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Nerve growth factor in the equine joint

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ABSTRACT

Nerve growth factor (NGF) is a neurotrophin with many functions. In humans, it is involved in inflammation, nerve growth, apoptosis and pain signalling. Increased concentrations of NGF in synovial fluid has been shown in humans and dogs with osteoarthritis. Despite osteoarthritis being a common problem in horses, no studies have previously been published on NGF in the equine joint. The aim of this study was to quantify NGF in equine synovial fluid from healthy joints, acutely inflamed septic joints and joints with structural changes associated with osteoarthritis. A secondary aim was to identify the localisation of NGF and its two receptors, TrkA and $p75^{NTR}$, in healthy and osteoarthritic articular cartilage. NGF concentrations in synovial fluid from osteoarthritic joints ($n = 27$), septic joints ($n = 9$) and healthy joints ($n = 16$) were determined by ELISA. In addition, articular cartilage from osteoarthritic and healthy joints was examined for NGF, TrkA and $p75^{NTR}$ using immunohistochemistry staining.

NGF was present in equine synovial fluid and articular cartilage. Compared to synovial fluid from healthy joints, NGF concentration was higher in synovial fluid from joints with structural osteoarthritic changes ($P = 0.032$) or acute septic inflammation ($P = 0.006$). In articular cartilage with severe osteoarthritic changes, there was more abundant positive immunohistochemistry staining for NGF and its receptors than in normal articular cartilage. Further studies should focus on identifying precursor forms of NGF, and on receptor expression and downstream signalling of TrkA and $P75^{NTR}$ in health and disease.

Introduction

Nerve growth factor (NGF) is a crucial molecule for nerve cell maturation and survival and has been extensively studied in humans and laboratory animals (Denk et al., 2017; Minnone et al., 2017). It binds to two membrane bound receptors, tyrosine kinase A (TrkA) and the pan-neurotrophin receptor $p75^{NTR}$. Interaction of NGF or precursor forms of NGF (proNGF) with these receptors can cause a range of effects from cell survival and neurite outgrowth to cell death through apoptosis (reviewed in Denk et al., 2017). NGF expression increases in many inflammatory diseases (reviewed in Minnone et al., 2017) and has important functions in the nervous system for the production of pain signals. In humans, mutational defects in NGF or its receptors cause severe loss of deep pain sensation with undiagnosed, non-painful, fractures as well as disabling joint disease as a consequence (Einarsdottir et al., 2004; Shaikh et al., 2017).

Human patients with osteoarthritis (OA) of the knee have been shown to have increasing synovial fluid concentrations of NGF with increased disease severity (Montagnoli et al., 2017). Osteoarthritis is a major cause for veterinary care and early retirement of sport horses (Egenvall et al., 2005, 2006) and a cause of chronic pain and compromised animal welfare. In order to advance treatment and rehabilitation of equine athletes there is a need to identify biomarkers of inflammation that can help the clinician diagnose and prognosticate stages of OA (McIlwraith et al., 2018). Nerve Growth Factor has shown promise as a marker of OA and a candidate for therapeutic intervention in other species

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(Isola et al., 2011; Stoppiello et al., 2014; Lascelles et al., 2015; Chen et al., 2017; Montagnoli et al., 2017) but the involvement of NGF in equine OA is currently unknown.

The aim of this study was to confirm the presence and compare the concentrations of NGF in equine synovial fluid from joints with structural changes associated with OA to acutely inflamed septic joints and healthy joints. A secondary aim was to identify the localisation of NGF and its receptors TrkA and $p75^{NTR}$ in healthy articular cartilage and articular cartilage with OA.

Materials and methods

Sample collection was approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (Approval number, 5.8.18-02896/2018; Approval date, 6 April, 2018). Written owner informed consent was obtained as required.

Horses

Synovial fluid was sampled from lame horses with OA or septic arthritis and from healthy controls. The horses in the OA group were admitted for lameness with an initial lameness or flexion test reaction of ≥2/5 (AAEP)[1] where the lameness or reaction to flexion could be abolished or ameliorated by ≥ 70% by intra-articular mepivacaine anaesthesia of the sampled joint. Synovial fluid was collected prior to the administration of local anaesthetic. All horses underwent radiographic examination of the sampled joint. Inclusion criteria for the OA group were radiographic findings of intra-articular osteophytes as evaluated by the attending veterinarian. Horses diagnosed with carpal chip fractures by radiographic examination as the first diagnostic procedure did not receive intra-articular anaesthesia in order to confirm that the sampled joint was the cause of the lameness, and were included without findings of osteophyte formation. Horses were excluded if they had received any intra-articular treatment or if they had been treated with systemic corticosteroids within three months prior to sampling.

Synovial fluid samples from horses with OA were centrifuged at 5700 g for 15 min and supernatant aliquots were frozen within hours of sampling. Some samples were initially frozen at −20 °C before moving to the −80 °C freezer, but no samples were thawed prior to the time of analysis.

Horses with septic arthritis were included if they were >1 year old and admitted to a referral hospital with a wound communicating with the sampled joint. The synovial fluid was macroscopically abnormal with >30 g/L of protein and >80% neutrophils (Cousty et al., 2017). The precise duration of the joint infections was not known but was estimated from the history to vary between a few hours to a few days. Synovial fluid from the septic group was often collected out of hours, and these samples were refrigerated overnight and frozen the following day. The septic synovial fluid samples were all stored at −20 °C until analysis.

Synovial fluid samples from healthy joints were collected from biobanks stored at the Swedish University of Agricultural Sciences. Horses had no history of lameness from the sampled joint and OA inflammatory changes had been excluded by synovial fluid analysis as well as by radiographic imaging (lateromedial, dorsopalmar, dorsolateral-palmaromedial oblique, palmarolateral-dorsomedial oblique and dorsoproximal-dorsodistal oblique at a 35° angle views; n = 6; Skiöldebrand et al., 2006), or by post mortem arthrotomy and visual inspection of the joint (n = 10). All synovial fluid samples had a normal macroscopic appearance with total protein values <25 g/L in untreated samples (Caron, 2011).

For immunohistochemistry, healthy articular cartilages and articular cartilages with OA were sampled. Healthy cartilage was macroscopically normal on post mortem arthrotomy. Articular cartilage with mild to moderate OA had visible abnormalities such as wear lines or fissures visible on post mortem arthrotomy. Full-thickness cartilage samples were obtained from the lateral or medial chondylar surface of the third metacarpal bone within 1–5 h of euthanasia and placed in 10% neutral buffered formalin for 48 h prior to paraffin embedding. Articular cartilages with severe OA were sampled from horses undergoing arthroscopic surgery for osteochondral carpal chip fractures. The cartilage-covered chip was retrieved and placed in formalin as described above. No samples were decalcified prior to embedding. In addition to the macroscopic examination, all sections were stained with hematoxylin-eosin and toluidine blue and examined by light microscopy prior to inclusion to confirm that they were representative for the OA and healthy articular cartilage groups (McIlwraith et al., 2010; Schmitz et al., 2010). Chondrocyte necrosis, chondrone formation, fissuring and focal cell loss were graded (McIlwraith et al., 2010) and results are presented in Table 1.

Synovial fluid analysis

Protein analysis was performed on untreated samples as single sample analysis with the biuret test (Coba's Mira instrumentation, Hoffman-LaRoche). Microscopic examination of synovial fluid including nucleated cell differential counts were performed by trained clinical pathologists.

ELISA

The NGF ELISA assay (Horse NGF ELISA kit, MBS040618, MyBioSource) was performed according to the kit manual. The pre-determined detection range was 15.6-500 ng/mL. Briefly, 50 μL of sample or standard were added to all plate wells except for the blanks, followed by 100 μL HRP-conjugate reagent to all wells. The plate was covered and incubated at 37 °C for 60 min, followed by washing four times with buffer. 50 μL of Chromogen Solution A and 50 μL of Chromogen Solution B were added to all wells, the plate was covered, gently swirled and incubated at 37 °C for 15 min. After incubation, 50 μL of Stop solution were added to all wells, and the optical density was read within 15 min at 450 nm with a plate reader (Infinite F50, Tecan). Inter- and intra-assay coefficient of variation (CV) was tested with an aliquoted synovial control sample run in duplicate on every plate. A four parameter Marquardt standard curve and the Magellan software (Tecan) were used for calculation of concentrations.

Immunohistochemistry

Slides were deparaffinised and rehydrated. Phosphate buffered saline was used for all washes and antibody dilutions and samples were handled in room temperature. After antigen retrieval in a 60 °C water bath for 2 h, endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 min. Non-specific binding was blocked with normal goat serum (X0907, Dako) for 30 min. Primary antibodies were diluted and added to the sections; NGF: 1:500 (polyclonal, H20/SC-548, Santa Cruz Biotech), TrkA: 1:1000 (polyclonal, LS-C389392, Nordic BioSite), $p75^{NTR}$: 1:4000 (polyclonal, ABIN1917233, Antibodies Online). Rabbit IgG (X0936, Dako) was run in parallel on all sections as a negative control, using identical protein concentrations as for the primary antibodies. The sections were incubated at 4 °C overnight, incubated with secondary antibody (EnVision K4003, Dako) for 30 min and

[1] See: American Association of Equine Practitioners. www.aaep.org/horsehealth/lameness-exams-evaluating-lame-horse (Accessed 9 October, 2020).

Table 1
Histopathology scoring of articular cartilage according to McIlwraith et al., 2010[a].

Cartilage classification	Chondrocyte necrosis	Chondrone formation	Fissuring	Focal cell loss	Total score
Healthy	0	0	0	0	0
Healthy	0	0	0	0	0
Healthy	0	0	0	0	0
Mild OA	4	3	3	3	13
Moderate OA	4	3	3	3	13
Moderate OA	4	3	3	4	14
Severe OA	4	3	4	4	15
Severe OA	4	3	4	4	15
Severe OA	4	4	4	4	16

OA, osteoarthritis.
[a] The classification of severity is not clearly reflected in the total score. The scoring system was developed for an osteochondral fragment model and scoring does not include if changes are focal (as in the samples graded as mild to moderate OA) or widespread throughout the whole sample (as in the samples graded as severe OA).

stained with 3,3′-diaminobenzidine tetrahydrochloride in organic solvent for 3 min (NGF and TrkA) or 6 min ($p75^{NTR}$). Nuclei were stained with Mayer's hematoxylin. Finally, sections were dehydrated and mounted. Equine spinal cord was used as a positive control for each antibody (Appendix A: Supplementary material).

For estimation of the proportion of positive chondrocytes in each articular cartilage sample, 200 chondrocytes were evaluated for positive immunohistochemistry staining on two separate occasions. Articular cartilage sections were reviewed by light microscopy by two evaluators, one of them a board-certified veterinary pathologist.

Statistical analysis

Statistical analysis of the synovial fluid NGF concentration was performed using a commercial statistical software program, JMP Pro 14.0 (JMP Nordics). Residuals were confirmed to be normally distributed. The concentration of NGF for the OA, septic and healthy joint groups were compared using ANOVA and least square means estimates. NGF was elected as the response variable with ELISA plate and diagnosis selected as model effects. Tukey's test was used for testing pairwise differences. In addition, NGF concentration for the OA and healthy carpal joint groups and fetlock joints were analysed using ANOVA and least square means estimates. NGF was elected as the response variable with ELISA plate, joint and diagnosis selected as model effects and differences were tested using the Student's t test. Correlation between age and the concentration of synovial fluid NGF in healthy horses was tested by bivariate analysis. Data is presented as mean (± standard deviation, SD) and significance was set at $P < 0.05$.

Results

ELISA

Twenty-seven horses met the inclusion criteria for the OA group. Nine horses were included in the septic group and 16 horses in the healthy group. Table 2 contains demographic details.

Synovial fluid concentration of NGF in the OA group was 57.78 ng/mL (±17.41), in the septic group 71.34 ng/mL (±20.95) and in the healthy group 46.32 ng/mL (±9.26; Fig. 1). NGF concentrations in the OA and septic groups were significantly higher compared to the healthy group ($P = 0.032$ and $P = 0.006$, respectively). There was no significant difference in NGF concentrations between the septic and the OA group ($P = 0.72$) or between carpal and fetlock joints ($P = 0.31$). There was no correlation between age and NGF concentration in synovial fluid.

The ELISA intra-assay CV for the three plates used was 1, 4 and 12%, respectively, and the inter-assay CV was 10%.

Immunohistochemistry

Appendix A Supplementary material contains demographic details. Three horses were included in each group. The healthy cartilage samples had no abnormal findings on light microscopy of hematoxylin-eosin and toluidine blue stained sections. The articular cartilage with mild to moderate OA changes had superficial fibrillation and loss of chondrocytes with adjacent chondrocyte cluster formations (chondrones) in a localised area (one horse) and as multifocal changes (two horses). These areas had decreased colouring of the matrix on toluidine blue staining.

Table 2
Demographics of horses sampled for ELISA.

Diagnosis (*n*)	Age (mean)	Breeds (*n*)	Sex (*n*)	Sampled joint (*n*)
OA (27)	2–22 (10)	Standardbred (3) Thoroughbred (1) Pony breed (5) Quarter Horse (1) Warmblood (17)	m (13) g (13) s (1)	Fetlock (10) Carpus (9) Tarsocrural (2) Tarsometatarsal (2) Coffin (2) Stifle (2)
Septic (9)	1–23 (8)	Standardbred (3) Pony breed (2) Warmblood (4)	m (6) g (3)	Fetlock (3) Carpus (2) Tarsocrural (3) coffin (1)
Healthy (16)	1–12 (4)	Standardbred (16)	m (8) g (3) s (5)	Fetlock (4) Carpus (12)

OA, osteoarthritis; m, mare; g, gelding; s, stallion.

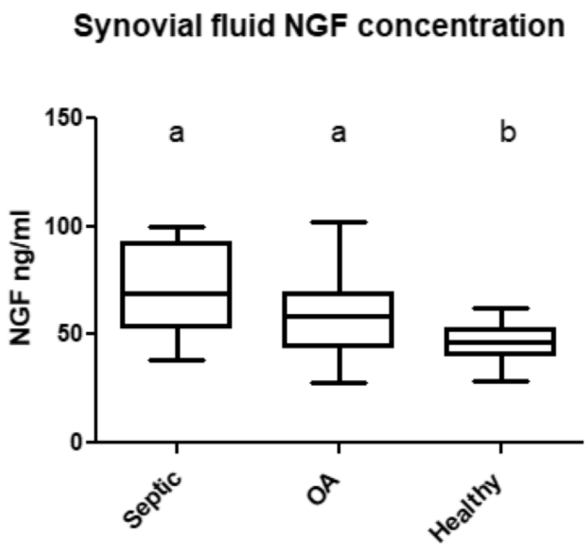

Fig. 1. Boxplot showing mean, interquartile and min-max ranges for synovial fluid nerve growth factor (NGF) concentrations in septic, osteoarthritic (OA) and healthy joints. Different letters indicate statistically significant differences in group means.

The articular cartilages with severe OA had fibrillation, areas of necrosis and of fibrous cartilage formation, as well as chondrone formation in all layers. These samples showed decreased toluidine blue staining, indicating marked matrix degeneration (Schmitz et al., 2010). Appendix A: Supplementary material contains examples of toluidine blue stained sections.

Immunohistochemistry for both NGF and TrkA showed increased number of staining chondrocytes with increasing disease severity, while the staining for $p75^{NTR}$ varied. Although $p75^{NTR}$ staining was more abundant in areas of cluster formation surrounding necrosis, there was not a consistent increase with increasing morphological disease severity.

For NGF, the healthy and the mild-moderate OA articular cartilages had similar staining patterns. In the superficial layer, nearly all chondrocytes had specific cytoplasmic immunostaining with only occasional cells without obvious staining present. In the middle and deeper layers, approximately 50% of the cells stained positive. The articular cartilages with severe OA did not have an intact superficial layer; hence, this could not be evaluated for staining characteristics. However, there were areas of fibrous cartilage formation and these cells did not stain positive for NGF. Positive staining was more abundant in the middle and deeper layers of the severe OA cartilage group compared to the healthy and mild-moderate OA cartilage groups, with more than 60% of the cells staining for NGF (Fig. 2).

For TrkA, staining patterns were similar to NGF with superficial cells in the healthy and mild-moderate OA articular cartilage groups showing positive cytoplasmic immunostaining. However, the staining of the middle and deep layers showed some variation. In the healthy cartilages, only occasional cells in the middle and deep layers stained, whereas the mild-moderate and the severe OA cartilage groups had more cells that stained positive. The increase in positively stained cells was marked in the articular cartilages with severe OA, compared to healthy cartilages. Chondrones were consistently positive for TrkA. Some articular cartilage samples also had non-specific staining of the extracellular matrix. This non-specific staining was most apparent in areas of cartilage necrosis in the severe OA articular cartilage group, but could also be seen as a very thin line in the most superficial layer of one healthy articular cartilage and one with mild OA, with no associated cartilage necrosis visible.

For $p75^{NTR}$, staining patterns were inconsistent within groups. All the healthy articular cartilages had only occasional cytoplasmic staining of cells in the middle and deep layers, but one horse had most of the superficial cells positive while two horses had positive staining of approximately 25% of the superficial cells. In the mild-moderate OA group, two samples had positive staining of more than 75% of the cells in the superficial layer and approximately 10-20% stained cells in the middle and deeper layers. In the third sample, less than 50% of the cells in the superficial layer stained positive and only occasional cells stained in the middle and deeper layers. However, there was a localised area of necrosis and cluster formation in this sample and in that area, all of the surrounding cells stained for $p75^{NTR}$. In the severe OA cartilage group, two samples had more than 60% of the cells staining positive. Most cells showed cytoplasmic staining, but there were also occasional chondrocytes with intranuclear staining. The third articular cartilage with severe OA had only a few positively stained cells.

Discussion

This study is the first to show the presence of NGF and its receptors (TrkA and $p75^{NTR}$) in equine joints. Concentrations of NGF were significantly higher in synovial fluid from joints with structural OA changes and joints with septic inflammation, compared to healthy joints. This is consistent with previous studies in dogs and humans, where joints with chronic OA had significantly higher synovial NGF concentrations than healthy control joints (Isola et al., 2011; Montagnoli et al., 2017). ELISA results were supported by immunohistochemistry; articular cartilage with severe OA showed more cells staining positive for NGF compared to normal articular cartilage. Moreover, given the role of NGF receptors in survival and apoptosis, the difference in receptor expression with more chondrocytes being positive for

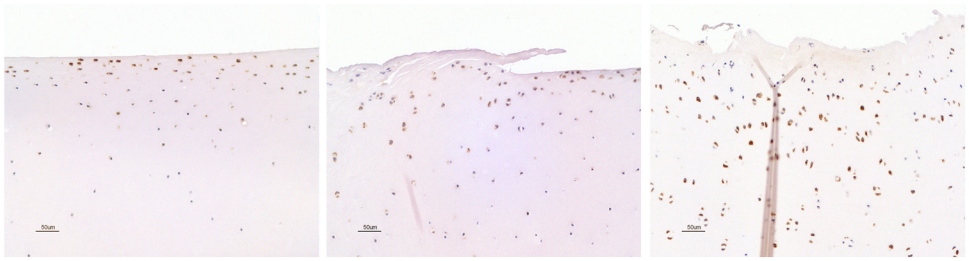

Fig. 2. Articular cartilage stained for nerve growth factor (x20). Left, normal; middle, mild osteoarthritis (OA); right, severe OA. Healthy and mild OA articular cartilage show similar staining patterns with positive chondrocyte staining of almost all cells in the superficial layer and in some cells of middle and deeper layers. In severe OA there is obvious staining of most chondrocytes in the middle and deeper layers.

TrkA in articular cartilage with severe OA, but marked variation in the expression of $p75^{NTR}$ is an interesting finding.

The NGF protein is secreted as a large molecule with several glycosylation sites, and post-translational processing involves glycosylation and splicing from the N-terminal into shorter proNGF forms and the mature NGF of 13 kDa (Fahnestock et al., 2004). The post-translational modification of NGF may differ between cell types depending on what arsenal of proteolytic enzymes are available, which means that different cell-lines are likely to have variations in what proNGF forms are expressed (Seidah et al., 1996; Reinshagen et al., 2000). Exact functions of the various forms of proNGF remain to be elucidated. It has been shown that proNGF function can change according to the relative expression of TrkA and $p75^{NTR}$. TrkA expression results in neurotrophic signalling, whereas a decrease in TrkA leads to apoptotic signalling (Ioannou and Fahnestock, 2017). The current understanding is that NGF binds preferentially to TrkA, while proNGF has been shown to have greater affinity for the $p75^{NTR}$ receptor (Lee et al., 2001; Clewes et al., 2008). Reasons for the differences in $p75^{NTR}$ expression in this study remain speculative and should be interpreted cautiously. Considering the interplay between TrkA and $p75^{NTR}$, it is possible that the lower $p75^{NTR}$ expression represents NGF signalling that stimulates healing and growth as supposed to apoptosis.

The NGF antibodies used in this study were directed at mature NGF and will not distinguish between mature and pro forms, which could potentially be a disadvantage in distinguishing the differences between NGF in synovial fluid and articular cartilage from healthy horses and those with OA, considering the different actions of proforms and mature NGF. Although the synovial fluid concentrations of NGF were different between healthy joints and those with OA, there was some overlap between the groups. Definition and quantification of proforms of NGF may have been more rewarding in terms of studying differences between these groups.

Synovial fluid from septically inflamed joints also had higher NGF concentrations than synovial fluid from healthy joints. It is not known if these horses had underlying OA of the sampled joint, as a complete lameness history and radiographs were not consistently obtained from these horses. However, it has been shown that NGF enhances neutrophil function in both murine models and humans (Kannan et al., 1991; Beigelman et al., 2009). In a carrageenan induced synovitis model in rats, NGF increased in synovial membranes within 24 h (Aloe et al., 1992). These studies indicate that NGF is not only involved in chronic OA processes but also has important functions in acute inflammation.

The horses in the healthy group included for synovial fluid analysis were younger (1–12 years) than the horses in the OA group (1–23 years), but the reverse was true for the cartilage samples, with older horses (4–12 years) in the healthy group than in the severe OA group (3–4 years). Age may have influenced the results and due to a relatively small sample size it was not possible to properly test the effect of age in the study material. However, there was no correlation between age and synovial fluid NGF concentrations in the healthy group, and it is unlikely to have affected the results of this study. Articular cartilage sections from both fetlock and carpal joints were included for immunohistochemistry as these two joints were not shown to differ statistically in synovial fluid NGF concentrations.

In the ELISA cohort, three horses with OA and three with septic joints had received systemic NSAIDs prior to sampling (data not shown). The horses with OA had not received treatment within 4 days, but the horses in the septic group had been treated the same day. The mean synovial fluid NGF concentrations (65.7 ng/mL for the NSAID-treated OA horses and 73 ng/mL for the NSAID-treated septic horses) were above the respective group means. It is possible that anti-inflammatory treatment decreased the NGF levels in the treated horses, however, it has not changed the final results of the study.

In humans, phase III studies of treatment with NGF antibody are being conducted, showing promising results in patients refractory to traditional treatments with NSAIDs and opioids (Dakin et al., 2019). Pilot studies in dogs with chronic lameness due to OA have also showed improvement after antibody treatment (Webster et al., 2014; Lascelles et al., 2015). A potential safety signal with rapidly progressing OA in human patients treated with NGF-antibody caused a temporary halt in trials in 2010. Causes for the rapid disease progression is still unknown, but combination with NSAIDs as well as neuropathic arthropathy and increased joint loading due to analgesia has been postulated (Hochberg, 2015).

NGF therapy could be an intriguing alternative to long-term NSAID treatment in horses. However, due to the varied effects of proNGF depending on the amount of TrkA receptors available (Ioannou, 2017), more research on receptor expression in different disease stages may be advisable before anti-NGF treatment in horses is considered.

Conclusions

In summary, NGF, TrkA and $p75^{NTR}$ is present in the equine joint and expression appears to be influenced by inflammation. The potential use of NGF as a biomarker or target for therapeutic intervention for OA pain should be further elucidated with emphasis on defining and quantifying the proNGF forms and the activation and downstream signalling of $p75^{NTR}$ and TrkA. Determining alterations of NGF and its proforms in other body fluids such as serum or saliva may provide a more accessible tool for future research in this area.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tvjl.2020.105579.

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