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Inflammatory mediators are potential biomarkers for extracorporeal shockwave therapy in horses

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Summary

Background: Extracorporeal shockwave therapy (ESWT) can potentially mask painful injuries in equine athletes. Tests to detect whether a horse has received ESWT prior to competition are needed. Extracorporeal shockwave therapy is known to affect inflammatory mediators in other species, and if these mediators are altered in the horse, these could serve as biomarkers of ESWT.
Objectives: To test the hypothesis that a single application of ESWT will alter the circulating protein concentrations of 10 inflammatory mediators in horse plasma.
Study design: Prospective repeated measures experimental study.
Methods: Eleven healthy horses were administered a single dose of ESWT on the dorsal surface of proximal MCIII. Blood samples were collected at −168, −144, −120, −96, −72, −70, −68, −66, −48, −24, −6, −4, −2, 0 h before and 2, 4, 6, 24, 48, 72, 96, 168, 336 and 504 h after ESWT. Plasma concentrations of interleukin 1 beta (IL-1β), IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-6, IL-10, IL-15, interferon gamma (IFN-γ), soluble toll-like receptor 2 (sTLR2) and tumour necrosis factor alpha (TNF-α) were measured to assess the effects of ESWT on these mediators.
Results: Baseline concentrations of inflammatory mediators did not change substantially during the week prior to ESWT. Plasma concentrations of five inflammatory factors changed following ESWT. IL-1β and IL-6 were significantly down-regulated ($P<0.01$), while TNF-α, IL-1RA and TLR2 were significantly up-regulated ($P<0.01$). The remaining cytokines were not significantly affected by ESWT.
Main limitations: This study was performed in a small number of sedentary, healthy pasture-kept horses using a single dose of ESWT applied to a single location. Additional studies are necessary to determine the effect of ESWT on inflammatory mediators in athletic horses undergoing treatment for musculoskeletal injuries.
Conclusions: Plasma concentrations of TNF-α, IL-1β, IL-1RA, IL-6 and TLR2 were significantly affected by ESWT, and deserve further investigation as possible biomarkers of ESWT.

Keywords: horse; extracorporeal shockwave therapy; immunoassay; cytokines; toll-like receptor

Introduction

Extracorporeal shock wave therapy (ESWT) involves the application of high pressure sonic waves that have a fast rise time and short duration [1,2]. In addition to stimulating healing and tissue regeneration, ESWT can significantly reduce pain associated with musculoskeletal disorders [3]. Focused ESWT directs the waves towards a focal point and has been used to treat a variety of orthopaedic conditions in horses including tendonitis, navicular syndrome, insertional desmopathy, stress fractures, back pain and osteoarthritis [4,5]. In horses, focused ESWT has been shown to have an analgesic effect when used to treat orthopaedic disease [6,7]. ESWT is banned for 7–10 days prior to competition by equine sports regulatory bodies because of the potential to mask pain in injured horses, theoretically increasing the possibility of a catastrophic breakdown during competition. However, there is currently no established method to detect if a horse has received ESWT during the prohibited period.

ESWT has been associated with changes in the expression of inflammatory mediators in vitro [8,9] and in vivo [10,11]. Pro-inflammatory mediators such as interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) promote inflammation [12] whereas anti-inflammatory mediators such as interleukin 1 receptor antagonist (IL-1RA), interleukin 4 (IL-4) and interleukin 10 (IL-10) suppress the activity of pro-inflammatory mediators [13]. Some cytokines, such as interleukin 6 (IL-6), have both pro- and anti-inflammatory properties [13]. In vivo studies in rats and humans have shown significant changes in the levels of inflammatory mediators in local tissue [14,15] and in urine for up to 14 days after ESWT [10]. It was hypothesised that changes in circulating inflammatory mediators may be detected in blood following a single application of ESWT to horses. To test this hypothesis, this study evaluated the effect of focused ESWT applied to the third equine metacarpal bone on the circulating protein concentrations of a variety of inflammatory mediators (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-15, TNF-α, interferon gamma [IFN-γ], IL-1RA, and soluble toll-like receptor 2 [sTLR2]).

Materials and methods

Animals

Horses were brought into temperature-controlled stalls 2 days before the study. Based on physical examination and routine blood chemistry analysis, all the horses in the study were in good health and were sound. The horses were no longer actively racing, were housed on pasture and were not exercised on a routine basis. Seven Thoroughbred and four Standardbred horses, six mares and five geldings, weighing 497 (450–558) kg, and ranging in age from 2 to 13 years, were used in the study. This number of animals was estimated to provide sufficient power (0.997–0.998; Supplementary Item 1).

Extracorporeal shockwave therapy (ESWT)

All horses were treated with a single dose of ESWT 10 cm distal to the carpus on the dorsal surface of the third metacarpal bone, lateral to the extensor tendon using a Duolith SD1 ESWT unit with a focused applicator[a]. Ultrasound transmission gel was applied before ESWT and the treatment was conducted in a focused circle area with a diameter of 2 cm. The dose chosen was the highest dose used to treat clinical patients with dorsal metacarpal disease: 2700 pulses at an energy flux density of 0.55 mJ/mm^2 and frequency of 3 Hz for a total of 48.56 J. The duration of application was approximately 15 min.

All animals were sedated prior to ESWT treatment with an i.v. dose of xylazine[b], detomidine or xylazine plus butorphanol according to the individual preference of the veterinarian administering ESWT.

Blood collection

Blood (10 mL) was collected at −168, −144, −120, −96, −72, −70, −68, −66, −48, −24, −6, −4, −2 and 0 h before and 2, 4, 6, 24, 48, 72, 96, 168, 336 and 504 h after completion of ESWT into heparinised collection tubes[c]. An indwelling 14-g catheter[d] was used for periods of frequent sampling (between the −24 and 24 h time points) while venipuncture of the left or right jugular vein was used for daily sampling for all horses. Prior to catheter placement, the jugular groove was clipped of hair, washed with sterile water and surgical soap (chlorhexidine gluconate, 4%)[e] and rinsed with a bactericide (chlorhexidine diacetate)[f] and 70% isopropyl alcohol. Samples were centrifuged for 10 min at 3000 ***g***, and aliquots of 1 mL of plasma were stored at −70°C until analysis.

Enzyme-linked immunosorbent assay (ELISA)

A dual sandwich ELISA was employed for quantification using commercial antibodies based on known equine specific protein sequences for IL-1β [16], IL-1RA [17], IL-2 [18], IL-4 [19], IL-6 [20], IL-10 [21], IL-15[22], TNF-α [23], IFN-γ [24]. Equine antigen-specific polyclonal antibodies (Abs)[g,h] were used as capture Abs, their biotinylated conjugates served as detection Abs, and recombinant equine proteins[g,h] served as calibrators using methodology developed in our laboratory [20]. Equine ELISA DuoSet kits[g] were employed to quantify IL-2, IL-4, IL-10, TNF-α and IFN-γ. One human DuoSet kit[g] was used to measure sTLR2 concentration. The assays used biotin–streptavidin chemical interaction to increase sensitivity of the measurement and an intensive wash was applied following each step of the procedure. Briefly, capture Ab was coated onto a 96-well microplate for 2 h and the plate was blocked with bovine serum albumin[i] to minimise nonspecific binding. A 100 μL aliquot of the test sample or standard was applied to each well, and the plate was incubated for 1 hour to ensure sufficient binding of the antigen to the Ab. A detection Ab was applied and, following a 1 hour incubation, streptavidin-horseradish peroxidase[g] was added. The substrate reagents (H_2O_2 and tetramethylbenzidine)[j] were applied to capture the immune-reactivity signals, and the inflammatory mediators were measured at OD_{450} in an iMark Microplate Reader[k]. The concentrations were determined based on a four-parameter logistic fit-curve by the Microplate Manager 6 software[k].

Data analysis

All statistical analysis was performed using Stata 15 statistical software[l]. A $P<0.05$ was set as the criterion for statistically significant differences. Maximum and minimum concentrations are based on descriptive statistics and reported as Mean ± 95% confidence interval (CI). Data were analysed using a repeated measure multilevel mixed effects linear regression model (Supplementary Item 2) with 10 unique dependent outcomes (IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, IL-15, IFNγ, TLR2, and TNFα). The fixed effects were group, interaction of group and time, and age as a confounder. Since the sedative was chosen at the discretion of the veterinarian, random effects were set on the level of animal nested within sedative. The mixed effects approach admitted the implications of subject level data correlation and by using robust variance-covariance estimation, the possibility of mild normality departures was admitted (Supplementary Item 3). Inference statistics are based on the model adjusted differences in plasma concentrations from pre- to post-ESWT.

Results

Unaffected cytokines

Plasma concentrations of IL-2, IL-4, IL-10, IL-15 and IFN-γ were not significantly affected by a single application of ESWT.

Tumour necrosis factor alpha

By 2 h following ESWT, the TNF-α plasma concentration was increased by 1034 pg/mL (95% CI: 553–1514 pg/mL; $P<0.001$). The maximum concentration of 4675 pg/mL (95% CI: 3280–6071 pg/mL) was reached at 24 h. This represents a 57% increase compared to the average pre-ESWT concentration for all horses ($n = 11$). No significant change in the concentration over time was observed during the pre- or post-ESWT period. Once elevated, TNF-α remained elevated for the duration of the study (Fig 1).

Interleukin 1 beta

Following ESWT, IL-1β plasma concentration was decreased by 1208 pg/mL (95% CI 621–1795 pg/mL; $P<0.001$). The minimum concentration of 2082 pg/mL (95% CI: 1046–3119 pg/mL) was reached at 72 h. This

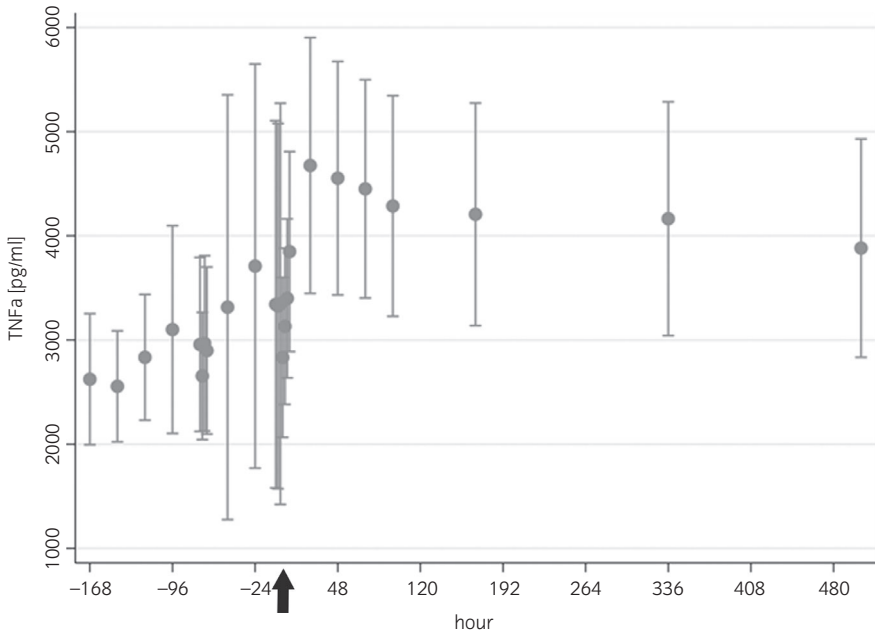

Fig 1: Plasma TNF-α concentrations in horses ($n = 11$) before (−168 ~ 0 h) and after ESWT (2 ~ 504 h). Data are presented as mean ± 95% CI for each time point. ESWT increased TNF-α plasma concentration for the duration of the study ($P<0.001$). Arrow indicates time of ESWT treatment.

represents a 61% decrease compared to the average pre-ESWT concentration for all horses (n = 11). No significant change in the concentration over time was observed during the pre-or post-ESWT period. Once decreased, IL-1β remained decreased for the duration of the study (Fig 2).

Interleukin 6

Following ESWT, IL-6 plasma concentration decreased by 802 pg/mL (95% CI: 568–1035 pg/mL; P<0.001). The minimum concentration of 1521 pg/mL (95% CI: 1214–1829 pg/mL) was reached at 2 h. No significant change in IL-6 over time was observed in the pre-ESWT period, however, during the post-ESWT period, a statistically significant increase was observed (0.54 pg/mL/h; P<0.05). Despite this slow increase, IL-6 did not return to baseline for the duration of the study (Fig 3).

Interleukin 1 receptor antagonist

In the period prior to ESWT, IL-1RA increased at a rate of 1.44 pg/mL/h (P = 0.001). Following ESWT, IL-1RA plasma concentration increased by 346 pg/mL (95% CI: 101–591 pg/mL; P<0.01). The maximum concentration of 5126 pg/mL (95% CI: 2880–7373 pg/mL) was reached at 4 h post-ESWT.

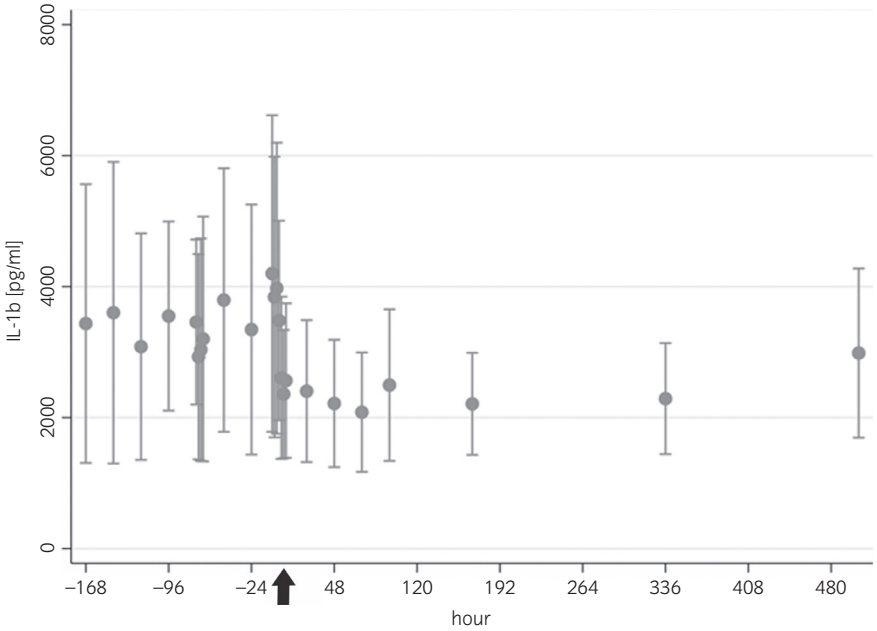

Fig 2: Plasma IL-1β concentrations in the horses (n = 11) before (−168 ~ 0 h) and after ESWT (2 ~ 504 h). Data are presented as mean ± 95% CI for each time point. ESWT decreased IL-1β plasma concentration for the duration of the study (P<0.001). Arrow indicates time of ESWT treatment.

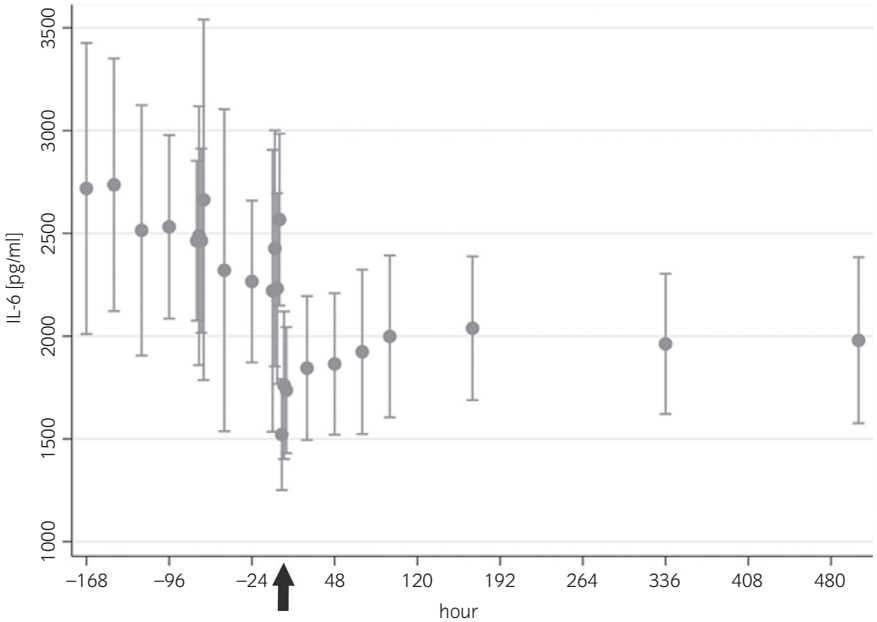

Fig 3: Plasma IL-6 concentration alterations in the horses (n = 11) before (−168 ~ 0 h) and after ESWT (2 ~ 504 h). Data are presented as mean ± 95% CI for each time point. ESWT decreased IL-6 plasma concentration (P<0.001) followed by a gradual increase during the post-ESWT period (0.54 pg/mL/h; P<0.05). Arrow indicates time of ESWT treatment.

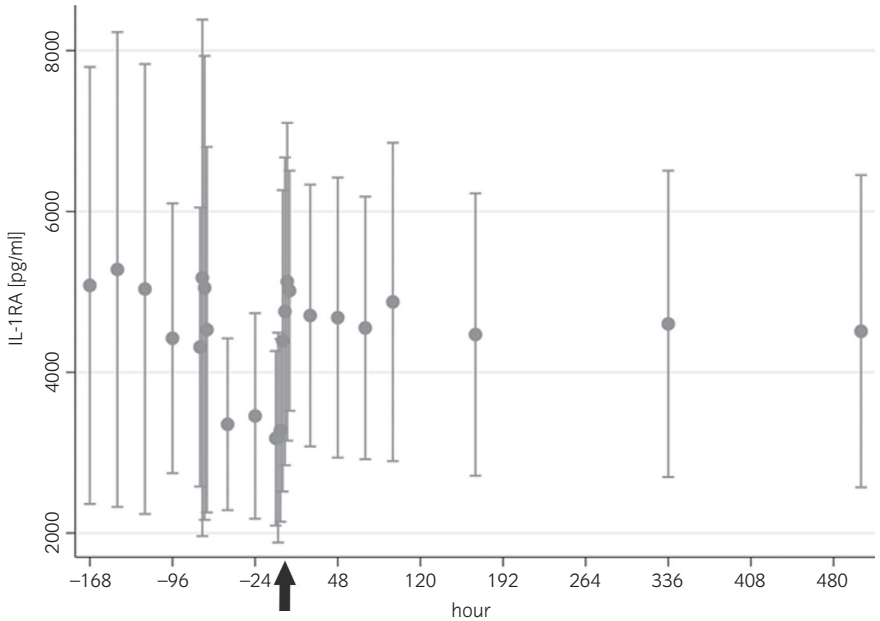

Fig 4: Plasma IL-1RA concentrations in the horses (n = 11) before (−168 ~ 0 h) and after ESWT (2 ~ 504 h). Data are presented as mean ± 95% CI for each time point. There was an overall small but significant increase in plasma IL-1RA during the pre-ESWT period (1.44 pg/mL/h; P = 0.001) followed by an abrupt increase after ESWT (P<0.01). Arrow indicates time of ESWT treatment.

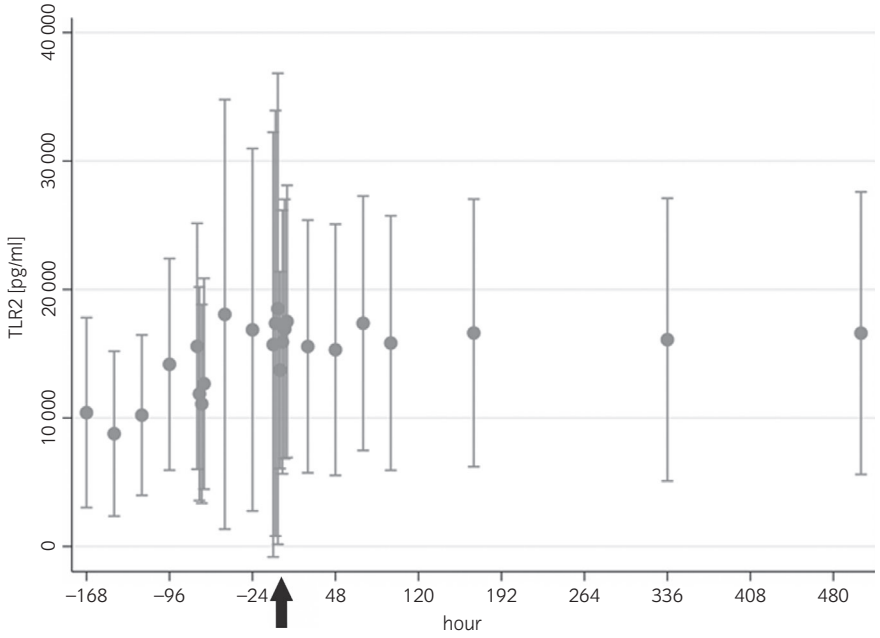

Fig 5: Plasma TLR2 concentrations in the horses (n = 11) before (−168 ~ 0 h) and after ESWT (2 ~ 504 h). Data are presented as mean ± 95% CI for each time point. ESWT increased TLR2 plasma concentration for the duration of the study (P<0.01).

Once increased, IL-1RA remained increased and did not significantly change with time for the remainder of the study (Fig 4).

Soluble toll-like receptor 2

Following ESWT, the plasma concentration of sTLR2 was significantly elevated by 1934 pg/mL (95% CI: 546–3322; P<0.01). The maximum concentration of 17,508 pg/mL (95% CI: 5452–29,565 pg/mL) was reached at 6 h. This represents a 30% increase compared to the average pre-ESWT concentration for all horses (n = 11). There was no change in the sTLR2 over time in the pre-and post-ESWT periods. Once increased, post-ESWT levels of TLR2 remained elevated throughout the remainder of the study (Fig 5).

Discussion

This is the first study to demonstrate that a single dose of ESWT given to a horse significantly alters inflammatory mediators circulating in the plasma. In the present study, five out of 10 circulating inflammatory mediators (TNF-α, Il-1β, IL-1RA, IL-6 and sTLR2) were significantly affected by a single

dose of focused ESWT. These mediators may represent biomarkers that could be used to develop a simple blood test to detect the illegal use of ESWT in equine athletes, and although these factors may not be specific to ESWT, further study of these factors is warranted.

In vivo studies report contrasting effects of focused ESWT on TNF-α expression. After application of focused ESWT to the kidneys in human patients, plasma TNF-α levels were not significantly altered [11] and TNF-α could not be detected in urine [10]. However, in a porcine model, TNF-α levels were significantly increased in urine but not in plasma after application of focused ESWT to the kidneys [25]. Focused ESWT suppressed TNF-α in surgically created skin wounds in rats, improving skin flap tissue survival [26]. It appears that response to ESWT may be complicated depending on application.

IL-1β plays an important role in pain induction as well as maintaining chronic pain, suggesting that this cytokine is a good therapeutic target for painful conditions [27]. In vivo studies in humans showed that IL-1β levels were not significantly different in plasma after focused ESWT administered to the kidneys [11]. In this study, plasma IL-1β concentration decreased significantly ($P<0.001$) following ESWT. This correlates well with findings that focused ESWT improves naturally occurring lameness in horses for up to 2 days [7].

As a natural inhibitor of IL-1 signalling, IL-1RA can reduce systemic inflammation and is therefore used in treating rheumatoid arthritis as well as equine lameness secondary to joint and soft tissue injury [28]. To the authors' knowledge, this is the first study describing the effect of ESWT on IL-1RA production. Plasma IL-1RA concentrations were significantly increased ($P<0.01$) following ESWT with the maximum concentration at 4 h, indicating that it may be a sensitive indicator of ESWT and the peak increase occurred in conjunction with TNF-α elevation. This result corresponded to attenuation of plasma IL-1β concentration in the treated horses, reflecting its anti-inflammatory role. Moreover, the peak increase in IL-1RA occurred earlier than IL-1β attenuation, suggesting a possible mechanism for IL-1β suppression via an increase in IL-1RA.

In the current study, up-regulation of TLR2 was observed, and the significance of TLR2 regulation by ESWT remains to be determined. This is the first study, to the authors' knowledge, to investigate the effect of ESWT on systemic TLR2 release.

In this study, plasma IL-6 concentrations decreased significantly following ESWT. Other studies, however, reported either increases both in vitro [8,9] and in vivo [10,15,25] or no significant differences in serum IL-6 levels in vivo [11,29] after application of focused ESWT. In vivo, a balance between pro- and anti-inflammatory cytokines is normally maintained [13], and the absolute effects on plasma concentrations measured in each in vivo study reflects the systemic release of cytokines for a specific model utilised. Thus, differing experimental conditions may explain why differing effects have been detected for these cytokines.

While our hypothesis was confirmed, further work is needed. The inflammatory mediator response to ESWT may be affected by the ESWT dose/dosing regimen, the anatomic location and tissue type to which it is applied, and the health and training level of the horse. Energy level and number of shocks have been demonstrated to directly influence changes in inflammatory mediators. For example, an in vivo study showed greater increases in renal tissue IL-6 concentration after 2000 pulses than 1000 pulses ($P<0.001$) when focused ESWT (lithotripsy) was applied to the porcine kidney [25]. An in vitro study showed that TGFβ1 gene expression increased after focused ESWT with 0.36 mJ/mm^2 and 100 impulses but decreased with 0.68 mJ/mm^2 and 250 impulses [30]. In our study, we used a dose used in our clinical practice to treat dorsal metacarpal disease in racehorses. This dose is higher than ESWT doses commonly applied to treat other orthopaedic conditions, and it is not known how the ESWT dose will affect inflammatory plasma biomarkers in horses.

Additionally, it is not clear if the effect of circulating cytokines to ESWT will be different in a healthy subject compared to one with clinical disease. In the present study, a single dose of ESWT was administered to healthy horses to provide proof of principle that changes in circulating inflammatory markers would be detectable in horse plasma following ESWT therapy. However, for these markers to be useful as a forensic test for ESWT, more work is needed to identify effects on these markers of musculoskeletal injury and the effects of multiple treatments.

Finally, exercise can induce a significant systemic inflammatory response by increasing mRNA levels of leucocyte IL-1β, IL-6 and TNF-α in horses [31]. Thus, the results of the current study may not represent the population at large, especially racehorses undergoing rigorous training, and population studies of all of these markers are ongoing to establish physiological ranges and longitudinal changes in individuals. Until these studies are completed, the use of plasma biomarkers to predict possible application of ESWT needs to be interpreted with caution.

Conclusions

Results of the present study showed that a single application of ESWT significantly affected circulating protein concentrations of TNF-α, IL-1RA, TLR2, IL-1β and IL-6, suggesting these inflammatory mediators may have potential value as biomarkers of ESWT. The data obtained serve as a benchmark for future studies to validate such biomarkers as possible predictors of ESWT. Further studies are needed to assess these biomarkers in equine athletes with musculoskeletal disease that receive ESWT.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

This study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Owner informed consent

Not applicable.

Sources of funding

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Authorship

J.W. Chen, L.R. Soma and M.A. Robinson designed the study. J.W. Chen and Z. Jiang executed the study. D. Stefanovski and R. Boston provided statistical analysis of the data. J. Haughan contributed to literature search and discussion. All authors contributed to data interpretation and the preparation of the manuscript.

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[e]Purdue Products LP, Stamford, Connecticut, USA.
[f]Fort Dodge Health, Fort Dodge, Iowa, USA.
[g]R&D Systems, Minneapolis, Minnesota, USA.
[h]KingFisher Biotech, St. Paul, Minnesota, USA.
[i]VWR International, West Chester, Pennsylvania, USA.

[j]Neogen Corporation, Lansing, Michigan, USA.
[k]BioRad, Hercules, California, USA.
[l]StataCorp, College Station, Texas, USA.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary item 1: Power analysis.
Supplementary item 2: Stata command.
Supplementary item 3: Quantile normal plots.