HIGHLIGHTED TOPIC | Molecular Adaptations to Exercise, Heat Acclimation, and Thermotolerance

Rapid cooling after acute hyperthermia alters intestinal morphology and increases the systemic inflammatory response in pigs

Jay S. Johnson, Avi Sapkota, and Donald C. Lay, Jr.
USDA-ARS Livestock Behavior Research Unit, West Lafayette, Indiana

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HYPERTHERMIA NEGATIVELY IMPACTS human and animal health, and extreme cases (i.e., heatstroke) can result in morbidity and mortality if recovery is not appropriately managed (18, 15, 28, 49). In the U.S. alone, 2,000 residents died per year from 2006 to 2010 because of heat-related illness (5), and global heat-related human deaths are expected to increase 10-fold by midcentury (20). In addition, it is estimated that the U.S. swine industry loses over $300 million per year because of hyperthermia-related losses in production as well as mortality (50). The effects of heat stress (HS) on human health and livestock production will likely worsen, as the frequency of heat waves is predicted to increase over the next century because of climate change, with days over 32.2°C increasing from 60 to 150 annually in the U.S. alone (52). Therefore, because of the expected increase in heat wave frequency and incidences of heat-related maladies for both humans and animals, there is an urgent need to develop and evaluate methods that mitigate the negative effects of heat illness and promote recovery.

Heatstroke is defined as an unchecked increase in body temperature that occurs when intrinsic or extrinsic heat generation overpowers homeostatic thermoregulation (49). To combat heat illness in humans, standard protocols call for cooling procedures that increase the temperature gradient (conduction), water vapor pressure (evaporation), and velocity of air between the skin and the surrounding area (convection) (8, 49, 56). Based on these principles, several techniques have been devised to return the body to euthermia, such as immersion in cold water, ice packs, cooling blankets, wetting the body surface while using fans, and chemically assisted cooling (3, 8, 10, 16, 49, 56). Although most techniques are effective in reducing rectal temperature (TR), many reports conflict regarding the most effective recovery method [as reviewed by Smith (49)], although it is generally accepted that rapid cooling is the most effective therapy (28), despite the fact that it may cause peripheral vasoconstriction and reduce heat dissipation capacity (56). Regardless of the cooling method, multiple organ dysfunction syndrome often occurs even after TR is lowered and intensive care is administered [as reviewed by Bouchama and Knochel (8)]. While specific reasons for this maladaptive response are unclear, previous reports suggest that pathophysiological responses to heatstroke may be due to a systemic inflammatory response that occurs after thermal injury (18, 34, 44) and is attributed to HS-induced enhanced intestinal permeability and increased endotoxin translocation into the bloodstream (27, 34, 40).

Although many studies [as reviewed by Smith (49)] have examined the effects of different cooling methods on reducing body temperature (specifically TR) in humans and rodent species, none to our knowledge has evaluated the direct effects of rapid cooling on the thermal response and intestinal morphol-
ogy in pigs, an animal model that shares a similar physiology and mass with humans. In a preliminary study, we determined that rapidly cooling pigs (immediate exposure to 21°C) after acute HS (constant 39°C) resulted in a 0.3°C increase in gastrointestinal tract temperature (T$_{GI}$) from maximum HS levels (unpublished data), which has obvious implications for cooling rate and could exacerbate the effects of HS on intestinal integrity. Therefore, the study objectives were to compare the direct effects of rapid cooling on body temperature response, intestinal morphology, and inflammatory response after acute hyperthermia in pigs. On the basis of our previous observations, we hypothesized that rapid cooling would directly cause peripheral vasoconstriction resulting in a reduction in heat dissipation capacity and increased intestinal temperature and damage.

MATERIALS AND METHODS

Animals and Study Design

All procedures involving animal use were approved by the Purdue University Animal Care and Use Committee (protocol no. 1410001136). Thirty-six crossbred (¼ Large White × ¾ Landrace × ½ Duroc) castrated male pigs ($88.7 \pm 1.6$ kg body weight (BW)) were housed in individual pens and allowed ad libitum access to feed and water. The diet consisted primarily of corn and soybean meal and was formulated to meet or exceed nutrient requirements [39]. Castrated male pigs were selected in an attempt to reduce the body temperature variability associated with gonadal steroid production, and pig weight was selected to represent the average body weight of U.S. adult males (88.9 kg; Ref. 9). One day before thermal treatments at 1500, 18 pigs in repetitions 1 and 2 were orally administered a calibrated thermodoneal temperature recorder (iButton, accuracy ±0.1°C; Dallas Semiconductor, Maxim, Irving, TX) and 18 pigs in repetitions 3 and 4 were orally administered a calibrated CorTemp temperature sensor (model HT150002, accuracy ±0.1°C; HQ, Palmetto, FL) to monitor $T_{GI}$. To improve data collection accuracy while taking multiple measurements during frequent blood sampling, and to determine the approximate location of temperature data recorders in the gastrointestinal tract, self-recording and temperature storing recorders (iButtons) were used in repetitions 1 and 2 because pigs in these repetitions were euthanized and collection from the gastrointestinal tract was possible. However, because no blood samples were collected in repetitions 3 and 4 (to reduce interaction with pigs during behavior monitoring) and pigs were not euthanized, CorTemp temperature sensors were utilized to determine $T_{GI}$ and recorders were able to pass naturally at the conclusion of the experiment with no long-lasting effects to pig health. The temperature sensors were validated to be of similar accuracy. In each environmental room [1 thermoneutral (TN) room and 1 HS room], % relative humidity (RH) and ambient temperature ($T_a$) were recorded every 5 min with two mounted data loggers (HOBO, data logger temp/RH; Onset, Bourne, MA) for the duration of the experiment.

To evaluate the direct effects of rapid cooling after acute hyperthermia, pigs were subjected to either TN ($n = 3$ reps; $88.0 \pm 1.9$ kg BW, 19.5 ± 0.1°C, 56.9 ± 1.1% RH) for 6 h or HS (36.4 ± 0.1°C, 31.2 ± 0.3% RH) for 3 h, followed by a 3-h recovery period of rapid cooling (HSRC; $n = 3$ rep; 89.7 ± 1.7 kg BW) or gradual cooling (HSGC; $n = 3$ rep; 88.3 ± 1.3 kg BW). Rapid cooling was achieved by moving pigs from the HS room directly into the TN room (1.5-m walking distance) and then pouring 15 gallons of ice water (4.0°C) on the backs of individual pigs every 30 min for 1.5 h (total of 4 times). Water was first poured between the shoulders and the bucket was subsequently moved down the back and ended at the tail, and this procedure lasted ~5 s. The approximate surface area exposed to ice water in HSRC pigs was 6.58 ± 0.12 m² (surface area = 0.0734 × BW$_{kg}$; Ref. 50). Gradual cooling was accomplished by reducing the HS room $T_a$ by 5.0°C every 30 min until TN environmental conditions (20.7 ± 0.3°C, 44.9 ± 0.5% RH) were reached (Fig. 1). All pigs survived the 3-h HS challenge and the 3-h recovery period. Throughout the 6 h of thermal treatments, respiration rate (RR), $T_r$, $T_{GI}$, and whole body skin temperature ($T_{SKIN}$) of individual pigs were measured every 15 min. Thermal measurements began 15 min after each period (HS and recovery) began so pigs could acclimate to their pens. In repetitions 3 and 4, pig behavior was recorded to quantify feeding and drinking behavior during the entire 6-h treatment period with ceiling-mounted cameras (Panasonic WV-CP254H, Matsushita Electric Industrial, Osaka, Japan) attached to a digital video recorder system. Video files were later split into six 1-h videos and analyzed in Observer XT 11.5 (Noldus, Wageningen, The Netherlands) by one trained individual using a continuous sampling technique. Recorded behaviors were feeding (head in feeder) and drinking (snout in contact with waterer), and the percentage of time each pig displayed the behavior was calculated. In all four repetitions, RR (breaths/min) was determined by counting flanks movement for 15 s and then multiplying by 4, $T_r$ was measured with a calibrated and lubricated thermistor thermometer (Cooper Atkins model TM99A, accuracy ±0.2°C; Middlefield, CT) inserted ~10 cm into the rectum of unrestrained pigs, and $T_{GI}$ was measured with iButtons (repetitions 1 and 2) or CorTemp temperature sensors (repetitions 3 and 4). Water drinking bouts were recorded for individual animals, and $T_{GI}$ data collected below biologically relevant levels for pigs (<36.0°C) during drinking bouts were removed from the final analysis. Whole body $T_{SKIN}$ was measured in all four repetitions by taking a broadside photo of individual pigs with an infrared camera (FLIR-T62101; FLIR Systems, Wilsonville, OR), and photos were analyzed with FLIR Tools software (version 2.1). A thermal circulation index (TCI) was calculated with $T_{GI}$, $T_{SKIN}$, and $T_a$ to quantify blood and heat transfer to the skin as described for livestock species [TCI = ($T_{SKIN}$ − $T_a$)/($T_{GI}$ − $T_{SKIN}$); Ref. 11].

Immediately after the 3-h recovery period at 180 min, pigs from repetitions 1 and 2 ($n = 6$ treatment) were humanely euthanized by captive bolt and exsanguination. Thermochron temperature recorders (iButtons) were collected from the gastrointestinal tract (located within the duodenum). Whole tissue samples from the duodenum (1 m anterior from the pyloric sphincter), ileum (1 m proximal from the ileo-caecal junction), colon (1 m proximal from the rectum), and liver were collected, immediately snap frozen in liquid nitrogen, and stored at −80°C. In addition, a portion of the duodenum, ileum, and colon from intestinal sections with minimal contents was placed in 10% formalin without rinsing or abrasion and preserved for histology.

![Fig. 1. Ambient temperature ($T_a$) by time point in the heat stress and recovery periods. TN Room, thermoneutral room; HS Room, heat stress room.](http://jap.physiology.org)
Blood Sampling and Analysis

Two blood tubes (serum and EDTA; 5 ml) were obtained from restrained pigs via jugular venipuncture (BD Vacutainers; Franklin Lakes, NJ; K-EDETA, serum) 1 day before thermal treatments at 1600, at 60 and 180 min during the HS period, and at 30 and 60 min during the recovery period. Plasma and serum were harvested by centrifugation at 4°C and 2,500 rpm for 15 min, divided into aliquots, and stored at −80°C. ELISA kits were used to determine plasma TNF-α (TNF-α Pig ELISA Kit; Abcam, Cambridge, MA) and IL-1β (IL-1β Pig ELISA Kit; Abcam) concentrations according to the manufacturer’s instructions. Serum endotoxin (LPS) concentrations were determined with a Limulus amebocyte lysate (LAL) endotoxin assay with a 1/1,000 dilution (Pierce LAL Chromogenic Endotoxin Quantitation Kit; Thermo Fisher Scientific, Rockford, IL) and processed according to the manufacturer’s instructions. Briefly, serum samples were diluted 1/10 in endotoxin-free water and then heated at 70°C for 20 min. After heating, samples were further diluted in endotoxin-free water to a 1/100 dilution. The intra-assay coefficients of variation were 7.9%, 13.2%, and 2.7% for endotoxin, TNF-α, and IL-1β, respectively, and the interassay coefficients of variation were 16.4%, 24.4%, and 7.61% for endotoxin, TNF-α, and IL-1β, respectively.

Histology

Duodenum, ileum, and colon tissue samples were placed into a 10% formalin solution for 24 h and then transferred to a 70% ethanol solution. Fixed samples were referred to the Purdue University Histology and Phenotyping Laboratory, where they were sectioned at 10-μm thickness and three random sections were stained with hematoxylin and eosin staining. Each section was imaged twice (n = 6 images/tissue per pig) with Q-capture Pro 6.0 software (Qimaging, Surrey, BC, Canada). One trained individual who was blind to treatments determined mean villus height (μm), mean villus width (μm), and percentage of total villi with autolysis [as described by Pearce and colleagues (42)] for duodenum and ileum samples and mean crypt depth (μm) for duodenum, ileum, and colon samples with ImageJ 1.47v software (National Institutes of Health, Bethesda, MD). Average villus height, villus width, crypt depth, and percent autolysis for each image were used in the final analysis.

Tissue TNF-α, IL-1β, and Hsp70 Analysis

Whole cell protein from duodenum, ileum, colon, and liver tissue was extracted with a commercially available extraction buffer (T-PER Tissue Protein Extraction Reagent; Life Technologies, Thermo Fisher Scientific) with protease and phosphatase inhibitors at a final concentration of 1× (HALT Protease and Phosphatase Inhibitor Cocktail, EDTA free; Thermo Fisher Scientific). The lysis buffer was added at a concentration of 20 mL/g tissue, and samples were homogenized on ice. Homogenized samples were centrifuged at 4°C for 20 min at 13,000 rpm. The resulting supernatant was divided into aliquots, and total protein concentration was determined by BCA protein assay (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific). ELISA kits were used to determine TNF-α (TNF-α Pig ELISA Kit; Abcam), IL-1β (IL-1β Pig ELISA Kit; Abcam), and Hsp70 (Porcine HSP 70 ELISA Kit; NEO Biolab, Cambridge, MA) concentrations according to the manufacturer’s instructions for tissue and cell lysates. All tissue and cell lysate samples were run on the same assay to avoid interassay expression. The intra-assay coefficients of variation were 8.3%, 7.5%, 4.9%, and 0.5% for Hsp70 in duodenum, ileum, colon, and liver tissue; 25.2%, 9.5%, 2.7%, and 1.6% for TNF-α in duodenum, ileum, colon, and liver tissue; and 0.8%, 7.4%, 4.0%, and 3.5% for IL-1β in duodenum, ileum, colon, and liver tissue.

Statistics

All data were analyzed with the PROC MIXED procedure in SAS 9.4 (SAS Institute, Cary, NC). Data within the HS and recovery periods were analyzed separately, and analyses were conducted with statistical model components including recovery treatment (TN, HSRC, HSGC), time point within period (15-180 min), and all possible interactions. The statistical model for HS and recovery was $Y_{ijk} = \mu + R_i + T_j + R \times T_j + e_{ijk}$, where $Y$ = parameter of interest, $\mu$ = mean, $R$ = recovery treatment, $T$ = time point within period, and $e$ = residual. For the HS and recovery period analyses, preplanned statistical comparisons were conducted for TN vs. HSRC and HSGC pigs and HSRC vs. HSGC pigs with the CONTRAST statement of SAS. Repetition was used as a random effect. Pretreatment blood samples were used as a covariate in all blood parameter analyses. Because no differences in time point were detected for blood parameters, it was removed from the model and only the recovery treatment effect was reported. For repeated analyses, each pig’s respective parameter was analyzed with repeated measures with an autoregressive covariance structure with time point within the HS or recovery periods as the repeated effect.

To compare data across periods, data within each period were averaged for individual pigs and statistical model components included recovery treatment (TN, HSRC, HSGC), period (HS, recovery), and all possible interactions. The statistical model for period analyses was $Y_{ijk} = \mu + R_i + P_j + R \times P_j + e_{ijk}$, where $Y$ = parameter of interest, $\mu$ = mean, $R$ = recovery treatment, $P$ = period, and $e$ = residual. All interactions, regardless of significance level, were included in the model, and repetition was used as a random effect. Data are presented as least-squares means, statistical significance was defined as $P \leq 0.05$, and a tendency was defined as $0.05 < P \leq 0.10$.

RESULTS

Gastrointestinal and rectal temperature. During the HS period, $T_R$ was greater ($P = 0.01; 1.22°C$) at every time point except at 15 min in HSRC and HSGC pigs compared with TN control pigs (Table 1; Fig. 2A). Although no differences were detected between HSRC and HSGC pigs, minimum and maximum $T_R$ were greater ($P = 0.01; 0.87 and 1.43°C$, respectively) for HSRC and HSGC pigs compared with TN control pigs (Table 1). $T_{GI}$ was greater ($P = 0.01$) at every time point during the HS period in HSRC and HSGC pigs ($1.61$ and $1.51°C$, respectively) compared with TN control pigs (Table 1; Fig. 2B). While minimum and maximum $T_{GI}$ were similar between HSRC ($40.10$ and $41.46°C$, respectively) and HSGC ($40.00$ and $41.50°C$, respectively) pigs, both minimum and maximum $T_{GI}$ were greater ($P = 0.01; 1.28$ and $1.83°C$, respectively) in HS-exposed compared with TN pigs (Table 1). No $T_R$ and $T_{GI}$ differences were detected between HSRC and HSGC pigs ($40.12$ and $40.88°C$, respectively) at any time point during the HS period (Table 1; Fig. 2A and B). Although $T_R$ and $T_{GI}$ were greater in HSGC and HSRC pigs compared with TN control pigs (Table 1), the overall $T_{GI}$ vs. $T_R$ difference was similar ($P = 0.44; 0.67°C$) in all recovery treatments and at every time point during the HS period (Table 1).

During the recovery period, overall $T_R$ was greater ($P = 0.01$) in HSRC ($39.38°C$) and HSGC ($39.74°C$) pigs compared with TN control pigs (Table 1; Fig. 2A). Minimum $T_R$ was greater ($P = 0.01; 0.42°C$) in HSRC and HSGC pigs compared with TN control pigs, but no differences were detected between HSRC ($38.90°C$) and HSGC ($38.98°C$; Table 1) pigs. Maximum $T_R$ was greater ($P = 0.01; 1.11°C$) in HSRC and HSGC pigs compared with...
TN control pigs; however, no differences were detected between HSRC (40.23°C) and HSGC (40.52°C) pigs (Table 1). A recovery treatment × time point interaction was detected \((P = 0.02)\), where \(T_R\) was reduced in HSRC compared with HSGC pigs from 30 to 90 min and was greater in HSRC and HSGC pigs compared with TN control pigs from 15 to 105 min. However, from 120 to 180 min, \(T_R\) in all recovery treatments were similar (Fig. 2A). Following a similar trend as \(T_R\), \(T_{GI}\) was greater overall \((P < 0.05)\) and maximum \(T_{SKIN}\) were similar between HSRC (40.23°C) and HSGC (40.52°C) pigs (Table 1). A

<table>
<thead>
<tr>
<th>Recovery period</th>
<th>TN</th>
<th>HSRC</th>
<th>HSGC</th>
<th>SD</th>
<th>R</th>
<th>T</th>
<th>R × T</th>
<th>Contrast 1</th>
<th>Contrast 2</th>
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<tr>
<td>(T_{GI}, {° C})</td>
<td>39.32(^a)</td>
<td>40.92(^b)</td>
<td>40.82(^b)</td>
<td>1.04</td>
<td>0.01</td>
<td>0.01</td>
<td>0.12</td>
<td>0.01</td>
<td>0.69</td>
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<td>Min (T_{GI}, {° C})</td>
<td>38.77(^a)</td>
<td>40.10(^b)</td>
<td>40.00(^b)</td>
<td>0.98</td>
<td>0.01</td>
<td>0.01</td>
<td>0.18</td>
<td>0.01</td>
<td>0.76</td>
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<tr>
<td>Max (T_{GI}, {° C})</td>
<td>39.65(^a)</td>
<td>41.46(^b)</td>
<td>41.50(^b)</td>
<td>1.08</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
<td>0.87</td>
</tr>
<tr>
<td>(T_R, {° C})</td>
<td>38.80(^a)</td>
<td>40.16(^b)</td>
<td>40.07(^b)</td>
<td>0.91</td>
<td>0.01</td>
<td>0.01</td>
<td>0.18</td>
<td>0.01</td>
<td>0.68</td>
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<td>Min (T_R, {° C})</td>
<td>38.51(^a)</td>
<td>39.57(^b)</td>
<td>39.18(^b)</td>
<td>0.67</td>
<td>0.01</td>
<td>0.01</td>
<td>0.07</td>
<td>0.01</td>
<td>0.87</td>
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<td>Max (T_R, {° C})</td>
<td>39.22(^a)</td>
<td>40.62(^b)</td>
<td>40.69(^b)</td>
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<td>(T_{SKIN}, {° C})</td>
<td>29.91(^a)</td>
<td>39.00(^b)</td>
<td>39.52(^b)</td>
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<td>Min (T_{SKIN}, {° C})</td>
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<td>37.15(^b)</td>
<td>36.96(^b)</td>
<td>5.54</td>
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<td>0.01</td>
<td>0.66</td>
</tr>
<tr>
<td>Max (T_{SKIN}, {° C})</td>
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<td>40.48(^b)</td>
<td>40.53(^b)</td>
<td>3.22</td>
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<td>0.01</td>
<td>0.32</td>
<td>0.01</td>
<td>0.83</td>
</tr>
<tr>
<td>(Δ T_{GI}) and (T_{SKIN}, {° C})</td>
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<td>0.74</td>
<td>0.77</td>
<td>0.70</td>
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<td>38(^a)</td>
<td>111(^b)</td>
<td>111(^b)</td>
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<td>0.01</td>
<td>0.01</td>
<td>0.11</td>
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<tr>
<td>TCL, AU</td>
<td>1.31(^a)</td>
<td>0.69(^b)</td>
<td>0.64(^b)</td>
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<td>0.06</td>
<td>0.02</td>
<td>0.96</td>
<td>0.01</td>
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</table>

**Table 1.** Thermal index differences in pigs rapidly or gradually cooled after acute hyperthermia

**Recovery treatment**

| TN, thermoneutral; HSRC, heat stress and rapid cooling; HSGC, heat stress and gradual cooling; R, recovery treatment; T, time; contrast 1, HSRC and HSGC vs. TN; contrast 2, HSRC vs. HSGC; \(T_{GI}\), gastrointestinal tract temperature; Min, minimum; Max, maximum; \(T_R\), rectal temperature; \(T_{SKIN}\), skin temperature; RR, respiration rate; TCL, thermal circulation index; AU, arbitrary units. Superscript letters \(a < b < c\) within a row indicate recovery treatment tendencies \((P < 0.05)\); superscript letters \(x < y\) within a time point indicate treatment tendencies \((0.05 < P < 0.10)\).

**Whole body skin temperature and thermal circulation index.** Overall, HS treatment increased \((P = 0.01; 9.35°C)\) \(T_{SKIN}\) of HSRC and HSGC pigs compared with TN control pigs at every time point measured, but no differences were detected between HSRC (39.00°C) and HSGC (39.52°C) pigs (Table 1; Fig. 2C). Although no differences were detected between HSRC and HSGC pigs, overall minimum and maximum \(T_{SKIN}\) were greater in HSRC and HSGC pigs compared with TN control pigs by 10.91 and 10.72°C and 6.35 and 6.41°C, respectively (Table 1). During the HS period, TCL tended to be reduced \((P = 0.06; 150.8\%)\) in HSRC and HSGC pigs compared with TN control pigs; however, no differences were detected between HSRC \((-0.69\%)\) and HSGC \((-0.64\%)\) pigs (Table 1; Fig. 2D).

During the recovery period, overall \(T_{SKIN}\) of HSRC pigs was greater \((P = 0.01; 5.63°C)\) and overall \(T_{SKIN}\) of HSGC pigs was reduced \((P = 0.01; 2.19°C)\) compared with TN control pigs (Table 1; Fig. 2C). In addition, overall \(T_{SKIN}\) of HSRC pigs was reduced \((P = 0.01\); 7.82°C) compared with HSGC pigs at every measured time point during recovery (Table 1; Fig. 2C). Although \(T_{SKIN}\) of HSRC pigs was reduced overall compared with TN control pigs during the recovery period, it was similar at 15, 45, 150, and 165 min (Fig. 2C). When considering overall minimum and maximum \(T_{SKIN}\) throughout the recovery period, HSRC pigs had reduced \((P = 0.01; 1.36\) and 1.55°C, respectively) and HSGC pigs had greater \((P = 0.01; 6.68\) and 2.56°C, respectively) minimum and maximum \(T_{SKIN}\) compared with TN control pigs (Table 1). Furthermore, minimum and maximum \(T_{SKIN}\) were reduced \((P = 0.01; 7.82°C)\) in HSRC compared with HSGC pigs (Table 1). During the recovery period, TCL was reduced \((P = 0.01; 53.5\%)\) in HSRC compared with both HSGC pigs and TN control pigs (Table 1; Fig. 2D).
Respiration Rate

During the HS period, overall RR was greater (P = 0.01; 73 breaths/min) in HSRC and HSGC pigs compared with TN control pigs; however, no differences were detected between HSRC and HSGC pigs (Table 1). Respiration rate was greater (P = 0.01) in HSGC pigs compared with HSRC pigs and TN control pigs by 22 and 28 breaths/min, respectively, during the recovery period, but no differences were detected between HSRC pigs and TN control pigs (Table 1).

Feeding and Drinking Behavior

During the HS period, no drinking differences were observed with any comparison (P = 0.84; 1.2% of hour; Fig. 3A); however, feeding was reduced (P = 0.01; 94%) in HSRC and HSGC pigs compared with TN control pigs (Fig. 3B). No hour or hour × recovery treatment differences were observed for feeding (P = 0.65) during the HS period (Fig. 3B).

In the recovery period, no recovery treatment (P = 0.59) or recovery treatment × hour (P = 0.22) effects were observed for drinking (Fig. 3A); however, an overall hour effect was detected, where drinking was greater (P = 0.01) during hour 3 compared with hours 1 and 2 by 68.3% and 79.9%, respectively (Fig. 3A). Overall, feeding was greater (P = 0.01; 240.7%) in TN and HSRC compared with HSGC pigs, but no differences were detected between HSRC and TN pigs during the recovery period (Fig. 3B). Although no time effect was observed (P = 0.49), a recovery treatment × time effect was detected, where feeding was greater during hours 1 and 2 (P = 0.05) in TN (11.43% and 13.65% of hour, respectively) and HSRC (20.5% and 19.7% of hour, respectively) compared with HSGC (0.01% and 0.10% of hour, respectively) pigs (Fig. 3B).

Overall, no differences (P = 0.56) in drinking were detected between the HS and recovery periods; however, feeding was greater (P = 0.01; 153.2%) during the recovery period compared with the HS period regardless of recovery treatment (Fig. 3B).

Blood Parameters

During the HS period, serum LPS was similar [P = 0.56; 56.2 endotoxin units (EU)/ml] in all pigs (Fig. 4A). TNF-α was greater (P = 0.04; 27.5%) in HSRC and HSGC pigs compared with TN control pigs during the HS period, but no differences were detected when comparing HSRC (37.9 pg/ml) vs. HSGC (37.3 pg/ml) pigs (Fig. 4B). Plasma IL-1β was similar (P = 0.61; 10.2 ± 14.8 pg/ml) in all treatment groups during HS (data not presented).
compared with HSRC pigs by 34.4% (Fig. 5C). No differences (P > 0.55) in villus autolysis were detected between TN (4.42 ± 7.46% autolysis), HSRC (11.96 ± 25.9% autolysis), and HSGC (2.04 ± 7.48% autolysis) pigs (Fig. 5).

Villus height in the ileum was decreased (P = 0.01; 38.9%) in HSRC compared with HSGC pigs and TN control pigs, but no differences were detected between HSGC and TN pigs (Fig. 5A). Ileal crypt depth was greater (P = 0.05; 19.5%) in HSRC pigs compared with HSGC and TN pigs; however, no differences were observed when comparing HSGC (137.8 ± 29.8 μm) and TN (139.2 ± 37.5 μm) pigs (Fig. 5B). The villus height-to-crypt depth ratio was reduced (P = 0.01; 46.0%) in HSRC compared with HSGC pigs and TN control pigs, but no differences were detected between HSGC (1.65 ± 0.44) and TN (1.85 ± 0.51) pigs (Fig. 5C). No differences (P > 0.42) in villus autolysis were detected between TN (1.26 ± 0.05) and HSGC (1.34 ± 0.01) pigs (Fig. 5). Colon crypt depth was decreased (P = 0.01) in HSRC (41.6%) and HSGC (32.7%) compared with TN control pigs (216.1 ± 49.9 μm; Fig. 5B). No differences were observed between HSRC (173.4 ± 56.7 μm) and HSGC (155.8 ± 30.9 μm) pigs (Fig. 5B).

Histology

In the duodenum, villus height was reduced (P = 0.01) in HSRC (40.7%) and HSGC (18.0%) compared with TN control pigs and was reduced (29.6%) in HSRC compared with HSGC pigs (Fig. 5A). Crypt depth in the duodenum tended to be greater (P = 0.06; 11.6%) in HSRC compared with HSGC and TN pigs; however, no differences were detected between HSGC pigs (188.8 ± 35.0 μm) and TN control pigs (190.0 ± 41.9 μm; Fig. 5B). The villus height-to-crypt depth ratio was reduced (P = 0.01) in HSRC (47.2%) and HSGC (19.7%) compared with TN control pigs and was reduced in HSRC
**Tissue Analysis**

In the liver, TNF-α levels tended to be greater \((P = 0.07; 20.1 \text{ pg/mg})\) in HSRC compared with HSGC pigs (Table 2); however, no differences were detected between HSRC and TN pigs (Table 2). TNF-α was decreased \((P = 0.03)\) in the duodenum and ileum of HSRC compared with HSGC pigs and TN control pigs by 41.8% and 21.3%, respectively, but no differences were detected between HSGC and TN pigs (Table 2). No colon TNF-α differences \((P = 0.26)\) were observed between recovery treatments (Table 2).

When comparing all recovery treatment groups, IL-1β was similar \((P = 0.24)\) in the liver \((8.54 \text{ pg/mg})\), duodenum \((51.81 \text{ pg/mg})\), and ileum \((64.25 \text{ pg/mg})\) tissue (Table 2). In the colon, IL-1β was greater \((P = 0.03)\) in HSRC \((66.78 \text{ pg/mg})\) and HSGC \((92.29 \text{ pg/mg})\) pigs compared with TN control pigs (Table 2). Furthermore, IL-1β was similar \((103.83 \text{ pg/mg})\) in the colon of HSGC and HSRC pigs (Table 2).

No Hsp70 recovery treatment differences \((P = 0.16)\) were detected in the liver \((1.20 \text{ ng/mg})\) or the colon \((12.13 \text{ ng/mg})\) (Table 2). In the duodenum, Hsp70 tended to be greater \((P = 0.10; 2.07 \text{ ng/mg})\) in HSRC and HSGC pigs compared with TN control pigs, but no differences were detected between HSRC and HSGC pigs (Table 2). Furthermore, in the ileum, Hsp70 tended to be greater \((P = 0.08)\) in HSRC \((79.9\%)\) and HSGC \((41.7\%)\) pigs compared with TN control pigs; however, no differences were detected between HSRC and HSGC pigs (Table 2).

**DISCUSSION**

Over the next century the frequency of intense heat waves is predicted to increase (52), and as a consequence it is likely that heat-related morbidity and mortality in humans and animals will become more widespread. Although current protocols (i.e., immersion in cold water, ice packs, cooling blankets, evaporative cooling, etc.) are successful in returning the body to euthermia after exertional or classic heatstroke \(\text{[based on TR; as reviewed by Smith (49)]}\), multiple organ dysfunction syndrome and death can often occur even after body temperature is reduced \((8, 25, 34)\). Therefore, it is imperative that heat illness mitigation and recovery methods are developed and improved to reduce the impact of acute hyperthermia on both human and animal health.

Hyperthermic mammals partition blood to the periphery in order to maximize heat dissipation through vasodilation \((6, 47)\). To support this blood redistribution, gastrointestinal tract vasoconstriction occurs \((47)\), leading to hypoxia and loss of intestinal integrity \((26, 33, 34, 41)\) since enterocytes are sensitive to oxygen and nutrient restriction \((46)\). In accordance with the aforementioned reports, morphological markers of reduced intestinal integrity and increased damage were ob-
served in the present study for pigs exposed to acute HS compared with TN control pigs. Specifically, HSRC and HSGC treatments reduced villus height in the duodenum and ileum, villus height-to-crypt depth ratio in the duodenum and ileum, and colon crypt depth compared with TN control pigs (Fig. 5), which are morphological measures associated with intestinal epithelium damage and integrity as previously described in HS-exposed pigs (41, 42, 57). HS-induced intestinal injury likely occurs before other organ damage because reduced blood flow affects the intestine first (41), and this damage may affect nutrient digestion and absorption (33), and increase intestinal permeability, leading to increased endotoxin translocation into the blood (25). In addition to the effects of HS, rapid cooling recovery treatment appears to have directly exacerbated these intestinal morphological changes as indicated by 27.6% and 33.6% reduction in villus height, 34.4% and 43.1% increase in villus height-to-crypt depth ratio, and 486% and 410% numerical increase in villus autolysis in the duodenum and ileum, respectively, compared with HSGC pigs (Fig. 5). Although the specific molecular mechanisms responsible for these physiological changes are currently unknown, several factors including thermal heat exchange and intestinal reperfusion may contribute to altered intestinal morphology in HSRC compared with HSGC pigs.

In the present study, 3 h of acute HS resulted in an increase in maximum $T_R$ and $T_Gt$ of HS-exposed pigs by 40.66 and 41.48°C, respectively, and this maximum increase was sustained for the final hour of the HS period immediately before recovery treatment. In addition, $T_{SKIN}$ of HSRC and HSGC pigs was increased compared with TN control pigs, indicating that body heat was being dissipated through the skin during the HS period, since increased $T_{SKIN}$ is associated with vasodilation to support enhanced body heat dissipation (6). Because vasoconstriction of the gastrointestinal tract occurs to support this vasodilation at the skin during HS (26, 47), it is likely that ischemia of the gut occurred during the HS period (22), which could partially contribute to the intestinal morphological differences observed in HSRC and HSGC pigs compared with TN control pigs.

During the recovery period, both rapid and gradual cooling procedures reduced $T_R$ and $T_Gt$ compared with the HS period, but $T_Gt$ was reduced more effectively by the rapid cooling treatment (Fig. 2A). This result was expected, as numerous reports (12, 13, 43) and reviews (28, 49) indicate that rapid cooling (i.e., ice water immersion or dousing to increase the temperature gradient between the core and the skin surface) is the most effective method of returning $T_R$ to euthermia after heatstroke or acute hyperthermia. However, in contrast to numerous reports in rodent heatstroke models [e.g., rats, mice, guinea pigs; as reviewed by Leon (28)] and a report in humans (36), pigs did not become hypothermic (based on $T_R$ and $T_Gt$) regardless of the recovery method used. While specific reasons are unclear, the greater skin surface area-to-body mass ratio and reduced skin thickness in rodents compared with pigs could be one explanation since they are associated with an enhanced ability to dissipate body heat through the skin (6). Therefore, when the thermal gradient is rapidly increased in rodents after heatstroke it may cause excessive heat loss that cannot be compensated for by an increase in metabolic heat production as suggested by Romanovsky and Blatteis (47), eventually resulting in hypothermia. Alternatively, it is possible that if pigs in the present study were more severely heat-stressed they might have become hypothermic during the recovery period. Nevertheless, because pigs and humans share a similar surface area-to-body mass ratio as well as similar skin thickness (49), it could be hypothesized that their core body temperature response would be similar during recovery from this level of acute hyperthermia.

Despite the fact that $T_R$ cooling time was faster in HSRC pigs (Fig. 2A), no overall recovery treatment differences were detected in $T_{Gt}$ during the recovery period, although there was a slight reduction for HSRC pigs in the first hour (Fig. 2B). While the initial decrease in $T_{Gt}$ during rapid cooling is in contrast to our preliminary data, a likely explanation is that the increased feeding behavior of HSRC pigs during the first 2 h of recovery (Fig. 3B) may have resulted in the initial $T_{Gt}$ reduction since data recorders were located in the duodenum distal to the stomach and feed was at TN room temperature. In addition, in light of our preliminary experiment in which pigs were fasted (unpublished data), it is likely that if feed were withheld during the recovery period in the present study, $T_{Gt}$ would have been even greater in HSGC pigs. Regardless of this initial decrease, increased $T_{Gt}$ was maintained overall during the recovery period for HSRC pigs despite the ice water treatment and rapid reduction in $T_R$, and this may be due to peripheral vasoconstriction that prevented effective heat dissipation through the skin. Extensive reviews by Smith (49) and Bouchama and Knochel (8) have suggested that, in practice, applying cold water or ice to the skin may trigger cutaneous vasoconstriction, therefore compromising blood flow and reducing effective heat dissipation in heatstroke victims. In the present study, $T_{SKIN}$ of rapidly cooled pigs was reduced to 27.22°C during the recovery period, and this reduction is lower than the 30.00°C $T_{SKIN}$ threshold proposed to prevent peripheral vasoconstriction (8). In addition to reducing $T_{SKIN}$, TCI was reduced in HSRC compared with HSGC pigs, further validating that the ability to dissipate heat through the skin was compromised in HSRC pigs despite the apparent enhanced

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Table 2. Tissue TNF-α, IL-1 β, and Hsp70 in pigs rapidly or gradually cooled after acute hyperthermia

<table>
<thead>
<tr>
<th>Recovery Treatment</th>
<th>TN</th>
<th>HSP</th>
<th>HSG</th>
<th>SD</th>
<th>R</th>
<th>Contrast 1</th>
<th>Contrast 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α, pg/mg</td>
<td>10.48</td>
<td>22.18</td>
<td>2.08</td>
<td>17.82</td>
<td>0.18</td>
<td>0.81</td>
<td>0.07</td>
</tr>
<tr>
<td>IL-1β, pg/mg</td>
<td>6.95</td>
<td>13.76</td>
<td>4.92</td>
<td>8.64</td>
<td>0.26</td>
<td>0.59</td>
<td>0.12</td>
</tr>
<tr>
<td>Hsp70, ng/mg</td>
<td>0.99</td>
<td>1.23</td>
<td>1.38</td>
<td>0.34</td>
<td>0.24</td>
<td>0.13</td>
<td>0.46</td>
</tr>
<tr>
<td>Duodenum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α, pg/mg</td>
<td>55.51</td>
<td>35.78</td>
<td>6.72</td>
<td>25.22</td>
<td>0.04</td>
<td>0.69</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-1β, pg/mg</td>
<td>43.13</td>
<td>58.03</td>
<td>54.26</td>
<td>25.42</td>
<td>0.60</td>
<td>0.34</td>
<td>0.81</td>
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<tr>
<td>Hsp70, ng/mg</td>
<td>1.00</td>
<td>2.09</td>
<td>2.05</td>
<td>1.19</td>
<td>0.24</td>
<td>0.10</td>
<td>0.95</td>
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<tr>
<td>Ileum</td>
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<tr>
<td>TNF-α, pg/mg</td>
<td>50.29</td>
<td>38.52</td>
<td>47.59</td>
<td>13.51</td>
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<td>IL-1β, pg/mg</td>
<td>55.39</td>
<td>76.35</td>
<td>61.00</td>
<td>21.71</td>
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<td>0.25</td>
<td>0.28</td>
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<tr>
<td>Hsp70, ng/mg</td>
<td>2.83</td>
<td>5.09</td>
<td>4.01</td>
<td>1.81</td>
<td>0.16</td>
<td>0.08</td>
<td>0.32</td>
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<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TNF-α, pg/mg</td>
<td>2.79</td>
<td>&lt;0.01</td>
<td>0.91</td>
<td>2.93</td>
<td>0.26</td>
<td>0.12</td>
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<tr>
<td>IL-1β, pg/mg</td>
<td>24.29</td>
<td>91.07</td>
<td>116.58</td>
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<tr>
<td>Hsp70, ng/mg</td>
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<td>13.31</td>
<td>12.74</td>
<td>4.78</td>
<td>0.33</td>
<td>0.15</td>
<td>0.78</td>
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</tbody>
</table>

Contrast 1. HSRC and HSGC vs. TN. contrast 2. HSRC vs. HSGC; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; Hsp70, heat shock protein 70. Superscript letters a < b within a row indicate recovery treatment differences (P ≤ 0.05).
thermal gradient [i.e., greater TGI-to-TSKIN ratio in HSRC (1.5:1) vs. HSGC pigs (1.2:1)]. All together, these data indicate that rapid cooling procedures caused peripheral vasoconstriction, which may have stimulated a rapid reperfusion of the gut with heated blood resulting in a sustained increase in TGI and likely contributed to the altered intestinal morphology and potential reduction in intestinal integrity in HSRC pigs.

Heat stress causes a well-documented reduction in feed intake in pigs (21), and in the present study HS treatment reduced feeding behavior (94%) in HSRC and HSGC pigs compared with TN control pigs; however, during the recovery period, feeding behavior rapidly rebounded to TN levels in the first 2 h for HSRC pigs and by hour 3 in HSGC pigs (Fig. 3B). Although it is possible that increased feeding behavior (i.e., feed intake) during the initial 2 h of recovery may have contributed to the altered intestinal morphology in HSRC compared with HSGC pigs (24), intestinal passage rate in pigs is estimated at 30 h (17), which is significantly longer than the 3-h recovery period during which pigs were monitored in the present study. Nevertheless, the increase in feeding behavior immediately following fasting could partially explain the altered intestinal morphology in HSRC pigs because of the associated increase in intestinal blood flow due to refeeding (4); however, it is important to note that HSGC pigs had a similar increase in refeeding behavior by hour 3 of recovery (Fig. 3B).

A repercussion of HS-induced intestinal damage is intestinal barrier dysfunction (i.e., increased intestinal permeability) that has the potential to induce pathological conditions in victims (25, 26, 40). Previous reports indicate that heatstroke victims do not necessarily succumb to hyperthermia but to a systemic inflammatory response that occurs after thermal injury (18, 25, 34, 44). These pathological conditions are thought to arise because of LPS leakage from damaged intestinal lumen into circulation (21, 25, 26, 40), which causes an elevated immune response (i.e., cytokine production; Refs. 7, 18) ultimately leading to multiple organ failure and cardiovascular collapse [as reviewed by Lambert (27)]. In the present study, although circulating LPS was similar in all recovery treatments during the acute HS period, it was increased by 60% in HSRC compared with HSGC and TN pigs during the recovery period, and this increase in circulating LPS was likely a consequence of increased intestinal permeability (25, 40). In accordance with the increase in circulating LPS during the recovery period, we observed a 37% increase in circulating TNF-α likely indicating an elevated immune response due to increased LPS presence in the body, and these observations are similar to those described by Bouchama and colleagues (7) in rodent species. However, despite the observed increase in circulating LPS and TNF-α concentrations, no differences were observed in circulating IL-1β in the present study, although there are many conflicting results regarding the presence and role of IL-1β after hyperthermia that could be due to species, sample collection timing, or use of anesthesia in experiments (18, 55).

For example, while some studies in rodent species describe an increase in circulating IL-1β during heatstroke (18, 30, 31, 32), others observe no differences in mice (29) or actually reduced levels in heat-stressed pigs (40). Regardless, the increase in circulating LPS and TNF-α observed in HSRC pigs could be indicative of a systemic inflammatory response initiated by the effects of acute hyperthermia and rapid cooling on intestinal epithelial damage.

In accordance with an increase in circulating cytokines, many studies [as reviewed by Leon (28)] report a corresponding elevation in tissue cytokine levels in hyperthermic animal models. In the present study, TNF-α tended to be greater in the liver of HSRC compared with HSGC pigs, and IL-1β was increased in the colon tissue of HSRC and HSGC pigs compared with TN control pigs. However, tissue TNF-α was actually reduced in the duodenum and ileum of HSRC compared with HSGC pigs (Table 2). While these observations are in contrast to the increase in circulating TNF-α (Fig. 4B), this difference may be associated with an increase in intestinal Hsp70 concentrations in HS-exposed pigs. Hsp70 protects against thermal stress (23), intestinal ischemia and reperfusion (45), and sepsis (19, 53), and Hsp70 synthesis is increased in direct proportion to HS severity (37). Furthermore, Hsp70 can reduce TLR-4 signaling in enterocytes (2), likely resulting in a decrease in NF-κB activation and proinflammatory response in the intestinal tissue of HSRC pigs; however, the reason why a similar reduction in tissue TNF-α did not occur for HSGC pigs is currently unclear. Alternatively, the peak immune response may have occurred before the time point when pigs were euthanized (i.e., the last blood sample was taken 60 min into recovery, and pigs were euthanized at 180 min), and a down-regulation of duodenal and ileal tissue cytokine response may have been measured in HSRC pigs (40, 55).

Several animal models have been developed to study the thermal and physiological responses to heatstroke, including mice (1, 18, 34), rats (1, 44), guinea pigs (1, 44), dogs (1), cats (1), rabbits (1, 30), and lower primate species (14); however, none to our knowledge has attempted to use pigs as experimental models for humans despite their physiological and anatomical similarities and the widespread use of pigs as a biomedical model for disease states (35, 38, 54). In the present study, rapid cooling successfully returned T R to eutermic levels [similar to reports in human subjects; as reviewed by Smith (49)]; however, in accordance with our preliminary data (unpublished data), rapid cooling recovery treatment after acute hyperthermia failed to effectively reduce T R, and this was likely a result of peripheral vasoconstriction that prevented effective heat dissipation through the skin. As a likely consequence, we observed an increase in morphological markers of intestinal epithelial damage and a corresponding elevation in circulating LPS and cytokines during the recovery period. Despite the fact that rapid cooling can increase heatstroke survivability (28), these results provide initial evidence (in a more advanced phylogenetic model) that current rapid cooling protocols may have unintended pathophysiological consequences that may contribute to multiple organ dysfunction syndrome that often occurs even after body temperature is reduced (8, 25, 34), which could lead to an increase in post-heatstroke recovery time and potential complications. With this in mind, it is important to recognize that some limitations to the pig model (such as their inability to sweat) may exist; however, we propose that this pig model may offer a close representation of thermal heat exchange and the corresponding biological consequences in humans suffering from heatstroke because of similar skin thickness and surface area-to-body mass ratio and their organ size and physiology (35, 38, 54). Furthermore, a benefit to the present study was the ability to gather core body
temperature data from multiple sources (i.e., gastrointestinal tract and rectally) under conditions of extreme HS in an animal model that has physiology and mass similar to humans, as well as the ability to collect tissue samples for molecular and histological analysis to determine the direct biological consequences of rapid cooling after acute hyperthermia, which would certainly not be possible or ethical in human subjects.

Conclusions

Despite the development of HS abatement strategies, extreme cases of hyperthermia can result in morbidity and mortality if proper recovery procedures are not implemented. In light of our previous observations that rapid cooling procedures increase TGI in pigs, we hypothesized that rapid cooling after acute hyperthermia would result in greater intestinal damage and increase bacterial translocation and the cytokine response in pigs. We have now demonstrated that rapid cooling procedures can directly prevent effective heat dissipation through the skin and this negatively impacts intestinal morphology, likely resulting in the greater circulating endotoxin and cytokine concentrations observed. Increased intestinal damage and cytokine production has obvious pathological implications for both human health and animal agriculture, and this study expands our knowledge of how heatstroke recovery methods directly affect both thermal status and mammalian biology. Furthermore, although the long-term biological effects of rapid cooling after acute hyperthermia are currently unknown, our future work will focus on evaluating the thermal and pathophysiological consequences.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

AUTHOR CONTRIBUTIONS

Author contributions: J.S.J. conception and design of research; J.S.J., A.S., and D.C.L. edited and revised manuscript; J.S.J., A.S., and D.C.L. performed experiments; J.S.J. and A.S. analyzed data; J.S.J. and A.S. interpreted results of experiments; J.S.J. prepared figures; J.S.J. drafted or endorsement by the U.S. Department of Agriculture.

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