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Oral zinc carnosine reduces multi-organ damage caused by gut ischemia/reperfusion in mice

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ABSTRACT

Gastrointestinal ischemia/reperfusion (I/R) injury occurs in multiple clinical situations. We examined effects of zinc carnosine (ZnC), a health food supplement claimed to possess gastrointestinal supportive activity, on I/R injury. AGS, RIE and Caco-2 cells exposed to hypoxia/normoxia (H/N) reduced cell viability by 50%. ZnC given prior- or during-hypoxia improved survival by 50–70% ($p < 0.01$) and truncated increase in transepithelial permeability (HRP passage) by 50% whereas carnosine or $ZnSO_4$ were ineffective. Induction of mesenteric I/R injury in mice caused severe jejunal mucosal and lung injury, increased lipid peroxidation (MDA), inflammatory infiltration (MPO) and circulating LPS and cytokines. ZnC (58 mg/kg/day, oral 7 days) reduced injury by 50%, reduced pro-apoptotic Caspase 3, 9 and Baxα, increased Bcl2 and Hsp70, and maintained tight junction ZO1 and Claudin1 levels (all $p < 0.01$ vs I/R alone). ZnC shows benefit over using zinc salt or carnosine. Oral ZnC may be a useful clinical approach for I/R injury.

1. Introduction

Chronic gastrointestinal ischemia/reperfusion (I/R) injury is involved in multiple clinical situations, such as neonatal necrotizing enterocolitis (Gregory, Deforge, Natale, Phillips, & Van Marter, 2011), radiation enteritis, chronic mesenteric ischemia (especially in the elderly) (Mensink, Moons, & Kuipers, 2011), cardiopulmonary disease and other conditions that reduce systemic blood flow such as shock states (Sastry et al., 2014), and intestinal transplantation or subsequent rejection (Lenaerts et al., 2013). In addition to local tissue injury, remote organs may be damaged by an excess inflammatory response resulting from release of inflammatory mediators and activation of leukocytes, due to the post-ischemic gut serving as a priming bed for circulating polymorphonuclear cells (Hassoun et al., 2012; Moore et al., 1994)

The combination of localized injury with an excessive systemic inflammatory response can result in a breakdown of gut mucosal integrity, increased gut permeability and leakage of luminal bacteria and other contents into the circulation resulting in repeated septicemic episodes. Current therapeutic options for chronic IR injury are limited. When possible, revascularization of partially occluded vessels provides relief, but if the underlying cause is not vascular occlusion or if surgery is not an option, current medicinal options of proton pump inhibitors, vasodilators, anticoagulation and antimicrobials have limited benefit (Eltzschig, 2004, 2014; Mensink et al., 2011)

There is currently a demand by the general public for more natural types of products, particularly for prolonged usage. Natural products with pharmaceutical activity are sometimes termed nutraceuticals (from nutrition and pharmaceuticals). Although artificially produced, zinc carnosine (ZnC) falls into this category and is freely available via the internet or through health food shops. ZnC is an artificially produced derivative of carnosine, where zinc and carnosine, a dipeptide comprising β-alanine and L-histidine are linked in a one-to-one ratio to provide a polymeric structure. We considered ZnC had potential value for I/R injury based on the findings that (A) Carnosine is naturally present in long-living cells such as muscle and nerves, where it probably has a role as an antioxidant (Boldyrev, 2005). (B) ZnC stimulates several aspects of gut mucosal integrity including increasing cell migration and proliferation (Mahmood et al., 2007). (C) A clinical trial of oral ZnC

Abbreviations: H/N, hypoxia/normoxia|(reperfusion); HRP, horse radish peroxidase; I/R, ischemia/reperfusion; MOF, multiple organ failure; RT, room temperature; SEM, standard error of mean; SFM, serum free medium; TEER, transepithelial electrical resistance; TNFα, Tumour Necrosis Factor alpha; ZnC, zinc carnosine.

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showed it prevented the rise in gut permeability caused by clinical doses of the NSAID indomethacin (Mahmood et al., 2007).

In the current series of studies, we examined whether ZnC administration influences gut I/R-induced injury and the likely molecular pathways involved in any effects seen. We also examined whether the macromolecular structure of ZnC was relevant it mediating its activity by examining its individual sub-components (tested as zinc sulphate and L-carnosine alone or in combination) in their ability to reduce Hypoxia (Ischemia)/ Normoxia (Reperfusion) (H/N) injury.

2. Materials and methods

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

2.1. Cell lines

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 (Fogh, Fogh, & Orfeo, 1977). AGS is derived from gastric adenocarcinoma of a 54-year-old female (Barranco, Townsend, & Casartelli, 1983) (ATCC), RIE1 is a spontaneously immortalized rat intestinal epithelial cell line (Blay & Brown, 1984) (ATCC). Cells were grown in DMEM or RPMI medium dependent on cell line.

2.2. Study 1. Effect of ZnC on cell survival following hypoxia (Ischemia)/ normoxia (Reperfusion) (H/N)

1A: Dose response study: To examine the effect of ZnC on H/N-induced cell injury in vitro, cells were set up in 96 well plates to 70–80% confluence and pre-incubated in serum free medium (SFM) containing glucose, with or without 10, 50, 100 or 200 μM ZnC (Xsto Solutions, New Jersey, USA) for 1 h (i.e. ZnC pre-treatment). The medium was subsequently replaced with SFM without glucose and placed in the hypoxia incubator (2% oxygen) for 4 h. At the end of the hypoxia period, medium was replaced with SFM containing glucose for 24 h at normoxia for the reperfusion period. All treatments were duplicated in a second set of identical plates under normoxic conditions throughout the same test period as controls.

1B: Efficacy of ZnC given pre-, during- or post-hypoxic period. We compared the potential benefit of giving ZnC prior to, during or following the hypoxic period. Cells were pre-treated with 100 μM ZnC for the 1 h before hypoxia (Pre), at the time of the start of hypoxic period (During, 2% oxygen) or added at the start of the return to normoxia stage (Post). The dose of 100 μM ZnC was chosen based on the optimal dose obtained from study 1A. To assess the effect of zinc and carnosine alone or in combination additional cells were incubated as above in an equivalent concentration of $ZnSO_4$ (100 μM), L-carnosine (100 μM) or $ZnSO_4$ (100 μM) + L-carnosine (100 μM). Cell Viability (MTT assay) and cell damage (LDH assay) were then assessed.

2.2.1. Cell viability (MTT) and lactate dehydrogenase (LDH) assays

Cell viability following H/N was assessed using a commercial MTT assay following manufacturer's instructions (Marshall, Goodwin, & Holt, 1995). The principal of the assay is that viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan which can be measured spectrophotometrically. Cell damage was also assessed using the activity of LDH released into the cell culture medium. LDH activity was determined using a Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Hemel Hempstead, UK) following manufacturer's instructions. For both assays results are expressed as percentage of the equivalent normoxic control, as mean ± SEM of six wells.

2.3. Study 2. Effect of ZnC on transepithelial permeability in cells exposed to H/N

Cells were grown to confluent monolayers in 6 well transwell plates. We examined the effect of 100 μM ZnC pre-treatment on transepithelial permeability induced by 1 h of hypoxia followed by 24 h of normoxia using Caco-2, polarising colonic adenocarcinoma cells. Timings were chosen based on preliminary studies showing that these conditions result in a five to six-fold increase in horse radish peroxidase (HRP) permeability but do not affect cell death, allowing a continuous monolayer to be maintained. The dose of 100 μM ZnC was chosen based on the results from study 1 examining cell viability. Two different published methods were used to determine changes in permeability, one followed change in transepithelial electrical resistance (TEER) and the other analysed the passage of horseradish peroxidase (HRP) across the epithelial layer. HRP (type II) is a nondigestible macromolecular protein (MW = 44 kD) and has previously been used as a tracer in evaluation of epithelial permeability (Davison, Marchbank, March, Thatcher, & Playford, 2016). Measurements were taken in triplicate from 4 wells per treatment. Results are expressed as mean ± SEM.

2.4. Study 3. Effect of ZnC on gut and distant organ injury in response to small intestinal I/R in mice

2.4.1. Animal experiments

All animal experiments were approved by local animal ethics committees (Queen Mary University of London's Animal Welfare and Ethical Review Board), performed at Queen Mary University of London, Barts & The London School of Medicine and Dentistry and covered by the appropriate project licences under the Home Office Animals Procedures Acts, 1986. Experiments were performed under terminal anethesia using ketamine and xylaxine.

2.4.2. I/R model

Male CD-1 mice (30–32 g) were randomly assigned to one of four groups (N = 8–11 per group); (A) I/R alone, (B) I/R + ZnC (58 mg/kg/day, in drinking water, 7 days pre-treatment) (C) Sham group animals, that underwent a laparotomy but no clamping of the SI. The 7-day duration of ZnC pre-treatment was based on our previous work examining the effect of ZnC on NSAID gut injury (Mahmood et al., 2007). All animals entered into the protocols survived the entire procedure and were included in the analyses.

Method of induction of I/R: Mice were anesthetised with ketamine (100 μg/g, ip) and xylazine (10 μg/g, ip). To induce ischemia, the midsection of SI along with its associated mesenteric vessels, comprising approximately the region 30–75% of total length (where entire length is defined as 100%, with proximal duodenum starting at 0% and end of SI as 100% distance) was clamped using non-traumatic surgical clamps (UK Quality Instruments, Ramsgate, Kent) for 30 min, followed by removal of the clamp and 3 h of reperfusion. Visual inspection of the previously clamped region of the gut was performed at the time of clamp removal to check that the segment became reperfused.

At the end of the study, under terminal anaesthesia, mice were killed by cervical dislocation and blood collected by cardiac puncture (approx. 1 ml). For intestinal tissues, the position of the various samples was defined by expressing its harvest site as a percentage total length at 50% and 90% small intestinal distance and can be considered as equivalent to jejunum and ileum respectively. 1 cm samples were collected from the various intestinal sites. The 50% SI collection sample was, therefore, in the middle of the clamped region that had undergone I/R and the 90% site was outside of the previously clamped region. Kidney, liver and lung tissue were also collected. All tissue harvested was split into two, half being snap frozen at −80 °C for further analysis, and the other half immediately fixed in 10% neutral buffered formalin for histopathological assessment.

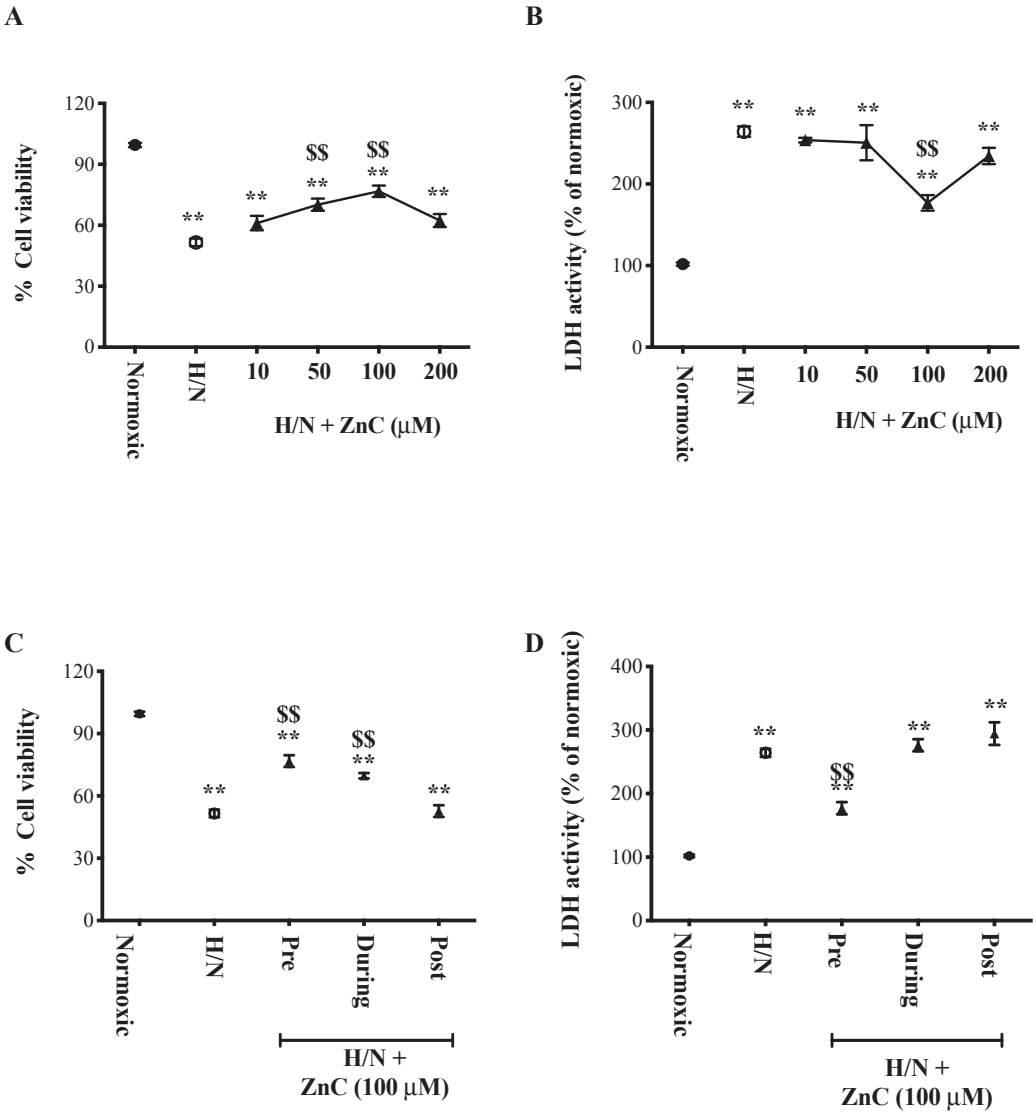

Fig. 1. Effect of hypoxia-normoxia (H/N) on cell viability and injury in presence and absence of ZnC. Cell viability using MTT assay (A, C) and cell damage measuring LDH activity (B, D) were determined for cells exposed to 4 h hypoxia (ischemia) and then returned to normoxia for 24 h (reperfusion). Values are expressed as percentage difference compared to cells incubated in serum free medium alone that were not exposed to H/N. A&B. Cells incubated under normoxic conditions throughout (●), subjected to H/N alone (○) and incubated in various concentrations of ZnC (▲), 1 h prior to undergoing H/N. C& D) The effect of adding ZnC (▲) pre hypoxia, during hypoxia or post hypoxia was compared to cells exposed to H/N alone (○). Results shown are for Caco-2 cells, RIE1 and AGS cells gave similar results. Results expressed as mean ± SEM. ** signifies $p < 0.01$ vs normoxic throughout, $$ signifies $p < 0.01$ vs H/N alone.

2.4.3. Histopathological assessment

Tissue was stained using hemotoxylin-eosin and scored in a blinded manner by two independent observers. The entire collected segments of intestine were assessed. Intestinal damage was assessed using the scoring method of Chiu et al (Chiu, McArdle, Brown, Scott, & Gurd, 1970) on a scale of 0 (normal) to 5 (damaged severely). 0- Normal mucosa and villi; 1- Development of subepithelial Gruenhagen's space at villus tips, often accompanied by capillary congestion; 2- Extension of subepithelial space with moderate lifting of epithelial layer from lamina propria; 3- Massive lifting down sides of villi, some denuded tips; 4- Denuded villi, with lamina propria and dilated capillaries exposed, increased cellularity of lamina propria may be seen; 5- Digestion and disintegration of the lamina propria, haemorrhage and ulceration.

Lung damage was scored using the method of Koksel et al. (2005) on a scale of 0 (no damage) to 3 (severe damage). 0- no damage; 1- mild neutrophil leukocyte infiltration and mild-moderate interstitial congestion; 2- moderate neutrophil leukocyte infiltration, perivascular edema formation and disintegration of the pulmonary structure; 3- dense neutrophil leukocyte infiltration and absolute destruction of pulmonary structure. Results are expressed as mean ± SEM.

2.4.4. Tissue processing for biochemical analysis.

Samples were homogenised on ice, in ice cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB), freeze thawed three times and briefly sonicated. An aliquot of the lysate was stored at −80 °C and the remainder centrifuged at 15,000 rpm for 20 min at 4 °C. Supernatants were collected and saved as cleared lysates. Total protein concentration of the lysates and cleared lysates was determined using a standard protein assay.

2.4.5. Myeloperoxidase (MPO) and MDA assays

MPO activity, used as a marker of neutrophilic infiltration, was measured as described previously (FitzGerald et al., 2004). Cleared tissue lysate was incubated with O-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide and change in absorbance at 460 nm recorded using a spectrophotometer. One unit of MPO activity was defined as that consuming 1 nmol of peroxide per minute at 22 °C. Results are expressed as U/μg of total protein, mean ± SEM from triplicate wells.

MDA levels were determined as a marker of lipid peroxidation from tissue lysates using the thiobarbituric acid method (Satoh, 1978). Results are expressed as nmol MDA/μg of total protein, mean ± SEM from triplicate wells.

2.4.6. Blood processing for LPS and cytokines

Blood was collected into EDTA-treated tubes and centrifuged for 10 min at 3000 rpm. Plasma was collected and frozen until analyses. LPS, IL6, IL1β and TNFα were analysed by commercial Elisa's (Antibodies-online.com) as per manufacturer's instructions

2.5. Study 4. Mechanisms of action of ZnC in the in vitro and in vivo studies

Having shown that ZnC reduced cell and organ damage in our in vitro and in vivo studies, samples from in vitro cell viability studies and whole animal I/R tissues were further analysed to examine ZnC's possible modes of action.

2.5.1. Cell apoptosis assays

Active caspase-3 and active caspase-9 were determined using methods described previously (Davison et al., 2016), using commercial colorimetric assay kits (BF3100 and BF10100, R&D Systems). Concentrations of the anti-apoptotic protein Bcl2 and the pro-apoptotic protein Baxα were determined in the same cell lysates as used for caspase analyses, using Duoset Elisa kits (R&D Systems Europe Ltd).

2.5.2. Measurements of HIF1α, VEGF, Hsp70 and ICAM-1

HIF1α, VEGF, Hsp70 and ICAM1 concentration in the cleared cell lysates was determined using Duoset Elisa kits as per the manufacturer's instructions (R&D Systems Europe). Hsp70 concentrations were determined using our previously published methods (Davison et al., 2016), with a Duoset Elisa kit (R&D Systems Europe).

2.5.3. Tight junction proteins

ZO1 and Claudin1 concentrations were determined using previously published methods (Davison et al., 2016), and standard ELISA kits (tight junction antibody samples pack 90–1200, Invitrogen)

2.6. Statistical analysis

All results are expressed as mean ± SEM. Statistics were performed using Graphpad Prism 8 version 8.3.1. Test for normality of data using Shapiro Wilks test showed equal variances between groups. Studies were analyzed using an ordinary one-way analysis of variance ANOVA. Comparisons between treatments was performed using a Tukey's multiple comparison test. Significance was determined at $P < 0.05$.

3. Results

3.1. Study 1. Effect of ZnC on cell survival following hypoxia (ischemia)/normoxia (reperfusion) (H/N)

Results shown in figures are for Caco-2 cells, RIE1 and AGS cells gave similar results.

1A: Dose response study: The addition of ZnC to cells not subjected to H/N did not affect MTT or LDH levels. Exposure of cells to H/N alone resulted in an approximate halving of cell viability (MTT values, Fig. 1A) and a threefold increase in LDH leakage (Fig. 1B). Addition of ZnC prior to H/N reduced cell death, with the most beneficial effect seen at 100 μM, reducing the fall in cell viability (MTT) and rise in LDH caused by H/N by about 50% (Fig. 1A&B).

1B: Efficacy of ZnC given pre-, during- or post-hypoxic period. When comparing the effect of ZnC (100 μM) administered pre-hypoxia, during hypoxia or post-hypoxia (at the reintroduction of normoxia stage), the greatest efficacy was seen if ZnC was given pre-hypoxia (Fig. 1C). Similar reciprocal changes were seen following changes in LDH (Fig. 1D). In contrast to the beneficial effect of adding ZnC, equimolar amounts of $ZnSO_4$ alone, carnosine alone or $ZnSO_4$ + carnosine in combination had no effect on H/N induced cell death or damage (Fig. 2A & B).

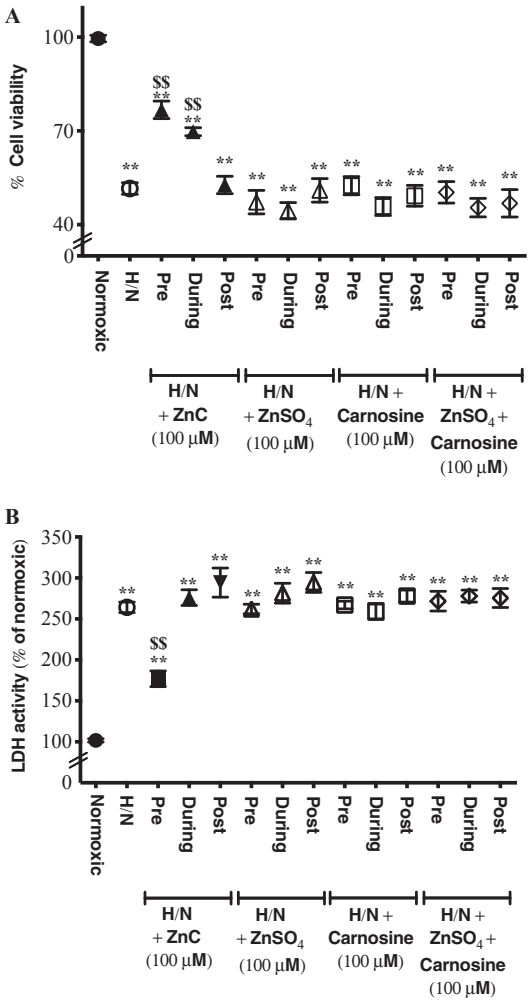

Fig. 2. Effect of hypoxia-normoxia (H/N) on cell viability and injury in presence and absence of $ZnSO_4$, carnosine alone and $ZnSO_4$ + carnosine. Cell viability using MTT assay (A) and cell damage measuring LDH activity (B) were determined for cells exposed to 4 h hypoxia (ischemia) and then returned to normoxia for 24 h (reperfusion). Values are expressed as percentage difference compared to cells incubated in serum free medium alone that were not exposed to H/N. Cells incubated under normoxic conditions throughout (●), and the effect of adding ZnC (▲), $ZnSO_4$ alone (Δ), carnosine alone (□) and $ZnSO_4$ + carnosine in combination (◇), pre hypoxia, during hypoxia or post hypoxia was compared to cells exposed to H/N alone (○). Results shown are for Caco-2 cells, RIE1 and AGS cells gave similar results. Results expressed as mean ± SEM. ** signifies $p < 0.01$ vs normoxic throughout, $$ signifies $p < 0.01$ vs H/N alone.

3.2. Study 2. Effect of ZnC on TEER and transepithelial permeability

The presence of ZnC given prior to H/N had no effect on TEER or HRP permeability under normoxic conditions. In monolayers subjected to H/N in the absence of ZnC, TEER fell to 81.3 ± 0.28% of baseline normoxic values at the end of the hypoxia period and fell further to 64.5 ± 0.4% of baseline normoxic values at the end of the "reperfusion" (return to normoxia) period. Pre-treatment with ZnC truncated this decrease in TEER by about 50% (Fig. 3A).

Similar reciprocal results were seen following permeability of HRP across monolayers. Control cells in serum free medium alone had a 4-fold increase in HRP permeability immediately after the hypoxic period, rising to a 6-fold increase at the end of the H/N period. Pre-

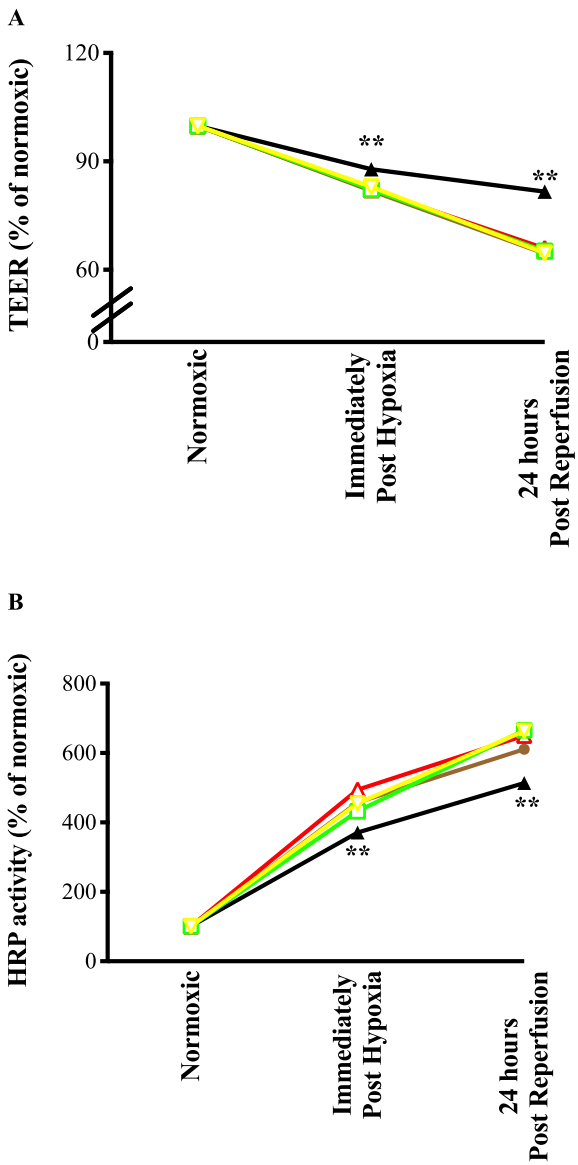

Fig. 3. Effect of ZnC on H/N- induced changes in TEER and transepithelial permeability. Polarised Caco-2 monolayers were exposed to H/N and the effect of ZnC on changes in TEER (A) and permeation of HRP through the monolayer (B) determined. Each monolayer had TEER and HRP permeability assessed at baseline ("normoxic"), at the end of 1 h incubation in a hypoxic chamber ("post hypoxia") and at the end of a 24 h period where they had been returned to normoxia, equivalent to a reperfusion period ("post reperfusion"). Monolayers incubated in serum free medium alone (●, brown line) had significantly greater increases in permeability compared to those that had ZnC (100 μmol▲, black line) added to the medium prior to H/N. Addition of equimolar (100 μmol) $ZnSO_4$ alone (Δ, red line), carnosine alone (□, green line) and $ZnSO_4$ + carnosine in combination (▽, yellow line) had no effect on H/N induced changes (results overlapping with values seen from incubation in medium alone). For both TEER and HRP permeability all samples and time points were significantly different from baseline normoxia values. Results expressed as mean ± SEM but note that SEMs are small and overlap with symbols. ** signifies $p < 0.01$ vs values seen in cells incubated in medium alone under the same conditions of hypoxia-reperfusion.

treatment with ZnC truncated the rise in HRP permeability by 24% at the end of the hypoxic period and by 20% at the final H/N time point (Fig. 3B). In contrast, addition of equimolar amounts of $ZnSO_4$ alone, carnosine alone or $ZnSO_4$ and carnosine together did not influence changes in TEER (Fig. 3A) or HRP permeability (Fig. 3B) caused by H/N.

3.3. Study 3. Effect of ZnC on gut and distant organ injury in response to small intestinal I/R

Histological damage and MPO and MDA tissue levels:

Intestinal tissue from 50% SI region of sham operated control animals showed normal histology (Fig. 4A), whereas the same region of animals that had undergone I/R showed extensive denudation and loss of villus structure and inflammatory infiltration (Fig. 4B). In contrast, animals that had received ZnC prior to I/R showed much less extensive damage (Fig. 4C). Tissue from the 90% SI length (not directly subjected to I/R) appeared histologically normal in all groups.

Lung tissue from sham operated control animals had normal histology or occasional mild increase in inflammatory infiltrate (Fig. 5A), whereas the I/R group showed marked pulmonary congestion and diffuse interstitial inflammatory cell infiltrate and areas of tissue destruction (Fig. 5B). ZnC pre-administration markedly truncated these effects (Fig. 5C).

Formal histological damage scoring using methods of Chiu (Chiu et al., 1970) for SI damage (Fig. 4D) and Koksel (Koksel et al., 2005) for lung damage (Fig. 5D) confirmed histological appearance. Pre-treatment with ZnC reduced the intestinal injury score at the 50% SI site and within the lungs by approx. 60% (all $p < 0.01$ vs I/R alone). Similar results were seen when MPO & MDA levels were assessed (Table 1). In both 50% SI and lung, MPO & MDA levels were increased in response to I/R and this increase was truncated in animals pre-treated with ZnC. Assessment of tissue from the 90% SI site (not directly subjected to I/R) showed no changes in MPO or MDA following I/R compared to sham operated animals (Table S1).

Histological examination of renal and liver tissue showed minimal changes, although biochemical assessment showed a small but significant increase in both MDA and MPO levels following I/R. These biochemical changes were truncated in animals that received ZnC pre-treatment (Table 1).

3.3.1. Plasma cytokine levels

Exposure of animals to I/R caused plasma LPS to increase about 2-fold and plasma IL6, TNFα and IL1β levels to increase about four to five-fold compared to sham operated animals. These increases were all truncated by about 50–70% if the animals had received ZnC pre-treatment (Fig. 6).

3.4. Study 4. Mechanisms of action of ZnC in the in vitro and in vivo studies

Results from N/H studies using cell culture and mouse mesenteric I/R tissue gave similar results (Fig. 7, Table 1, and Table S2) and are therefore discussed together.

Subjecting cells or 50% SI tissue to periods of hypoxia/ischemia followed by normoxia/ reperfusion caused:

- Increased pro-apoptotic molecules Caspase 3 & 9
- Increased pro-apoptotic Baxα
- Reduced anti-apoptotic peptide Bcl2
- Reduced tight junction proteins ZO1 and Claudin 1
- Increased cell adhesion molecule ICAM-1 (Table 1)
- Increased HIF1α & VEGF

Subjecting animals to mesenteric I/R also caused increased Hsp70 and ICAM-1 expression in the lung tissue (Table 1). In the same animals, no changes were seen in Caspase 3, 9, Bcl2 or Baxα in the lung, kidney, liver or in the non-clamped SI region (90% SI, Table S1).

ZnC pre-treatment of cells or animals caused:

- Truncation of rise of pro-apoptotic molecules Caspase 3 & 9
- Truncation of rise of pro-apoptotic Baxα
- Truncation of fall in anti-apoptotic peptide Bcl2

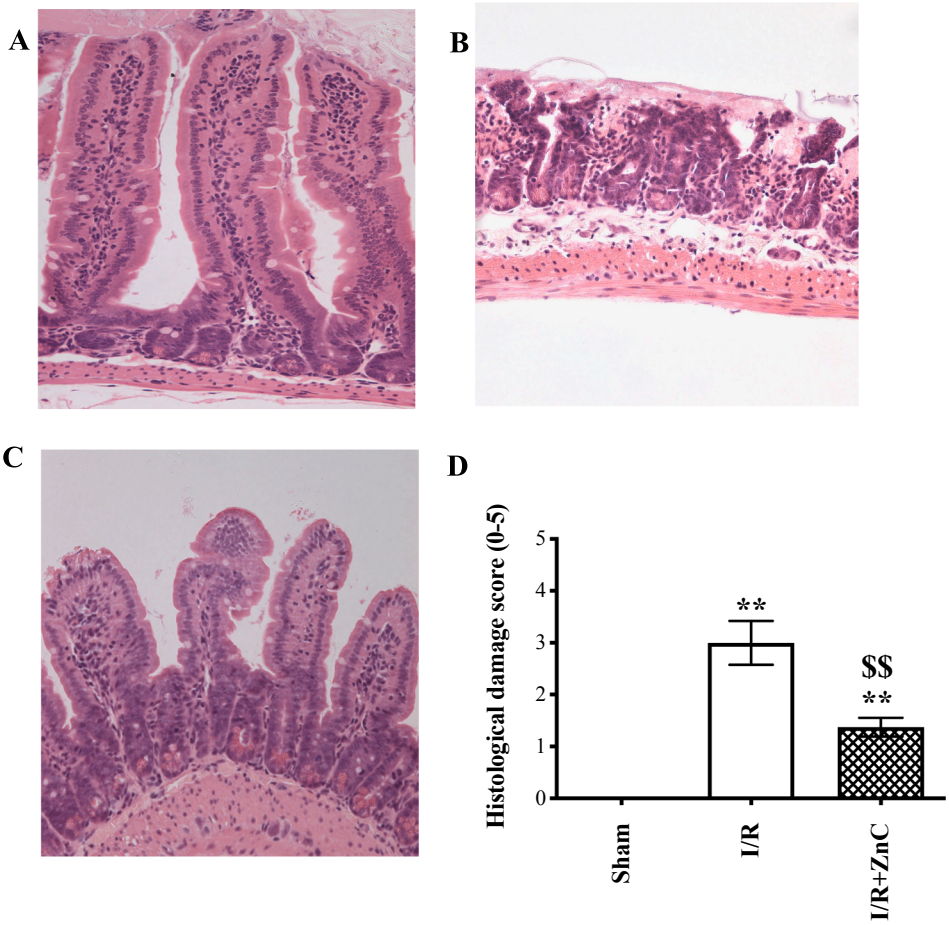

Fig. 4. Effect of ZnC pre-treatment on histology and damage score in SI following I/R. Mice underwent a sham (laparotomy only) procedure or were subjected to 30 min mesenteric ischemia followed by 3 h of reperfusion (I/R). Some animals also received ZnC (58 mg/kg/day, in drinking water for 7 days pre-I/R). Photomicrographs (original magnification 200X) of intestinal tissue from 50% SI region A) sham operated control animal, B) animal that had undergone I/R protocol, C) animal pre-treated with ZnC prior to I/R. D) Formal histological damage scoring used method of Chiu et al. (1970). ** signifies $p < 0.01$ vs sham operated animals, $$ signifies $p < 0.01$ vs I/R alone.

- Truncation of fall in tight junction proteins ZO1 and Claudin 1
  - Did not affect the increase in cell adhesion molecule ICAM-1
  - Caused further increase in VEGF.
  - Caused further increase in Hsp70

4. Discussion

Using a combination of in vitro and in vivo models we showed that ZnC reduced I/R-induced gut injury. The protective pathways stimulated in response to oral ZnC reduced I/R-induced apoptosis, increased cellular protective proteins such as Hsp70 and enhanced tight junction structure. The protective effect of ZnC were seen in all three cell lines tested. This demonstrates our findings have applicability across the entire gastrointestinal tract and are not species dependent, although caution always must be shown when extrapolating from in vitro cancer cell lines to the human in vivo situation. Some of the pathophysiological processes that occur due to I/R are mucosal epithelial cell damage, increased apoptosis, loss of basement membrane integrity and disruption of barrier function. This promotes bacterial and luminal content translocation with induction of local production of cytokines, resulting in chronic low-grade systemic inflammation and episodes of septicaemia (Eltzschig & Collard, 2004; Stallion et al., 2005) Our results examining changes in cell survival and transepithelial resistance in response to ZnC showed positive effects on these processes. We also found the macromolecule ZnC was more effective than an equimolar dose of $ZnSO_4$ alone, carnosine alone or the combination. This may be due, in part, to physicochemical aspects of ZnC, an idea supported by the finding that orally administered ZnC adheres to ulcerative areas of the bowel more effectively than $ZnSO_4$ or carnosine alone (Furuta et al., 1995).

The zinc and carnosine components of ZnC both possess potential protective effects, with their relative importance varying dependent on the model being examined. Zinc is an essential trace element involved in numerous physiological functions including stimulating cell proliferation and reducing apoptosis through pathways including activation of MMPs and the ERK1/2 cascades (For review see Nuttall & Oteiza, 2012). Carnosine is abundant in skeletal muscle and possesses anti-glycation, anti-stress, antioxidant and hydroxyl radical scavenging activity (For detailed reviews see Boldyrev, Aldini, & Derave, 2013, Kawahara, Tanaka, & Kato-Negishi, 2018).

Several different in vivo methods are available for inducing gut I/R, the majority involving temporary complete occlusion of mesenteric arteries +/-veins (see ref Gonzalez, Moeser, & Blikslager, 2014). All have the limitation of being acute rather than chronic I/R injury. We chose segmental mesenteric occlusion as it allows easy identification of intestine outside of the I/R area that can be used as a control to differentiate local versus systemically mediated injury. The most severe damage was in the intestinal segment that had undergone direct I/R and systemic injury was confirmed with raised plasma cytokine levels and lung, liver and kidney markers of injury. Pre-treatment with ZnC significantly reduced histological and biochemical markers of injury in blood, gut, lung, and kidney.

Administration of ZnC reduced apoptosis (as measured by caspases) and increased anti-apoptotic signaling molecules. These effects were mediated locally as distant organs and non-clamped regions of bowel did not show any similar changes. HIF1α and VEGF expression reduces apoptosis, stimulates angiogenesis and causes immune modulation (Cummins, Keogh, Crean, & Taylor, 2016; Karhausen et al., 2004), suggesting that Hsp70, HIF1α and VEGF may all have relevance to the protective effects seen.

ICAM-1 stabilises cell–cell interactions and facilitates leukocyte

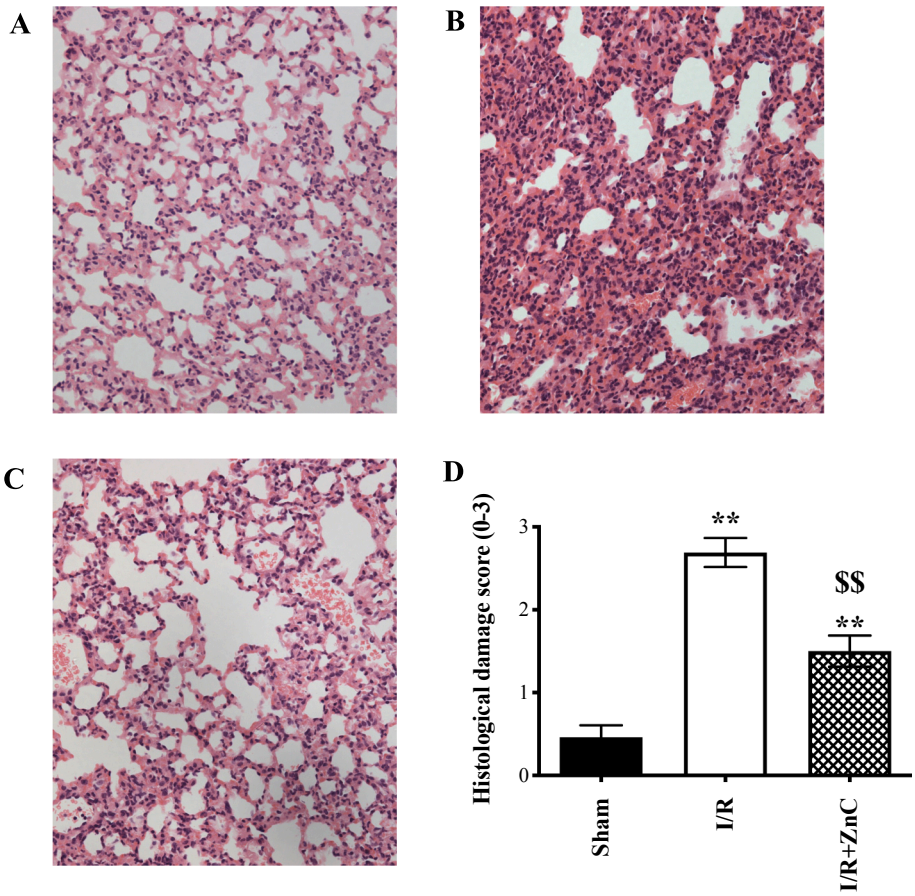

Fig. 5. Effect of ZnC pre-treatment on histology and damage score in lung following I/R. Same protocol as Fig. 4. Photomicrographs (original magnification 200X) of lung tissue (A) sham operated control animal, (B) animal that had undergone I/R protocol, (C) animal pre-treated with ZnC prior to I/R. (D) Formal histological lung damage scoring used method of Koksel et al. (2005). ** signifies $p < 0.01$ vs sham operated animals, $$ signifies $p < 0.01$ vs I/R alone.

endothelial transmigration.

Its upregulation in the lungs resulting from gut I/R is thought be an important signalling mechanism involved in pulmonary leukocyte infiltration (Zhao, Montalto, Pfeiffer, Hao, & Stahl, 1985). ZnC administration did not affect ICAM levels post I/R, suggesting that the pulmonary protective effects of ZnC must be through other processes, such as via the reduced circulating cytokines demonstrated. Intestinal epithelial tight junctions are multiprotein complexes that act as selective barriers. The truncation of falls in ZO1 and Claudin 1 caused by ZnC administration may have contributed to the enhanced integrity seen in the TEER and HRP permeability studies. ZnC has been shown to activate the Nrf2/HO-1 signalling pathway, which may reduce the expression of inflammatory mediators, such as NO and iNOS (Ooi, Chan, & Sharif, 2017). In addition, ZnC has been shown to increase phosphorylation of Smad1, a signal-transducing molecule (Maeno et al., 2004) and activate the ERK1/2 cascade system causing multiple effects on transcription factors and scaffolding proteins (Lu & Xu, 2006; Orton et al., 2005).

Chronic gastrointestinal ischemia/reperfusion (I/R) injury results from a variety of causes and in contrast to the acute I/R situation, diagnosis in adults is often delayed. When the underlying cause of I/R is vascular in origin, symptoms may include post-prandial abdominal pain, sometimes termed *abdominal angina* resulting from periods of relative ischemia followed by return to vascular sufficiency, exacerbating the I/R mediated injury. Periods of increased gut permeability may also result in septicemic episodes requiring antibiotics. In some cases, revascularization deals with the primary cause but when surgical intervention is not possible, pharmacological options have limited value and include anticoagulation, proton pump inhibitors, vasodilators, antimicrobials and inflammatory modulators (Jiang et al., 2012; Kalogeris, Baines, Krenz, & Korthuis, 2016; Mensink et al., 2011). ZnC offers a novel approach working through mechanisms distinct from the current alternatives and can be administered orally, surviving passage through the gastrointestinal tract. Previous clinical studies examining the effect of ZnC on gut integrity for conditions such as exercise induced hyperpermeability (Davison et al., 2016) have used a dose of 75 mg/day, which is within recommended daily allowances for zinc intake, limiting risks of side effects.

In summary, our studies showed that orally administered ZnC protects gut and distant organs from mesenteric I/R-induced injury. ZnC may provide a novel approach to the prevention and treatment of a wide variety of gut conditions which have I/R as a component of their pathophysiology. Further studies including clinical trials appear warranted

Author contributions

TM contributed to study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript and statistical analysis. RJP contributed to study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript, critical revision of the manuscript for important intellectual content, obtained funding and study supervision.

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Table 1
Effect of gut ischemia–reperfusion (I/R) ± pre-administration of ZnC on injury and protective pathways in 50% SI, lung, renal and liver tissue. Data presented as mean ± SEM. * and ** signifies $p < 0.05$ and $p < 0.01$ vs Sham operated animals (laparotomy only), $$ signifies $p < 0.05$ and < 0.01 vs I/R.

50% SI	Sham	I/R	I/R + ZnC
MPO (U/μg of protein)	0.162 ± 0.006	0.776 ± 0.012**	0.306 ± 0.02** $$
MDA (nmol/μg of protein)	0.241 ± 0.007	2.148 ± 0.067**	0.951 ± 0.080** $ $
Hsp70 (pg/μg total protein)	18.93 ± 0.29	30.80 ± 0.39**	37.13 ± 0.67** $$ ++
ICAM-1 (pg/μg total protein)	1.29 ± 0.161	2.443 ± 0.285**	2.537 ± 0.236**
Lung	**Sham**	**I/R**	**I/R + ZnC**
MPO (U/μg of protein)	0.219 ± 0.021	1.481 ± 0.045**	1.001 ± 0.029** $ $
MDA (nmol/μg of protein)	0.281 ± 0.019	1.082 ± 0.026**	0.763 ± 0.011** $ $
Hsp70 (pg/μg total protein)	21.25 ± 0.589	29.99 ± 0.79**	36.33 ± 0.18** $$
ICAM-1 (pg/μg total protein)	3.22 ± 0.31	5.85 ± 0.14**	5.51 ± 0.12**
KIDNEY	Sham	I/R	I/R + ZnC
MPO (U/μg of protein)	0.166 ± 0.008	0.526 ± 0.041**	0.343 ± 0.015** $ $
MDA (nmol/μg of protein)	0. 291 ± 0.013	0.557 ± 0.040**	0.481 ± 0.014**
Hsp70 (pg/μg total protein)	36.0 ± 0.55	34.5 ± 0.93	36.3 ± 2.96
ICAM-1 ((pg/μg total protein)	5.24 ± 0.13	4.96 ± 0.16	5.35 ± 0.10
LIVER	Sham	I/R	I/R + ZnC
MPO (U/μg of protein)	1.92 ± 0.05	2.29 ± 0.06**	2.03 ± 0.04** $$ ++
MDA (nmol/μg of protein)	0.25 ± 0.007	0.34 ± 0.005**	0.28 ± 0.004** $$
Caspase 3 activity (Absorbance change)	0.024 ± 0.0008	0.024 ± 0.001	0.025 ± 0.0007
Caspase 9 activity (Absorbance change)	0.023 ± 0.002	0.024 ± 0.001	0.025 ± 0.002

Ethics statement

All animal experiments were approved by local animal ethics committees (Queen Mary University of London's Animal Welfare and Ethical Review Board), performed at Queen Mary University of London, Barts & The London School of Medicine and Dentistry and covered by the appropriate project licences under the Home Office Animals Procedures Acts, 1986. Experiments were performed under terminal anethesia using ketamine and xylaxine.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: PepZin GI™ (zinc carnosine) is manufactured and trademarked by Hamari Chemicals Ltd., Osaka, Japan. Xsto Solutions, New Jersey, are USA based suppliers and distributors of PepZin GI™. This does not alter our adherence to publication policies on sharing data and materials.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104361.

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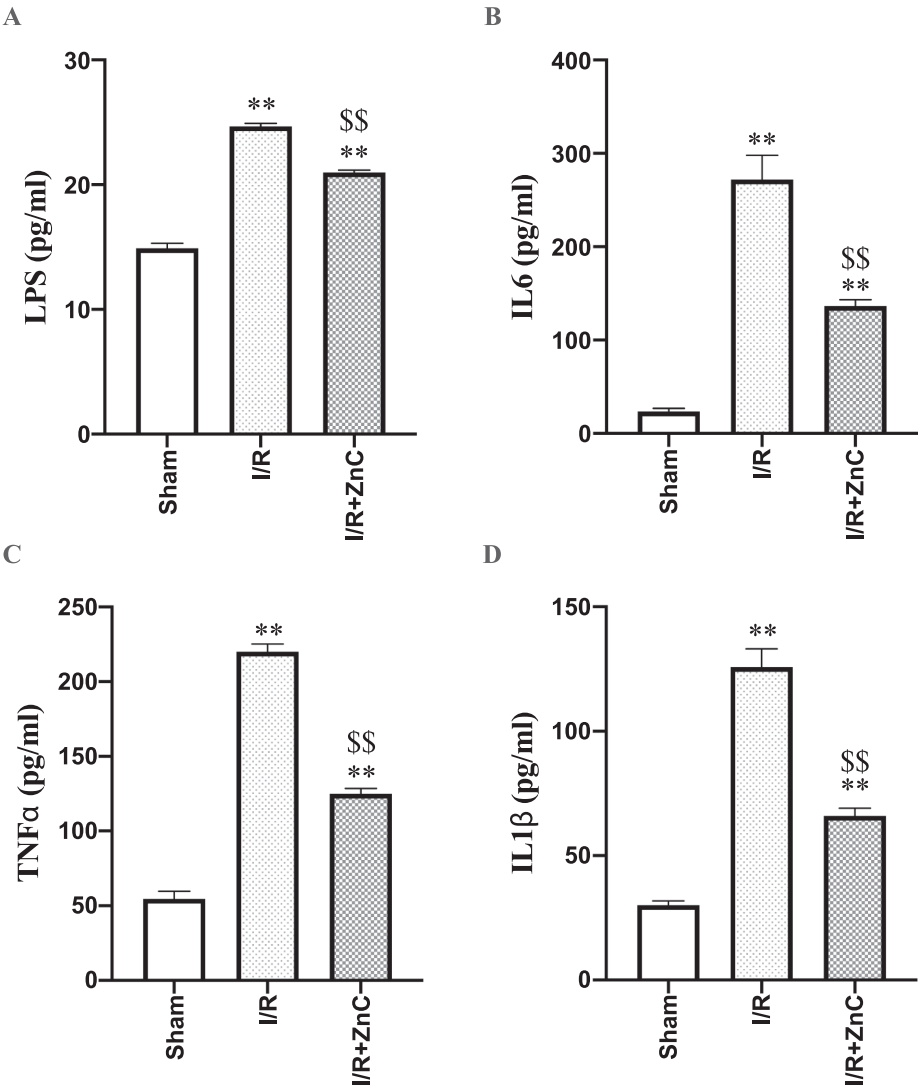

Fig. 6. Plasma LPS and cytokine levels in animals undergoing I/R. Same protocol as Fig. 4. Plasma was collected at the end of the I/R protocol for animals that received sham operation, I/R alone, or I/R with ZnC pre-treatment. LPS (A) and cytokine analyses, IL6 (B), TNFα (C) and IL1β (D). * and ** signifies $p < 0.05$ and $p < 0.01$ vs sham operated animals, $$ signifies $p < 0.01$ vs I/R alone.

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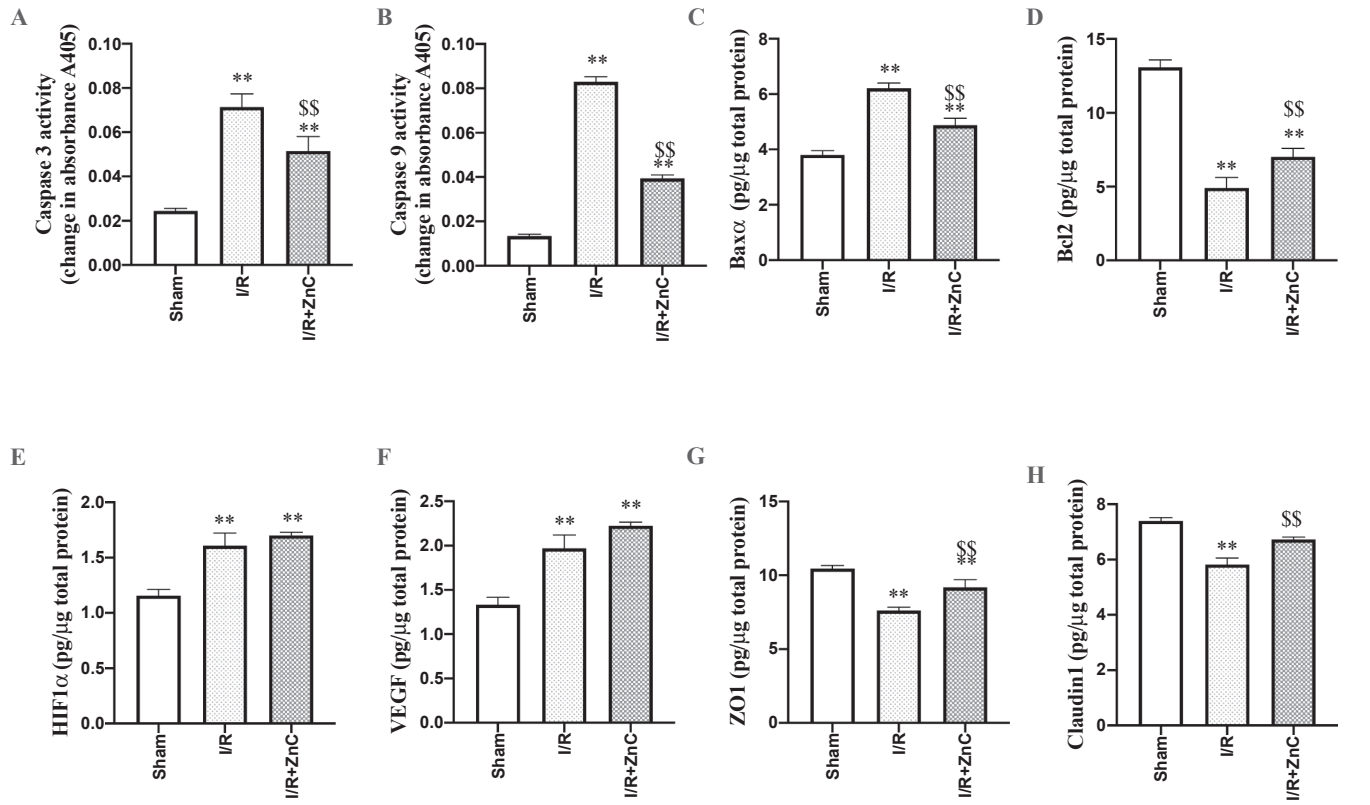

Fig. 7. Molecular mechanisms of action of ZnC on mice SI undergoing I/R. Same protocol as Fig. 4. Cleared cell lysates were collected from the 50% SI site of animals that had received sham operation, I/R alone, or I/R with ZnC pre-treatment. These were analysed for changes in apoptotic molecules Caspase 3 (A), Caspase 9 (B), Baxα (C) and Bcl2 (D). HIF1α (E), VEGF (F). Tight junction molecules ZO1 (G) and Claudin1 (H) levels were also measured. * or ** signifies $p < 0.05$ and < 0.01 vs sham operated animals, $$ signifies $p < 0.01$ when comparing the effect of ZnC pre-treatment against animals that had undergone I/R alone.