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

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Running title: ZnC +/- bovine colostrum and gut permeability.

Abbreviations: Bax α ; 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, VO₂max ; maximal oxygen uptake, NSAID; nonsteroidal anti-inflammatory, pSer; phosphorylated Serine, pTyr; phosphorylated Tyrosine, ZnC; zinc carnosine, ZO1; zona occludens protein 1

Clinical trial registration: ISRCTN, study ID ISRCTN51159138

(<http://www.isrctn.com/ISRCTN51159138>).

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1 ABSTRACT

2 **Background:** Heavy exercise causes gut symptoms and, in extreme cases, “heat stroke” due
3 to increased intestinal permeability of luminal toxins.

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

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

12 **Results:** Body temperature increased 2°C and gut permeability (5 hour urinary
13 lactulose:rhamnose ratios) increased 3-fold following exercise (0.32 ± 0.016 baseline to $1.0 \pm$
14 0.017 at +14 days, $p < 0.01$). ZnC or colostrum truncated rise by 70% after 14 days treatment.
15 Combination treatment gave additional benefit and truncated exercise induced increase at +2
16 day (30% reduction, $p < 0.01$). 2°C temperature rise in in vitro studies caused doubling of
17 apoptosis and reduced epithelial resistance 3-4-fold. ZnC or colostrum truncated these effects
18 (35-50%) with greatest response seen with combination treatment (all $p < 0.01$). Mechanisms
19 of action included increasing Hsp70 and truncating temperature-induced changes in B cell
20 leukemia/lymphoma-2 associated X protein-alpha (Bax α) and B-cell lymphoma 2 (Bcl-2). ZnC
21 also increased total occludin and reduced phosphorylated Tyrosine (pTyr)-occludin, pTyr-
22 occludin and phosphorylated Serine (pSer)-occludin, enhancing tight junction formation and
23 stabilisation.

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

26 personnel.

27 **Keywords:** Repair; gut growth; injury; nutraceutical; clinical trial

28 INTRODUCTION

29 Several stresses affect the integrity of the intestinal barrier including prolonged strenuous
30 exercise (1), heat stress (2) and drugs such as nonsteroidal anti-inflammatory agents
31 (NSAIDs)(3). Loss of intestinal integrity may result in passage of luminal endotoxins into the
32 circulation, causing an inflammatory cascade, exacerbating loss of barrier function. This can
33 result in severe systemic effects (4), such as in exertional heat stroke, associated with
34 hyperthermia, multi-organ failure and endotoxemia. Similar processes have relevance for
35 many athletes involved in heavy exercise such as long-distance running where gastrointestinal
36 symptoms including cramps, diarrhoea, nausea, and bleeding are commonly reported (5, 6).
37 These symptoms are probably due to a combination of reduced splanchnic blood flow (7),
38 hormonal changes, altered gut permeability, and increased body temperature.

39
40 Pharmacological options to reduce these problems are limited, particularly in competitive
41 athletics. There is, therefore, interest in using natural or naturally derived products. One
42 product already commercially available is zinc carnosine (ZnC), where zinc and carnosine are
43 linked in a polymeric one-to-one ratio and is currently marketed as a zinc dietary supplement
44 with “added value for gastric health”. Combining zinc with carnosine has potential
45 advantages over simple zinc supplementation as carnosine is a dipeptide (comprising β -
46 alanine and l-histidine) that is naturally present in long living cells such as muscle and nerves,
47 where, among other actions, it probably has a role as an antioxidant (8).

48

49 We previously showed ZnC stimulates several aspects of gut mucosal integrity, including
50 stimulating cell migration and proliferation in vitro and reducing the amount of gastric and

51 small intestinal injury caused by NSAIDs in rats and mice (9). Furthermore, using normal
52 volunteers, we demonstrated that ZnC prevented the rise in gut permeability caused by
53 clinical doses of the NSAID indomethacin (9). Its potential value in decreasing gut
54 permeability associated with heavy exercise and its mechanism of actions are, however,
55 unknown.

56

57 We now examine the effect of oral ZnC on gut permeability and exercise-induced temperature
58 rise in subjects undertaking heavy exercise and compared effects of ZnC alone with taking it
59 in combination with bovine colostrum, a rich source of growth factors and immune
60 modulators (10). Our previous studies utilising colostrum alone showed benefit in reducing
61 exercise induced increased gut permeability in athletes, but only after prolonged (14 days)
62 administration (11). Colostrum given alone, therefore, also provided a useful positive control.

63

64 To examine some of the mechanisms by which protective effects were mediated, we
65 performed a series of in vitro studies using two human intestinal cell lines focusing on the
66 effect of a temperature rise to 39°C (similar to that seen in athletes undergoing the in vivo
67 studies) on apoptosis, epithelial barrier resistance, heat shock protein 70 (Hsp70) expression
68 and tight junction (TJ) proteins in the presence and absence of test compounds.

69

70 **MATERIALS & METHODS**

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

72

73 **A) CLINICAL STUDY: EFFECT OF ZNC AND COLOSTRUM ON EXERCISE-** 74 **INDUCED CHANGES IN HUMAN GUT PERMEABILITY.**

75

76 Zinc carnosine (Lonza Nutrition Inc USA) and indistinguishable placebo capsules were used
77 for clinical study. Colostrum (Neovite brand lactose-reduced colostrum) and placebo were
78 provided by Colostrum UK, London. Placebo used in place of colostrum was isoenergetic and
79 isomacronutrient milk protein concentrate at 80% protein content (principally casein) and was
80 indistinguishable in appearance and taste from the colostrum powder, which was the form
81 administered.

82

83 **Ethical Approval:** All procedures were conducted according to the Declaration of Helsinki.

84 Ethics approval was obtained from Aberystwyth University Ethics Committee.

85

86 **Subjects:** Eight healthy males took part and all were active individuals who exercised
87 regularly 4 or more times per week (4 participants were runners, 1 cyclist, 1 lacrosse player, 1
88 footballer, 1 rugby player). Physical parameters were: mean age 25, range 19-33; height 1.78
89 ± 0.02 m; body mass 80.1 \pm 2.5 kg; BMI 24.98 \pm 0.17 kg/m²; maximal oxygen uptake
90 (VO₂max) 59.6 \pm 1.8 ml/kg/min; peak speed in VO₂max test 18 \pm 0.4 km/h; running speed at
91 80% VO₂max 13.5 \pm 0.03 km/h; (values mean \pm SEM). Subjects completed a pre-exercise
92 screening questionnaire (Physical Activity Readiness Questionnaire: PAR-Q) before
93 participating in each test. VO₂max exercise assessments were performed by standard methods
94 as reported previously (9) on day -5 of each arm to ensure consistency of the 80% VO₂max
95 protocol on day +2 and +14 (**Figure 1 and 2**).

96

97 **Preparation of subjects for the exercise study:** Subjects completed a 24 h food diary on the
98 day before the main exercise trial in the first arm of the trial and repeated this diet in the
99 subsequent arms.. All trials were performed after an overnight fast of at least 10 h. Subjects

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

105 Subjects sat for 10 min before baseline venous blood sample (pre-exercise) was taken.

106 Subjects then ran on the treadmill, with 1% grade, for 20 min at a constant speed equivalent to
107 80% VO₂max, as determined from preliminary tests. Expired gas was analyzed during
108 exercise using an online breath-by-breath system (Jaeger Oxycon Pro. Hoechberg, Germany).

109 Core body temperature, heart rate, and rating of perceived exertion were recorded every 5 min
110 during the trial. After completing the run, subjects were quickly seated and a second blood
111 sample (post-exercise) was obtained (within 5 minutes). Subjects then emptied their bladder
112 before consuming the intestinal permeability test drink and commencing with a 5-h urine
113 collection to determine intestinal permeability.

114

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

121 Maximal oxygen uptake (VO₂max) was assessed on day -5 for each arm of the study. Gut
122 permeability assessments were performed under non- exercise conditions on day -2 and 0 (to

123 confirm stable baseline) and immediately following the standardised exercise (treadmill
124 running 20 min at 80% VO_2max) protocol on day +2 and +14.

125 Oral supplements consisted of 37.5 mg ZnC + 10 g placebo, 10 g bovine colostrum + placebo
126 capsule, 37.5 mg ZnC + 10 g bovine colostrum and 10 g placebo + placebo capsule, taken
127 twice per day. The capsules (ZnC) and powder (colostrum) or their placebo equivalents were
128 taken just prior to breakfast or evening meal. The doses were chosen based on the results of
129 pilot in vitro studies (**Supplemental Material 1, Supplemental Figure 1**).

130

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

135

136 **B) IN VITRO STUDIES**

137 To investigate mechanisms by which test compounds influenced gut permeability in the
138 clinical study, we performed a series of experiments examining the effect of a 2°C rise (from
139 37 to 39°C).

140

141 **Cell lines:** HT29 is derived from colorectal adenocarcinoma of 44-year-old Caucasian female
142 (ATCC)(13). Caco-2 is derived from colorectal adenocarcinoma of 72-year-old male (ATCC)
143 and exhibits tight junctions and desmosomes between adjacent cells and grows as polarized
144 monolayers (14).

145

146 **Transepithelial permeability assays:** The influence of temperature changes on
147 transepithelial permeability in the presence and absence of test factors were determined using
148 two different methods. One determined changes in transepithelial electrical resistance using
149 our previously published methods (11). The other analysed the passage of horseradish
150 peroxidase (HRP) across the epithelial layer using standard methods (15). To enhance any
151 effects seen, the above experiments were also performed in low calcium medium (0.9 mM) in
152 addition to normal calcium medium (1.7 mM).

153

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

157

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

165

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

169

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

181

182 **Statistical analyses**

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

190

191 **RESULTS**

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

194 As expected, rating of perceived exertion expressed during exercise, heart rate (mean rise
195 106 ± 2 BPM, from 73 ± 1 to 179 ± 1 BPM), lactate concentrations (mean rise 5.76 ± 0.31 mM,
196 from 1.10 ± 0.07 to 6.86 ± 0.31 mM), core temperature (mean rise 1.59 ± 0.04 °C, from
197 36.75 ± 0.02 to 38.33 ± 0.05 °C), VO_2 , VCO_2 and respiratory exchange ratio, all rose in response
198 to exercise (all $p < 0.01$). The presence of supplements had no significant effect on results.
199 $\text{VO}_{2\text{max}}$ assessments on day -5 of each arm and 80% $\text{VO}_{2\text{max}}$ protocol on day 2 and 14 were
200 not different between the four arms (**Table 1**).

201
202 Baseline permeability expressed as the ratio of Lactulose/Rhamnose under the curve values
203 were similar at the beginning of each study arm (**Figure 3**). Permeability increased about 3-
204 fold in response to exercise during the placebo arm (rising from 0.318 ± 0.016 , initial baseline
205 value, to 0.979 ± 0.026 at day +2 and 1.000 ± 0.017 at day +14 (both $p < 0.01$ vs baseline).

206 Expressing results as Lactulose/Rhamnose % urinary excretion ratios gave equivalent results
207 (Supplemental Material 1, **Supplemental Figure 2**).

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

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

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

220

221 **B. IN VITRO STUDIES**

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

228

229 **Apoptosis:** Both ELISA and western blot analysis showed that increasing incubation
230 temperature caused an approximate 2-fold increase in active caspase 3 and 9 expression at the
231 8 h time points (**Figure 5 and Supplemental Figure 3**). Addition of ZnC, colostrum or ZnC
232 + colostrum had no significant effect on caspase expression when incubated at 37°C.
233 However, the co-presence of ZnC, colostrum or the combination all significantly reduced
234 caspase 3 and 9 expression compared to cells grown in medium alone at 39°C (all $p < 0.01$).
235 These changes were specific as they were not seen when the caspase-3 or caspase-9 inhibitor
236 were also added to the cells (**Supplemental Figure 4**).

237

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

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

244

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

251

252 Raising incubation temperature caused increased Hsp70 levels from 139 ± 1 to 181 ± 3 pg/ml
253 (p<0.01) after 4 h (Figure 6C). Adding ZnC alone or colostrum alone increased Hsp70 levels
254 at both 37°C and 39°C above values seen in cells grown in medium alone (all p<0.05).

255 Compared to giving either test compound alone, additional increases were found when ZnC
256 and colostrum were added together in cells at 39°C at both time points (Figure 6C+D).

257

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

260

261 ZO1: Total ZO1 increased in response to temperature rise and were not affected by test
262 factors (**Figure 7A**). P-Tyr-ZO1 were reduced by temperature rise and presence of colostrum
263 or combination treatment reduced levels further (Figure 7B). P-Ser-ZO1 was reduced by
264 temperature rise. The co-presence of test factors increased p-Ser-ZO1 levels at both 37 and
265 39°C (Figure 7C). Analyses using Western blotting and densitometry showed similar results
266 (**Supplemental Figure 5**).

267

268 Occludin: Total occludin increased in response to temperature rise. Presence of ZnC,
269 colostrum or combination all increased total occludin levels at 37°C. At 39°C all test factors
270 caused additional rises in total occludin levels compared to cells in medium alone (**Figure**
271 **8A**). Increased temperature caused p-Tyr-occludin to rise but presence of test factors reduced
272 p-Tyr-occludin levels at both 37 and 39°C with largest fall seen in cells treated with ZnC +
273 colostrum (Figure 8B). P-Ser-occludin levels were reduced in response to temperature rise
274 and presence of test factors caused further reductions in p-Ser-occludin ratios, with the largest
275 fall seen with combination treatment (Figure 8C). Analyses using Western blotting and
276 densitometry showed similar results (**Supplemental Figure 6**).

277

278 Claudin-1: Total claudin-1 was not affected by temperature change or test factors (**Figure**
279 **9A**). P-Tyr-claudin-1 levels rose in response to temperature increase and there was a small but
280 significant truncation of the rise in the presence of ZnC alone or in combination with
281 colostrum (Figure 9B). P-Ser-claudin-1 was not significantly affected by temperature rise or
282 presence of test factors (Figure 9C). Analyses using Western blotting and densitometry
283 showed similar results (**Supplemental Figure 7**).

284

285

286 DISCUSSION

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

292

293 Numerous exercise protocols are used by exercise physiologists. We chose a 20-min run at
294 80% VO_2max protocol as we have previous experience of this (11), it allows a crossover
295 study design to be used in a relatively short period, reliably increases gut permeability by 2-3
296 fold and increases core temperature by 1.5–2°C. Assessment of intestinal permeability by
297 quantitating unmediated absorption of at least two sugars of different sizes provides a
298 sensitive index of intestinal damage as we and others have previously shown (11, 12, 16).

299

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

307

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

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

315

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

326

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

332

333 Hsps maintain cellular homeostasis during normal cell growth and enhance survival during
334 and after various cellular stresses (19). Increased Hsp expression may be one mechanism
335 through which thermo-tolerance occurs in animals and cells (20). Hsp70 is increased in

336 response to temperature rises as a homeostatic mechanism for maintaining viability under
337 conditions that increase the accumulation of damaged proteins. Our finding that ZnC induced
338 Hsp70 expression at 37°C and caused additional increases when added at 39°C suggests that
339 this pathway may have relevance to our results. Importantly, our in vitro results were
340 demonstrated reproducing the temperature rise seen in the clinical study (to ~39°C), and seen
341 in most athletes during standard performance, rather than the typical 41.5°C used in rat
342 models of hyperthermia that results in massive breakdown of mucosal integrity.

343

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

357

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

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

365

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

378

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

386 endotoxin levels. It would also be of interest to examine additional markers of cellular
387 integrity and enterocyte permeability such as I-FABP, although it seems likely that later blood
388 samples and potentially a longer exercise protocol than that used in our studies would be
389 required to demonstrate such changes (24). Additional studies could also include the
390 relevance of hypoxia on paracellular and cellular integrity when cells are stressed by hypoxia
391 alone and in combination with temperature rises. Our current studies focusing on temperature
392 change builds on previous work showing ZnC prevents NSAID gut damage. It would
393 therefore be of interest to examine its effects on other gut disorders such as inflammatory
394 bowel disease where uncontrolled inflammatory response combined with disruption of
395 epithelial integrity is a major factor.

396

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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.

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Table 1. VO₂max and 80% VO₂ max exercise assessments.

	Day -5 of trial VO ₂ max protocol	Day 2 of trial 80% VO ₂ max protocol	Day 14 of trial 80% VO ₂ max 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)

¹There 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% $\dot{V}O_{2\max}$ 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°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 Bax α , 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 Bax α , (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 \pm 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 \pm 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.

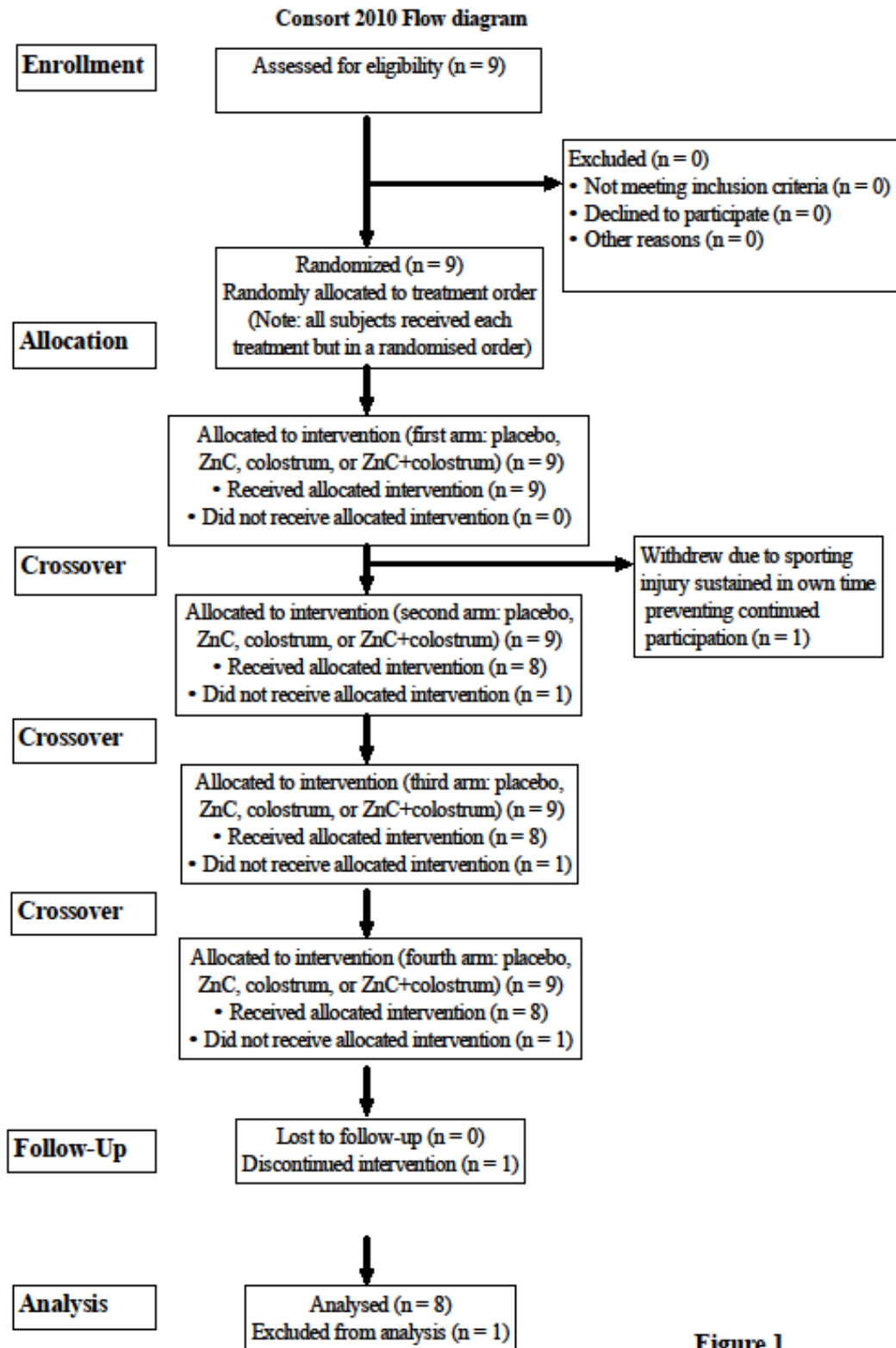


Figure 1

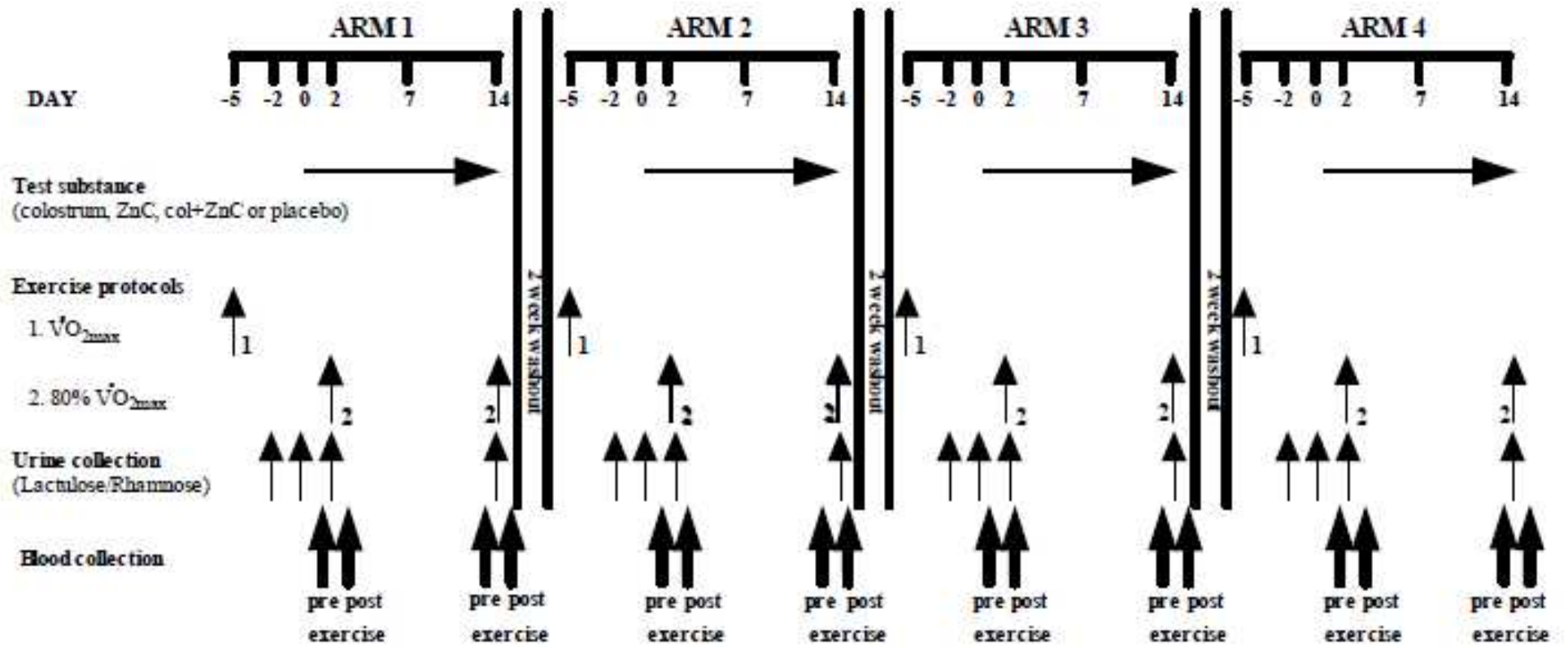


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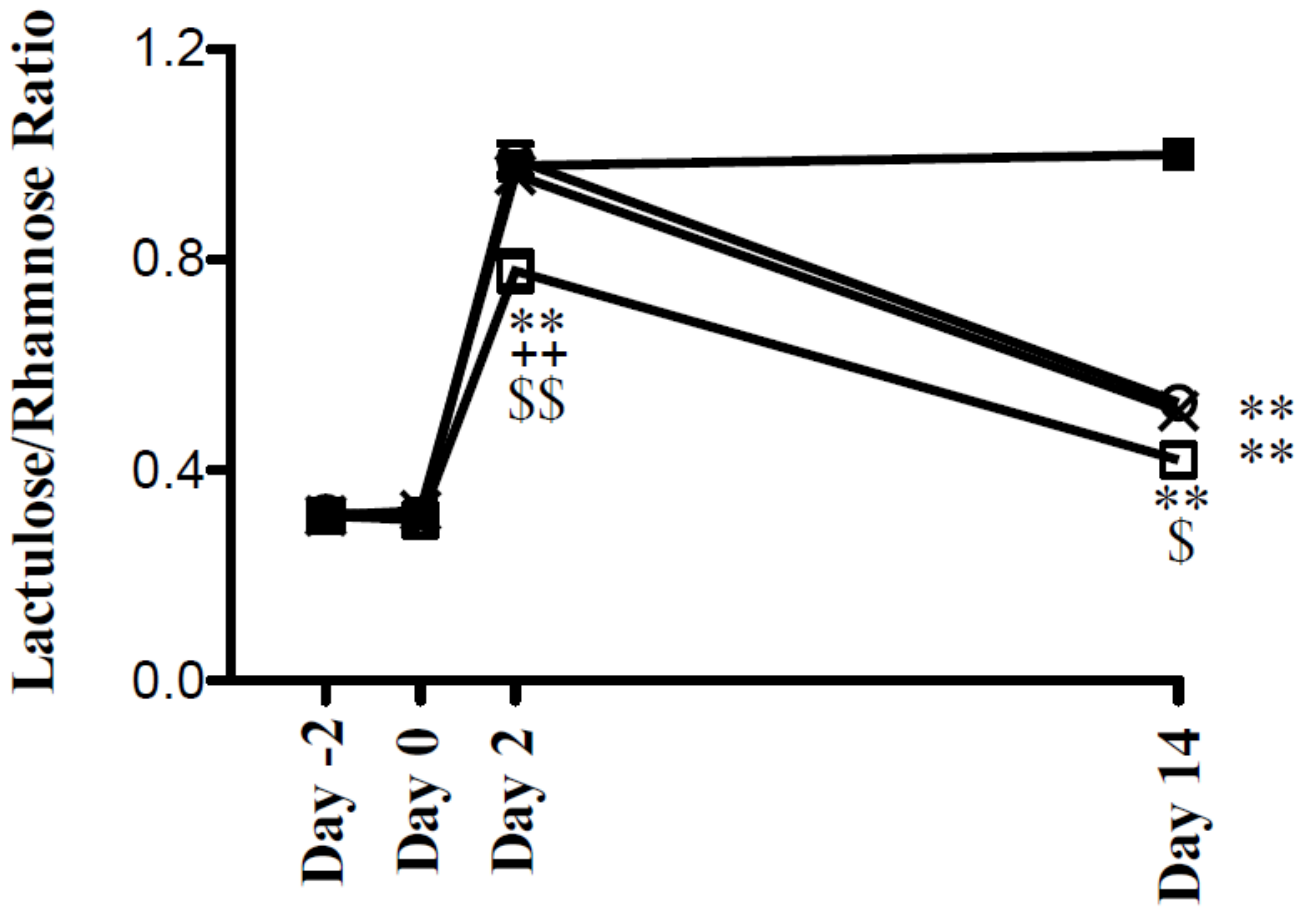


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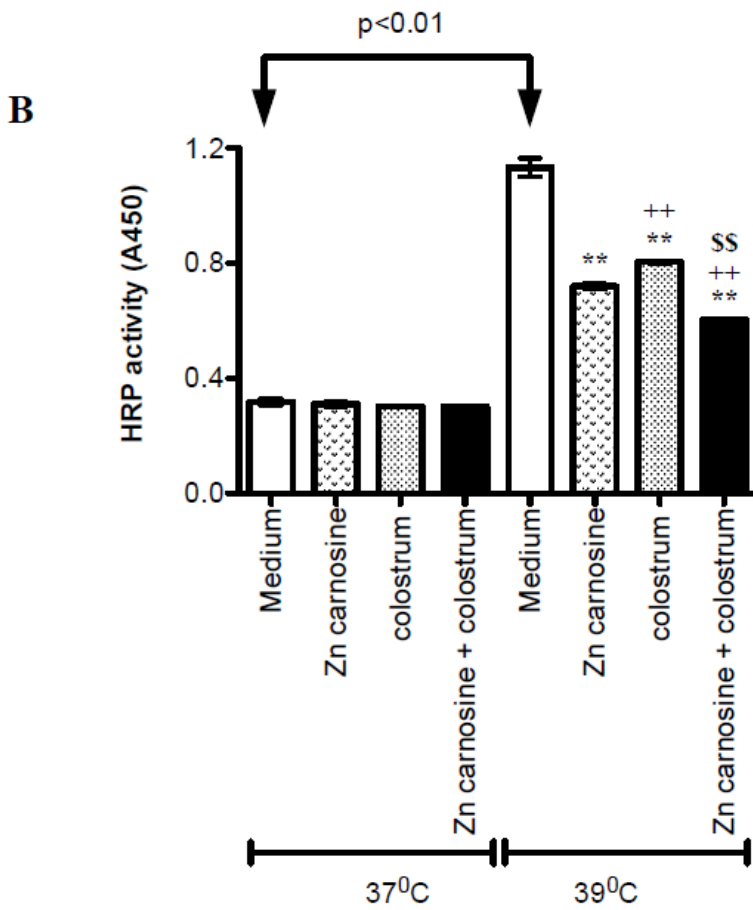
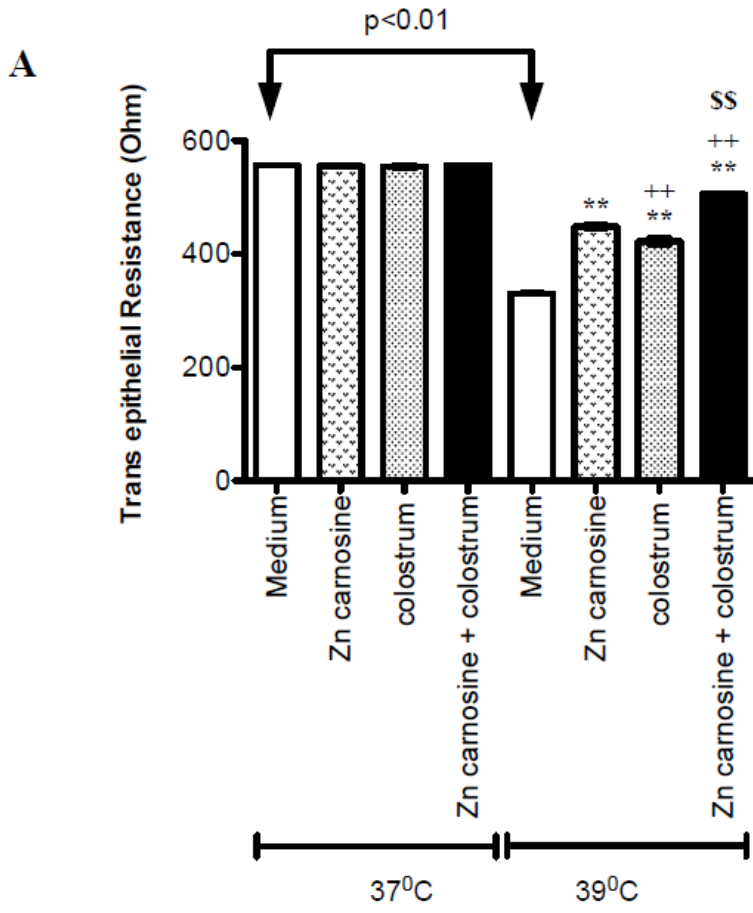


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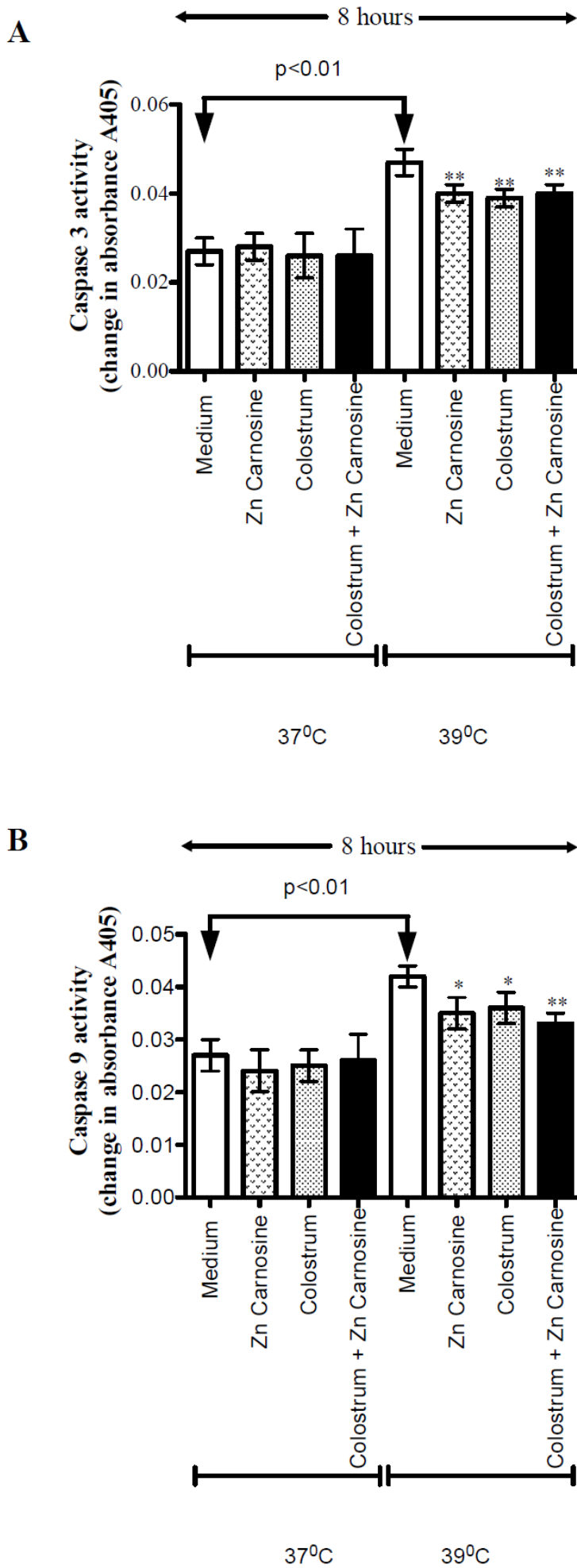


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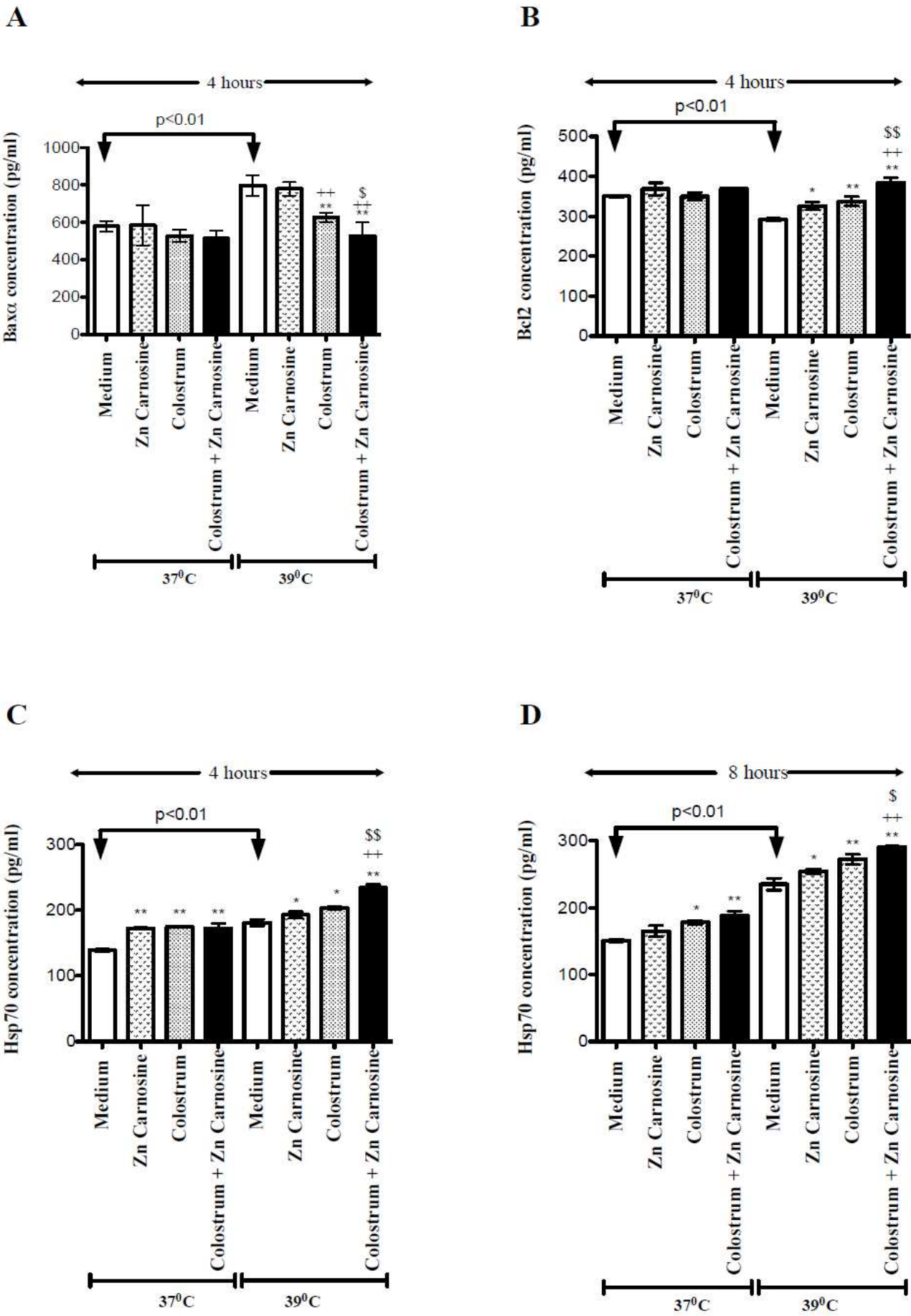


Figure 6

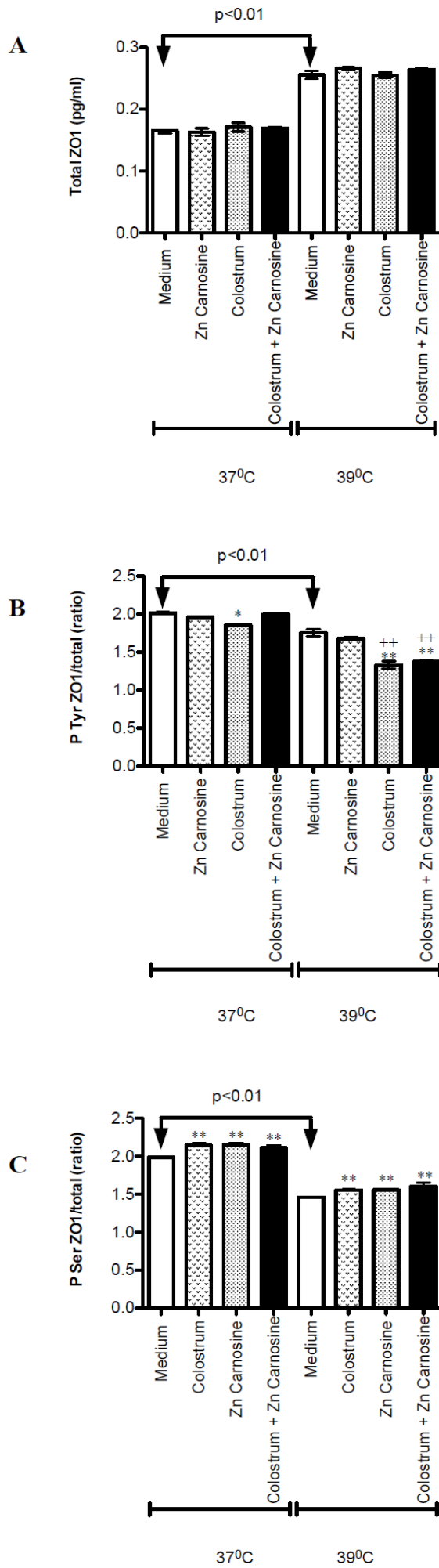


Figure 7

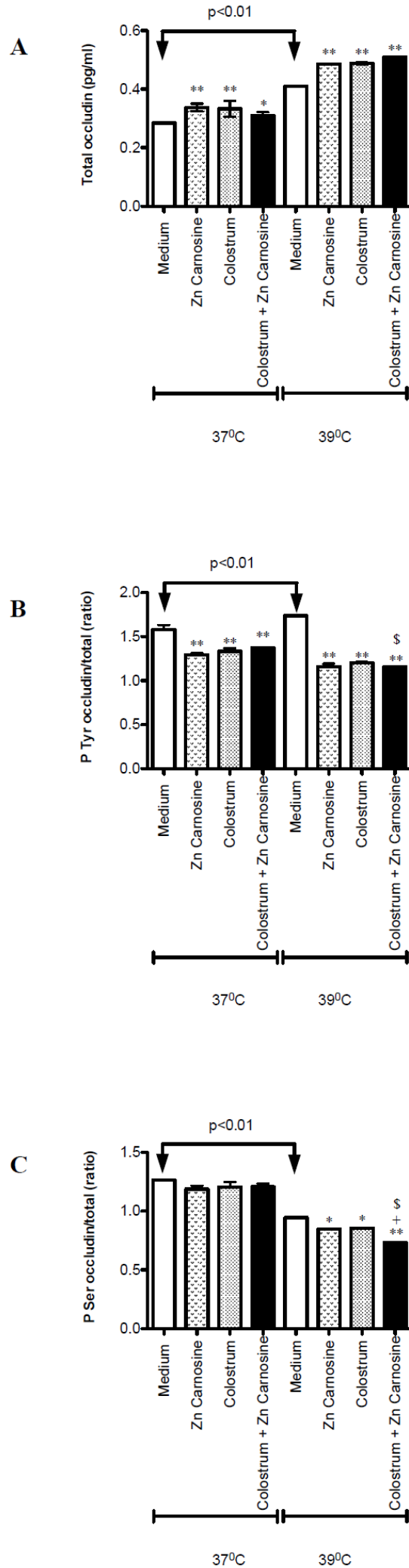


Figure 8

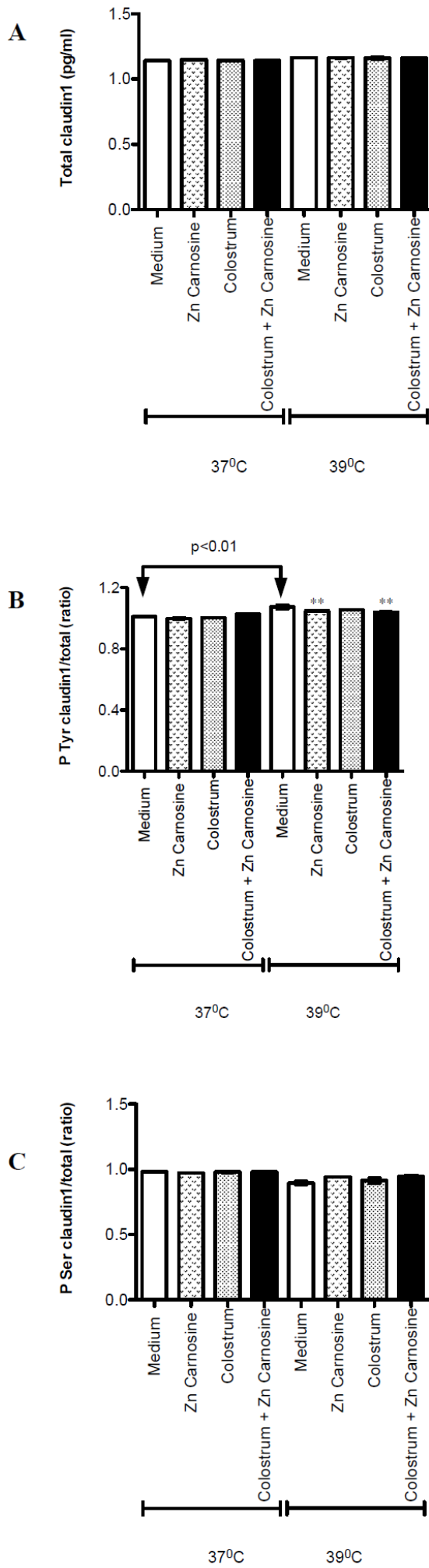


Figure 9