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

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Abbreviations: Baxα; B cell leukemia/lymphoma-2 associated X protein-alpha, Bcl-2; B-cell lymphoma 2, HRP; horseradish peroxidase, HPLC; high pressure liquid chromatography, Hsp; heat shock protein, VO_2max ; maximal oxygen uptake, NSAID; nonsteroidal anti-inflammatory, pSer; phosphorylated Serine, pTyr; phosphorylated Tyrosine, ZnC; zinc carnosine, ZO1; zona occludens protein 1

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ABSTRACT

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

Objective: To examine whether zinc carnosine (ZnC) a health food product taken alone or in combination with bovine colostrum, a natural source of growth factors, moderated such effects.

Design: 8 volunteers completed a four-arm double-blind, placebo-controlled, crossover protocol (14 days placebo, ZnC, colostrum, ZnC + colostrum) prior to standardized exercise undertaken 2 and 14 days after starting treatment. Changes in epithelial resistance, apoptosis signalling molecules and tight junction protein phosphorylation in response to 2°C rise were determined using Caco-2 & HT29 intestinal cells.

Results: Body temperature increased 2°C and gut permeability (5 hour urinary lactulose:rhamnose ratios) increased 3-fold following exercise (0.32 ± 0.016 baseline to 1.0 ± 0.017 at +14 days, $p<0.01$). ZnC or colostrum truncated rise by 70% after 14 days treatment. Combination treatment gave additional benefit and truncated exercise induced increase at +2 day (30% reduction, $p<0.01$). 2°C temperature rise in *in vitro* studies caused doubling of apoptosis and reduced epithelial resistance 3-4-fold. ZnC or colostrum truncated these effects (35-50%) with greatest response seen with combination treatment (all $p<0.01$). Mechanisms of action included increasing Hsp70 and truncating temperature-induced changes in B cell leukemia/lymphoma-2 associated X protein-alpha (Baxα) and B-cell lymphoma 2 (Bcl-2). ZnC also increased total occludin and reduced phosphorylated Tyrosine (pTyr)-claudin, pTyr-occludin and phosphorylated Serine (pSer)-occludin, enhancing tight junction formation and stabilisation.

Conclusion: ZnC taken alone or with colostrum increased epithelial resistance and tight junction structure and may have value for athletes and preventing heat stroke in military

personnel.

Keywords: Repair; gut growth; injury; nutriceutical; clinical trial

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 agents (NSAIDs)(3). Loss of intestinal integrity may result in passage of luminal endotoxins into the circulation, causing an inflammatory cascade, exacerbating loss of barrier function. This can result in severe systemic effects (4), such as in exertional heat stroke, associated with hyperthermia, multi-organ failure and endotoxemia. Similar processes have relevance for many athletes involved in heavy exercise such as long-distance running where gastrointestinal symptoms including cramps, diarrhoea, 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.

Pharmacological options to reduce these problems are limited, particularly in competitive athletics. There is, therefore, interest in using natural or naturally derived products. One product already commercially available is zinc carnosine (ZnC), where 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”. Combining zinc with carnosine has potential advantages over simple zinc supplementation as carnosine is a dipeptide (comprising β-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 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, using normal volunteers, we demonstrated that ZnC prevented the rise in gut permeability caused by clinical doses of the NSAID indomethacin (9). Its potential value in decreasing gut permeability associated with heavy exercise and its mechanism of actions are, however, unknown.

We now examine the effect of oral ZnC on gut permeability and exercise-induced temperature rise in subjects undertaking heavy exercise and compared effects of ZnC alone with taking it in combination with bovine colostrum, a rich source of growth factors and immune modulators (10). Our previous studies utilising colostrum alone showed benefit in reducing exercise induced increased gut permeability in athletes, but only after prolonged (14 days) administration (11). Colostrum given alone, therefore, 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 using two human intestinal cell lines focusing on the effect of a temperature rise to 39^{o}C (similar to that seen in athletes undergoing the *in vivo* studies) on apoptosis, epithelial barrier resistance, heat shock protein 70 (Hsp70) expression and tight junction (TJ) proteins in the presence and absence of test compounds.

MATERIALS & METHODS

Chemicals were purchased from Sigma (Poole, Dorset) unless otherwise stated.

A) CLINICAL STUDY: EFFECT OF ZNC AND COLOSTRUM ON EXERCISE-INDUCED CHANGES IN HUMAN GUT PERMEABILITY.

Zinc carnosine (Lonza Nutrition Inc USA) and indistinguishable placebo capsules were used for clinical study. Colostrum (*Neovite* brand lactose-reduced colostrum) and placebo were provided by Colostrum UK, London. Placebo used in place of colostrum was isoenergetic and isomacronutrient milk protein concentrate at 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 Aberystwyth University Ethics Committee.

Subjects: Eight healthy males took part and all were active individuals who exercised regularly 4 or more times per week (4 participants were runners, 1 cyclist, 1 lacrosse player, 1 footballer, 1 rugby player). Physical parameters were: mean age 25, range 19-33; height 1.78 ± 0.02 m; body mass 80.1±2.5 kg; BMI 24.98±0.17 kg/m^2; maximal oxygen uptake (VO_2max) 59.6±1.8 ml/kg$^{/}$min; peak speed in VO_2max test 18±0.4 km/h; running speed at 80% VO_2max 13.5±0.03 km/h; (values mean ± SEM). Subjects completed a pre-exercise screening questionnaire (Physical Activity Readiness Questionnaire: PAR-Q) before participating in each test. VO_2max exercise assessments were performed by standard methods as reported previously (9) on day -5 of each arm to ensure consistency of the 80% VO_2max protocol on day +2 and +14 (**Figure 1 and 2**).

Preparation of subjects for the 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 of at least 10 h. Subjects

reported at 07:00 for all trials and self-positioned a rectal thermistor (Grant Instruments, Cambridge, England), 10 cm beyond the anal sphincter, and positioned a telemetric heart rate monitor transmitter band (Polar S610i, Polar Electro Oy, Tampere, Finland). Core temperature (*Tcore*) was recorded using an electronic data logger (Squirrel SQ2020, Grant Instruments, Cambridge, England).

Subjects sat for 10 min before baseline venous blood sample (pre-exercise) was taken. Subjects then ran on the treadmill, with 1% grade, for 20 min at a constant speed equivalent to 80% VO_2max, as determined from preliminary tests. Expired gas was analyzed during exercise using an online breath-by breath system (Jaeger Oxycon Pro. Hoechberg, Germany). 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 (post-exercise) was obtained (within 5 minutes). Subjects then emptied their bladder before consuming the intestinal permeability test drink and commencing with a 5-h urine collection to determine intestinal permeability.

Study design: In a four-arm double-blind placebo controlled randomised crossover design, subjects received oral supplementation twice a day for 14 days with a 14 day washout period between each study arm (Figure 1 and 2). Each arm was administered in a randomised fashion using the web site *randomization.com* (4x4 blocks). Timing was based on our previous studies using this type of protocol that had demonstrated was sufficient time to ensure baseline permeability values returned back to normal (11).

Maximal oxygen uptake (VO_2max) was assessed on day -5 for each arm of the study. Gut permeability assessments were performed under non- exercise conditions on day -2 and 0 (to

confirm stable baseline) and immediately following the standardised exercise (treadmill running 20 min at 80% VO_2max) protocol on day +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, taken twice per day. The capsules (ZnC) and powder (colostrum) or their placebo equivalents were taken just prior to breakfast or evening meal. The doses were chosen based on the results of pilot in vitro studies (**Supplemental Material 1, Supplemental Figure 1**).

Analytical methods: Intestinal permeability was assessed using our previously published protocol, equipment and methods (11). Results are expressed as a simple area under the curve ratios as described by us previously (11) and also as ratio of percentage of ingested sugar excreted in the urine as used by some other groups (12).

B) IN VITRO STUDIES

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

Cell lines: HT29 is derived from colorectal adenocarcinoma of 44-year-old Caucasian female (ATCC)(13). Caco-2 is derived from colorectal adenocarcinoma of 72-year-old male (ATCC) and exhibits tight junctions 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 using two different methods. One determined changes in transepithelial electrical resistance using our previously published methods (11). The other analysed the passage of horseradish peroxidase (HRP) across the epithelial layer using standard methods (15). To enhance any effects seen, the above experiments were also performed in low calcium medium (0.9 mM) in addition to normal calcium medium (1.7 mM).

Heat shock protein 70 (Hsp70) assay: Effects of temperature change and various test factors on cell lysate Hsp70 levels were determined using our previously published methods (11), using a Duoset Elisa kit (DYC1663-2, R&D Systems Europe, Abingdon, UK).

Cell apoptosis assays: Effects of temperature change and the various test factors on cell lysate levels of active caspase-3 (an effector caspase) and caspase-9 (an initiator caspase) were determined using methods previously described (11), using commercial colorimetric assay kits (BF3100 and BF10100, R&D Systems). In addition, Westerns were performed using caspase-3 (sc-7272, Santa Cruz) and caspase-9 (sc-81589) antibodies capable of detecting both pro-caspase and active caspase. Films were scanned and mean signal density of each band determined using Adobe Photoshop.

Concentrations of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Baxα were determined in the same cell lysates as used for caspase analyses, using Duoset Elisa kits (DYC827B-2 and DYC820-2, respectively, R&D Systems Europe Ltd).

TJ protein and phosphorylation assessments: Effects of temperature change were assessed using standard assays and commercial kits: occludin, zona occludens protein 1 (ZO1) and claudin-1 (tight junction antibody samples pack 90-1200, Invitrogen), tyrosine, serine and threonine phosphorylation levels were measured by standard ELISA (anti-phosphothreonine ab9337, anti-phosphotyrosine ab9318 and anti-phosphoserine ab9332, all Abcam, Cambridge UK). In addition, Western analyses were performed for total occludin, ZO1 and claudin-1 and a commercial kit (35050, Thermo Scientific). Immunocomplexes were prepared from lysates by incubation with relevant TJ antibody and analysed by Western using anti-phosphothreonine , anti-phosphotyrosine or anti-phosphoserine and a commercial kit (35050, Thermo Scientific). Films were scanned and the mean signal density of each band was determined using Adobe Photoshop.

Statistical analyses

All values are expressed as the mean $\pm$ SEM unless stated. For in vitro studies, a JMP statistical package (version 10) was used to perform three way ANOVA with temperature, treatment and time as factors. For the clinical study, a three way ANOVA with treatment (arm), permeability and time as factors was performed. Where a significant effect was seen ($p<0.05$), individual comparisons were performed using t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA, a method equivalent to repeated measures analyses (11).

RESULTS

A) CLINICAL STUDY: EFFECT OF ZNC AND COLOSTRUM ON EXERCISE-INDUCED CHANGES IN HUMAN GUT PERMEABILITY.

As expected, rating of perceived exertion expressed during exercise, heart rate (mean rise 106±2 BPM, from 73±1 to 179±1 BPM), lactate concentrations (mean rise 5.76±0.31 mM, from 1.10±0.07 to 6.86±0.31 mM), core temperature (mean rise 1.59±0.04 ˚C, from 36.75±0.02 to 38.33±0.05 ˚C), VO_2, VCO_2 and respiratory exchange ratio, all rose in response to exercise (all $p<0.01$). The presence of supplements had no significant effect on results. VO_2max assessments on day -5 of each arm and 80% VO_2max protocol on day 2 and 14 were not different between the four arms (**Table 1**).

Baseline permeability expressed as the ratio of Lactulose/Rhamnose under the curve values were similar at the beginning of each study arm (**Figure 3**). Permeability increased about 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 (both $p<0.01$ vs baseline). Expressing results as Lactulose/Rhamnose % urinary excretion ratios gave equivalent results (Supplemental Material 1**, Supplemental Figure 2**).

After 2 days treatment, ingestion of ZnC alone or colostrum alone did not significantly reduce the rise in exercise induced permeability compared to placebo. In contrast, ingestion of ZnC + colostrum attenuated this increase in permeability by 30% ($p<0.01$ versus the other treatment groups arms at the same time point).

After 14 days treatment, the increase in permeability caused by exercise was reduced by 71% in the ZnC alone arm, 68% in the colostrum alone arm and by 85% in the ZnC + colostrum arm (Figure 3, all $p<0.01$ vs placebo at same time point). ZnC + colostrum was significantly better at truncating the rise in permeability induced by exercise than using colostrum alone ($p<0.05$) and although it had a greater reductive effect than using ZnC alone, this difference was not statistically significant at the <0.05 level. ($p=0.069$).

The order in which the arms were administered did not influence results (although numbers are too small to perform detailed statistical analysis).

B. IN VITRO STUDIES

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

Apoptosis: Both ELISA and western blot analysis showed that increasing incubation temperature caused an approximate 2-fold increase in active caspase 3 and 9 expression at the 8 h time points (**Figure 5** and **Supplemental Figure 3**). Addition of ZnC, colostrum or ZnC + colostrum had no significant effect on caspase expression when incubated at 37°C. However, the co-presence of ZnC, colostrum or the combination all significantly reduced caspase 3 and 9 expression compared to cells grown in medium alone at 39°C (all $p<0.01$). These changes were specific as they were not seen when the capase-3 or caspase-9 inhibitor were also added to the cells (**Supplemental Figure 4**).

The 2°C rise caused increased Baxα concentration from 578.6±16.7 to 797.4±29.7 pg/ml ($p<0.01$) at the 4 h time point (**Figure 6A**). Addition of ZnC alone did not affect Baxα expression at either 37 or 39°C whereas colostrum alone did reduce the temperature-induced rise in Baxα. A significant further decrease in Baxα concentration was seen when ZnC and

colostrum were added together at 39°C ($p<0.01$ vs ZnC alone, Figure 6A). Similar results were seen after 8h (data not shown).

The 2°C rise resulted in a decrease of Bcl2 levels from 350 ± 2 to 292 ± 2 pg/ml to ($p<0.01$) after 4 h (Figure 6B). Addition of ZnC, colostrum or the combination did not affect Bcl2 levels at 37°C. At 39°C, the presence of ZnC or colostrum alone significantly attenuated the temperature-induced decrease in Bcl2 levels and an additive/synergistic effect was seen when ZnC and colostrum were added together, completely preventing the temperature induced decline in Bcl2. Similar results were seen after 8h (data not shown).

Raising incubation temperature caused increased Hsp70 levels from 139 ±1 to 181±3 pg/ml ($p<0.01$) after 4 h (Figure 6C). Adding ZnC alone or colostrum alone increased Hsp70 levels at both 37°C and 39°C above values seen in cells grown in medium alone (all $p<0.05$). Compared to giving either test compound alone, additional increases were found when ZnC and colostrum were added together in cells at 39°C at both time points (Figure 6C+D).

TJ protein expression and phosphorylation: As results at 4h were similar to those at 8h, they are reported together below.

ZO1: Total ZO1 increased in response to temperature rise and were not affected by test factors (**Figure 7A**). P-Tyr-ZO1 were reduced by temperature rise and presence of colostrum or combination treatment reduced levels further (Figure 7B). P-Ser-ZO1 was reduced by temperature rise. The co-presence of test factors increased p-Ser-ZO1 levels at both 37 and 39°C (Figure 7C). Analyses using Western blotting and densitometry showed similar results (**Supplemental Figure 5**).

Occludin: Total occludin increased in response to temperature rise. Presence of ZnC, colostrum or combination all increased total occludin levels at 37˚C. At 39˚C all test factors caused additional rises in total occludin levels compared to cells in medium alone (**Figure 8A**). Increased temperature caused p-Tyr-occludin to rise but presence of test factors reduced p-Tyr-occludin levels at both 37 and 39˚C with largest fall seen in cells treated with ZnC + colostrum (Figure 8B). P-Ser-occludin levels were reduced in response to temperature rise and presence of test factors caused further reductions in p-Ser-occludin ratios, with the largest fall seen with combination treatment (Figure 8C). Analyses using Western blotting and densitometry showed similar results (**Supplemental Figure 6**).

Claudin-1: Total claudin-1 was not affected by temperature change or test factors (**Figure 9A**). P-Tyr-claudin-1 levels rose in response to 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 temperature rise or presence of test factors (Figure 9C). Analyses using Western blotting and densitometry showed similar results (**Supplemental Figure 7**).

DISCUSSION

Using a combination of a clinical trial and *in vitro* experiments, we showed that ZnC attenuates the exercise-induced increase in gut permeability through mechanisms that include reducing temperature-induced apoptosis, induction of Hsp70 and modulation of TJ protein expression and phosphorylation. Enhanced results were seen if the ZnC was co-administered with another natural bioactive nutriceutical product; bovine colostrum.

Numerous exercise protocols are used by exercise physiologists. We chose a 20-min run at 80% VO_2max protocol as we have previous experience of this (11), it allows a crossover study design to be used in a relatively short period, reliably increases gut permeability by 2-3 fold and increases core temperature by 1.5–2°C. Assessment of intestinal permeability by quantitating unmediated absorption of at least two sugars of different sizes provides a sensitive index of intestinal damage as we and others have previously shown (11, 12, 16).

Subjects' VO_2max and speed at 80% VO_2max remained consistent for all arms and similar exercise-induced changes in core temperatures were observed in each study arm. The protective effect of test substances could, therefore, not be attributed to changing core temperature during exercise. Gut permeability increased 3-fold in response to exercise in the placebo control arm, as expected using this protocol (11). These changes in gut permeability are similar to those reported by us previously in subjects ingesting clinically relevant doses of the NSAID indomethacin (9), which is known to cause small intestinal injury (17).

Similar levels of protection, as determined by gut permeability, were seen when either ZnC or colostrum were administered alone with no protective effect seen after 2 days treatment but reducing permeability values by 70% after 14 days treatment. At this +14 day time point,

additional advantage was seen with combination treatment and, possibly more importantly in regards to the use by athletes or military entering a high temperature environment, combination treatment also attenuated exercise induced gut permeability after only 2 days treatment.

We then 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 two well validated complementary models to examine changes in trans-epithelial resistance by following changes in electrical resistance (11) and passage of a large molecule (HRP) across polarized monolayers of human colonic cancer cells (15). We have experience of studying effects of proteins in these systems and it removes confounding factors such as changes in blood flow. The results were consistent with the clinical trial; temperature rise was associated with increased permeability but this effect could be attenuated by the co-presence of ZnC +/- colostrum, with greatest effects seen with combination treatment. These effects are likely to be due, at least in part, to effects on paracellular permeability, such as alteration in TJs (18) and changes in apoptosis.

Temperature rise is a well-known trigger of apoptosis (11), and we measured active caspase-3 and 9 to examine potential effects of test compounds. We showed that this 2°C rise was sufficient to increase apoptosis and that ZnC truncated this response, possibly by maintaining levels 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.

Hsps maintain cellular homeostasis during normal cell growth and enhance survival during and after various cellular stresses (19). Increased Hsp expression may be one mechanism through which thermo-tolerance 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 suggests that this pathway may have relevance to our results. Importantly, our *in vitro* results were demonstrated reproducing the temperature rise seen in the clinical study (to ~39°C), and seen in most athletes during standard performance, rather than the typical 41.5°C used in rat models of hyperthermia that results in massive breakdown of mucosal integrity.

Intestinal epithelial TJs are multi-protein complexes that connect adjacent cells on apical and lateral membranes and act as selective barriers. TJ integrity is regulated by assembly of extracellular loops of transmembrane proteins occludin and claudin and several intracellular plaque proteins such as ZO-1 which link to the actin cytoskeleton. TJ function is regulated by changes in both absolute amounts and degree of phosphorylation at specific residues. In general terms, increased expression of occludin, claudin and/or ZO-1 increase TJ formation and increase resistance (for good overview see 21, 22). Increased total occludin in response to ZnC can, therefore, be considered as potentially beneficial. Tyr phosphorylation of any of the three TJ proteins assessed hinders TJ formation, reducing epithelial resistance. Our finding that ZnC reduced pTyr levels of claudin and occludin should therefore enhance TJ formation although it should be noted that the changes in claudin phosphorylation in response to treatment were small and, therefore, of unclear significance. Similarly, our finding that ZnC reduced phosphorylation of serine in occludin should also enhance TJ formation.

We showed that the overall effect of giving bovine colostrum alone or ZnC alone were similar in reducing exercise induced permeability. Both compounds increased Hsp70 levels and reduced heat induced apoptosis, although the signalling processes somewhat different with

colostrum, but not ZnC, reducing the temperature- induced rise in Baxα levels. Analyses of TJ modulation also showed broadly similar results in phosphorylation effects on the TJ proteins although some differences, such as reduced pTyr of ZO-1 by colostrum, but not by ZnC, were seen.

There is currently demand by the general public for more natural types of products, often termed “alternative-”,“complementary-” therapies or “nutriceuticals” (from nutrition and pharmaceuticals). Because of their natural origin, the general public often assume they are safe and may take high doses for prolonged periods. Caution needs to shown, however, as there is biological activity in many of these products, such as colostrum which is rich in multiple growth factors. (see 23) The general principals of using the lowest dose for the shortest time possible, therefore, seems appropriate. In the current studies, ZnC was administered at 37.5 mg twice daily, giving total daily dose of zinc of 16 mgs/day. Current recommendations for daily zinc intake are 5.5-9.5mg (male) and 4-7mg (female) from UK food standards authority and 11mg (male) and 8 mg (female) from US NIH with daily upper recommended limits being 25mg/day in UK and 40 mg/day in US. The regimen used in the current studies is therefore well within safety guidelines.

The findings of additive or synergistic effects (dependent on parameter) are particularly relevant in the clinical study as it was only combination treatment that was effective after 2 days treatment. This suggests that short courses, taken for a few days before embarking on prolonged heavy exercise (such as athletic events or military manoeuvres in hot climates) could provide optimal results while minimising dosing. Further studies appear warranted to explore these findings. These could include examination of athletes undertaking prolonged strenuous exercise, such as a marathon where it would also be of interest to examine blood

endotoxin levels. It would also be of interest to examine additional markers of cellular integrity and enterocyte permeability such as I-FABP, although it seems likely that later blood samples and potentially a longer exercise protocol than that used in our studies would be required to demonstrate 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. Our current studies focusing on temperature change builds on previous work showing ZnC prevents NSAID gut damage. It would therefore be of interest to examine its effects on other gut disorders such as inflammatory bowel disease where uncontrolled inflammatory response combined with disruption of epithelial integrity is a major factor.

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Author contributions: GD, TM, RJP designing research studies, GD, TM, DSM, RT conducting experiments, GD, TM, DSM, RT acquiring data, GD, TM, RJP analyzing data, and GD, TM, RJP writing the manuscript.

REFERENCES

1) Lambert GP. Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: the 'canary in the coal mine' during exercise-heat stress? *Med Sport Sci.* 2008;53:61-73.

2) Lambert GP. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *J Anim Sci.* 2009;87:E101-108.

3) Playford RJ, MacDonald CE, Calnan DP Floyd DN, Podas T, Johnson W, Wicks AC, Bashir O, Marchbank T. Co-administration of the health food supplement, bovine colostrum, reduces the acute non-steroidal anti-inflammatory drug-induced increase in intestinal permeability. *Clin Sci (Lond).* 2001;100:627-633.

4) Doig CJ, Sutherland LR, Sandham JD, Fick GH, Verhoef M, Meddings JB.Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. *Am J Respir Crit Care Med*. 1998;158:444-451.

5) Pals KL, Chang RT, Ryan AJ, Gisolfi CV. Effect of running intensity on intestinal permeability. *J Appl Physiol*. 1997;82:571-76.

6) de Oliveira EP, Burini RC, Jeukendrup A. Gastrointestinal complaints during exercise: prevalence, etiology, and nutritional recommendations. *Sports Med* 2014;44:S79-85

7) van Wijck K, Lenaerts K, Grootjans J, Wijnands KA, Poeze M, van Loon LJ, Dejong CH, Buurman WA. Physiology and pathophysiology of splanchnic hypoperfusion and intestinal

injury during exercise: strategies for evaluation and prevention. *Am J Physiol.* 2012;303:G155–168.

8) Boldyrev AA. Protection of proteins from oxidative stress: a new illusion or a novel strategy? *Ann N Y Acad Sci.* 2005;1057:193-205.

9) Mahmood A, FitzGerald AJ, Marchbank T Ntatsaki E, Murray D, Ghosh S, Playford RJ. Zinc carnosine, a health food supplement that stabilises small bowel integrity and stimulates gut repair processes. *Gut.* 2007;56:168-75.

10) Playford RJ, Macdonald CE, Johnson WS. Colostrum and milk-derived growth factors for the treatment of gastrointestinal disorders. *Am J Clin Nutr* 2000;72:5–14.

11) Marchbank T, Davison G, Oakes JR, Ghatei MA, Patterson M, Moyer MP, Playford RJ. The nutriceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes. *Am J Physiol Gastrointest Liver Physiol.* 2011;300:G477-84.

12) Miki K, Butler R, Moore D, Davidson G. Rapid and simultaneous quantification of rhamnose, mannitol, and lactulose in urine by HPLC for estimating intestinal permeability in pediatric practice. *Clin Chem.* 1996;42:71-75.

13) Abaan OD, Polley EC, Davis SR, Zhu YJ, Bilke S, Walker RL, Pineda M, Gindin Y, Jiang Y, Reinhold WC, et al. The exomes of the NCI-60 panel: a genomic resource for cancer biology and systems pharmacology. *Cancer Res.* 2013;73:4372-4382.

14) Fogh J, Fogh JM, Orfeo T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.* 1977;59:221-226.

15) Yang PC, He SH, Zheng PYJ. Investigation into the signal transduction pathway via which heat stress impairs intestinal epithelial barrier function. *Gastroenterol Hepatol.* 2007;22:1823-31.

16) Bjarnason I, Macpherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 1995;108:1566–1581.

17) Petruzzelli M, Vacca M, Moschetta A, Cinzia Sasso R, Palasciano G, van Erpecum KJ, Portincasa P. Intestinal mucosal damage caused by non-steroidal anti-inflammatory drugs: role of bile salts. *Clin Biochem* 2007;40:503–510.

18) Blume LF, Denker M, Gieseler F, Kunze T. Temperature corrected transepithelial electrical resistance (TEER) measurement to quantify rapid changes in paracellular permeability. *Pharmazie* 2010;65:19–24.

19) Rokutan K. Role of heat shock proteins in gastric mucosal protection. *J Gastroenterol Hepatol* 2000;15:D12–D19.

20) Moseley PL, Gapen C, Wallen ES, Walter ME, Peterson MW. Thermal stress induces epithelial permeability. *Am J Physiol Cell Physiol* 1994;267:C425–C434.

21) Zuhl M1, Schneider S, Lanphere K. Exercise regulation of intestinal tight junction proteins. Br J Sports Med. 2014;48:980-986

22) Fanning AS, Mitic LL, Anderson JM Transmembrane. Proteins in the Tight Junction Barrier. *J Am Soc Nephrol* 1999;10:1337–1345.

23) Ghosh S, Playford RJ. Bioactive natural compounds for the treatment of gastrointestinal disorders. *Clin Sci (Lond).* 2003;104:547-56.

24) van Wijck K1, Lenaerts K, van Loon LJ, Peters WH, Buurman WA, Dejong CH. Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. *PLoS One.* 2011;6:e22366. .

Table 1. VO_2max and 80% VO2 max exercise assessments.

	Day -5 of trial VO_2max protocol	Day 2 of trial 80% VO_2max protocol	Day 14 of trial 80% VO_2max protocol
Placebo +Placebo	5.01 (4.44 – 5.11)	3.73 (3.31 – 3.89)	3.61 (3.26 – 3.79)
Colostrum + Placebo	4.77 (4.50 – 4.93)	3.54 (3.25 - 3.77)	3.48 (3.19 – 3.69)
ZnC + Placebo	4.73 (4.57 – 5.06)	3.53 (3.34 – 3.78)	3.45 (3.29 – 3.73)
ZnC + Colostrum	4.78 (4.61 – 4.89)	3.458 (3.35 – 3.70)	3.54 (3.36 – 3.58)

[1]There were no significant differences between any of the treatment arms.
[2] Data shown as median and interquartile range (n=8)
[3] Data analyzed by 3-way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA.

FIGURE LEGENDS

Figure 1. Consort Flowchart for randomised trial.

Figure 2. Schematic of trial design.

Each subject took part in a double blind cross over protocol. Subjects received oral supplementation twice a day with ZnC, bovine colostrum, ZnC + bovine colostrum or placebo for 2 weeks with a 2-week washout in between study arms. The timings used to determine $\dot{V}O_2$max and to undertake 80% $\dot{V}O_2$max protocols, gut permeability assessments (involving 5 h urine collection) and blood samples are shown.

Figure 3. Gut permeability assessments during trial shown in Figure 2.

Two baseline assessments (no exercise) were performed before each arm of the study. Tests products were started on day 0. The other 2 assessments were performed at the end of 2 and 14 days ingestion of placebo (■), ZnC (**X**), colostrum (❍) or ZnC + colostrum (□) immediately after the subject had followed a 20 min 80% VO_2max protocol. Results are expressed as Lactulose/Rhamnose area under the curve ratio. Data expressed as mean +/-SEM (n=8). Data analysed by 3 way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA. ** signifies $p<0.01$ compared the placebo arm at the same timepoint, ++ signifies $p<0.01$compared to ZnC arm at that timepoint, $ and $$ signifies $p<0.05$ and $p<0.01$, respectively, compared to colostrum arm at that timepoint .

Figure 4. Effect of ZnC +/- colostrum on temperature induced changes in transepithelial electrical resistance and permeability to Horse radish peroxidase using Caco-2 monolayers.

A) Transepithelial resistance was measured in confluent polarised monolayers after incubating at 37 or 39^{o}C for 8 hours.

B) Permeability through the monolayers was also assessed by the measurement of passage of HRP into the basal medium, having been added to the apical medium at time zero.

Data expressed as mean +/-SEM (n=4). Data analysed by 3 way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA. ** signifies $p<0.01$, respectively compared to medium alone at the same temperature, + and ++ signifies $p<0.05$ and $p<0.01$ compared to ZnC alone at the same temperature. $$ signifies $p<0.01$ compared to colostrum alone . $p<0.01$ for all test conditions, 37°C vs 39°C

Figure 5. Effect of ZnC +/- colostrum on temperature induced apoptosis, active caspase 3 and 9.

Caco-2 cells were incubated at 37 or 39°C for 8 hours in medium alone or with ZnC, colostrum or ZnC + colostrum. Changes in apoptosis were determined using active caspase-3 (**A**) & 9 (**B**) assay kits, following changes in absorbance at 405 nM. Studies were also analysed using western analysis and showed similar results (Supplemental Figure 3). Data expressed as mean +/-SEM (n=3). Data analysed by 3 way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA. * and ** signifies $p<0.05$ and $p<0.01$ compared to medium alone at the same temperature and timepoint, respectively. $p<0.01$ for all test conditions 37°C vs 39°C

Figure 6. Effect of ZnC +/- colostrum on temperature induced changes in the pro-apoptotic protein Baxα, the anti-apoptotic protein Bcl-2 and heat shock protein expression (Hsp70).

Caco-2 cells were incubated at 37 or 39°C in medium alone or with ZnC, colostrum or ZnC + colostrum. Changes in Baxα, (**A**), Bcl-2 (**B**) after 4h are shown. Changes seen in Hsp70 after 4h (**C**) or 8h **(D**) incubation at these two temperatures are also shown. Similar results were seen using HT29 cells (data not shown)

Data expressed as mean +/-SEM (n=3). Data analysed by 3 way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA.* and ** signifies $p<0.05$ and $p<0.01$, respectively compared to medium alone at the same temperature and timepoint,++ signifies $p<0.01$ compared to ZnC alone at the same temperature and timepoint, $ and $$ signifies $p<0.05$ and $p<0.01$, respectively compared to colostrum alone at the same temperature and timepoint. $p<0.01$ for all test conditions, 37°C vs 39°C

Figure 7. Effect of ZnC +/- colostrum on temperature induced changes of ZO1 protein levels and phosphorylation.

Cells were incubated in the presence of test factors for 8 h at either 37 or 39°C.

A) Total ZO1, B) Phospho-tyrosine ZO1, C) Phospho-serine ZO1 analysed by Elisa. Studies using Western blotting and densitometry gave similar results (Supplemental Figure 5). Data expressed as mean +/-SEM (n=3). Data analysed by 3 way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA. ** signifies

p<0.01, respectively compared to medium alone at the same temperature, ++ signifies p<0.01, respectively compared to ZnC alone at the same temperature.

Figure 8. Effect of ZnC +/- colostrum on temperature induced changes of occludin protein levels and phosphorylation.

Cells were incubated in the presence of test factors for 8 h at either 37 or 39°C.

A) Total occludin, B) Phospho-tyrosine occludin, C) Phospho-serine occludin analysed by Elisa. Studies using Western blotting and densitometry gave similar results (Supplemental Figure 6). Data expressed as mean +/-SEM (n=3). Data analysed by 3 way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA. * and ** signifies p<0.05 and p<0.01, respectively compared to medium alone at the same temperature, + signifies p<0.05, respectively compared to ZnC alone at the same temperature, $ signifies p<0.05 compared to colostrum alone at the same temperature.

Figure 9. Effect of ZnC +/- colostrum on temperature induced changes of claudin 1 protein levels and phosphorylation.

Cells were incubated in the presence of test factors for 8 h at either 37 or 39°C.

A) Total claudin-1, B) Phospho-tyrosine claudin-1, C) Phospho-serine claudin-1 analysed by elisa. Studies using Western blotting and densitometry gave similar results (Supplemental Figure 7). Data expressed as mean +/-SEM (n=3). Data analysed by 3 way ANOVA followed by t-tests based on the group means, residual and degrees of freedom obtained from the

ANOVA. ** signifies $p<0.01$, respectively compared to medium alone at the same temperature.

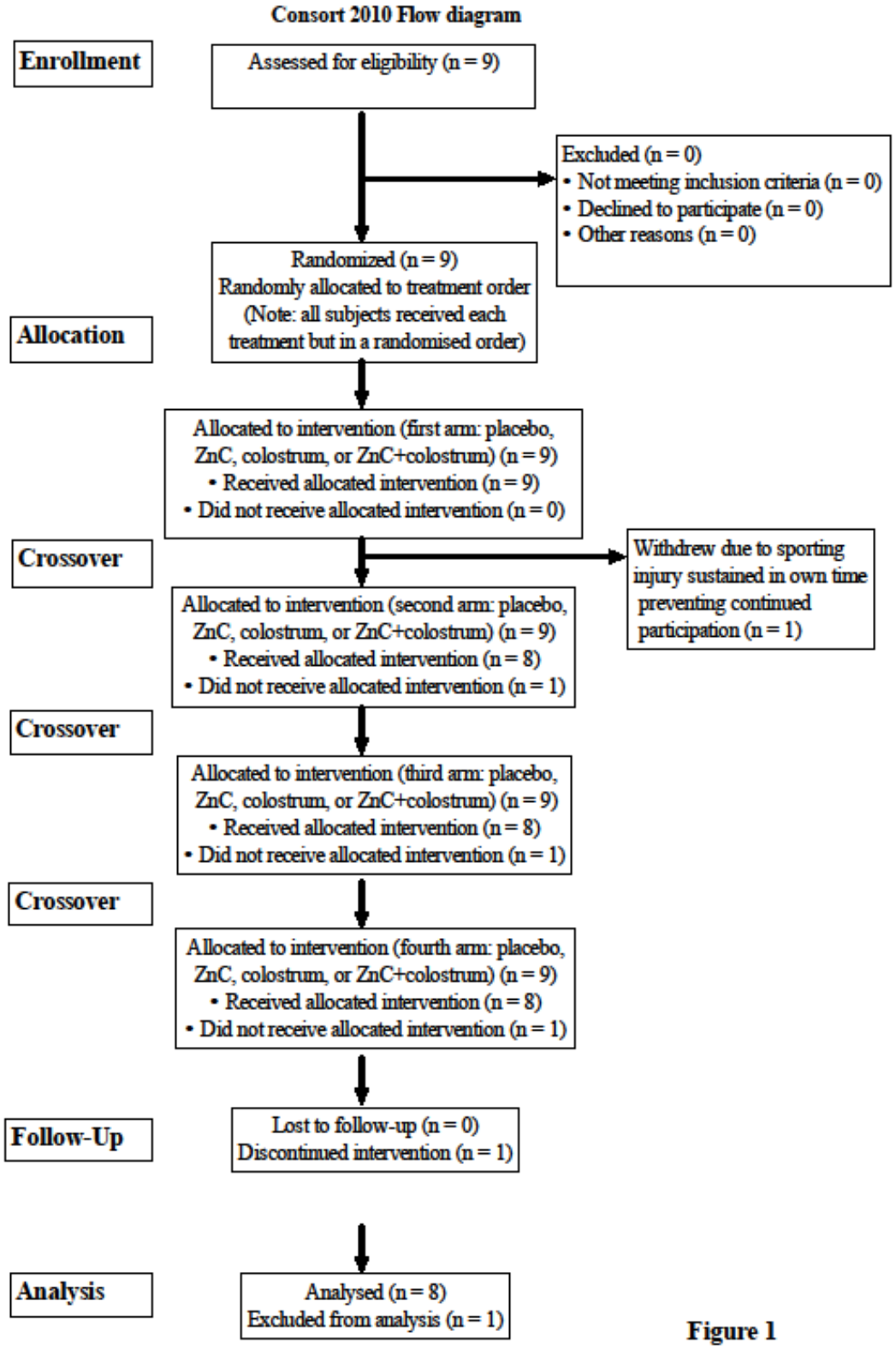

Consort 2010 Flow diagram
Enrollment
Assessed for eligibility (n = 9)
Excluded (n = 0)
• Not meeting inclusion criteria (n = 0)
• Declined to participate (n = 0)
• Other reasons (n = 0)
Allocation
Randomized (n = 9)
Randomly allocated to treatment order
(Note: all subjects received each treatment but in a randomised order)
Allocated to intervention (first arm: placebo, ZnC, colostrum, or ZnC+colostrum) (n = 9)
• Received allocated intervention (n = 9)
• Did not receive allocated intervention (n = 0)
Crossover
Withdrew due to sporting injury sustained in own time preventing continued participation (n = 1)
Allocated to intervention (second arm: placebo, ZnC, colostrum, or ZnC+colostrum) (n = 9)
• Received allocated intervention (n = 8)
• Did not receive allocated intervention (n = 1)
Crossover
Allocated to intervention (third arm: placebo, ZnC, colostrum, or ZnC+colostrum) (n = 9)
• Received allocated intervention (n = 8)
• Did not receive allocated intervention (n = 1)
Crossover
Allocated to intervention (fourth arm: placebo, ZnC, colostrum, or ZnC+colostrum) (n = 9)
• Received allocated intervention (n = 8)
• Did not receive allocated intervention (n = 1)
Follow-Up
Lost to follow-up (n = 0)
Discontinued intervention (n = 1)
Analysis
Analysed (n = 8)
Excluded from analysis (n = 1)

Figure 1

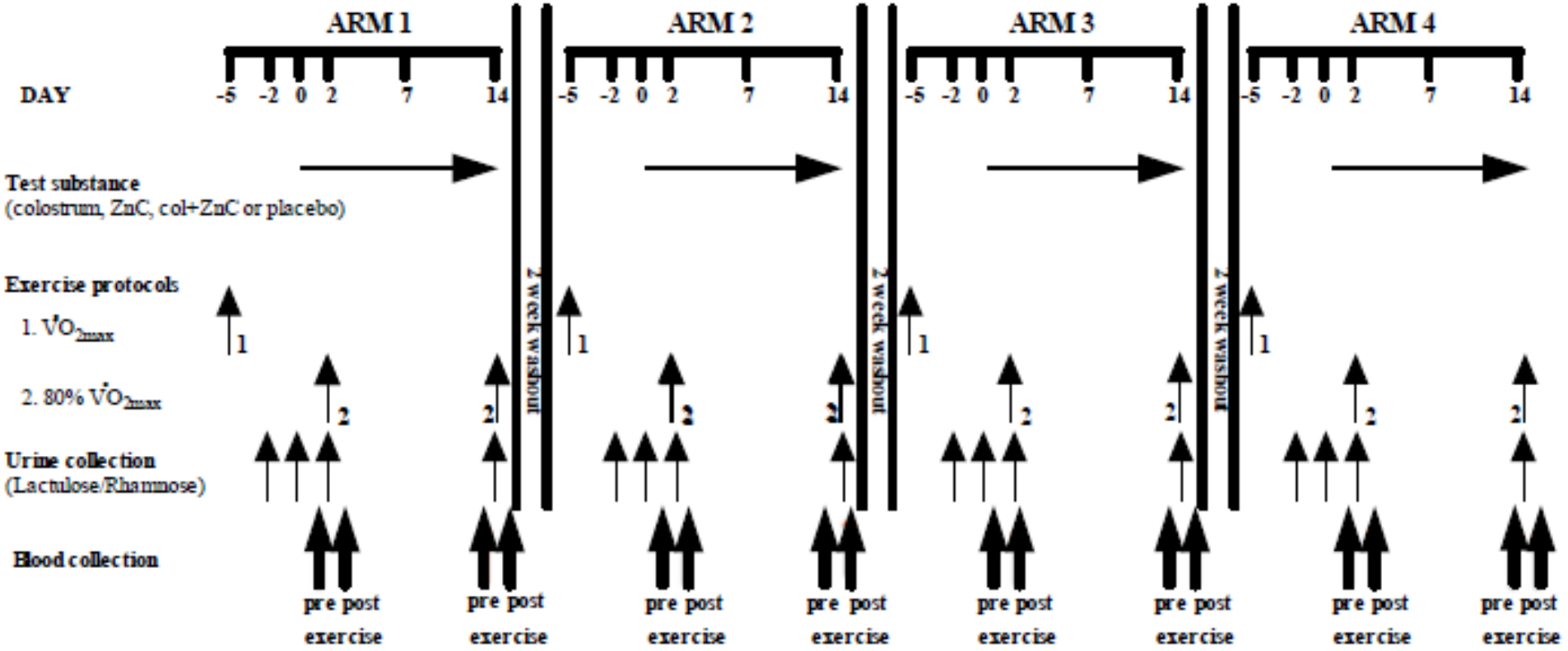

ARM 1
ARM 2
ARM 3
ARM 4
DAY
-5 -2 0 2 7 14
2 week washout
Test substance
(colostrum, ZnC, col+ZnC or placebo)
Exercise protocols
1. V̇O2max
2. 80% V̇O2max
Urine collection
(Lactulose/Rhamnose)
Blood collection
pre post
exercise

Figure 2

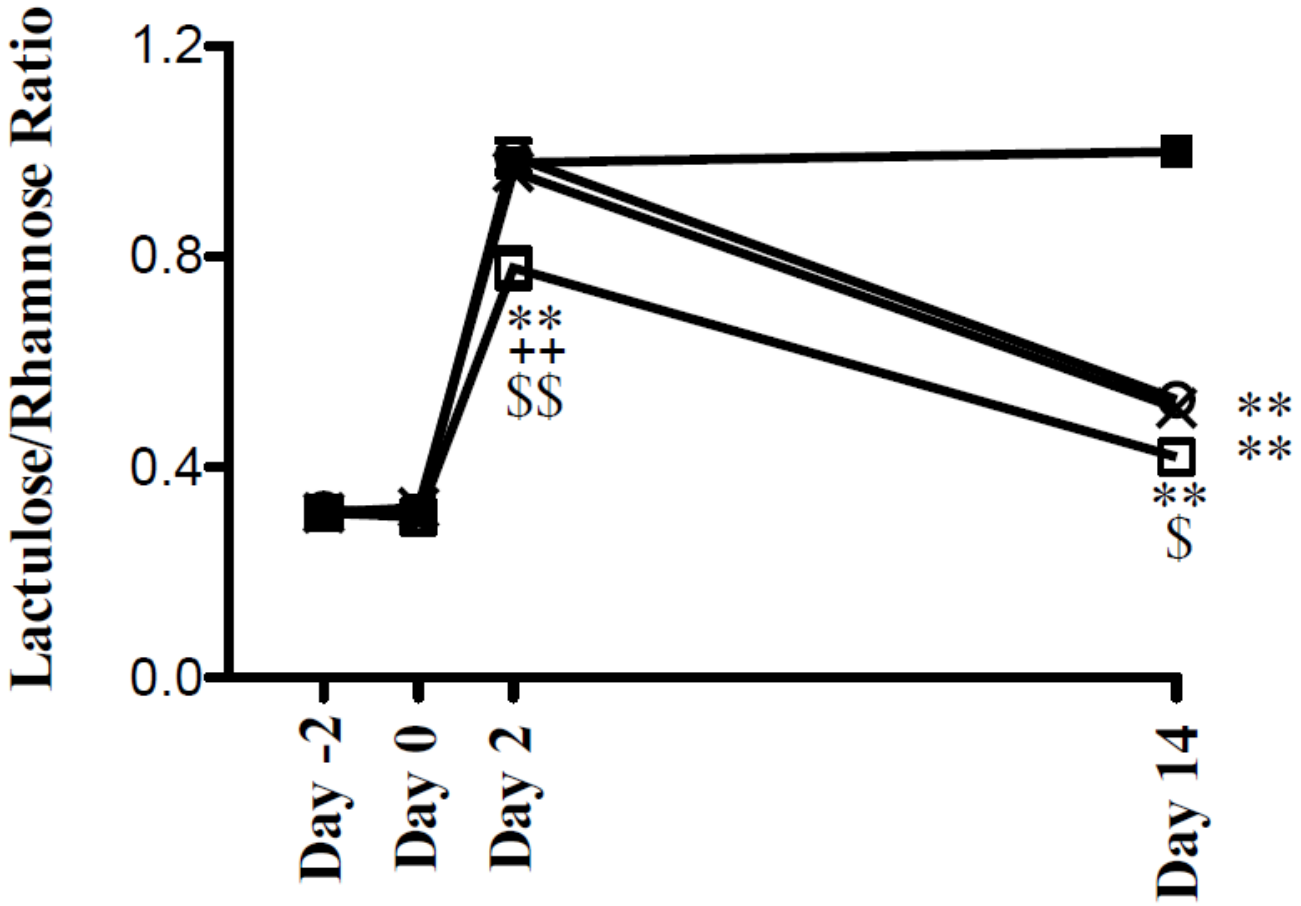


Figure 3

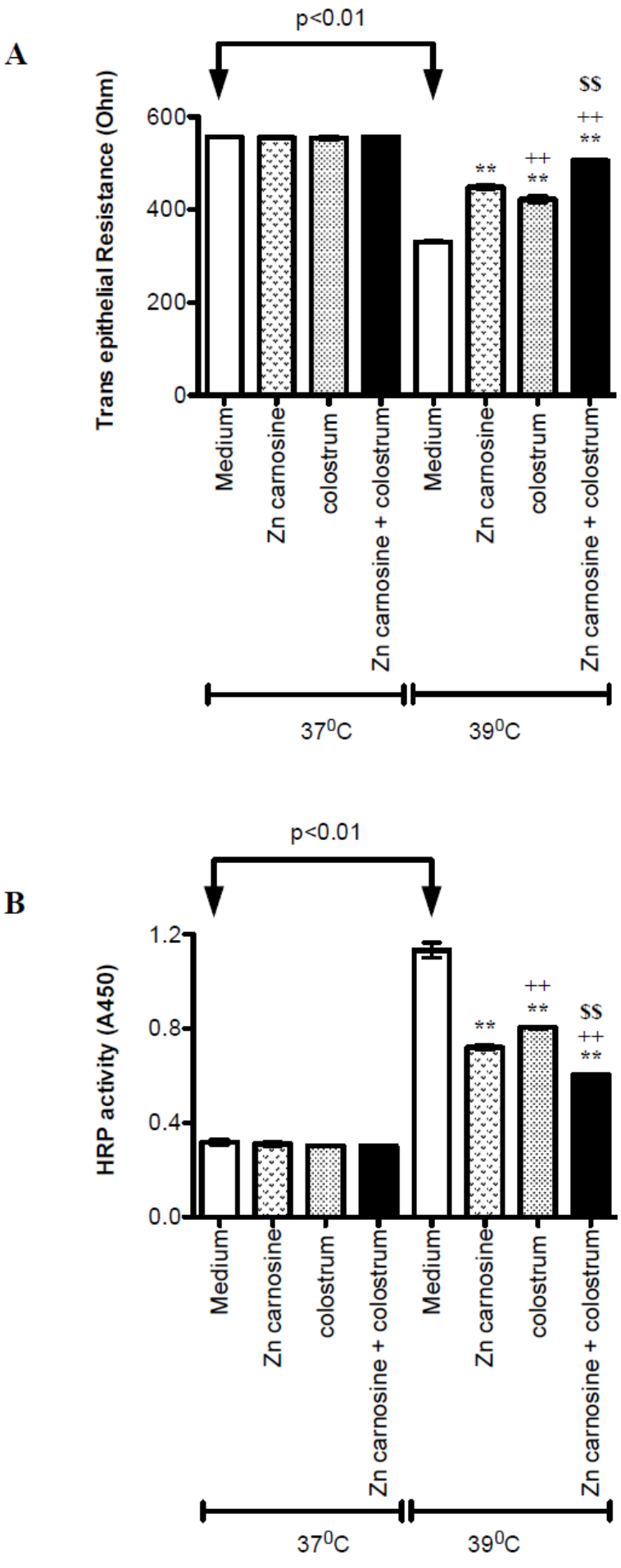
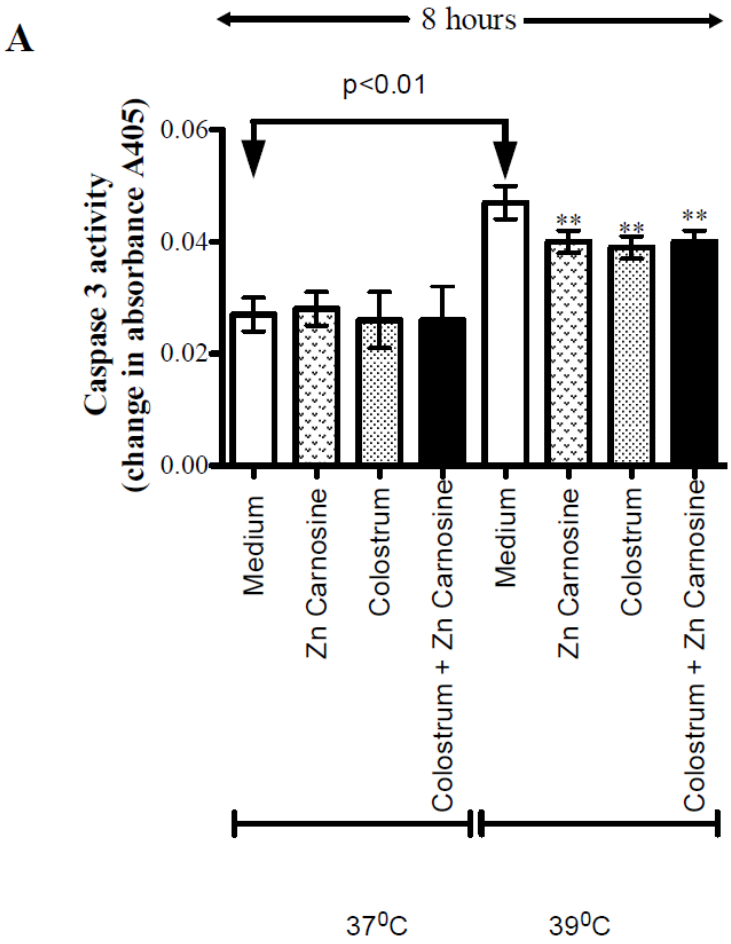

A
p<0.01
Trans epithelial Resistance (Ohm)
600
400
200
0
$$
++
**
**
++
**
Medium
Zn carnosine
colostrum
Zn carnosine + colostrum
Medium
Zn carnosine
colostrum
Zn carnosine + colostrum
37⁰C
39⁰C
B
p<0.01
HRP activity (A450)
1.2
0.8
0.4
0.0
**
++
**
$$
++
**
Medium
Zn carnosine
colostrum
Zn carnosine + colostrum
Medium
Zn carnosine
colostrum
Zn carnosine + colostrum
37⁰C
39⁰C

Fig 4


A
8 hours
p<0.01
Caspase 3 activity
(change in absorbance A405)
0.06
0.04
0.02
0.00
**
**
**
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C

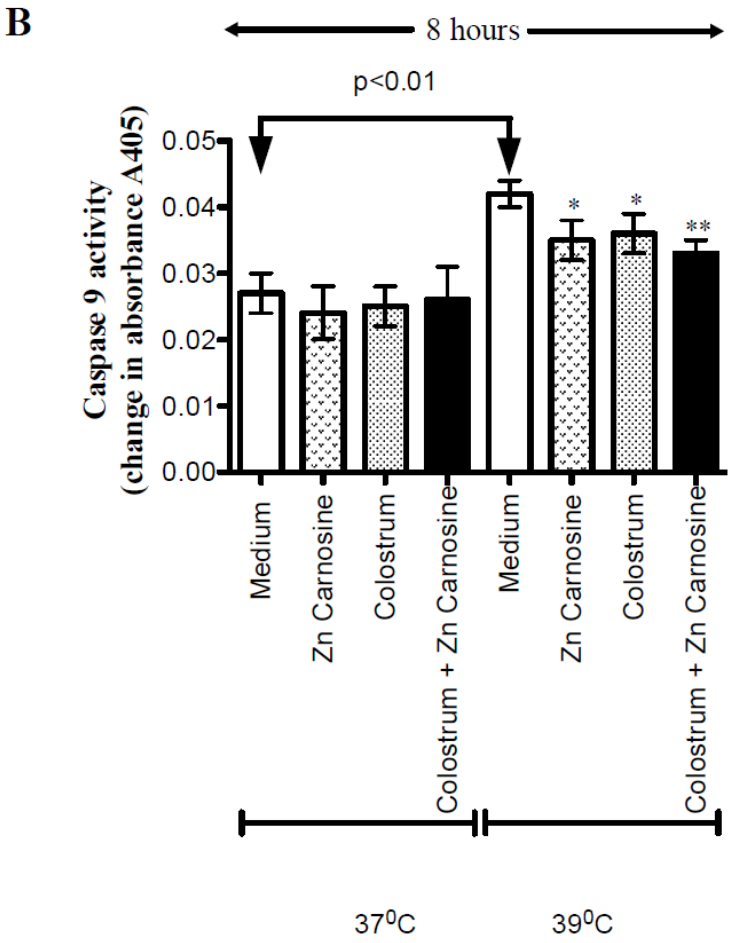

B
8 hours
p<0.01
Caspase 9 activity
(change in absorbance A405)
0.05
0.04
0.03
0.02
0.01
0.00
*
*
**
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C

Fig 5

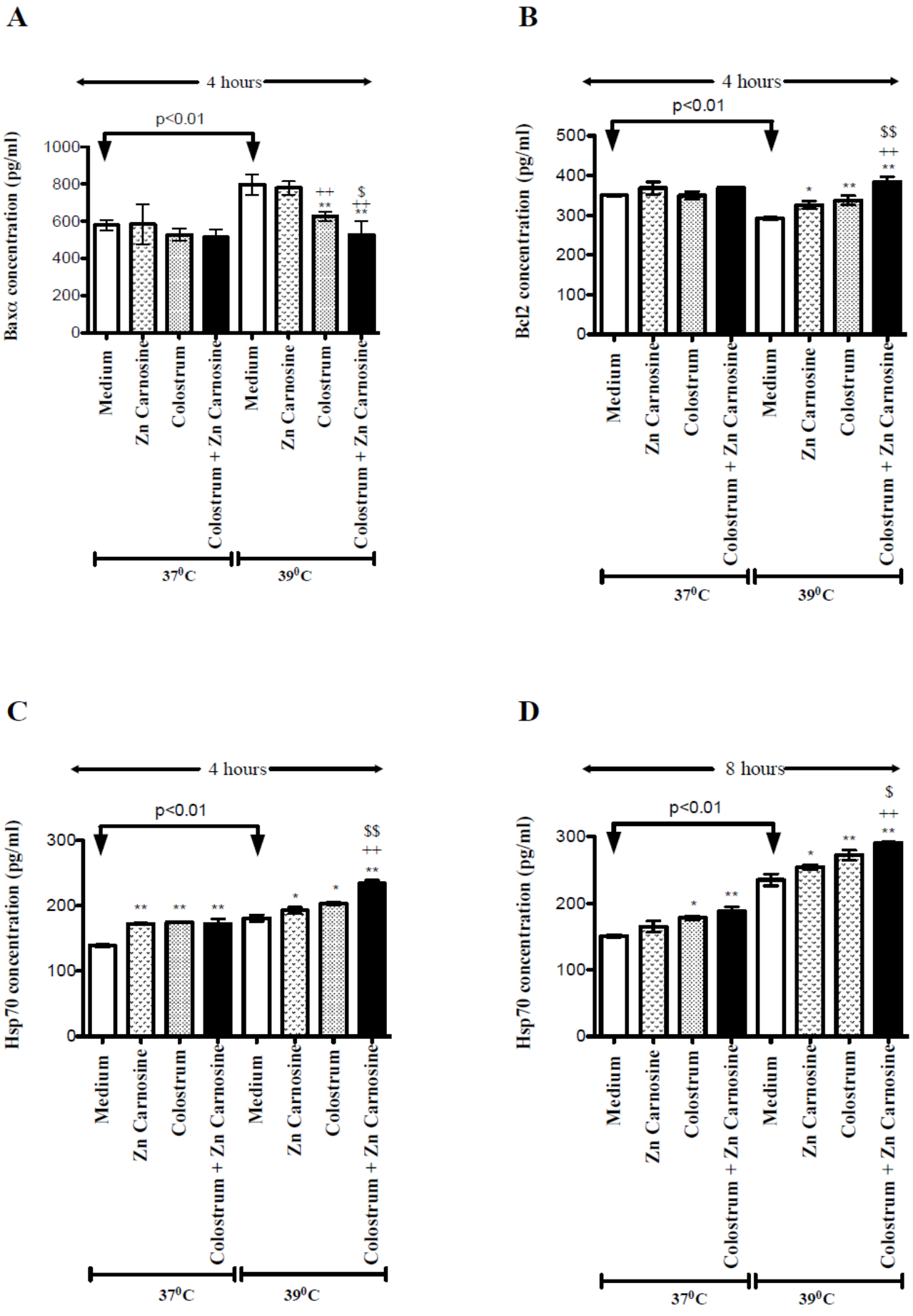

A
4 hours
p<0.01
Baxα concentration (pg/ml)
1000
800
600
400
200
0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
B
4 hours
p<0.01
Bcl2 concentration (pg/ml)
500
400
300
200
100
0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
C
4 hours
p<0.01
Hsp70 concentration (pg/ml)
300
200
100
0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
D
8 hours
p<0.01
Hsp70 concentration (pg/ml)
300
200
100
0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C

Figure 6

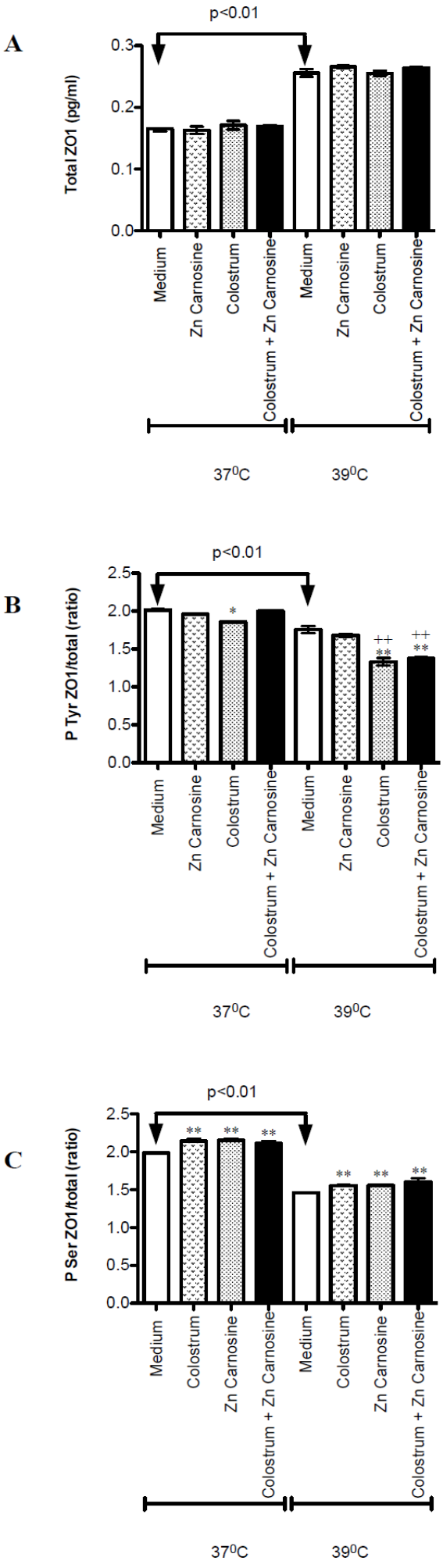

A
p<0.01
Total ZO1 (pg/ml)
0.3
0.2
0.1
0.0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
B
p<0.01
P Tyr ZO1/total (ratio)
2.5
2.0
1.5
1.0
0.5
0.0
*
++
**
++
**
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
C
p<0.01
P Ser ZO1/total (ratio)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
**
**
Medium
Colostrum
Zn Carnosine
Colostrum + Zn Carnosine
Medium
Colostrum
Zn Carnosine
Colostrum + Zn Carnosine
37⁰C
39⁰C

Figure 7

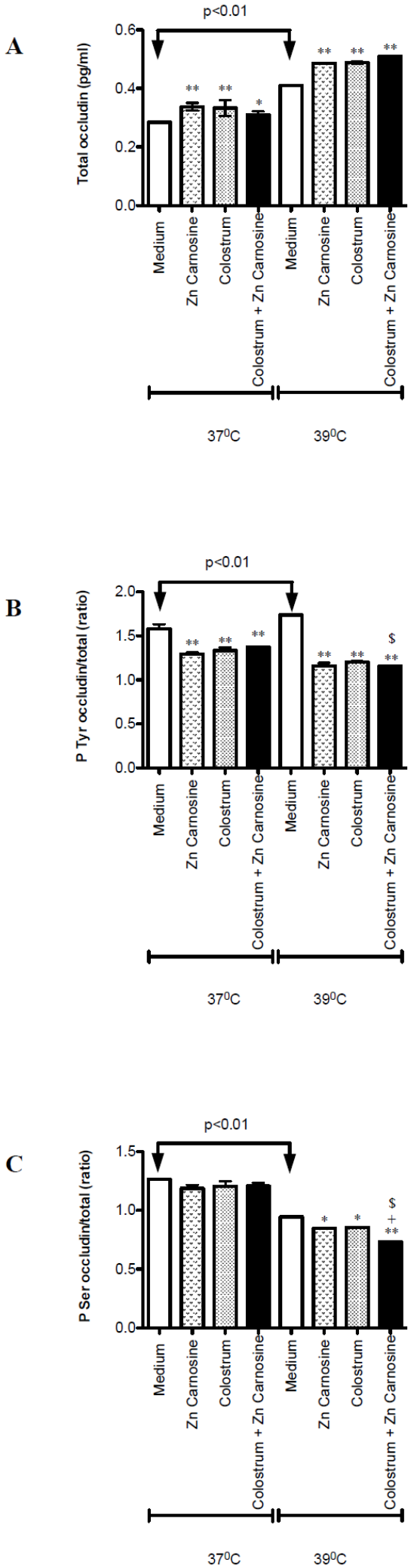

A
p<0.01
Total occludin (pg/ml)
0.6
0.4
0.2
0.0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
B
p<0.01
P Tyr occludin/total (ratio)
2.0
1.5
1.0
0.5
0.0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
C
p<0.01
P Ser occludin/total (ratio)
1.5
1.0
0.5
0.0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C

Figure 8

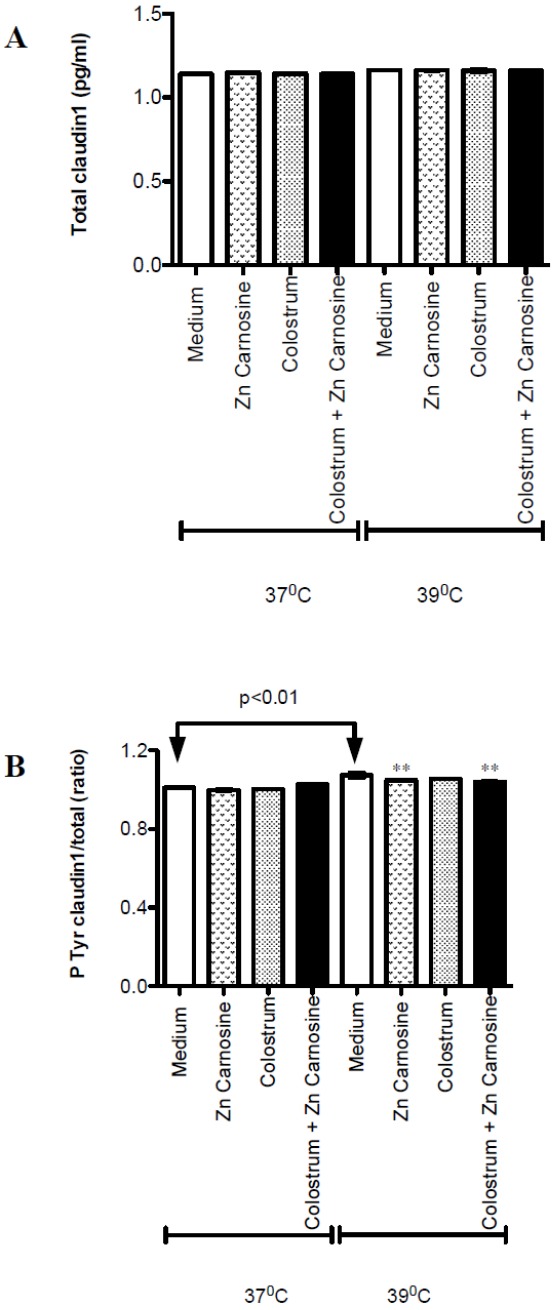

A
Total claudin1 (pg/ml)
1.5
1.0
0.5
0.0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
B
P Tyr claudin1/total (ratio)
p<0.01
1.2
0.8
0.4
0.0
**
**
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C
C
P Ser claudin1/total (ratio)
1.5
1.0
0.5
0.0
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
Medium
Zn Carnosine
Colostrum
Colostrum + Zn Carnosine
37⁰C
39⁰C

Figure 9