Zinc carnosine works with bovine colostrum in truncating heavy exercise–induced increase in gut permeability in healthy volunteers

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ABSTRACT

Background: Heavy exercise causes gut symptoms and, in extreme cases, heat stroke that is due to the increased intestinal permeability of luminal toxins.

Objective: We examined whether zinc carnosine (ZnC), a health-food product taken alone or in combination with bovine colostrum (a natural source of growth factors), would moderate such effects.

Design: Eight volunteers completed a 4-arm, double-blind, placebo-controlled crossover protocol (14 d of placebo, ZnC, colostrum, or ZnC plus colostrum) before undertaking standardized exercise 2 and 14 d after the start of treatment. Changes in epithelial resistance, apoptosis signaling molecules, and tight junction (TJ) protein phosphorylation in response to a 2°C rise in body temperature were determined with the use of Caco-2 and HT29 intestinal cells.

Results: Body temperature increased 2°C, and gut permeability (5-h urinary lactulose:rhamnose ratios) increased 3-fold after exercise (from 0.32 ± 0.016 baseline to 1.0 ± 0.017 at 14 d; P < 0.01). ZnC or colostrum truncated the rise by 70% after 14 d of treatment. The combination treatment gave an additional benefit, and truncated exercise induced increase at 2 d (30% reduction; P < 0.01). A 2°C temperature rise in in vitro studies caused the doubling of apoptosis and reduced epithelial resistance 3–4-fold. ZnC or colostrum truncated these effects (35–50%) with the greatest response seen with the combination treatment (all P < 0.01). Mechanisms of action included increasing heat shock protein 70 and truncating temperature-induced changes in B cell leukemia/lymphoma-2 associated X protein α and B cell lymphoma 2. ZnC also increased total occludin and reduced phosphorylated tyrosine claudin, phosphorylated tyrosine occludin, and phosphorylated serine occludin, thereby enhancing the TJ formation and stabilization.

Conclusion: ZnC, taken alone or with colostrum, increased epithelial resistance and the TJ structure and may have value for athletes and in the prevention of heat stroke in military personnel. This trial was registered at www.isrctn.com as ISRCTN51159138.

Keywords: clinical trial, gut growth, injury, nutriceutical, repair

INTRODUCTION

Several stresses affect the integrity of the intestinal barrier including prolonged strenuous exercise (1), heat stress (2), and drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) (3). A loss of intestinal integrity may result in the passage of luminal endotoxins into the circulation, thereby causing an inflammatory cascade and an exacerbating loss of barrier function. These developments can result in severe systemic effects (4) such as an exertional heat stroke, which is associated with hyperthermia, multiorgan failure, and endotoxemia. Similar processes have relevance for many athletes who are involved in heavy exercise, such as long-distance running, in which gastrointestinal symptoms including cramps, diarrhea, nausea, and bleeding are commonly reported (5, 6). These symptoms are probably due to a combination of reduced splanchnic blood flow (7), hormonal changes, altered gut permeability, and increased body temperature.

Pharmacologic options to reduce these problems are limited particularly in competitive athletics. Therefore, there is interest in the use of natural or naturally derived products. One product that is already commercially available is zinc carnosine (ZnC), in which zinc and carnosine are linked in a polymeric one-to-one ratio, and is currently marketed as a zinc dietary supplement with “added value for gastric health.” The combination of zinc with...
carnosine has potential advantages over simple zinc supple-
mentation because carnosine is a dipeptide (comprising \(\beta\)-alanine and L-histidine) that is naturally present in long-living cells such as muscle and nerves, where, among other actions, it probably has a role as an antioxidant (8).

We previously showed ZnC stimulates several aspects of the gut mucosal integrity including stimulating cell migration and proliferation in vitro and reducing the amount of gastric and small-intestinal injury caused by NSAIDs in rats and mice (9). Furthermore, with the use of normal volunteers, we showed that ZnC prevented the rise in gut permeability caused by clinical doses of the NSAID indomethacin (9). However, the potential value of ZnC in decreasing gut permeability associated with heavy exercise and its mechanism of actions are unknown.

In the current study, we examined the effect of oral ZnC on gut permeability and an exercise-induced temperature rise in subjects who were undertaking heavy exercise and compared effects of ZnC alone with those of taking ZnC in combination with bovine colostrum, which is a rich source of growth factors and immune modulators (10). Our previous studies that used colostrum alone showed a benefit in reducing exercise-induced increased gut permeability in athletes but only after prolonged (14 d) administration (11). Therefore, colostrum given alone also provided a useful positive control.

To examine some of the mechanisms by which protective effects were mediated, we performed a series of in vitro studies with the use of 2 human intestinal cell lines with the focus on the effect of a temperature rise to \(39^\circ\)C (similar to that shown in athletes who underwent the in vivo studies) on apoptosis, epithelial barrier resistance, the expression of heat shock protein 70 (Hsp70), and tight junction (TJ) proteins in the presence and absence of test compounds.

METHODS

This trial was registered at www.isrctn.com as ISRCTN51159138. Chemicals were purchased from Sigma unless otherwise stated.

Clinical study: effect of ZnC and colostrum on exercise-induced changes in human gut permeability

ZnC (Hamari–Xsto Solutions) and indistinguishable placebo capsules were used for the clinical study. Colostrum (Neovite-brand lactose-reduced colostrum) and the placebo were provided by Colostrum UK. The placebo that was used in place of colostrum was an isoenergetic and isomacronutrient milk-protein concentrate at an 80% protein content (principally casein) and was indistinguishable in appearance and taste from the colostrum powder, which was the form administered.

Ethical approval

All procedures were conducted according to the Declaration of Helsinki. Ethics approval was obtained from the Aberystwyth University Ethics Committee.

Subjects

Eight healthy men took part in the study, and all subjects were active individuals who exercised regularly ≥4 times/wk (4 participants were runners, one participant was a cyclist, one participant was a lacrosse player, one participant was a footballer, and one participant was a rugby player). Physical variables were as follows: mean age: 25 y (range: 19–33 y); mean ± SEM height: 1.78 ± 0.02 m; body mass: 80.1 ± 2.5 kg; BMI (in kg/m²): 24.98 ± 0.17; maximal oxygen uptake (VO₂ max): 59.6 ± 1.8 mL · kg⁻¹ · min⁻¹; peak speed in the VO₂ max test: 18 ± 0.4 km/h; and running speed at 80% of the VO₂ max: 13.5 ± 0.03 km/h. Subjects completed a pre-exercise screening questionnaire (the Physical Activity Readiness Questionnaire) before participating in each test.

V₀₂ max exercise assessments were performed with the use of standard methods, as reported previously (9), on day −5 of each arm to ensure the consistency of the 80% of the VO₂ max protocol on days 2 and 14 (Figures 1 and 2).

Preparation of subjects for exercise study

Subjects completed a 24-h food diary on the day before the main exercise trial in the first arm of the trial and repeated this diet in the subsequent arms. All trials were performed after an overnight fast ≥10 h. Subjects reported at 0700 for all trials and self-positioned a rectal thermistor (Grant Instruments) 10 cm beyond the anal sphincter and positioned a telemetric heart-rate monitor transmitter band (Polar S610i; Polar Electro Oy). The core temperature was recorded with the use of an electronic data logger (Squirrel SQ2020; Grant Instruments).

Subjects sat for 10 min before a baseline venous blood sample (pre-exercise) was taken. Subjects ran on the treadmill with a 1% grade for 20 min at a constant speed that was equivalent to 80% of the VO₂ max as determined from preliminary tests. Expired gas was analyzed during exercise with the use of an online breath-by-breath system (Jaeger Oxycon Pro.). The core body temperature, heart rate, and rating of perceived exertion were recorded every 5 min during the trial. After completing the run, subjects were quickly seated, and a second blood sample (postexercise) was obtained (within 5 min). Subjects emptied their bladders before consuming the intestinal permeability test drink and commencing with a 5-h urine collection to determine intestinal permeability.

Study design

In a 4-arm, double-blind, placebo-controlled, randomized crossover design, subjects received oral supplementation 2 times/d for 14 d with a 14-d washout period between each study arm (Figures 1 and 2). Each arm was administered in a randomized fashion with the use of the web site randomization.com (4 × 4 blocks). Timing was based on our previous studies that used this type of protocol that had shown the sufficient amount of time to ensure baseline permeability values returned back to normal (11).

FIGURE 2 Schematic of trial design. Each subject took part in a double-blind crossover protocol. Subjects received oral supplementation 2 times/d with ZnC, bovine colostrum, ZnC plus bovine colostrum, or placebo for 2 wk with a 2-wk washout period between study arms. The schedule used to determine the VO₂ max and to undertake the 80% of VO₂ max protocols, gut-permeability assessments (involving a 5-h urine collection), and blood samples is shown.

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VO₂ max was assessed on day −5 for each arm of the study. Gut-permeability assessments were performed under nonexercise conditions on days −2 and 0 (to confirm a stable baseline) and immediately after the standardized exercise (treadmill running for 20 min at 80% of the VO₂ max) protocol on days 2 and 14.

Oral supplements consisted of 37.5 mg ZnC + 10 g placebo, 10 g bovine colostrum + placebo capsule, 37.5 mg ZnC + 10 g bovine colostrum, and 10 g placebo + placebo capsule, each of which were taken 2 times/d. The capsules (ZnC) and powder (colostrum) or their placebo equivalents were taken just before breakfast or the evening meal. The doses were chosen on the basis of the results of pilot in vitro studies (Supplemental Material 1).

### Analytic methods

Intestinal permeability was assessed with the use of our previously published protocol, equipment, and methods (11). Results are expressed as simple AUC ratios as described by us previously (11) and also as the ratio of the percentage of ingested sugar excreted in the urine as has been used by some other groups (12).

### In vitro studies

To investigate mechanisms by which test compounds influenced gut permeability in the clinical study, we performed a series of experiments that examined the effect of a 2°C rise (from 37°C to 39°C).

### Cell lines

HT29 is derived from the colorectal adenocarcinoma of a 44-yr-old Caucasian woman (American Type Culture Collection) (13). Caco-2 is derived from the colorectal adenocarcinoma of a 72-yr-old man (American Type Culture Collection) and exhibits TJs and desmosomes between adjacent cells and grows as polarized monolayers (14).

### Transepithelial permeability assays

The influence of temperature changes on transepithelial permeability in the presence and absence of test factors were determined with the use of 2 different methods. One method determined changes in the transepithelial electrical resistance with the use of our previously published methods (11). The other method analyzed the passage of horseradish peroxidase (HRP) across the epithelial layer with the use of standard methods (15). To enhance any effects shown, the previously detailed experiments were also performed in low-calcium medium (0.9 mmol/L) in addition to normal-calcium medium (1.7 mmol/L).

### Hsp70 assay

Effects of the temperature change and various test factors on cell lysate Hsp70 concentrations were determined with the use of our previously published methods (11) with a Duoset Elisa kit (DYC1663-2; R&D Systems Europe).

### Cell apoptosis assays

Effects of the temperature change and various test factors on cell lysate concentrations of active caspase-3 (an effector caspase) and caspase-9 (an initiator caspase) were determined according to methods that were previously described (11) with the use of commercial colorimetric assay kits (BF3100 and BF10100; R&D Systems). In addition, Western blots were performed with the use of caspase-3 (sc-7272; Santa Cruz Biotechnology Inc.) and caspase-9 (sc-81589; Santa Cruz Biotechnology Inc.) antibodies that are capable of detecting both procaspase and active caspase. Films were scanned, and the mean signal density of each band was determined with the use of the Analytic methods

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Day −5 of trial</th>
<th>Day 2 of trial</th>
<th>Day 14 of trial</th>
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<td>VO₂ max protocol</td>
<td>80% of the VO₂ max protocol</td>
<td>80% of the VO₂ max protocol</td>
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<tr>
<td>Placebo plus placebo</td>
<td>5.01 (4.44–5.11)</td>
<td>3.73 (3.31–3.89)</td>
<td>3.61 (3.26–3.79)</td>
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<tr>
<td>Colostrum plus placebo</td>
<td>4.77 (4.50–4.93)</td>
<td>3.54 (3.25–3.77)</td>
<td>3.48 (3.19–3.69)</td>
</tr>
<tr>
<td>ZnC plus placebo</td>
<td>4.73 (4.57–5.06)</td>
<td>3.53 (3.34–3.78)</td>
<td>3.45 (3.29–3.73)</td>
</tr>
<tr>
<td>ZnC plus colostrum</td>
<td>4.78 (4.61–4.89)</td>
<td>3.45 (3.35–3.70)</td>
<td>3.54 (3.36–3.58)</td>
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</table>

1All values are medians (IQRs); units are L/min. n = 8. Data were analyzed with the use of a 3-factor ANOVA followed by t tests on the basis of the group means, residual, and df obtained from the ANOVA. There were no significant differences between any of the treatment arms. VO₂ max, maximal oxygen uptake; ZnC, zinc carnosine.
Concentrations of the antiapoptotic protein B cell lymphoma 2 (Bcl-2) and the proapoptotic protein B cell leukemia/lymphoma-2 associated X protein-α (Baxα) were determined in the same cell lysates as was used for caspase analyses with the use of Duoset ELISA kits (DYC827B-2 and DYC820-2, respectively; R&D Systems Europe Ltd).

**TJ protein and phosphorylation assessments**

Effects of the temperature change were assessed with the use of standard assays and commercial kits [occludin, zona occludens protein 1 (ZO1), and claudin-1 (TJ antibody samples pack 90–1200; Invitrogen)], tyrosine, serine, and threonine phosphorylation concentrations were measured with the use of a standard ELISA (antiphosphothreonine ab9337, antiphosphotyrosine ab9318, and antiphosphoserine ab9332; Abcam). In addition, Western blot analyses were performed for total occludin, ZO1, and claudin-1 with the use of a commercial kit (35050; Thermo Scientific). Immunocomplexes were prepared from lysates by incubation with the relevant TJ antibody and analyzed by Western blot analysis with the use of antiphosphothreonine, antiphosphotyrosine, or antiphosphoserine and a commercial kit (35050; Thermo Scientific). Films were scanned, and the mean signal density of each band was determined with the use of the Adobe Photoshop program.

**Statistical analyses**

All values are expressed as means ± SEMs unless otherwise stated. For in vitro studies, a JMP statistical package (SAS version 10) was used to perform a 3-factor ANOVA with temperature, treatment, and time as factors. For the clinical study, a 3-factor ANOVA with treatment (arm), permeability, and time as factors was performed. When a significant effect was seen (P < 0.05), individual comparisons were performed with the use of t tests on the basis of the group means, residual, and df obtained from the ANOVA, which is a method that is equivalent to repeated-measures analyses (11).

**RESULTS**

**Clinical study: effect of ZnC and colostrum on exercise-induced changes in human gut permeability**

As expected, ratings of the perceived exertion expressed during exercise, heart rate (mean rise: 106 ± 2 beats/min; from 73 ± 1 to 179 ± 1 beats/min), lactate concentrations (mean rise: 5.76 ± 0.31 mmol/L; from 1.10 ± 0.07 to 6.86 ± 0.31 mmol/L), core temperature (mean rise: 1.59 ± 0.04°C; from 36.75°C ± 0.02°C to 38.33°C ± 0.05°C), VO₂, carbon dioxide uptake, and the respiratory exchange ratio all rose in response to exercise (all P < 0.01). The presence of supplements had no significant effect on the results. VO₂ max assessments on day 2 and at 80% of the VO₂ max protocol on days 2 and 14 were not different between the 4 arms (Table 1). Baseline permeability expressed as the ratio of lactulose: rhamnose AUC values was similar at the beginning of each study arm (Figure 3). Permeability increased ~3-fold in response to exercise during the placebo arm [rising from 0.318 ± 0.016 (initial baseline value) to 0.979 ± 0.026 at day 2 and 1.000 ± 0.017 at day 14 (compared with baseline, both P < 0.01)]. With the results expressed as lactulose:rhamnose percentage of urinary excretion ratios gave equivalent results (Supplemental Material 1, Supplemental Figure 2).
**Compared with medium alone at the same temperature and time point,** the basis of the group means, residual, and df obtained from the ANOVA. Data were analyzed with the use of a 3-factor ANOVA followed by use of Western analysis and showed similar results (Supplemental Figure 3).

Changes in apoptosis were determined with the use of active caspase-3 (A) and caspase-9 (B) assay kits after changes in the A405. Studies were also analyzed with the use of caspase-3 or caspase-9 inhibitor was added to the cells (Figure 4). The 2°C rise caused an increased Bax concentration from 578.6 ± 16.7 to 797.4 ± 29.7 pg/mL (P < 0.01) at the 4-h time point (Figure 6A). The addition of ZnC alone did not affect Bax expression at either 37°C or 39°C, whereas colostrum alone reduced the temperature-induced rise in Bax. A significant further decrease in the Bax concentration was seen when ZnC and colostrum were added together at 39°C (all P < 0.01). These changes were specific because they were not seen when the capase-3 or caspase-9 inhibitor was added to the cells (Supplemental Figure 4).

The 2°C rise resulted in a decrease of Bcl-2 concentrations from 350 ± 2 to 292 ± 2 pg/mL (P < 0.01) after 4 h (Figure 6B). The addition of ZnC, colostrum, or the combination did not affect Bcl-2 concentrations at 37°C. At 39°C, the presence of ZnC or colostrum alone significantly attenuated the temperature-induced decrease in Bcl-2 concentrations, and an additive, synergistic effect was seen when ZnC and colostrum were added together, which completely prevented the temperature-induced decline in Bcl-2. Similar results were seen after 8 h (data not shown).

**A**

![Figure 5: Mean ± SEM (n = 3) effects of ZnC, colostrum, and ZnC plus colostrum on temperature-induced apoptosis and active caspase-3 and caspase-9. Caco-2 cells were incubated at 37°C or 39°C for 8 h in medium alone or with ZnC, colostrum, or ZnC plus colostrum. Changes in apoptosis were determined with the use of active caspase-3 (A) and caspase-9 (B) assay kits after changes in the A405. Studies were also analyzed with the use of Western analysis and showed similar results (Supplemental Figure 3). Data were analyzed with the use of a 3-factor ANOVA followed by t tests on the basis of the group means, residual, and df obtained from the ANOVA. *P < 0.05, **P < 0.01. For all test conditions at 37°C compared with 39°C, P < 0.01. A405, absorbance at 405 nm; ZnC, zinc carnosine.](https://academic.oup.com/ajcn/article-abstract/104/2/526/4564661)

**B**

![Figure 6:](https://academic.oup.com/ajcn/article-abstract/104/2/526/4564661)

After 2 d of treatment, the ingestion of ZnC alone or of colostrum alone did not significantly reduce the rise in exercise-induced permeability compared with that with the placebo. In contrast, the ingestion of ZnC plus colostrum attenuated this increase in permeability by 30% (compared with the other treatment-group arms at the same time point, P < 0.01).

After 14 d of treatment, the increase in permeability caused by exercise was reduced by 71% in the ZnC-alone arm, by 68% in the colostrum-alone arm, and by 85% in the ZnC plus colostrum arm (compared with placebo at same time point, all P < 0.01) (Figure 3). ZnC plus colostrum was significantly better at truncating the rise in permeability induced by exercise than was the use of colostrum alone (P < 0.05), and although it had a greater reductive effect than with the use of ZnC alone, this difference was NS at the <0.05 level (P = 0.069). The order in which the arms were administered did not influence results (although numbers were too small to perform a detailed statistical analysis).

**In vitro studies**

**Transepithelial permeability**

Results of the examination of electrical resistance (Figure 4A) or the passage of HRP (Figure 4B) confirmed the protective effects of the test substances. With the use of this protocol, the combination of ZnC plus colostrum resulted in a significant beneficial effect (77% attenuation of increased permeability caused by the temperature rise), which was greater than that seen when cells were incubated with either ZnC (52% attenuation) or colostrum (41% attenuation) given alone (Figure 4).

**Apoptosis**

Both the ELISA and Western blot analyses showed that increasing the incubation temperature caused an ~2-fold increase in active caspase-3 and caspase-9 expression at the 8-h time points (Supplemental Figure 3, Figure 5). The addition of ZnC, colostrum, or ZnC plus colostrum had no significant effect on caspase expression when incubated at 37°C. However, the co-presence of ZnC, colostrum, or ZnC plus colostrum significantly reduced caspase-3 and caspase-9 expression compared with that of cells grown in medium alone at 39°C (all P < 0.01). These changes were specific because they were not seen when the caspase-3 or caspase-9 inhibitor was added to the cells (Supplemental Figure 4).
Raising the incubation temperature caused increased Hsp70 concentrations from 139 ± 1 to 181 ± 3 pg/mL (P < 0.01) after 4 h (Figure 6C). Changes in Hsp70 after 4 h (C) or 8 h (D) of incubation at these 2 temperatures are also shown. Similar results were seen with the use of HT29 cells (data not shown). Data were analyzed with the use of a 3-factor ANOVA followed by t tests on the basis of the group means, residual, and df obtained from the ANOVA. *Compared with medium alone at the same temperature and time point, **P < 0.05, ***P < 0.01. #Compared with ZnC alone at the same temperature and time point, $P < 0.05,$$P < 0.01. For all test conditions at 37°C compared with at 39°C, P < 0.01. Bax, B cell leukemia/lymphoma-2 associated X protein; Bcl2, B cell lymphoma 2; Hsp70, heat shock protein 70; ZnC, zinc carnosine.

**FIGURE 6** Mean ± SEM (n = 3) effects of ZnC, colostrum, and ZnC plus colostrum on temperature-induced changes in the proapoptotic protein Bax, the antiapoptotic protein Bcl2, and heat shock protein expression (Hsp70). Caco-2 cells were incubated at 37°C or 39°C in medium alone or with ZnC, colostrum, or ZnC plus colostrum. Changes in Bax (A) and Bcl2 (B) after 4 h are shown. Changes in Hsp70 after 4 h (C) or 8 h (D) of incubation at these 2 temperatures are also shown. Similar results were seen with the use of HT29 cells (data not shown). Data were analyzed with the use of a 3-factor ANOVA followed by t tests on the basis of the group means, residual, and df obtained from the ANOVA. *Compared with medium alone at the same temperature and time point, **P < 0.05, ***P < 0.01. #Compared with ZnC alone at the same temperature and time point, $P < 0.05,$$P < 0.01. For all test conditions at 37°C compared with at 39°C, P < 0.01. Bax, B cell leukemia/lymphoma-2 associated X protein; Bcl2, B cell lymphoma 2; Hsp70, heat shock protein 70; ZnC, zinc carnosine.

TJ protein expression and phosphorylation

Because results at 4 h were similar to those at 8 h, they are reported together.

**TJ protein expression and phosphorylation**

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**ZO1**

Total ZO1 increased in response to the temperature rise and were not affected by test factors (Figure 7A). Phosphorylated tyrosine (p-Tyr) ZO1 was reduced by the temperature rise and presence of colostrum, and the combination treatment reduced concentrations further (Figure 7B). Phosphorylated serine (p-Ser) ZO1 was reduced by the temperature rise. The co-presence of test factors increased p-Ser–ZO1 concentrations at both 37°C and 39°C (Figure 7C). Analyses with the use of Western blotting and densitometry showed similar results (Supplemental Figure 5).
Occludin

Total occludin increased in response to the temperature rise. The presence of ZnC, colostrum, or the combination all increased total occludin concentrations at 37°C. At 39°C, all test factors caused additional rises in total occludin concentrations compared with those of cells in medium alone (Figure 8A). The increased temperature caused p-Tyr occludin to rise, but the presence of test factors reduced p-Tyr–occludin concentrations at both 37°C and 39°C with the largest fall shown in cells treated with ZnC plus colostrum (Figure 8B). p-Ser–occludin concentrations were reduced in response to the temperature rise, and the presence of test factors caused further reductions in p-Ser–occludin ratios with the largest fall shown with the combination treatment (Figure 8C). Analyses with the use of Western blotting and densitometry showed similar results (Supplemental Figure 6).

Claudin-1

Total claudin-1 was not affected by the temperature change or test factors (Figure 9A). p-Tyr–claudin-1 concentrations rose in response to the temperature increase, and there was a small but significant truncation of the rise in the presence of ZnC alone or in combination with colostrum (Figure 9B). p-Ser claudin-1 was not significantly affected by the temperature rise or the presence of test factors (Figure 9C). Analyses with the use of Western blotting and densitometry showed similar results (Supplemental Figure 7).

DISCUSSION

With the use of a combination of a clinical trial and in vitro experiments, we showed that ZnC attenuated the exercise-induced increase in gut permeability through mechanisms that included reducing temperature-induced apoptosis, the induction of Hsp70, and the modulation of TJ protein expression and phosphorylation. Enhanced results were shown when the ZnC was co-administered with the other natural bioactive nutriceutical product bovine colostrum.

Numerous exercise protocols are used by exercise physiologists. We chose a 20-min run at 80% of the \( V_{\text{O2max}} \) protocol because we had previous experience with this protocol (11), which allowed a crossover-study design to be used in a relatively short period, reliably increased gut permeability 2–3-fold, and increased the core temperature by 1.5–2°C. The assessment of intestinal permeability by quantitating unmediated absorption of \( \text{xylose} \) of different sizes provided a sensitive index of intestinal damage as we and others have previously shown (11, 12, 16).

The subject \( V_{\text{O2max}} \) and speed at 80% of the \( V_{\text{O2max}} \) remained consistent for all arms, and similar exercise-induced changes in core temperatures were observed in each study arm. Therefore, the protective effect of test substances could not be attributed to the changing core temperature during the exercise. Gut permeability increased 3-fold in response to exercise in the placebo control arm as expected with the use of this protocol (11). These changes in gut permeability were similar to those reported by us previously in subjects who ingested clinically relevant doses of the NSAID indomethacin (9), which is known to cause a small intestinal injury (17).
Similar levels of protection, as determined by gut permeability, were seen when either ZnC or colostrum was administered alone with no protective effect shown after 2 d of treatment but with a reduction in permeability values by 70% after 14 d of treatment. At this 14-d time point, an additional advantage was shown with the combination treatment, and possibly more importantly, in regards to the use by athletes or military personnel who are entering a high-temperature environment, the combination treatment also attenuated the exercise-induced gut permeability after only 2 d of treatment.

We undertook a series of in vitro studies to examine the effect of the core-temperature rise on gut integrity in a controlled environment. We used 2 well-validated complementary models to examine changes in transepithelial resistance by following the changes in electrical resistance (11) and the passage of a large molecule (HRP) across polarized monolayers of human colonic cancer cells (15). We have the experience of studying the effects of proteins in these systems, and the use of these in vitro models removes confounding factors such as changes in blood flow. The results were consistent with those of the clinical trial; the temperature rise was associated with increased permeability, but this effect could be attenuated by the presence of ZnC, colostrum, or ZnC plus colostrum with greatest effects shown with the combination treatment. These effects were likely to be due, at least in part, to effects on the paracellular permeability such as the alteration in TJs (18) and changes in apoptosis.

A temperature rise is a well-known trigger of apoptosis (11), and we measured active caspase-3 and caspase-9 to examine potential effects of the test compounds. We showed that this 2°C rise was sufficient to increase apoptosis, and ZnC truncated this response possibly by maintaining concentrations of the anti-apoptotic protein Bcl-2. An additive effect was seen in the maintenance of Bcl-2 when ZnC and colostrum were added together.

Heat shock proteins maintain cellular homeostasis during normal cell growth and enhance survival during and after various cellular stresses (19). An increased expression of heat shock proteins may be one mechanism through which thermotolerance occurs in animals and cells (20). Hsp70 is increased in response to temperature rises as a homeostatic mechanism for maintaining viability under conditions that increase the accumulation of damaged proteins. Our finding that ZnC induced Hsp70 expression at 37°C and caused additional increases when added at 39°C suggested that this pathway may have relevance with our results. Our in vitro results were shown by reproducing the temperature rise seen in the clinical study (to ~39°C) and in most athletes during standard performance rather than at the typical 41.5°C that has been used in rat models of hyperthermia that results in a massive breakdown of mucosal integrity.

Intestinal epithelial TJs are multiprotein complexes that connect adjacent cells on apical and lateral membranes and act as selective barriers. TJ integrity is regulated by the assembly of extracellular loops of transmembrane proteins occludin and claudin and several intracellular plaque proteins such as ZO1, which link to the actin cytoskeleton. TJ function is regulated by changes in both absolute amounts and the degree of phosphorylation at specific residues. In general terms, the increased expression of occludin, claudin, or ZO1 increases TJ formation and resistance (for a good overview, see references 21 and 22). Therefore, increased total occludin in response to ZnC can be

FIGURE 8 Mean ± SEM (n = 3) effects of ZnC, colostrum, and ZnC plus colostrum on temperature-induced changes of occludin protein concentrations and phosphorylation. Cells were incubated in the presence of test factors for 8 h at either 37°C or 39°C. Total occludin (A), p-Tyr occludin (B), and p-Ser occludin (C) were analyzed with the use of an ELISA. Studies that were analyzed with the use of Western blotting and densitometry gave similar results (Supplemental Figure 6). Data were analyzed with the use of a 3-factor ANOVA followed by t tests on the basis of the group means, residual, and df obtained from the ANOVA. **Compared with medium alone at the same temperature, *P < 0.05, **P < 0.01. *Compared with ZnC alone at the same temperature, P < 0.05. Comparred with colostrum alone at the same temperature, P < 0.05. p-Ser, phosphorylated serine; p-Tyr, phosphorylated tyrosine; ZnC, zinc carnosine.
considered potentially beneficial. Tyrosine phosphorylation of any of the 3 TJ proteins assessed hinders TJ formation, thereby reducing epithelial resistance. Therefore, the reduction of p-Tyr concentrations of claudin and occludin by ZnC should enhance the TJ formation, although, in the current study, the changes in claudin phosphorylation in response to treatment were small and, therefore, of unclear significance. Similarly, because ZnC reduced the phosphorylation of serine in occludin, TJ formation should also be enhanced.

We showed that the overall effect of giving bovine colostrum or ZnC alone were similar in reducing exercise-induced permeability. Both compounds increased Hsp70 concentrations and reduced heat-induced apoptosis although the signaling processes were somewhat different with colostrum but not with ZnC, whereby there was a reduction in the temperature-induced rise in Bax concentrations. Analyses of TJ modulation also showed broadly similar results in phosphorylation effects on the TJ proteins although some differences, such as reduced p-Tyr of ZO1 by colostrum but not by ZnC were shown.

There is currently a demand by the general public for more natural types of products, which are often termed alternative or complementary therapies or nutriceuticals (from nutrition and pharmaceuticals). Because of their natural origin, the general public often assume that they are safe and may take high doses for prolonged periods. However, caution needs to be taken because there is biological activity in many of these products such as colostrum, which is rich in multiple growth factors (23). Therefore, general principals of the use of the lowest dose for the shortest time possible seem appropriate. In the current studies, ZnC was administered at 37.5 mg 2 times/d, which provided a total daily dose of 16 mg Zn/d. Current recommendations for daily zinc intake are 5.5–9.5 mg (for men) and 4–7 mg (for women) from a United Kingdom food-standards authority and 11 mg (for men) and 8 mg (for women) from the US NIH with daily upper recommended limits being 25 mg/d in the United Kingdom and 40 mg/d in the United States. Therefore, the regimen that was used in the current studies was well within the safety guidelines.

The findings of additive or synergistic effects (dependent on the variable measured) were particularly relevant in the clinical study because it was only the combination treatment that was effective after 2 d of treatment. This result suggests that short courses taken for a few days before embarking on prolonged heavy exercise (such as athletic events or military maneuvers in hot climates) could provide optimal results while minimizing dosing. Additional studies appear warranted to explore these findings. These trials could include the examination of athletes undertaking prolonged strenuous exercise such as a marathon, in which case it would also be of interest to examine blood endotoxin concentrations. It would also be of interest to examine additional markers of cellular integrity and enterocyte permeability such as intestinal fatty acid-binding protein although it seems likely that later blood samples and a potentially a longer exercise protocol than was used in our studies would be required to show such changes (24). Additional studies could also include the relevance of hypoxia on paracellular and cellular integrity when cells are stressed by hypoxia alone and in combination with temperature rises.

In conclusion, our current studies focusing on temperature changes build on previous work that showed ZnC prevents NSAID gut damage. Therefore, it would be of interest to examine the
effects of ZnC on other gut disorders, such as inflammatory bowel disease, in which an uncontrolled inflammatory response combined with the disruption of epithelial integrity is a major factor.

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