In vitro effects of poly(ADP-ribose) polymerase inhibitors on the production of tumor necrosis factor-α by interferon-γ– and lipopolysaccharide-stimulated peripheral blood mononuclear cells of horses

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OBJECTIVE
To evaluate effects of poly(ADP-ribose) polymerase-1 (PARP1) inhibitors on the production of tumor necrosis factor-α (TNF-α) by interferon-γ (IFN-γ)– and lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMCs) of horses as an in vitro model of inflammation in horses.

SAMPLE
1,440 samples of PBMCs from 6 healthy research horses.

PROCEDURES
From heparinized whole blood samples, PBMC cultures were obtained. An initial dose-response trial on 48 PBMC samples from 2 horses (24 samples each) was used to determine concentrations of IFN-γ and LPS for use as low- and high-level stimulation concentrations. Seventy-two PBMC samples from 6 horses were assigned equally to 1 of 4 PARP1 inhibition categories: no PARP1 inhibitor (PARP1 inhibition control); 2-((*R*)-2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carbozamide dihydrochloride (ABT888);4-(3-(1-(cyclopropanecarbonyl)piperazine-4-carbonyl)-4-fluorobenzyl)phthalazin-1(2*H*)-one (AZD2281); or *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylacetamide hydrochloride (PJ34). Samples of PBMCs from each horse and each PARP1 inhibition category were then assigned to 1 of 3 levels of IFN-γ and LPS stimulation: none (control), low stimulation, or high stimulation. After a 24-hour incubation period, a TNF-α ELISA was used to measure TNF-α concentration in the supernatant. Results were compared across treatments and for each horse. Data were analyzed with repeated-measures ANOVA.

RESULTS
Median TNF-α concentration was significantly lower for PJ34-treated, high-level stimulated PBMCs than for PARP1 inhibition control, high-level stimulated PBMCs; however, no other meaningful differences in TNF-α concentration were detected among the inhibition and stimulation combinations.

CONCLUSIONS AND CLINICAL RELEVANCE
Findings suggested that PJ34 PARP1 inhibition may reduce TNF-α production in horses, a potential benefit in reducing inflammation and endotoxin-induced damage in horses. (*Am J Vet Res* 2019;80:663–669)

In horses, endotoxemia, such as with strangulating and inflammatory lesions of the small intestines and large colon, is a leading cause of morbidity and death.[1,2] Ischemia-reperfusion injury and severe inflammation lead to intestinal mucosal injury with increased absorption of endotoxins and subsequent systemic inflammatory response syndrome that can lead to multiple organ failure. Methods to decrease intestinal IRI and inflammation and the effects of endotoxins on systemic inflammation may improve the prognosis for affected horses.

Poly(ADP-ribose) polymerase-1, which is the most abundant, ubiquitous, and studied member of the large family of poly(ADP-ribose) polymerase enzymes,[3] is involved in the IRI process. When activated in response to DNA damage from oxidative, nitrosative, genotoxic, oncogenic, thermal, inflammatory, or metabolic stress, PARP1 induces synthesis of poly(ADP-ribose) polymers, with the oxidized form of nicotinamide adenine dinucleotide serving as the donor of ADP-ribose units.

ABBREVIATIONS

ABT888	2-((*R*)-2-methylpyrrolidin-2-yl)-1*H*-benzimidazole-4-carbozamide dihydrochloride
AZD2281	4-(3-1-(cyclopropanecarbonyl)piperazine-4-carbonyl)-4-fluorobenzyl)phthalazin-1(2*H*)-one
IFN-γ	Interferon-γ
IRI	Ischemia-reperfusion injury
LPS	Lipopolysaccharide
PARP1	Poly(ADP-ribose) polymerase-1
PBMC	Peripheral blood mononuclear cell
PJ34	*N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylacetamide hydrochloride
TNF-α	Tumor necrosis factor-α

The poly(ADP-ribose) polymers and other acceptor proteins (eg, histones, DNA repair proteins, transcription factors, and chromatin modulators) then attach to the polymerase,[3-5] resulting in rapid depletion of the intracellular pools of ATP and oxidized nicotinamide adenine dinucleotide, which in turn leads to decreased rates of glycolysis and mitochondrial respiration and potentially culminates in energy crisis–induced cell necrosis.[6] Poly(ADP-ribose)-dependent cell death may also be caused by cellular translocation of the apoptosis-inducing factor from the mitochondrion to the nucleus, which occurs in a poly(ADP)-ribosylation–dependent manner and leads to DNA fragmentation and cell death. In addition, PARP1 activation plays a central role in inflammation through different pathways.[7-9] For instance, PARP1 induces release of high mobility group box 1, a proinflammatory mediator that can induce macrophage activation and TNF-α production.[10] Activation of PARP1 is also involved in inflammatory responses mediated by nuclear factor κ-light-chain-enhancer of activated B cells and in responses with secretion of other proinflammatory mediators (eg, TNF-α, interleukin-1β, P-selectin, intracellular adhesion molecule, and inducible nitric oxide synthetase).[11] As expected, considering the involvement of PARP1 in the inflammatory cascade and cell death, activation of PARP1 is involved in processes such as IRI, endotoxemia, and hemorrhagic shock. Therefore, PARP1 inhibition may be a strategy for decreasing IRI, inflammation, and the effects of endotoxin on systemic inflammation.

Numerous studies[12-16] show that the absence or pharmacologic inhibition of PARP1 is responsible for several protective effects on gut epithelium, such as improved mucosal barrier function, decreased inflammatory cell infiltration, lowered proinflammatory cytokines production, and reduced tissue damage. Because PARP1 inhibition may afford partial protection against endotoxemia in horses by decreasing capillary leakage and permeability, use of PARP1 along with current treatments for IRI and endotoxemia in affected horses may be beneficial. Species-specific research, however, is needed because the extent of endotoxin response varies among species and individuals.[17] Thus, research investigating the role of PARP1 inhibition on LPS-stimulated PBMCs of horses is desirable.

We hypothesized that for horses, TFN-α production would be lower in IFN-γ– and LPS-stimulated PBMCs exposed to versus not exposed to PARP1 inhibitors and that efficacy differed among PARP1 inhibitors. The aim of the present study was to evaluate effects, including potential protective effects, of PARP1 inhibitors on the production of TNF-α by IFN-γ– and LPS-stimulated PBMCs as an in vitro model of inflammation in horses.

Materials and Methods

Sample collection

Blood samples were aseptically collected from the jugular veins of 6 healthy adult horses from our research herd. For each horse, an 18-gauge needle attached to a 7-inch extension set was used to collect approximately 480 mL of blood in 8 sterile 60-mL syringes, each containing 5,000 U of sodium heparin. Filled syringes were sealed with sterile caps and transported on ice to the laboratory. The Institutional Animal Care and Use Committee of the University of Pennsylvania had approved all procedures involving the study animals.

Cultures of PBMCs

Heparinized blood samples from each horse were diluted in a ratio of 1:1 with sterile PBSS containing no calcium and no magnesium.[a] The diluted blood was then gently pipetted onto, but not mixed with, a high-density (1.077 g/mL) solution[b] of polysucrose and sodium diatrizoate in 50-mL conical tubes. The ratio of diluted blood to high-density solution was 3:2. The prepared samples were centrifuged at 400 × *g* for 30 minutes at room temperature in a centrifuge[c] set for slow or no brake. After centrifugation, the resulting distinct layer of WBCs at the interface of diluted blood and high-density solution was collected with manual pipetting, washed 4 times with sterile PBSS,[a] then resuspended in a balanced salt solution[d] that was supplemented with 10% fetal bovine serum[e] and 1% penicillin–streptomycin.[f] Concentration and vitality of PBMCs in each resuspended preparation were determined with trypan blue[g] exclusion.[18]

Peripheral blood mononuclear cells at a concentration of 1 million cells/mL were added to cell culture well plates[h] pretreated with fibronectin.[i] The seeded well plates were then incubated[j] at 37°C and 5% CO_2 for 1 hour to allow PBMCs to adhere to the well plates. The shape of adherent cells changed from round to polygonal, typical of confluent cells. Supernatant containing nonadherent cells was removed, and adherent PBMCs were washed with a balanced salt solution that was supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Afterward, 1 mL of a fresh supply of this supplemented solution was added to each well.

Determining IFN-γ and LPS stimulation challenge concentrations

In an initial dose-response trial, PBMCs from 2 healthy horses were stimulated with various assigned concentrations of IFN-γ[k] and LPS[l] combined in a supplemented salt solution (10 µL/well). These prepared well plates were incubated for 24 hours, which was sufficient time to allow cellular attachment but not cellular proliferation. After incubation at 37°C and 5% CO_2 for 24 hours, the PBMCs were detached from the culture plate wells with a cell lifter, and the supernatant along with the PBMCs of each well were transferred to individual conical-bottom centrifuge tubes and centrifuged at 400 × *g* for 5 minutes. The supernatant of each tube was separated into 3 aliquots of 100 µL and stored at –80°C until analysis with a TNF-α ELISA.[19,m] The ELISA kits were stored and used according to the manufacturer's instructions. The

absorbance, a direct indication of TNF-α concentration, was measured with a spectrophotometer[n] and an ELISA plate reader set at A450 nm minus A550 nm.[n] A standard curve was run with each assay. On the basis of results of this initial dose-response trial, reproducible low-level and high-level stimulation challenge concentrations of IFN-γ and LPS were identified for use in the rest of the study.

Inhibition of PARP1

Samples of PBMCs from each horse were assigned to 1 of 4 categories of PARP 1 inhibition: no PARP1 inhibitor (control), PJ34,[o] ABT888,[p] or AZD2281.[q] The PARP1 inhibitors were added at a final concentration of 1μM to cell culture plate wells containing PBMCs. Samples from each horse and each inhibition category were then assigned to 1 of 3 levels of IFN-γ and LPS stimulation: none (control), low, or high, as determined in the initial dose-response trial. Ten microliters of the assigned concentrations of IFN-γ and LPS in a supplemented salt solution was added to each sample, with controls receiving the same volume of solution without IFN-γ or LPS. After 24 hours of incubation at 37°C and 5% CO_2, the PBMCs were detached from the bottom of the wells with a cell lifter, then the supernatant and PBMCs of each well were transferred to individual conical-bottom centrifuge tubes and centrifuged at 400 × *g* for 5 minutes. The supernatant of each tube was separated into 3 aliquots of 100 μL and stored at –80°C until analysis with a TNF-α ELISA. Three independent repetitions were conducted for each of the combinations of PARP1 inhibition and IFN-γ and LPS stimulation of PBMCs from each horse.

Statistical analysis

Results, in median and range, of each PARP1 inhibition treatment and IFN-γ and LPS stimulation level were compared with results obtained for the PARP1 controls for the given IFN-γ and LPS stimulation level. In addition, data were aggregated, grouping all of the TNF-α ELISA results obtained from inhibition experiments (TREAT variable = 1), compared with results from PARP1 controls (TREAT variable = 0), so that the general effect of PARP1 inhibition could be investigated independently of particular inhibitors. Data were analyzed with repeated measures ANOVA, including the Shapiro-Wilk test to assess the normality of data. Concentration of TNF-α was used as a dependent variable, and horse identification was used as a random effect. An open-source software program[r] was used for analyses. Values of $P < 0.05$ were considered significant.

Results

Samples

The PBMCs were isolated from heparinized blood samples from 6 healthy adult research horses (3 Thoroughbred mares and 3 Standardbred geldings). Median age of the horses was 4.5 years (range, 2 to 15 years). The initial dose-response trial used PBMCs from 2 of these Thoroughbred mares: a 9-year-old and a 15-year-old. The 6 horses were part of our research herd, and previous CBC results for the herd indicated that the mean ± SD monocyte count was $0.3 \times 10^3 \pm 0.09 \times 10^3$ cells/μL.

Stimulation with IFN-γ and LPS

For each of the 12 combinations of IFN-γ and LPS concentrations evaluated, a total of 48 samples of PBMCs (24 samples from each of 2 horses) were tested in triplicate in an initial dose-response trial to determine which combinations of IFN-γ and LPS concentrations to use as the low- and high-level stimulation challenges for the rest of the study **(Table 1)**. Two combinations of IFN-γ and LPS concentrations were determined to be reproducible low- and high-level stimulation challenges and were therefore used along with a control (no IFN-γ and no LPS) in the rest of the study.

Low-level stimulation consisted of incubation of PBMCs in a solution containing 1 μg of LPS/mL and

Table 1—The 12 combinations of IFN-γ and LPS concentrations tested as stimulation challenges in an initial dose-response trial to assess TNF-α production by IFN-γ– and LPS-stimulated PBMCs from 2 healthy research horses.

Test concentration combinations		Response	
LPS (μg/mL)	IFN-γ (U/mL)	Median TNF-α (pg/mL)	Range of TNF-α (pg/mL)
0.0	0.0	198.24	168.57–489.75
0.1	0.0	10,712.63	9,124.41–24,318.01
1	0.0	11,797.60	10,003.09–29,016.23
10	0.0	10,605.04	9,035.98–25,748.40
0.0	10	387.85	364.16–870.18
0.1	10	13,375.96	11,353.29–23,511.47
1*	10*	9,338.42	8,082.32–25,667.82
10	10	7,894.73	6,740.32–28,224.94
0.0	100	122.94	121.84–212.68
0.1	100	10,285.01	8,836.83–26,239.34
1	100	9,327.11	8,051.38–27,200.49
10†	100†	14,262.31	12,010.16–30,093.23

*Concentration combination selected as the low-level stimulation. †Concentration combination selected as the high-level stimulation.

10 U of IFN-γ/mL. Median TNF-α concentration in the initial dose-response trial with low-level stimulation of PMBCs from 2 horses was 9,338.42 pg/mL (range, 8,082.32 to 25,667.82 pg/mL; Table 1). Median TNF-α concentration for low-level stimulation and no PARP1 inhibition treatment of PMBCs from 6 horses was 20,500 pg/mL (range, 1,950 to 64,010 pg/mL; **Figure 1**).

High-level stimulation consisted of incubation of PBMCs in a solution containing 10 µg of LPS/mL and 100 U of IFN-γ/mL. Median TNF-α concentration in the initial dose-response trial with PMBCs of 2 horses was 14,262.31 pg/mL (range, 12,010.16 to 30,093.23 pg/mL; Table 1). Median TNF-α concentration for high-level stimulation and no PARP1 inhibition treatment of PMBCs from 6 horses was 47,030 pg/mL (range, 10,820 to 97,160 pg/mL; Figure 1).

Inhibition of PARP1

For each of the 12 combinations of PARP1 inhibition category and IFN-γ and LPS stimulation level, 72 PBMC samples (12 samples from each of 6 horses) were tested in triplicate. Median TNF-α concentration for PBMCs assigned to the stimulation control (no IFN-γ and no LPS) was not significantly ($P > 0.05$) different between the PARP1 inhibition control (67.50 pg/mL; range, 16.85 to 770.90 pg/mL) and the other PARP1 treatment categories (ABT888, 45.90 pg/mL [range, 14.60 to 445.00 pg/mL]; AZD2281, 52.90 pg/mL [range, 13.80 to 1,489 pg/mL]; and PJ34, 67.00 pg/mL [range, 11.58 to 1,158 pg/mL]). In addition, results of repeated measures ANOVA comparisons indicated that median TNF-α concentration did not differ significantly ($P > 0.05$) between the low-level versus high-level stimulation of PBMCs treated with PARP1 inhibitors. Although median TNF-α concentration did not differ substantially for low-level stimulated PBMCs treated with ABT888 (18,320 pg/mL; range, 2,628 to 109,600 pg/mL), AZD2281 (16,570 pg/mL; range, 4,037 to 119,500 pg/mL), or PJ34 (13,250 pg/mL; range, 913.1 to 71,740 pg/mL), compared with the corresponding PARP1 control (20,500 pg/mL; range, 1,950 to 64,010 pg/mL; **Figure 2**), the median TNF-α concentration for high-level stimulated PBMCs treated with PJ34 (29,480 pg/mL; range, 3,715 to 74,570 pg/mL) was significantly ($P = 0.022$) lower than that for the corresponding PARP1 control (47,030 pg/mL; range, 10,820 to 97,160 pg/mL; **Figure 3**). However, median TNF-α concentra-

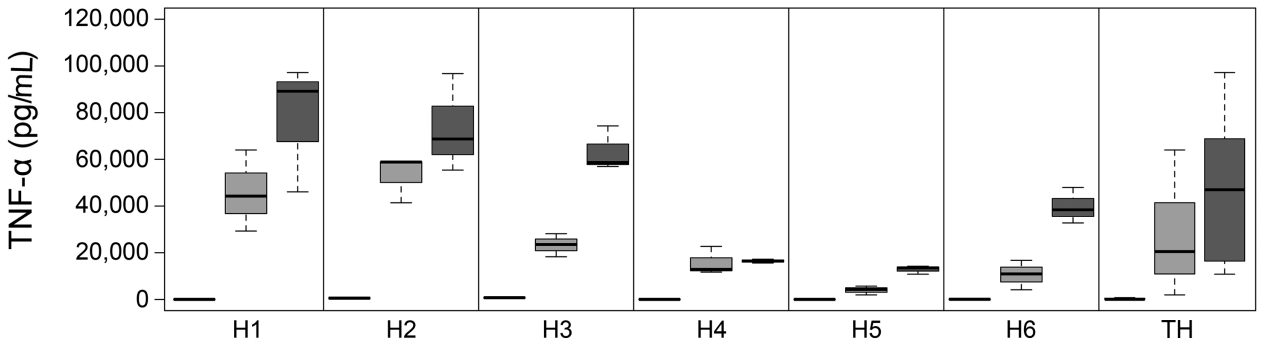

Figure 1—Box-and-whisker plots of TNF-α concentrations (measured by ELISA) stratified by level (none [unshaded], low [light gray], and high [dark gray]) of IFN-γ and LPS stimulation of PBMC samples from 6 healthy research horses. Each box represents the first to third quartiles, the line in the box represents the median, and whiskers represent the range of TNF-α concentrations. H = Horse. TH = Total horses.

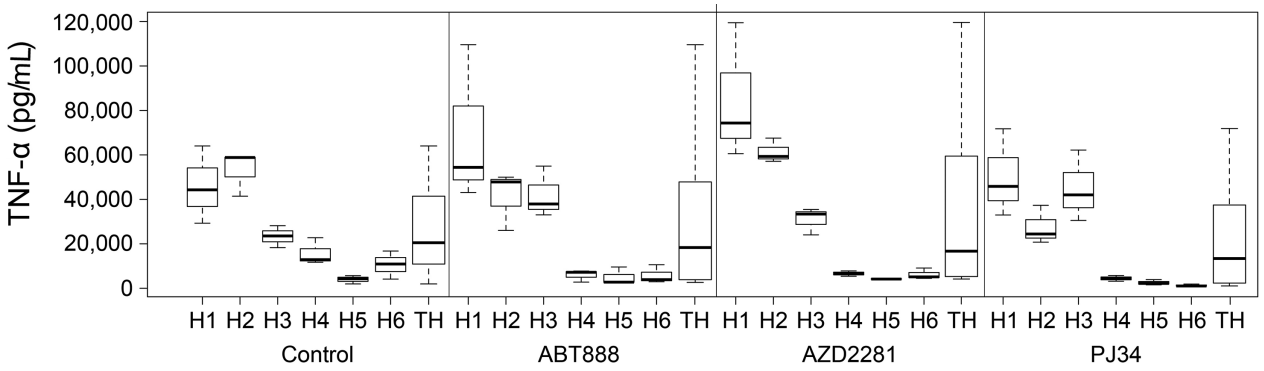

Figure 2—Box-and-whisker plots of TNF-α concentrations (measured by ELISA) stratified by PARP1 inhibitor treatment category (control [none], ABT888, AZD2281, or PJ34) of low-level IFN-γ– and LPS-stimulated samples of PBMCs from the horses described in Figure 1. TH = Total horses. ***See*** Figure 1 for the remainder of the key.

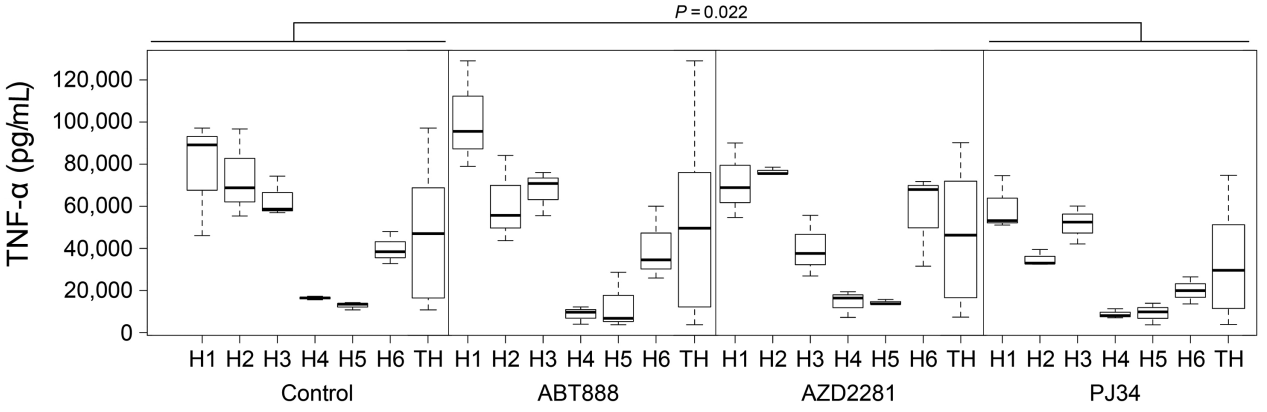

Figure 3—Box-and-whisker plots of TNF-α concentrations (measured by ELISA) stratified by PARP1 inhibitor (control [none], ABT888, AZD2281, or PJ34) treatment of high-level IFN-γ– and LPS-stimulated samples of PBMC from horses described in Figures 1 and 2. Each box represents the 1st to 3rd quartiles, the line in the box represents the median, and the whiskers represent the range of TNF-α concentrations. **See** Figure 2 for the key.

tion for high-level stimulated PBMCs treated with ABT888 (49,620 pg/mL; range, 3,768 to 129,100 pg/mL) or AZD2281 (46,150 pg/mL; range, 7,220 to 90,080 pg/mL) did not differ substantially from the corresponding PARP1 control.

Discussion

To our knowledge, the present study was the first in vitro model of inflammation in horses with the goal of evaluating production of TNF-α by IFN-γ– and LPS-stimulated PBMCs in the absence or presence of PARP1 inhibitors. The finding that only PJ34-treated, high-level–stimulated PBMCs had lower median TNF-α concentration than did the corresponding control supported our hypothesis that efficacy differed among PARP1 inhibitors. The finding also supported our hypothesis that TFN-α production would be lower in IFN-γ– and LPS-stimulated PBMCs exposed to PARP1 inhibitors, compared with results for those not exposed; however, no meaningful effect was observed with ABT888 or AZD2281. Hence, PJ34 may be a suitable PARP1 inhibitor for further in vivo evaluation of endotoxemia in horses.

A recent in vitro study[20] shows the importance of the PARP1 pathway in response to oxidative damage and the benefits of inhibiting the pathway in horses. In that study,[20] all PARP1 inhibitors tested, including PJ34, ABT888, and AZD2281, prevented PARP1 activation and poly(ADP-ribose) accumulation in response to hydrogen peroxide–induced oxidative damage. Findings of the present study were consistent regarding the notion that PARP1 inhibitors could potentially serve as novel treatments of IRI in horses.

Results of the present study indicated that ABT888 and AZD2281 did not have meaningful effects on TNF-α production by IFN-γ– and LPS-stimulated PBMCs of horses and supported findings of a previous study[21] that indicate neither AZD2281 nor 3-aminobenzamide (another PARP1 inhibitor) has any protective effects on TNF-α–induced necroptosis. Investigators of that study[21] concluded that TNF-α–induced necroptosis and PARP1-mediated necrosis operate independent pathways to programmed cell death, which cannot be influenced by AZD2281 or 3-aminobenzamide.

In the present study, findings indicated that only treatment with PJ34 had a significant effect and was consistent with results of other studies[21–24] that show a protective role of PJ34; however, the mechanisms of action for PJ34 are unclear and might have other molecular targets in addition to PARP1 that may explain its effect. In addition, dose-dependent effects of PJ34 prevented macrophage stimulation, prevented LPS-induced loss of cell viability, and suppressed production of several inflammatory mediators, including TNF-α[24]; therefore, PJ34 appears to be protective in endotoxemic inflammation and subsequent bacterial translocation, conditions that commonly occur in gastrointestinal tracts of horses during IRI.

Studies[25,26] of pigs show that PJ34 may be beneficial in reducing the effects of endotoxemia, and findings from laboratory animal models of endotoxemia support this notion.[27–32] In LPS-challenged mice, treatment with PJ34 reduced the inflammatory response and extent of typical signs of endotoxemia, including reduction in cellular lesions and death.[28] In addition, PJ34 reduced TNF-α concentration in serum and reduced activation of nuclear factor κ-light-chain-enhancer of activated B cells in liver tissue, diminishing the inflammatory response in LPS-treated mice.[27,28] Further, PJ34 treatment also improved vascular hyporesponsiveness and hemodynamic function after administration of LPS in rats.[30,31] A recent study[33] in rats shows that PARP1-mediated regulated necrosis in the intestinal epithelial barrier has an important role in intestinal injury owing to IRI and that inhibition of this PARP1-mediated regulated necrosis by treatment with PJ34 confers intestinal protection.

We recognize that the present study had important limitations. The sample size was small, a common limitation of studies involving large animals, and increasing the sample size would increase the statistical power. Although no in vitro model could fully represent the

complexity of events that take place in vivo, findings of the present study provided further information regarding PARP1 inhibitor effects in horses. In addition, our findings combined with previously published reports could help to improve understanding of the actions of inflammatory mediators at the cellular and subcellular levels. However, in vivo studies could be designed to evaluate beneficial effects, routes of administration, and dosages of PARP1 inhibitors in horses. In addition, analyses of PARP1 cleavage as an indicator of commencing apoptosis in PBMC samples from all horses could be performed to screen for changes in apoptotic level with and without PARP1 inhibition. Further, our protocol included an incubation period after PBMC treatment of only 24 hours; however, a 24-hour incubation period was chosen because it was sufficient to allow attachment, but not proliferation, of PBMCs and because use of a 24-hour incubation period allowed consistency with another study.[24]

Findings suggested that PJ34 PARP1 inhibition may reduce TNF-α production in horses, a potential benefit in reducing inflammation and endotoxin-induced damage in horses. Further investigation of the potential use of PJ34 for treating intestinal IRI and endotoxemia in horses is warranted.

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The authors declare that there were no conflicts of interest.

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Footnotes

a. Gibco DPBS with no calcium and no magnesium, Mediatech Inc, Manassas, Va.
b. Ficoll-Hypaque, GE Healthcare Bio-Sciences AB, Uppsala, Sweden.
c. Centrifuge 5810R, Eppendorf AG, Hauppage, NY.
d. Gibco HBSS with no calcium, magnesium, or phenol red, Thermo Fisher Scientific Inc, Waltham, Mass.
e. Hyclone fetal bovine serum, SH30070, Thermo Fisher Scientific Inc, Waltham, Mass.
f. Gibco penicillin-streptomycin (10,000 U/mL), Thermo Fisher Scientific Inc, Waltham, Mass.
g. Gibco trypan blue solution, 0.4%, Thermo Fisher Scientific Inc, Waltham, Mass.
h. Costar clear TC-treated 12-well plates, Corning Inc, Corning, NJ.
i. Human fibronectin, No. 365008, BD Biosciences, San Jose, Calif.
j. Heraeus HERAcell 150, DJB Labcare Ltd, Newport Pagnell, Buckinghamshire, England.
k. Recombinant human INF-γ, No. HC2030, Hycult Biotech Inc, Wayne, Pa.
l. LPS from *Escherichia coli* O55:B5, No. L2880, Millipore Sigma, St Louis, Mo.
m. Equine TNFα ELISA Reagent Kit, ESS0017, Thermo Scientific, Rockford, Ill.
n. Benchmark Plus Microplate Spectrophotometer System, Bio-Rad Laboratories Inc, Hercules, Calif.
o. PJ-34, Enzo Life Sciences Inc, Farmingdale, NY.
p. Veliparib, Enzo Life Sciences Inc, Farmingdale, NY.
q. Olaparib, Selleck Chemicals, Houston, Tex.
r. R: a language and environment for statistical computing, version 3.2.0, R Foundation for Statistical Computing, Vienna, Austria. Available at: www.r-project.org. Accessed January 21, 2019.

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