

# The nutraceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes

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<sup>1</sup>Digestive Diseases, Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine, Queen Mary University of London, London; <sup>2</sup>Department of Sport and Exercise Science, Aberystwyth University, Aberystwyth;

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**Marchbank T, Davison G, Oakes JR, Ghatei MA, Patterson M, Moyer MP, Playford RJ.** The nutraceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes. *Am J Physiol Gastrointest Liver Physiol* 300: G477–G484, 2011. First published December 9, 2010; doi:10.1152/ajpgi.00281.2010.—Heavy exercise causes gut symptoms and, in extreme cases, “heat stroke” partially due to increased intestinal permeability of luminal toxins. We examined bovine colostrum, a natural source of growth factors, as a potential moderator of such effects. Twelve volunteers completed a double-blind, placebo-controlled, crossover protocol (14 days colostrum/placebo) prior to standardized exercise. Gut permeability utilized 5 h urinary lactulose-to-rhamnose ratios. In vitro studies (T84, HT29, NCM460 human colon cell lines) examined colostrum effects on temperature-induced apoptosis (active caspase-3 and 9, Bax $\alpha$ , Bcl-2), heat shock protein 70 (HSP70) expression and epithelial electrical resistance. In both study arms, exercise increased blood lactate, heart rate, core temperature (mean 1.4°C rise) by similar amounts. Gut hormone profiles were similar in both arms although GLP-1 levels rose following exercise in the placebo but not the colostrum arm ( $P = 0.026$ ). Intestinal permeability in the placebo arm increased 2.5-fold following exercise ( $0.38 \pm 0.012$  baseline, to  $0.92 \pm 0.014$ ,  $P < 0.01$ ), whereas colostrum truncated rise by 80% ( $0.38 \pm 0.012$  baseline to  $0.49 \pm 0.017$ ) following exercise. In vitro apoptosis increased by 47–65% in response to increasing temperature by 2°C. This effect was truncated by 60% if colostrum was present (all  $P < 0.01$ ). Similar results were obtained examining epithelial resistance (colostrum truncated temperature-induced fall in resistance by 64%,  $P < 0.01$ ). Colostrum increased HSP70 expression at both 37 and 39°C ( $P < 0.001$ ) and was truncated by addition of an EGF receptor-neutralizing antibody. Temperature-induced increase in Bax $\alpha$  and reduction in Bcl-2 was partially reversed by presence of colostrum. Colostrum may have value in enhancing athletic performance and preventing heat stroke.

repair; gut growth; injury; clinical trial

SEVERAL STRESSES AFFECT the integrity of the intestinal barrier. These include prolonged strenuous exercise (10), heat stress (11), and drugs such as nonsteroidal anti-inflammatory agents. Loss of intestinal barrier integrity leading to increased intestinal permeability may result in passage of luminal endotoxins into the circulation. This, in turn, results in an inflammatory cascade, exacerbating the loss of barrier function and, in severe cases, resulting in severe systemic effects.

Severe cases of decompensation can result in the life-threatening condition of exertional heat stroke, associated with

hyperthermia, multiorgan failure, and endotoxemia. In addition, it is increasingly recognized that similar processes have relevance for many athletes involved in heavy exercise such as long-distance running. Gastrointestinal symptoms including cramps, diarrhea, nausea, and bleeding are commonly reported by long-distance runners (16). These symptoms are likely to be due to a combination of reduced splanchnic blood flow, hormonal changes, altered gut permeability, and increased body temperature.

Pharmacological options to reduce these problems are limited, particularly in competitive athletics, and there is therefore great interest in the use of natural products. One such product, that is already commercially available, is bovine colostrum. Colostrum is the first milk produced after birth and is particularly rich in immunoglobulins, antimicrobial peptides (e.g., lactoferrin, lactoperoxidase), and other bioactive molecules including growth factors (20). In combination with the milk that is subsequently produced, it is important for the nutrition, growth, and development of the newborn infant and contributes to the immunological defense of the neonate. Some studies suggest it may be of value in eliminating infection and stimulating growth of the neonatal gastrointestinal tract (25, 26). Its value in the prevention and treatment of adult gastrointestinal injury is, however, largely unexplored.

We have previously shown, using a combination of in vitro and in vivo studies, that a commercially available defatted bovine colostrum preparation can reduce NSAID-induced upper intestinal gut injury in rats, mice, and humans (19, 21). Its potential value in decreasing gut permeability associated with heavy exercise is unknown. We have now progressed our studies to examine the effect of orally administered bovine colostrum on gut permeability, exercise-induced temperature rise, and gut hormonal profile for subjects undertaking heavy exercise. To gain greater understanding as to how any protective effects of colostrum are elicited, we also performed studies examining the effect of temperature rises (similar to those seen in athletes undergoing the in vivo studies) on the amount of apoptosis, epithelial barrier resistance and expression of heat shock protein (HSP) using gut cells in vitro in the presence and absence of colostrum.

## MATERIALS AND METHODS

All chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. Neovite brand lactose-reduced colostrum and the placebo were provided by importers Colostrum UK, London.

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### Colostrum

NZMP colostrum high-protein powder for use in the studies was produced by Fonterra, New Zealand. The powder is naturally low in casein and high in whey proteins and rich in immunoglobulins (15–20%) and growth factors, and it contained 1.3% fat and 9.1% lactose. This form of colostrum is commercially available as a health food in the USA, UK, and the rest of Europe and is marketed as a general “health-promoting” product, particularly suitable for athletes. The total protein content of the colostrum was 80%. The concentrations of the various growth factors present in the colostrum preparation are incompletely defined but include IGF-I at 213 ng/g, TGF- $\beta_1$  at 113 ng/g, and TGF- $\beta_2$  at 441 ng/g.

### Placebo

The placebo was an isoenergetic and isomacronutrient milk protein concentrate (skimmed milk with lactose sugar membrane filtered off) at 80% protein content (principally casein).

### Cell Lines

The HT29 cell line is an epithelial cell line derived from a colorectal adenocarcinoma of a 44-yr-old Caucasian woman (LGC Standards, Teddington, Middlesex, UK).

The T84 cell line is an epithelial cell line derived from a colorectal carcinoma of a 72-yr-old man (LGC Standards). This line exhibits tight junctions, and desmosomes between adjacent cells and cells grow as polarized monolayers (6).

The NCM460 cell line is an epithelial cell line derived from the normal colon of a 68-year-old Hispanic man (INCELL; Ref. 15). It is not infected or transfected with any exogenous genetic information. The NCM460 cell line was received by a materials transfer agreement with INCELL, San Antonio, TX. The cells were routinely propagated under standard conditions in M3:10 medium (INCELL).

### Ethical Approval

Appropriate ethical approval was obtained from the Aberystwyth University ethics committee, and all subjects gave written, informed consent.

### Clinical Study: Effect of Colostrum on Exercise-Induced Changes in Human Gut Permeability

**Subjects.** Twelve healthy male subjects volunteered to take part in the study. All subjects were regular exercisers and took part in running as part of their training; seven were runners, two participated in boxing, and three participated in rugby. Their physical parameters were as follows: mean age 26, range 19–38; height  $1.77 \pm 0.11$  m; body mass  $74.7 \pm 11.6$  kg; body mass index  $23.7 \pm 1.5$  kg/m<sup>3</sup>; maximal oxygen uptake ( $\dot{V}O_{2\max}$ )  $53.3 \pm 6.8$  ml·kg<sup>-1</sup>·min<sup>-1</sup>; peak

speed in ramp test  $18.5 \pm 1.5$  km/h; running speed at 80% peak oxygen uptake  $12.6 \pm 1.4$  km/h (values are means  $\pm$  SD).

**Design.** In a double-blind crossover design, subjects received oral supplementation with 20 g/day bovine colostrum or the isoenergetic and isomacronutrient placebo. Supplements were taken daily for 14 days before the main exercise trials began. For each subject, there was a 14-day washout period between the two arms of the trial; this timing was based on our previous studies examining changes in gut permeability (21). All procedures were then repeated, including the preliminary baseline assessment to complete the protocol. Subjects completed a preexercise screening questionnaire (Physical Activity Readiness Questionnaire: PAR-Q) before participating in each test.

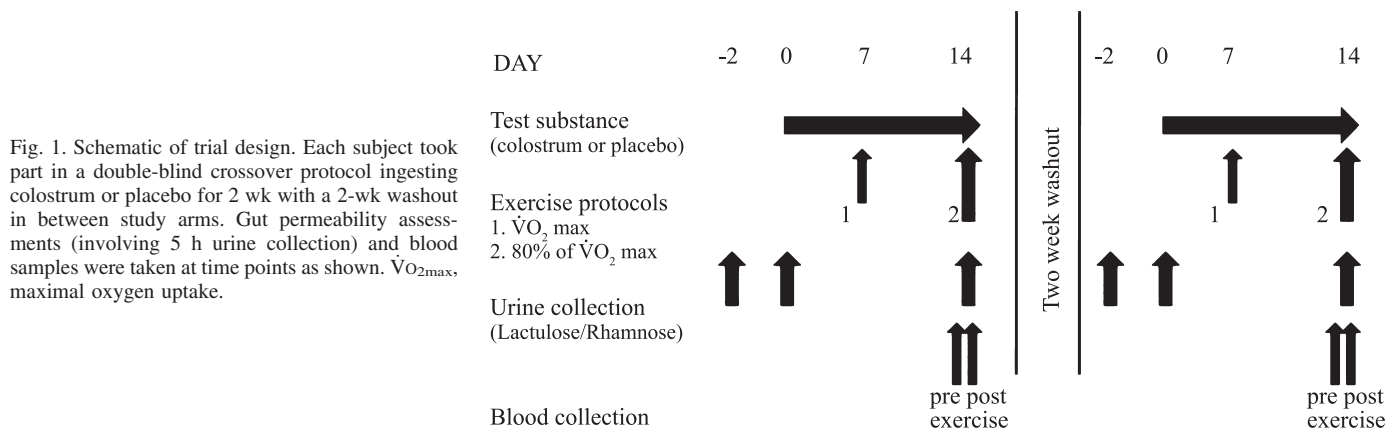
**Testing protocols.** All subjects visited the laboratory on eight separate occasions, four for each half (phase) of the crossover study (Fig. 1). Subjects completed a 24-h food diary on the day before the exercise trial in the first arm and repeated this diet during the second arm. No alcohol consumption, NSAID consumption, or unaccustomed or strenuous exercise was permitted during this period. Baseline permeability assessments were performed on day -2 and day 0, after which the colostrum or placebo was started. All urine collections were performed following an overnight fast. The final urine collection of each arm was collected immediately after the exercise protocol on day 14.

$\dot{V}O_{2\max}$  exercise assessments were performed by standard methods as reported previously (4) on day 7 of each arm to ensure consistency of the 80%  $\dot{V}O_{2\max}$  protocol on day 14.

**Day 14 protocol.** Core body temperature was assessed via a rectal thermistor (Grant Instruments, Cambridge, UK), positioned 10 cm beyond the anal sphincter and continuously recorded using an electronic data logger (Squirrel SQ2020, Grant Instruments). Heart rate was determined by use of a telemetric heart rate monitor transmitter band (Polar S610i, Polar Electro Oy, Tampere, Finland).

Subjects sat for 10 min before a baseline venous blood sample (preexercise) was taken. Subjects then ran on the treadmill, with 1% grade, for 20 min at a constant speed equivalent to 80%  $\dot{V}O_{2\max}$ , as determined from the preliminary tests. Expired gas was analyzed during the 5th, 10th, 15th, and 20th minute of exercise. 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. A maximum of 3 min elapsed between the end of exercise and collection of the sample. Subjects then emptied their bladder before consuming the intestinal permeability test drink and commencing with a 5-h urine collection to determine intestinal permeability.

**Blood measures.** Blood was collected in heparinized tubes for determination of hematocrit, blood glucose, and lactate concentrations. Trasylol (0.2 ml per 10 ml blood) was immediately added to the remaining heparinized blood, mixed, centrifuged at 1,500 g for 10 min



at 4°C and plasma separated and stored at -80°C for later analysis of gut hormones.

**Analytical methods.** Hemoglobin, hematocrit, lactate, and glucose concentrations were determined by using standard auto analyzers. The coefficient of variation was 1.5, 1.7, 1.3, and 0.3% for the measurement of blood lactate, glucose, hematocrit, and hemoglobin, respectively.

**Gut hormone concentrations.** Plasma VIP, PYY, ghrelin, and glucagon-like peptide (GLP-1) immunoreactivities were determined as previously described by radioimmunoassay (1, 9, 13, 17). All samples were assayed in one assay to avoid interassay variation.

**Intestinal permeability.** Permeability was assessed using our previously published protocol, equipment, and methods (21). Briefly, following an overnight fast, subjects emptied their bladders and then drank a standardized sugar solution containing lactulose 5 g, mannitol 2 g, and rhamnose 1 g in a total of 450 ml water (calculated osmolality 69 mosmol/kg). Subjects were allowed unlimited intake of fluid after the first hour of the test to ensure adequate urine output. The urine was collected and pooled over the next 5 h and total volume was recorded. Aliquots were centrifuged briefly to remove gross debris and the supernatant was frozen at -20°C until later analysis. The various sugars were separated by HPLC and quantitated by use of a pulsed amphoteric detector. With this technique, sugars are oxidized on the gold electrode at the working potential (0.05 V), the current produced being a measure of the amount of sugar present in the sample.

Results are expressed in two forms, one as simple area under the curve ratios as described by us previously (21). The other is as a ratio of percentage of ingested sugar excreted in the urine as has been used by some other groups (12).

### In Vitro Studies

**Background to studies.** The exercise protocol of the clinical study resulted in a change of ~1.4°C (range 0.92–1.98°C) in core body temperature. To begin to examine whether this might explain the change in permeability seen as a result of exercise, we used a series of in vitro studies to look at changes in apoptosis, mucosal resistance, and HSP expression. The influence of the presence of colostrum on any effects seen were also determined.

**Effect of colostrum on temperature-induced apoptosis.** T84 and HT29, human colonic carcinoma, and NCM460, normal human colon, cells were seeded at  $5 \times 10^5$  cells/well in six-well plates in DMEM or M3 medium (INCELL) containing 10% FCS and grown for 24 h. Cells were then treated for 4 h with medium containing FCS alone or with the addition of colostrum (4%) or placebo (4%) with or without EGF receptor (EGFR)-neutralizing antibody (1 µg/ml, R&D Systems Europe, Abingdon, UK). Plates were incubated at either 37 or 39°C for 4 h. This timing was based on that of previous studies (5). Cells were washed in ice-cold PBS and lysed in lysis buffer for 5 min on ice. Lysates were cleared by centrifugation at 10,000 g for 10 min at 4°C. Protein concentrations were determined by a standard BCA method (Pierce, Rockford, IL). Activity of caspase-3, an effector caspase, and caspase-9, an initiator caspase, was measured in cell lysates by use of caspase-3 and caspase-9 colorimetric assay kits (BF3100 and BF10100, respectively, R&D Systems) following manufacturer's instructions. Additional wells containing cell lysates had the specific caspase-3 inhibitor (Ac-DEVD-CHO) or caspase-9 inhibitor (Z-LEHD) added to show that any activity detected was caspase-3 or caspase-9 specific. The concentration of the antiapoptotic protein Bcl-2 and the proapoptotic protein Baxα in the lysates was determined by using Duoset ELISA kits as per manufacturer's instructions (DYC827B-2 and DYC820-2, respectively, R&D Systems Europe). We used 100 µg of protein in triplicate wells for each treatment.

**Effect of colostrum on temperature-induced changes in monolayer resistance.** Influence of temperature changes ± presence of colostrum on monolayer resistance were determined by previously published methods (5). Briefly, T84, polarizing colonic adenocarcinoma, cells

were grown to confluence and resistance was monitored daily via a Millicell-ERS (electrical resistance system), three measurements per well. When a consistent mean resistance of  $528 \pm 3.1 \Omega$  was obtained wells were incubated in DMEM F12 with FCS alone or also containing colostrum (4%) or placebo (4%). Plates were incubated at either 37 or 39°C for 4 h. Resistance was determined at 2 and 4 h. Following the final resistance measurement cells were washed in ice-cold PBS, lysed in lysis buffer (50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4) for 5 min on ice. Lysates were cleared by centrifugation at 10,000 g for 10 min at 4°C. Protein concentrations were determined by a standard BCA method (Pierce). Caspase-3 activity was determined as described above.

**Effect of colostrum on HSP70.** HT29, human colonic carcinoma, and NCM460, normal human colon, cells were seeded at  $5 \times 10^5$  cells/well in six-well plates in DMEM or M3 medium containing 10% FCS, and were grown for 24 h. Cells were then treated for 4 h with DMEM containing FCS alone or also containing colostrum (4%) or placebo (4%) with or without EGFR-blocking/neutralizing antibody (1 µg/ml, R&D Systems Europe). Plates were incubated at either 37 or 39°C for 4 h. Cells were washed in ice-cold PBS, lysed in lysis buffer (50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4) for 5 min on ice. Lysates were cleared by centrifugation at 10,000 g for 10 min at 4°C. Protein concentrations were determined by a standard BCA method (Pierce). HSP 70 (HSP70) concentration in the lysates was determined by using a Duoset ELISA kit as per manufacturer's instructions (DYC1663-2, R&D Systems Europe). We used 100 µg of protein in triplicate wells for each treatment.

### Statistics

All values are expressed as means ± SE. Two-way ANOVA was used with exercise or temperature and presence of colostrum as factors. Where a significant effect was seen ( $P < 0.05$ ), individual comparisons were performed via *t*-tests based on the group means, residual, and degrees of freedom obtained from the ANOVA, a method equivalent to repeated-measures analyses.

## RESULTS

### Physiological Responses to Exercise

As expected, rating of perceived exertion expressed during exercise, respiratory exchange ratio, heart rate, lactate concentrations (Table 1), core temperature (mean rise 1.4°C in both arms, Fig. 2A),  $\dot{V}O_2$ , and  $\dot{V}CO_2$  all rose in response to exercise (all  $P < 0.01$ ). Presence of colostrum had no effect on results. Day 7  $V_{max}$  assessments at day 7 of placebo or colostrum treatment were not different between the two arms (data not shown).

### Gut Hormone Concentrations

Mean plasma VIP levels increased slightly following exercise in both arms of the study (rise by ~2 pmol/l), as did plasma PYY levels (rise of ~4 pmol/l). However, neither of

Table 1. Effect of colostrum on exercise-induced changes in gut hormone and lactate levels

|                 | Placebo Arm  |              | Colostrum Arm |              |
|-----------------|--------------|--------------|---------------|--------------|
|                 | Preexercise  | Postexercise | Preexercise   | Postexercise |
| PYY, pmol/l     | 22.9 ± 1.6   | 27.1 ± 2.9   | 22.2 ± 2.5    | 24.8 ± 2.7   |
| VIP, pmol/l     | 7.4 ± 1.4    | 9.8 ± 2.0    | 6.7 ± 1.6     | 9.0 ± 1.7    |
| Ghrelin, pmol/l | 466.6 ± 35.0 | 425.7 ± 30.7 | 443.3 ± 31.7  | 435.7 ± 33.6 |
| Lactate, mmol/l | 0.67 ± 0.07  | 3.44 ± 0.57* | 0.76 ± 0.09   | 3.06 ± 0.54* |

Values are means ± SE. \* $P < 0.01$  vs. preexercise value.

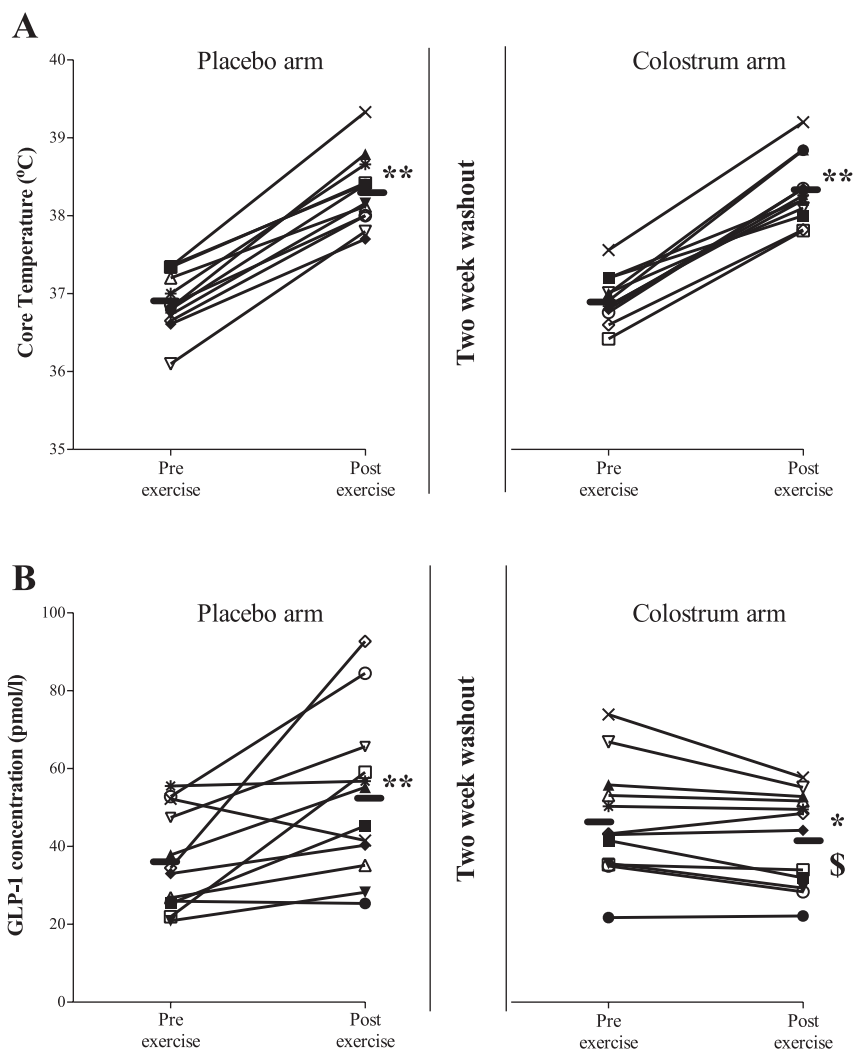


Fig. 2. Core temperature (A) and plasma GLP-1 (B) values taken immediately before and after exercise protocol in each arm of the study. Black bars show mean value for each time sampled. \*\* $P < 0.01$  and \* $P < 0.05$  different from preexercise value for that arm. \$ $P < 0.05$  compared with equivalent period in opposite arm.

these changes reached statistical significance ( $P = 0.17$  and  $0.18$  respectively) and copresence of colostrum had no additional effect (Table 1). Plasma ghrelin concentrations also showed a slight nonsignificant decrease in response to exercise. There was a small but significant rise in plasma GLP-1 concentrations following exercise in the placebo arm of the study ( $36.2 \pm 3.7$  pmol/l to  $52.5 \pm 6.0$  pmol/l,  $P = 0.006$ , Fig. 2B). In contrast, GLP-1 plasma levels decreased slightly following exercise in the colostrum-treated arm ( $46.3 \pm 4.2$  to  $42.1 \pm 3.5$  pmol/l,  $P = 0.018$ ).

#### Intestinal Permeability

Baseline permeability values were similar at the beginning of each study arm (Fig. 3). Permeability increased  $\sim 2.5$ -fold in response to exercise during the placebo arm (rising from  $0.38 \pm 0.012$ , initial baseline value, to  $0.92 \pm 0.014$ ,  $P < 0.01$ ) (Fig. 3, left). In contrast, this rise in exercise induced permeability was truncated by  $\sim 80\%$  if colostrum had been taken. Statistical analyses using presence of colostrum and time as factors showed significant effects of time (i.e., exercise,  $F_{2, 71} = 108.66$ ,  $P < 0.0001$ ), presence of colostrum ( $F_{1, 71} = 48.6$ ,  $P < 0.001$ ), and an interaction between the two ( $F_{2, 42} = 46.8$ ,  $P < 0.0001$ ). This showed that the rise in intestinal permea-

bility caused by exercise was truncated by the presence of colostrum ( $P < 0.01$ ). The order in which placebo and colostrum were administered did not influence results (although numbers are too small to perform detailed statistical analysis).

Expressing results as lactose-to-rhamnose % urinary excretion ratios, rather than area under curve values, unsurprisingly, gave equivalent results with values as follows: The two baseline results within a study arm were similar (placebo arm baselines being  $0.0231 \pm 0.0003$  and  $0.0229 \pm 0.0004$  and colostrum arm baselines being  $0.0222 \pm 0.0003$  and  $0.0224 \pm 0.0001$ ). Values postexercise in the placebo arm were about twofold higher than baseline ( $0.0421 \pm 0.0002$ ), but there was only a minor rise in values in response to exercise when the same subjects had ingested colostrum (rising to  $0.0261 \pm 0.0005$ ,  $P < 0.001$  vs. postexercise value during placebo arm).

#### In Vitro Studies

**Effect of colostrum on temperature-induced apoptosis.** Colostrum  $\pm$  EGFR-blocking/neutralizing antibody had no effect on caspase-3 or caspase 9 activity in HT29 or NCM460 cells when incubated at  $37^{\circ}\text{C}$  (Fig. 4A). Increasing the temperature to  $39^{\circ}\text{C}$  resulted in significant increases in caspase-3 and -9 activity when cells were incubated in medium  $\pm$  placebo.

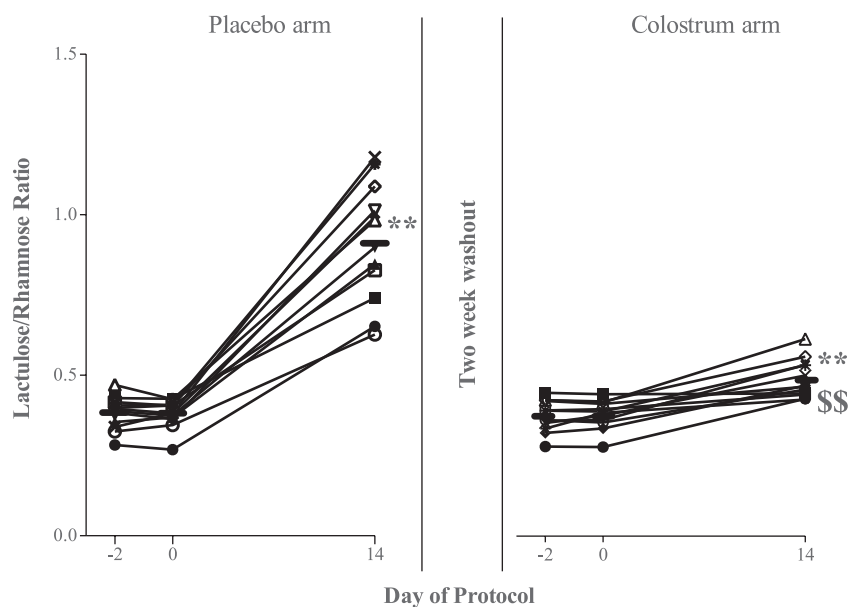


Fig. 3. Gut permeability assessments during trial shown in Fig. 1. Two baseline assessments (no exercise) were performed before each arm and at the end of 14 days ingestion of placebo or colostrum. The third value for each arm of the study was performed immediately after the subject had followed a 20-min 80% maximal oxygen uptake ( $\dot{V}O_{2max}$ ) protocol. Black bars show mean value for each time period. \*\* $P < 0.01$  different from baseline for that arm. \$\$ $P < 0.01$  compared with equivalent period in opposite arm.

However, copresence of colostrum significantly truncated the rise in caspase-3 and -9 activity in both cell lines ( $P < 0.01$ ). Presence of the EGFR-blocking/neutralizing antibody removed the changes in caspase-3 and 9 activity caused by presence of colostrum. Changes in absorbance (used as a marker of caspase-3 and -9 activity) were shown to be specific as these

effects were not seen when the caspase-3 or caspase-9 inhibitor were also added to the cells (data not shown).

To examine the mechanism of action of the temperature-induced antiapoptotic effects of colostrum, we examined changes in the proapoptotic factor Bax $\alpha$  and the antiapoptotic factor Bcl-2. For both HT29 and NCM460 cell lines, similar results were seen.

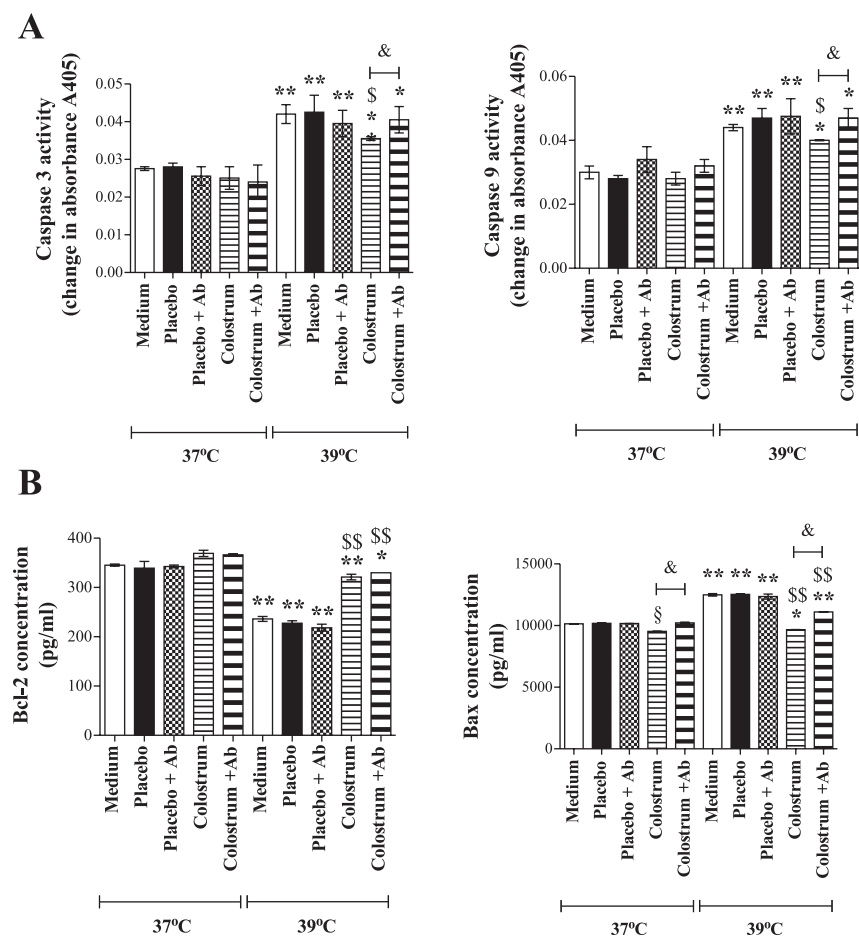


Fig. 4. A: effect of colostrum on temperature-induced apoptosis. HT29 cells were incubated at 37 or 39°C in medium alone, with placebo (4%) or with colostrum (4%). Additional wells had an EGF receptor (EGFR)-neutralizing antibody added. Changes in apoptosis were determined by use of active caspase-3 and 9 assay kits, following changes in absorbance at 405 nM. Similar results were seen using NCM460 cells (data not shown). B: effect of colostrum on temperature-induced changes in the proapoptotic protein Bax $\alpha$  and the antiapoptotic protein Bcl-2. Experimental protocol as for A. Results shown are for NCM460 cells, similar results were seen using HT29 cells (data not shown). \*\* $P < 0.01$  and \* $P < 0.05$  different from 37°C equivalent. \$\$ $P < 0.01$  compared with medium alone at 37°C. \$\$\$ $P < 0.01$  and \$ $P < 0.05$  compared with medium alone at 39°C. & $P < 0.01$  compared with colostrum without EGFR-neutralizing antibody.

At 37°C colostrum reduced Bax $\alpha$ , and this effect was removed if the EGFR-neutralizing antibody was present. In contrast, at 37°C, Bcl-2 was not influenced by presence of colostrum  $\pm$  EGFR-neutralizing antibody (Fig. 4B).

Incubating the cells at 39°C caused Bax $\alpha$  levels to increase and Bcl-2 to decrease. These effects were markedly truncated in the copresence of colostrum. Addition of the EGFR-neutralizing antibody had no effect on the changes in Bcl-2 caused by the colostrum but did remove most of the protective effect on Bax $\alpha$  (Fig. 4B).

**Effect of colostrum on temperature-induced resistance.** Transepithelial resistance was similar in wells incubated at 37°C in the presence of medium alone or with colostrum or placebo added (Fig. 5). Cells incubated at 39°C in medium or with placebo had a 22% lower resistance value. In contrast, the decrease in transepithelial resistance caused by incubating the cells at 39°C, rather than at 37°C, was truncated by 58% if colostrum was present (Fig. 5).

**Effect of colostrum on HSP70.** HSP70 could be detected in both HT29 and NCM460 cells following incubation in medium alone at 37°C. This level was increased by  $\sim$ 25% following 4 h incubation at 39°C (Fig. 6,  $P < 0.001$ ). At 37°C the presence of placebo did not have a significant effect on HSP70 levels ( $P = 0.302$  and  $0.28$ , respectively); however, the presence of colostrum significantly increased HSP70 levels ( $P < 0.001$ ). Incubation at 39°C caused a significant increase in HSP70 levels in both the placebo and colostrum treatment groups in both cell lines ( $P < 0.001$ ). At 39°C, the rise in HSP70 levels in cells incubated in the presence of placebo was similar to that seen to cells incubated in medium alone ( $P < 0.001$ ). In contrast, cells incubated at 39°C in the presence of colostrum showed a markedly enhanced increase in HSP70 expression above values seen in the control or placebo groups ( $P < 0.001$ , Fig. 6). The presence of the EGFR-neutralizing antibody significantly reduced the colostrum-induced increase in HSP70 levels at 37 and 39°C in both of the cell lines ( $P < 0.001$ ).

## DISCUSSION

Using a combination of a clinical trial and in vitro experiments, we have shown that bovine colostrum reduced the

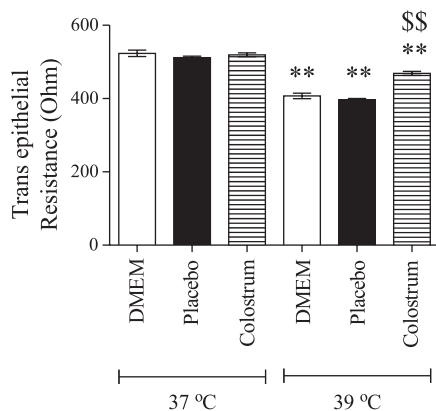


Fig. 5. Effect of colostrum on temperature-induced changes in cellular resistance. The polarizing human colonic cell line T84 were grown as confluent polarized monolayers on Transwell permeable supports until resistance  $>500 \Omega$ . Cells were then incubated at 37 or 39°C for 4 h. Resistance was measured by using a Millicell-electrical resistance system at 4 h.  $**P < 0.01$  different from 37°C equivalent.  $$$$P < 0.01$  compared with medium alone at 39°C.

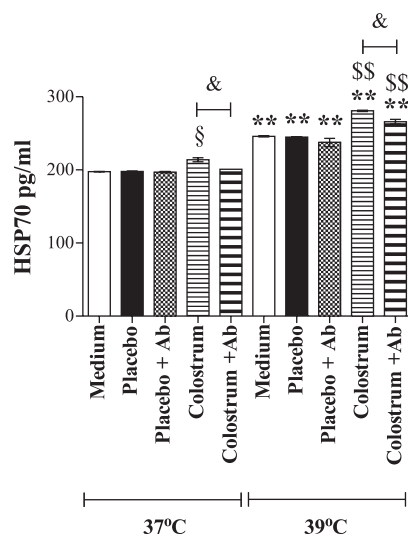


Fig. 6. Effect of colostrum on heat shock protein expression (HSP70). Cells were incubated in similar conditions to Fig. 4A. Presence of colostrum increased HSP70 expression at both 37 and 39°C. These effects were truncated in the presence of an EGFR-neutralizing antibody. Results shown are for NCM460 cells, similar results were seen using HT29 cells (data not shown).  $**P < 0.01$  different from 37°C equivalent.  $$$$P < 0.01$  compared with medium alone at 37°C.  $$$$P < 0.01$  compared with medium alone at 39°C.  $\&P < 0.01$  compared with colostrum without EGFR-neutralizing antibody at same temperature.

exercise-induced increase in gut permeability, possibly through mechanisms including reducing temperature-induced apoptosis and induction of HSP.

Numerous exercise protocols are used by sports physiologists. We chose a 20-min, intensive 80%  $\dot{V}O_{2\max}$  protocol to allow a crossover protocol to be used in a relatively short period. Our preliminary studies had shown that this standard regimen was sufficient to increase gut permeability and to increase core temperature by  $\sim 1.5$ – $2^\circ\text{C}$  (data not shown) and we therefore used this protocol. Assessment of intestinal permeability by quantitating unmediated absorption of at least two sugars of different sizes provides a sensitive index of intestinal damage (2). In a previous study using this method of assessing permeability (21), we included lactulose as the disaccharide probe and rhamnose and mannitol as two alternative monosaccharide probes in a hypo-osmolar formulation. Both rhamnose and mannitol have been widely used and provide similar information regarding changes in “paracellular pathways.” However, subsequent analyses showed that the “mannitol peak” in urine samples is sometimes obscured by overlap from other urinary constituents, and we therefore only presented lactulose and rhamnose data (21). To maintain consistency between studies (including osmolality), we therefore used the same mixture as previously but again only present lactulose-to-rhamnose ratios.

Subjects in both arms of the study had similar  $\dot{V}O_{2\max}$  assessment values after 7 days ingestion of colostrum or placebo and showed similar changes in core temperature, cardiovascular parameters, and gut hormone profiles (with exception of GLP-1) in response to exercise at the assessments at the end of the 14-day study arms. Studies from other groups suggest that exercise causes a modest rise in GLP-1 as seen in the placebo arm of our studies (24). This was not seen when

colostrum was coadministered although caution must be shown in regard to this finding since the baseline value was slightly higher.

Gut permeability increased ~2.5-fold in response to exercise in the control arm. These degrees of changes were similar to those seen by us previously in subjects ingesting clinically relevant doses of the nonsteroidal anti-inflammatory drug indomethacin (21), which is known to cause small intestinal injury (18). In contrast, when subjects had ingested colostrum rather than placebo, they showed a markedly truncated increase in gut permeability following exercise.

To investigate possible mechanisms for this effect of colostrum, we undertook a series of *in vitro* studies that allowed us to examine the effect of the core temperature rise on gut integrity in a controlled environment. One model involved following changes in electrical resistance in polarized monolayers of human colonic cancer cells. This model was chosen because we have experience of studying effects of proteins in this model and it removes confounding factors such as changes in gut hormones and blood flow. The results from these studies were consistent with the clinical trial; temperature rise was associated with decreased resistance but this effect could be abrogated by the copresence of colostrum. These effects are likely to be due, at least in part, to effects on paracellular permeability, such as alteration in tight junctions (3) and acute cytotoxicity.

Temperature rise is a well-known trigger of apoptosis (5), and we used well-validated assay systems (active caspase-3 and 9) to examine potential effects of colostrum. These studies suggested that this degree of temperature rise was sufficient to increase apoptosis and that the copresence of colostrum truncated this response. Use of an EGFR-neutralizing antibody showed that an EGFR ligand present in the colostrum was at least partially responsible. Analyses of two of the key proteins involved in the extrinsic cellular apoptosis pathway showed that colostrum decreased the proapoptotic protein Bax $\alpha$ , even at 37°C. This effect was partially mediated through an EGFR ligand.

Analyses of changes in the antiapoptotic protein Bcl-2 showed that colostrum truncated the decrease in Bcl-2 caused by a rise in temperature. This effect of colostrum was due to factors other than its EGF ligand components since there was no effect caused by addition of the EGFR-neutralizing antibody.

Our studies suggested that a further mechanism by which these antiapoptotic effects may have been mediated was through induction of HSP. HSPs are important in the maintenance of cellular homeostasis during normal cell growth and for survival during and after various cellular stresses. The HSP70 family functions as a molecular chaperone and reduces stress-induced denaturation and aggregation of intracellular proteins. In addition to its chaperoning activities, HSP70 has been suggested to exert its cytoprotective action by protecting mitochondria and by interfering with the stress-induced apoptotic program (22). As for the caspase results, use of an EGFR-neutralizing antibody showed that an EGFR ligand present in the colostrum was at least partially responsible for these changes in HSP levels.

Increases in HSP expression have been proposed to be one mechanism through which thermotolerance occurs in both animals and cells (14). In this process, prior exposure to

moderate temperature rise causes the cells to be primed to withstand a subsequent more extreme temperature stress. Our finding that colostrum induces HSP expression even at 37°C and causes an even more pronounced increase when the cells are incubated at 39°C suggests that this pathway may have relevance to our results and merits further study. Importantly, our results were demonstrated in the *in vitro* studies by 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.

Although there is currently insufficient evidence to conclusively establish the value of bovine colostrum to enhance performance, it is being used increasingly by track and field athletes. Of the studies that have been performed, its major value may be in the context of athletes undertaking high-intensity training and in aiding recovery (23). Our finding that bovine colostrum stabilizes gut integrity and may induce thermotolerance would be compatible with this idea.

The colostrum preparation used in the present study contains multiple factors that may be important in mediating these effects, including epidermal growth factor, transforming growth factors- $\alpha$  and - $\beta$ , and interleukin-1 $\beta$ . In addition, it also contains other bioactive molecules such as amino acids, lipids and fatty acids. The use of “natural” products, such as this colostrum preparation, has several potential advantages. We have previously shown that they possess greater intrinsic stability against luminal digestion than isolated individual peptides (8) and that combination therapy with peptides provides the potential for the stimulation of gut stabilization or healing in a synergistic fashion (7). Furthermore, there is currently a demand from the general public for more natural types of products, which are usually considered as “alternative therapy,” but which can possess potent biological activity. Products such as these are often termed “nutriceuticals” (from nutrition and pharmaceuticals). This aspect is especially relevant for athletes where risks of accusations of “doping” have serious consequences. It is, therefore, of relevance that this product has been certified by a company (HFL Sports Science UK, Fordham, Cambridgeshire) that has ISO 17025 accreditation for testing sports supplements to control contamination with banned substances to ensure compliance with the World Anti-Doping Agency.

In conclusion, our findings show that, in a physiologically relevant sports model, colostrum appears beneficial in maintaining gut stability. Mechanisms may include reduced apoptosis and paracellular permeability. Further studies involving more prolonged exercise protocols and colostrum’s value in extreme heat stress situations appear justified to examine potential value in athletes and other subjects, such as members of armed forces, subjected to such stresses.

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## REFERENCES

1. Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR. Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 89: 1070–1077, 1985.
2. Bjarnason I, Macpherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 108: 1566–1581, 1995.
3. Blume LF, Denker M, Gieseler F, Kunze T. Temperature corrected transepithelial electrical resistance (TEER) measurement to quantify rapid changes in paracellular permeability. *Pharmazie* 65: 19–24, 2010.
4. Carter H, Jones AM, Barstow TJ, Burnley M, Williams CA, Doust JH. Oxygen uptake kinetics in treadmill running and cycle ergometry: a comparison. *J Appl Physiol* 89: 899–907, 2000.
5. Dokladny K, Moseley PL, Ma TY. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *Am J Physiol Gastrointest Liver Physiol* 290: G204–G212, 2006.
6. Dharmasathaphorn K, McRoberts JA, Mandel KG, Tisdale LD, Masui H. A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am J Physiol Gastrointest Liver Physiol* 246: G204–G208, 1984.
7. Hagiwara T, Shinoda I, Fukuwatari Y, Shimamura S. Effects of lactoferrin and its peptides on proliferation of rat intestinal epithelial cell line, IEC-18, in the presence of epidermal growth factor. *Biosci Biotechnol Biochem* 59: 1875–1881, 1995.
8. Khan Z, Macdonald C, Wicks AC, Holt MP, Floyd D, Ghosh S, Wright NA, Playford RJ. Use of the 'nutriceutical', bovine colostrum, for the treatment of distal colitis: results from an initial study. *Aliment Pharmacol Ther* 16: 1917–1922, 2002.
9. Kreymann B, Williams G, Ghatei MA, Bloom SR. Glucagon-like peptide-1 7–36: a physiological incretin in man. *Lancet* 2: 1300–1304, 1987.
10. Lambert GP. Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: the 'canary in the coal mine' during exercise-heat stress? *Med Sport Sci* 53: 61–73, 2008.
11. Lambert GP. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *J Anim Sci* 87: E101–E108, 2009.
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* 42: 71–75, 1996.
13. Mitchell SJ, Bloom SR. Measurement of fasting and postprandial plasma VIP in man. *Gut* 19: 1043–1048, 1978.
14. Moseley PL, Gopen C, Wallen ES, Walter ME, Peterson MW. Thermal stress induces epithelial permeability. *Am J Physiol Cell Physiol* 267: C425–C434, 1994.
15. Moyer MP, Manzano LA, Merriman RL, Stauffer JS, Tanzer LR. NCM460, a normal human colon mucosal epithelial cell line. *In Vitro Cell Devel Biol Anim* 32: 315–317, 1996.
16. Pals KL, Chang RT, Ryan AJ, Gisolfi CV. Effect of running intensity on intestinal permeability. *J Appl Physiol* 82: 571–576, 1997.
17. Patterson M, Murphy KG, Le Roux CW, Ghatei MA, Bloom SR. Characterization of ghrelin-like immunoreactivity in human plasma. *J Clin Endocrinol Metab* 90: 2205–2211, 2005.
18. 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* 40: 503–510, 2007.
19. Playford RJ, Floyd DN, Macdonald CE, Calnan DP, Adenekan RO, Johnson W, Goodlad RA, Marchbank T. Bovine colostrum is a health food supplement which prevents NSAID-induced gut damage. *Gut* 44: 653–658, 1999.
20. Playford RJ, Macdonald CE, Johnson WS. Colostrum and milk-derived growth factors for the treatment of gastrointestinal disorders. *Am J Clin Nutr* 72: 5–14, 2000.
21. 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)* 100: 627–633, 2001.
22. Rokutan K. Role of heat shock proteins in gastric mucosal protection. *J Gastroenterol Hepatol* 15: D12–D19, 2000.
23. Shing CM, Jenkins DG, Stevenson L, Coombes JS. The influence of bovine colostrum supplementation on exercise performance in highly trained cyclists. *Br J Sports Med* 40: 797–801, 2006.
24. Ueda S, Yoshikawa T, Katsura Y, Usui T, Nakao H, Fujimoto S. Changes in gut hormone levels and negative energy balance during aerobic exercise in obese young males. *J Endocrinol* 201: 151–159, 2009.
25. Xanthou M, Bines J, Walker WA. Human milk and intestinal host defense in newborns: an update. *Adv Pediatr* 42: 171–200, 1995.
26. Xu RJ. Development of the newborn GI tract and its relation to colostrum/milk intake: a review. *Reprod Fertil Dev* 8: 35–48, 1996.