attenuated in EAT of dogs with CES (3.07 ± 2.29, 95% CI: 1.80-3.34 vs 9.83 ± 8.42, 95% CI: 3.36-16.30, **: $P = .007$). Inflammatory stimulation of EAT explant cultures resulted in an enhanced release of IL-6 (LPS: 5491.55 ± 4438, 95% CI: 833.7-10 149; HMGB1: 1001.78 ± 522.2, 95% CI: 518.8-1485; PBS: 310.9 ± 98.57, 95% CI: 228.5-393.3, ***: $P < .001$).

Conclusion and Clinical Importance: Expression profile of inflammatory adipo(-cyto) kines by EAT is influenced from compressive forces acting in dogs with CES and might contribute to amplification of pain.

KEYWORDS

damage-associated molecular patterns, degenerative lumbosacral stenosis, high mobility group box 1, inflammation, interleukin 6, intervertebral disc, leptin, spinal pain, tumor necrosis factor

Abbreviations: CES, cauda equina syndrome; CGRP, calcitonin gene-related peptide; CRP, C-reactive protein; DAMP, damage-associated molecular pattern; DLSS, degenerative lumbosacral stenosis; EAT, epidural adipose tissue; FCS, fetal calf serum; HMGB1, high mobility group box-1; IL, interleukin; IVD, intervertebral disc; LPS, lipopolysaccharide; RAGE, receptor for advanced glycation end products; SAT, subcutaneous adipose tissue; T2W, T2-weighted; TLR, toll-like receptor; TNFα, tumor necrosis factor alpha.

1 | INTRODUCTION

Degenerative diseases of the vertebral column, such as degenerative lumbosacral stenosis (DLSS), represent an increasing welfare concern in dogs.[1,2] Stenotic lesions located at the lumbosacral transition can result in compression of spinal nerves of the *cauda equina*. The clinical manifestation of the compression is referred to as cauda equina syndrome (CES) consisting of pain and hyperesthesia in certain movements (eg, jumping and climbing), sensory nerve deficits, and motor impairment of the tail and hind limbs.[1]

Although the pathophysiological mechanisms underlying degeneration of intervertebral disc (IVD) were intensively studied,[2-4] the lack of direct correlation between the degree of nerve compression and the severity of clinical signs was not systematically investigated until now. In this context, the inflammatory milieu resulting from nerve damage upon compression and ischemia might play a critical role. In models of experimental spinal stenosis, researchers detected infiltration of immune cells into the *cauda equina*, accompanied by an upregulation of inflammatory mediators, such as cytokines and prostaglandin E2.[5,6] Two studies investigating inflammatory processes within the epidural space in dogs with IVD extrusion observed an infiltration of neutrophils and macrophages[7] as well as an altered cytokine expression profile in the epidural material including cells of the extruded disc.[8] Cytokines and prostaglandins, as well as endogenous toll-like receptor (TLR) agonists (eg, high mobility group box-1 protein [HMGB1]) further promote local inflammation[9] and are capable of modulating excitability of nociceptive neurons, leading to peripheral sensitization.[10-14]

Adipose tissue from various locations exerts endocrine and paracrine capacities by secretion of adipokines and adipocytokines.[15-18] Obesity is now accepted to induce a chronic low-grade inflammation with direct impact on the febrile response,[16] arthritis,[17,19,20] and IVD degeneration.[21-23] Adipose tissue and adipokines not only exert systemic effects, but further impact local inflammatory processes, for example, in human arthritis[19] or canine cruciate ligament disease.[24] The role of epidural adipose tissue (EAT) in disorders related to the vertebral column, such as CES, has been poorly investigated.

In this study, we aimed to investigate the expression of inflammatory mediators in EAT of dogs with CES and to evaluate the inflammation-induced production of cytokines by EAT upon stimulation with damage- (DAMPs) or pathogen-associated molecular patterns.[25]

2 | MATERIALS AND METHODS

2.1 | Animals and diagnostic workup

Fifteen dogs were included in the study (4 German Shepherd dogs, 3 Golden retrievers, 2 Labrador retrievers, 2 Great Danes, 1 Large Münsterländer, 1 Weimaraner, 1 Belgian Shepherd, and 1 mixed-breed dog). The median age of the dogs was 7.5 years (2.5-11). There were 6 female and 9 male dogs. The median body weight was 30.8 kg (25-44 kg). Dogs pretreated with anti-inflammatory drugs were excluded from the study. Clinical history of all dogs were consistent with CES,[26] such as gait abnormalities in the hindlimbs (n = 3), toe dragging (n = 3), reluctance of jumping or climbing stairs (n = 6), pain at the caudolumbar region (n = 7), and a flaccid tail (n = 3). Neurologic examination was carried out by a board-certified neurologist (ECVN) and revealed hyperesthesia during palpation of the lumbosacral region (n = 12), proprioceptive deficits (n = 4), and reduced spinal reflexes of the hind limbs (n = 1).

Diagnosis was confirmed using magnetic resonance imaging (3.0 Tesla, MAGNETOM Verio, Siemens Healthcare) under general anesthesia. Multi-planar T2-weighted (T2W; Figure 1A), T1-weighted before and after contrast as well as Short Inversion Time Inversion Recovery sequences of the lumbar and sacral vertebral column were acquired. Dorsal bulging of the *anulus fibrosus* into the vertebral canal with varying degrees of displacement of the epidural fat and a signal loss of the normally hyperintensive *nucleus pulposus* on T2W sequences as a sign of IVD dehydration was noticed in all cases (Figure 1A).

Confirmation of CES was followed by a standard dorsal laminectomy[27] on the following day. Subcutaneous adipose tissue (SAT) and EAT that was removed as a standard procedure during the surgical approach to achieve adequate exposure of the subcutaneous fascias, the *cauda equina*, and the IVD (Figure 1B) was sterilely handed over from the surgical team. All owners gave their written consent to tissue sampling for scientific purposes. Epidural adipose tissue and SAT were immediately deep-frozen at −80°C for subsequent real-time quantitative polymerase chain reaction (RT-qPCR) or collected for cultivation of fat explants.

Subcutaneous adipose tissue and EAT were also taken from 9 middle to large breed dogs euthanized for medical reasons unrelated to the lumbosacral neural system or chronic pain. Again, owners gave their consent for tissue sampling after euthanasia. Collection and processing were executed analogically to the CES group and within 1 hour postmortem.

2.2 | Real-time quantitative polymerase chain reaction

To detect changes in relative expression of inflammatory mediators, we extracted mRNA of EAT and SAT from 15 dogs with CES and 9 control dogs. Extraction of mRNA was performed with TRIzol (Invitrogen, Carlsbad, California) according to the manufacturers' protocol. Concentrations of mRNA were equalized to 250 ng/μL and 4 μL (= 1 μg of total RNA) were applied for reverse transcription in a total reaction volume of 20 μL with reverse transcriptase (50 U), dNTP mix (10 mM), and random hexamers (50 μM; all: Applied Biosystems, Foster City, California). Relative quantification of mRNA was performed in duplicates employing the StepOnePlus Real-Time PCR System with TaqMan Gene Expression Assays and TaqMan MasterMix (all: Applied Biosystems). Four suggested housekeeping genes (CANX, β-actin, GAPDH, B2M) were analyzed using the NormFinder software, revealing CANX as the most stable

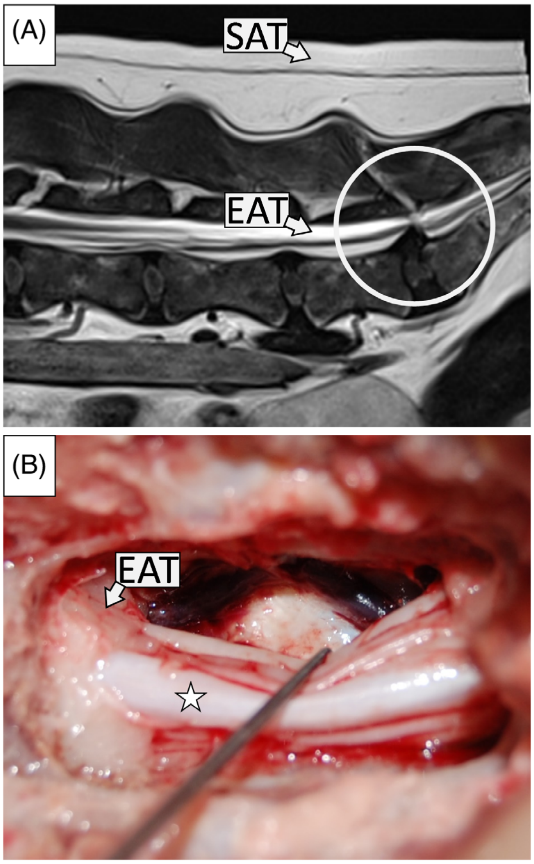

FIGURE 1 Representative images from magnetic resonance imaging diagnostics and dorsal laminectomy surgery. A, Sagittal T2-weighted section of the lumbosacral junction (circle) of a dog presented with signs of lumbosacral pain indicating a protrusion of the intervertebral disc with compression of the *cauda equina* and epidural adipose tissue (EAT). SAT, subcutaneous adipose tissue, B, Photograph taken during dorsal laminectomy in a dog with cauda equina syndrome, highlighting the close proximity of EAT to *cauda equina* nerve roots (star).

reference gene. Results were analyzed using the $2^{-(\Delta\Delta Ct)}$ method and are presented as the x-fold increase compared to the sample with the lowest expression, given a designated value of 1. The following TaqMan Gene Expression Assays were used to determine relative gene expression of inflammatory target genes: TNFα: cf02628236_m1, IL-6: cf02624153_m1, IL-10: cf02624265_m1, TLR-4: cf02622203_g1, HMGB1: cf02688763_g1, RAGE: cf02626372_g1, leptin: cf02692890_m1, CGRPα: cf04947276_m1, substance P: cf02701359_m1, CRP: cf04947508_m1, CANX: cf02679196_m1, β-actin: cf02689313_m1, GAPDH: cf04419463_gH, B2M: cf02659077_m1.

2.3 | Fat explant cultures

Epidural adipose tissue was abundant enough to prepare explant cultures from 6 of the dogs with CES. Cultivation of adipose tissue was performed as previously described for the rat.[18,25] After 1 washing step in ice-cold phosphate-buffered saline (PBS; Capricorn Scientific GmbH, Ebsdorfergrund, Germany), the tissue was transferred into sterile falcon tubes filled with ice-cold Hank's Balanced Salt Solution (HBSS, Ca^{2+}- and Mg^{2+}-free, Biochrom GmbH, Berlin, Germany), supplemented with penicillin (100 U/mL)/streptomycin (0.1 mg/mL) and HEPES (15 mM; Thermo Fisher Scientific, Langenselbold, Germany). Samples were cut into slices of similar weight to get up to 6 replicates per animal for cultivation and subsequent inflammatory stimulation (see below). On average, fat explants of SAT weighed 85.57 ± 28.97 mg (mean ± SD), whereas EAT explants weighed 25.70 ± 10.15 mg (mean ± SD). No significant weight differences were detectable among the 3 stimulation groups (PBS, lipopolysaccharide [LPS], HMGB1). For cultivation, each explant was transferred into 1 well of a 12-well plate filled with 2 mL of pre-warmed (37°C) cultivation medium consisting of DMEM/F12 medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F12; Invitrogen, Darmstadt, Germany) supplemented with fetal calf serum (FCS; 5%), penicillin (100 U/mL)/streptomycin (0.1 mg/mL), and HEPES (15 mM). Epidural adipose tissue and SAT were strictly separated and cultured in distinct cultivation plates. After 1 day of cultivation at 37°C in humidified atmosphere of 5% CO_2 and 95% air, fat explant cultures were used for inflammatory stimulation.

2.4 | Inflammatory stimulation

To investigate inflammation-induced production of cytokines tumor necrosis factor alpha (TNFα) and interleukin (IL)-6 by epidural and SAT, explant cultures were stimulated with exogenous (LPS) and endogenous (HMGB1) agonists of the TLR-4, inducing a robust inflammatory response. Therefore, fat explants were washed with FCS-free DMEM/F12 medium supplemented with penicillin (100 U/mL)/streptomycin (0.1 mg/mL), and HEPES (15 mM) and incubated with either LPS (0.1 μg/mL; *Escherichia coli* serotype O111:B4; Sigma-Aldrich Chemie GmbH, Munich, Germany) or HMGB1 (1 μg/mL; disulfide high-mobility group box-1, LPS-free; HMGBiotech S.r.l., Milan, Italy) or PBS dissolved in FCS-free medium with supplements. All doses were chosen according to established protocols and previous studies.[9,25,28] After 24 hours of stimulation, supernatants were collected and stored at −20°C for subsequent cytokine measurements.

2.5 | Measurements of cytokine release (TNFα, IL-6)

To determine the concentrations of released cytokines TNFα and IL-6 from EAT and SAT, we performed specific bioassays that are able to detect even low amounts of both cytokines.[12,28] Both bioassays have previously been described in detail[29,30] and were applied for samples of dogs.[31,32] Briefly, the TNFα bioassay is based on the concentration-dependent cytotoxic effect of TNFα on the fibrosarcoma cell line WEHI 164 subclone 13. Applying a dimethylthiazol-diphenyl tetrazolium bromide (MTT) colorimetric assay and an international standard (murine TNFα standard: code 88/532, National Institute for Biological Standards and Control [NIBSC], South Mimms, UK), the

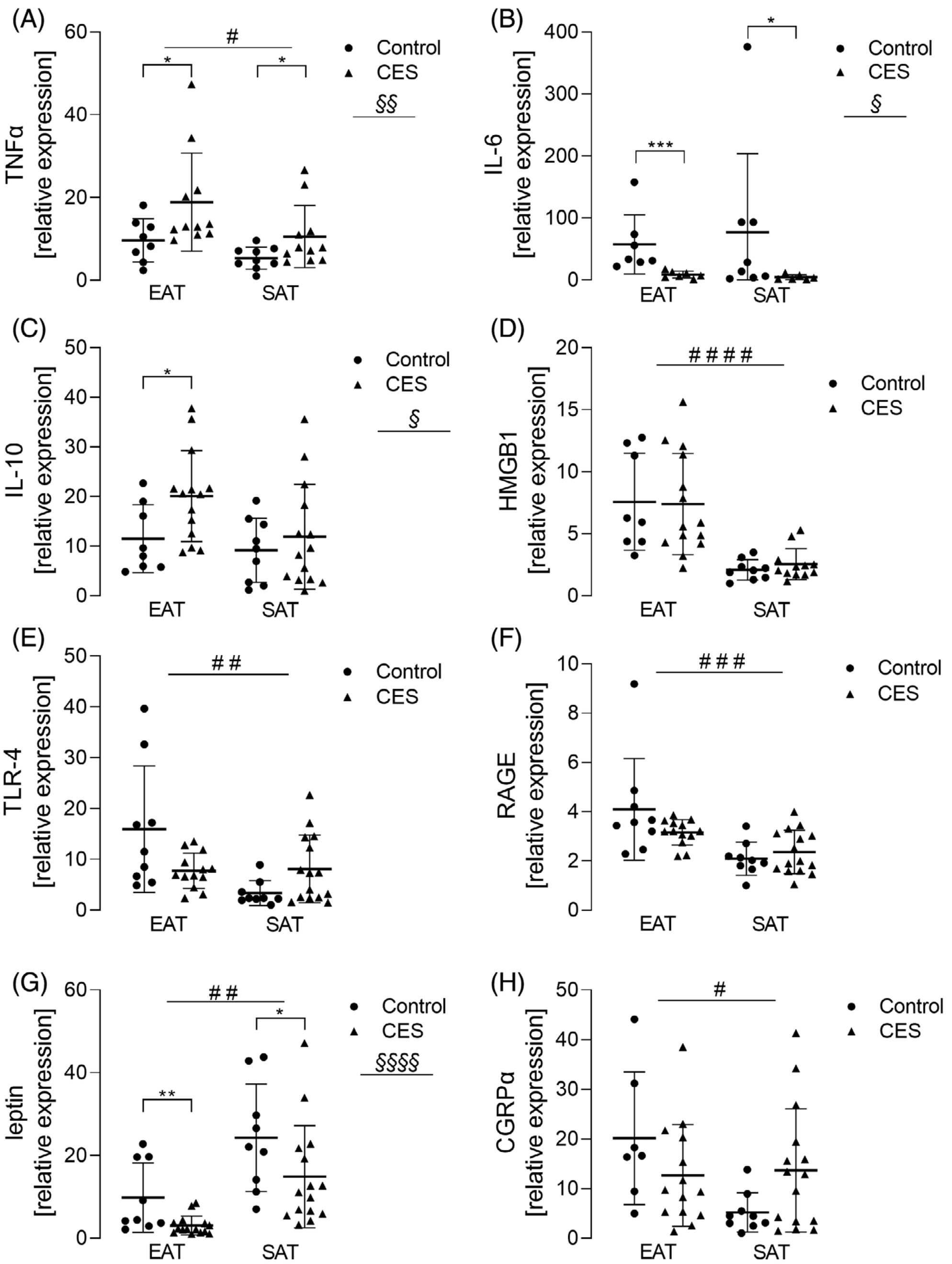

FIGURE 2 Expression of inflammatory mediators and receptors in epidural adipose tissue (EAT) of dogs with cauda equina syndrome (CES). The impact of CES (triangles) on epidural (EAT) and subcutaneous adipose tissue (SAT) was investigated by means of RT-qPCR and analyzed applying a 2-way ANOVA and Mann-Whitney tests. Results revealed an enhanced expression of TNFα (A) and IL-10 (C), as well as an attenuated expression of IL-6 (B) and leptin (G) in dogs with CES (main effect *disease*: §). Compared to SAT, samples from EAT showed an enhanced expression of HMGB1 (D), TLR-4 (E), RAGE (F), and CGRPα (H), but a reduced expression of leptin (G; main effect *tissue*: #). Direct comparison between groups indicate a CES-associated increase of TNFα and IL-10 and a reduced expression of IL-6 and leptin in EAT (*). Graphs show the mean ± SD with symbols indicating results of independent samples. §, #, *: $P < .05$; §§, ##, **: $P < .01$; ###, ***: $P < .001$, §§§§, ####: $P < .0001$.

concentration of the released TNFα can be calculated.[33] This bioassay is capable of detecting low amounts of TNFα from 6.0 pg/mL. The B9 hybridoma cell line shows an IL-6 dependent cell growth and can therefore be applied to determine concentrations of released IL-6 in culture supernatants.[34] Using an international standard (human IL-6 standard: code 89/548, NIBSC), concentrations can be quantified with a detection limit of 3.0 international units (I.U.) IL-6. Concentrations of released cytokines were adjusted to the weight of the respective tissue explant. Results present the concentration of released IL-6 in relation to the mean weight of the respective tissue (for EAT: 25.7 mg, for SAT: 85.6 mg). Therefore, a statistical analysis comparing released amounts of cytokines from SAT with EAT cultures is not applicable.

2.6 | Evaluation and statistics

Relative gene expression of inflammatory mediators was examined in EAT and SAT of 15 dogs with CES and compared to 9 control dogs. Statistical outliers were identified using the ROUT method with a Q-value of 0.5% and removed before further analysis. Results were analyzed using a 2-way ANOVA (main effects of *tissue* (#) and *disease* (§)). For direct comparison between 2 groups, the Mann-Whitney test was applied (*). Data of released IL-6 and TNFα result from 6 to 9 fat explant cultures originating from 3 to 5 independent experiments. Results of cytokine release from cultures stimulated with LPS or HMGB1 were compared to PBS-treated controls using the Mann-Whitney test (*). All data are presented as means ±SD with the respective results of single samples presented as symbols. Data analysis and graphical illustrations were performed using the software of Excel 2016 and PowerPoint 2016 (both: Microsoft Corporation, Redmond, Washington) and Prism 9.0 (GraphPad Software, Inc, San Diego, California).

3 | RESULTS

3.1 | Expression of inflammatory mediators in epidural and SAT

In EAT of dogs with CES, TNFα was significantly enhanced (Figure 2A: main effect *disease*: §§: $P = .008$) with an increased CES-associated expression in EAT and SAT (EAT: control: 9.66 ± 5.22, 95% CI: 5.29-14.02 vs CES: 18.88 ± 11.87, 95% CI: 10.90-26.86, *: $P = .04$; SAT: control: 5.36 ± 2.69, 95% CI: 3.29-7.43 vs CES: 10.57 ± 7.50, 95% CI: 5.53-15.62, *: $P = .03$). Expression of IL-6 was reduced in dogs with CES (Figure 2B: main effect *disease*: §: $P = .04$, EAT: control: 57.51 ± 47.76, 95% CI: 13.34-101.7 vs CES: 8.53 ± 5.50, 95% CI: 3.45-13.62; SAT: control: 77.07 ± 126.6, 95% CI: −28.80 to 183.0 vs CES: 4.54 ± 4.09, 95% CI: 0.24-8.83). Dogs with CES had an enhanced expression of IL-10 (Figure 2C: main effect *disease*: §: $P = .04$). Direct comparison revealed a significant upregulation of IL-10, exclusively in EAT (Figure 2C: control: 11.52 ± 6.82, 95% CI: 5.82-17.22 vs CES: 20.1 ± 9.15, 95% CI: 14.82-25.39, *: $P = .03$), whereas no significant differences were detectable for SAT (control: 9.18 ± 6.45, 95% CI: 4.23-14.14 vs CES: 11.93 ± 10.57, 95% CI: 5.83-18.03). Expression of HMGB1 was not affected by the main factor *disease* (Figure 2D). However, our results provide evidence for an enhanced expression in EAT compared to SAT (Figure 2D: main effect *tissue*: ####: $P < .0001$; EAT: control: 7.58 ± 3.91, 95% CI: 4.31-10.85 vs CES: 7.40 ± 4.07, 95% CI: 5.04-9.75; SAT: control: 2.10 ± 0.82, 95% CI: 1.47-2.73 vs CES: 2.56 ± 1.25, 95% CI: 1.76-3.35). High mobility group box-1 can act on cell surface receptors, such as TLR-4 and RAGE (receptor for advanced glycation end products) to induce intracellular inflammatory signaling cascades.[35] No significant main effect *disease* was detectable for TLR-4, but EAT showed an enhanced expression compared to SAT (Figure 2E: main effect: *tissue*: ##: $P = .006$; EAT: control: 15.90 ± 12.44, 95% CI: 6.34-25.46 vs CES: 7.71 ± 3.47, 95% CI: 5.61-9.81; SAT: control: 3.34 ± 2.44, 95% CI: 1.47-5.22 vs CES: 8.08 ± 6.64, 95% CI: 4.40-11.75). Expression of RAGE was significantly higher in samples from EAT compared to SAT (Figure 2F: main effect *tissue*: ###: $P = .0002$). However, RAGE expression was not altered in dogs with CES compared to control dogs (EAT: control: 4.10 ± 2.07, 95% CI: 2.51-5.69 vs CES: 3.16 ± 0.52, 95% CI: 2.85-3.47; SAT: control: 2.10 ± 0.67, 95% CI: 1.58-2.61 vs CES: 2.36 ± 0.88, 95% CI: 1.88-2.85). The adipokine leptin was downregulated in CES dogs (Figure 2G: main effect *disease*: §§§§: $P < .0001$), whereas its expression was higher in SAT compared to EAT (main effect *tissue*: ##: $P = .008$). Direct comparison revealed an attenuated expression of leptin upon CES in EAT (control: 9.83 ± 8.42, 95% CI: 3.36-16.30 vs CES: 3.07 ± 2.29, 95% CI: 1.80-4.34, **: $P = .007$), as well as SAT (control: 24.26 ± 12.99, 95% CI: 14.27-34.25 vs CES: 14.89 ± 12.35, 95% CI: 8.05-21.73; *: $P = .05$). Expression of CGRPα (calcitonin gene-related peptide) was enhanced in EAT compared to SAT (Figure 2H: main effect *tissue*: #: $P = .05$; EAT: control: 20.13 ± 13.37, 95% CI: 7.77-32.49 vs CES: 12.64 ± 10.26, 95% CI: 6.74-18.56; SAT: control: 5.20 ± 3.93, 95% CI: 2.18-8.22 vs CES: 13.67 ± 12.39, 95% CI: 6.81-20.53). Moreover, relative expression of substance P and C-reactive protein (CRP) were examined, but the amount of expressed mRNA was too low for sufficient replication in the applied protocol (data not shown).

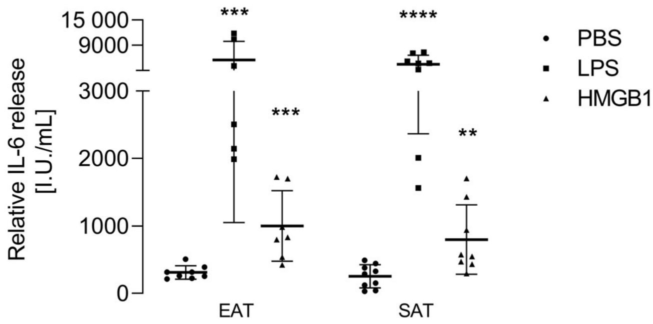

FIGURE 3 Inflammation-induced release of IL-6 in epidural adipose tissue (EAT) and subcutaneous adipose tissue (SAT) explant cultures of dogs. Explant cultures of EAT and SAT were stimulated with lipopolysaccharide (LPS, squares), high mobility group box-1 (HMGB1, triangles), or phosphate-buffered saline (PBS, circles) for 24 hours to determine inflammation-induced release of cytokines. Stimulation with both inflammatory mediators resulted in enhanced release of IL-6 into culture supernatants compared to PBS-treated controls (**: $P < .01$; ***: $P < .001$; ****: $P < .0001$). Graphs show the mean ± SD with symbols indicating results of single explant cultures.

3.2 | Cytokine release by explant cultures of EAT and SAT upon inflammatory stimulation

Supernatants of EAT and SAT explant cultures were collected after inflammatory stimulation with HMGB1 or LPS to determine release of TNFα and IL-6 by specific bioassays. TNFα was detectable in supernatants of all LPS-treated explant cultures (EAT: LPS: 219.9 ± 131.4, 95% CI: 125.9-313.9; SAT: 250.3 ± 148.0, 95% CI: 126.6-374.1). However, concentrations in supernatants of PBS- or HMGB1-treated groups remained below the detection limit of 6.0 pg/mL. It was, therefore, not possible to statistically evaluate effects of inflammatory stimulation on TNFα release. However, the observed robust increase above the detection limit compared to nondetectable concentrations in the PBS-treated groups indicates a LPS-induced release of TNFα (data not shown). The release of IL-6 was detectable in all treatment groups (Figure 3). In EAT, stimulation with LPS and HMGB1 resulted in significantly higher concentrations of IL-6 in supernatants compared to the PBS-treated controls (PBS: 310.9 ± 98.57, 95% CI: 228.5-393.3 vs LPS: 5491.55 ± 4438, 95% CI: 833.7-10 149, ***: $P = .0007$; PBS vs HMGB1: 1001.78 ± 522.2, 95% CI: 518.8-1485, ***: $P = .0006$).

Cultured SAT also showed LPS- and HMGB1-induced release of IL-6 into supernatants (PBS: 254.09 ± 171.8, 95% CI: 122.1-386.1 vs LPS: 4496.84 ± 2132, 95% CI: 2715-6279, ****: $P < .0001$, PBS vs HMGB1: 800.68 ± 514.8, 95% CI: 370.3-1231, **: $P = .004$).

4 | DISCUSSION

In this study, we examined the production of inflammatory adipokines and adipocytokines by EAT in dogs with CES. The results indicate an upregulation of TNFα and IL-10, as well as an attenuated expression of leptin in EAT of dogs with CES compared to controls. Additionally, tissue-specific differences in the expression of adipokines and adipocytokines between EAT and SAT were detectable. Stimulation with HMGB1 and LPS in cultured EAT explants induced a release of pro-inflammatory cytokines, TNFα and IL-6.

4.1 | Expression of adipokines and adipocytokines by EAT and SAT in the context of CES

In the recent decades, adipose tissue gained attention as potent modulator of systemic[15,16] and local inflammatory processes.[17,20,24] It has to be noted that adipose tissue not only consists of vacuole-containing adipocytes, but also a majority of stromal-vascular cells, such as immune cells.[36] Adipocytes are capable of secreting adipokines, such as leptin,[37] whereas resident macrophages are the principal source of cytokines, like TNFα and IL-6.[38] Mechanical challenges alter adipocytes' metabolic functions in vitro and lead to a production of cytokines and chemokines, as well as fibrotic mediators.[39] An enhanced infiltration of immune cells as well as fibrosis of EAT was previously observed in an experimental model of IVD herniation in dogs.[40] Moreover, infiltration of neutrophils and macrophages into the epidural space was detected in another study investigating dogs with IVD extrusion.[7] However, in a follow-up study, the researchers observed a downregulation of classical pro-inflammatory mediators, such as TNFα, IL-6, or IL-1β, whereas chemokine ligand 2 (CCL2) was upregulated.[8] One study investigating humans with radiculopathy caused by herniated discs also detected enhanced levels of TNFα in periradicular adipose tissue.[41] The presence of inflammatory irritants around the nerve roots of the *cauda equina* is capable of augmenting local nerve damage in dogs with experimental mechanical compression.[42] A main finding of this study is an upregulation of pro-inflammatory TNFα in EAT of dogs with CES (Figure 2A). Tumor necrosis factor alpha is a potent modulator of nociceptive signaling, leading to signs of hyperalgesia.[43] Indeed, TNFα is involved in mechanisms of peripheral[12,44] and central sensitization.[28,45] An enhanced production of pro-inflammatory mediators by resident and infiltrating immune cells in EAT could directly or indirectly affect nerve roots of the *cauda equina* and, therefore, clinical signs of pain.

We further detected an enhanced expression of the anti-inflammatory cytokine IL-10 in EAT samples from dogs of the CES groups (Figure 2C). Interleukin 10 has previously been described as the "master regulator of immunity,"[46] emphasizing its important roles in limiting inflammatory processes. Its upregulation in macrophages is mediated by similar activating transcription factors as for TNFα, such as nuclear factor (NF)-κB, NF-IL-6, or signal transducer and activator of transcription 3.[47] Therefore, an increase in IL-10 expression supports the hypothesis of augmented inflammatory processes in EAT of dogs with CES.

The functions of leptin are diverse and receptors for leptin have been detected on various cell types from several tissues, including peripheral sensory neurons[48,49] as well as neurons and glial cells in the spinal cord.[50-52] In the context of neuropathic pain, most studies implicate a pro-inflammatory function of leptin by augmenting production of cytokines by glial cells[51] and enhancing spinal excitation.[52,53] In contrast, 1 other study indicates a function of leptin to improve the recovery from spinal cord injury (SCI).[54,55] Our results provide evidence for an attenuated expression of leptin in EAT, as well as SAT of dogs with CES (Figure 2G). These results correlate to previous studies investigating leptin expression in adipose tissue or circulating leptin concentrations in experimental models of SCI.[56,57] In contrast, in humans with chronic SCI, the opposite effects were observed.[58] In this context, it has to be noted that experimental animals in the mentioned studies lost weight as a consequence of SCI intervention, whereas humans with chronic SCI had a significant higher BMI than controls. Changes in leptin levels in these studies could therefore be related to the body mass, and not necessarily to the injury itself but the functional significance of leptin for CES in dogs remains to be further investigated.

Results of this study further implicate differences in expression of inflammatory target genes with regard to the source of adipose tissue. Fat depots from distinct regions, for example, SAT or visceral adipose tissue differ in function and gene expression profiles.[36,59] Moreover, inflammation-induced expression and release of adipokines and adipocytokines is location- and age-dependent.[25] We present an enhanced expression of HMGB1 (####) and associated receptors TLR-4 ($P = .09$) and RAGE (##) in EAT compared to SAT (Figure 2D-F). The endogenous DAMP, HMGB1 is released upon tissue injury or inflammation and involved in adipose tissue inflammation,[60,61] IVD degeneration,[62,63] and persistent pain.[14] Via the receptors RAGE or TLR-4, it activates immune cells and, thereby, promotes tissue inflammation and immune cell infiltration.[61] However, the expression of HMGB1 was not altered in dogs affected by CES compared to healthy controls (Figure 2D). Overall, the presented data provide evidence for an altered gene expression in EAT of dogs with CES that might contribute to the effects of nerve root compression to facilitate pain.[64]

4.2 | LPS and HMGB1 induce the release of cytokines from cultured EAT explants

To test the hypothesis of an inflammation-induced production of inflammatory mediators by EAT, we harvested adipose tissue from dogs and performed in vitro stimulation with HMGB1 and

LPS. This method has previously been used to study effects of inflammation on rat adipose tissue from several locations.[18,25] Indeed, incubation of EAT in the presence of both inflammatory mediators resulted in an enhanced release of IL-6 (Figure 3), whereas TNFα was only detectable in supernatants of LPS-stimulated fat explants. These results support the data obtained in RT-qPCR experiments (Figure 2) and implicate a role of EAT on the local inflammatory milieu by paracrine secretion of pro-inflammatory mediators.

4.3 | Limitations and outlook

This study provides novel insights in a potential role of EAT in CES-associated spinal inflammation and pain in dogs. However, it has to be noted that there are some limitations. The number of investigated animals was representative, but still relatively small and heterogeneous. In future studies, study and control groups should ideally be standardized regarding their age and sex, as both can potentially influence the inflammatory response. Age-dependent changes in secretion of adipokines and adipocytokines have previously been shown in rodent models.[25] In the context of pain, recent studies in experimental models have revealed several sex-specific mechanisms contributing to chronic pain.[65,66] Our results provide evidence for an impact of DLSS on the expression of inflammatory mediators in EAT. However, it remains unclear if these mediators are responsible for CES-related hyperalgesia and to which extent they promote local inflammation.

5 | CONCLUSIONS

The presented results provide evidence that compression acting during DLSS alters the inflammatory state of EAT, indicated by an altered expression profile of cytokines and adipokines. An injury-induced production of cytokines by EAT could affect neuronal transmission of nociceptive signals and together with further resident and infiltrating cells, contribute to CES-associated pain. Therefore, we suggest to consider EAT as an immunological active tissue with a potential role in the pathophysiology of DLSS.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Tissue investigated in this study was removed during surgery and not explicitly for scientific purposes. Approval by the regional authority is not required for usage of otherwise discarded tissue. All clients gave their consent for the use for scientific purposes.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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