



Disturbance of oxidant/antioxidant balance, acute phase response and high mobility group box–1 protein in acute undifferentiated diarrhea in crossbred piglets

U K DE¹, REENA MUKHERJEE², S NANDI³, BHARAT BHUSHAN⁴, U DIMRI⁵ and A K VERMA⁶

Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 7 January 2014; Accepted: 18 April 2014

Key words: Ceruloplasmin, Haptoglobin, HMGB1, Oxidative stress, Piglet diarrhoea

Piglet diarrhoea is a major cause of high morbidity and mortality in the swine production farm, resulting in significant magnitude of economic losses. The common enteric pathogens responsible for piglet diarrhea are virus (transmissible gastroenteritis, porcine epidemic diarrhea virus, porcine circo virus-2 and rotavirus), bacteria (enterotoxigenic *Escherichia coli*, *Salmonella* sp, *Clostridium perfringens*), and parasites (*Isospora suis* and *Cryptosporidium parvum*) apart from many other pathogens (Katsuda *et al.* 2006). Release of some molecules following disruption of intestinal mucosa aggravates the pathogenesis of enteritis. The role of high mobility group box 1 (HMGB1) protein, which is released in response to activated signals by pathogen derived toxins and virus stimuli (Wang *et al.* 2006), in the pathogenesis of gastrointestinal disorders was investigated (Dai *et al.* 2010, Luan *et al.* 2010). The acute phase proteins are considered to be involved in the restoration of homeostasis and restraint of microbial growth before animals develop acquired immunity to infection (Petersen *et al.* 2004). Haptoglobin (Hp) and ceruloplasmin (Cp), the porcine acute phase proteins, are well studied in piglet (Zyczko and Zyczko 2005, Grau-Roma *et al.* 2009). Kim *et al.* (2012) suggested that tissue damage attributed by oxidative stress and reactive oxygen species (ROS) plays a key role in pathogenesis in enteric diseases of farm animals. The nitric oxide (NO) contributes to oxidative stress in conjunction with other ROS in the states of inflammation (Lubos *et al.* 2008). To the best of our knowledge, there are no such reports and detail studies describing the role of HMGB1, acute phase proteins and oxidative stress in acute undifferentiated piglet diarrhea. Therefore, the present study was undertaken to examine whether acute undifferentiated diarrhea influences these

levels in crossbred suckling piglets and that these may be targets for supportive therapy.

Animals: Crossbred piglets (Landrace × indigenous) from the institute's experimental swine production farm were included for the present study. The piglets and dam were kept in same farrowing pens with concrete floor and piglets were allowed to suck adlib dam's milk to the age of 7 weeks. The overall management practices were identical for all piglets. The pregnant dams were not immunized with *E. coli* K99+ vaccine and any viral vaccine, nor were additional supplementation of antioxidant practiced in the farm. Fifteen piglets between 0–15 days old of age, irrespective of sex and body weight, that were suffering from acute diarrhoea were randomly used for sampling. The diarrhoea was diagnosed on the basis of clinical symptoms such as frequency of defecation (>5 times in a day), consistency (profuse, watery, with or without mucus), dehydration score (prominent protruding vertebrae and pelvic bones and retracted eyes), dullness and weakness. Another 15 healthy piglets of either sex of same age group were also randomly selected to serve as controls.

Collection and processing of samples: Blood samples (5 ml) from both groups of piglets were collected from anterior vena cava in sterile vials containing disodium salt of ethylene diamine tetraacetic acid (Na₂-EDTA) as anticoagulant. Samples were immediately centrifuged at 200g for 10 min to separate plasma and stored at -20°C till analysis.

Oxidative stress markers

Assay for malondialdehyde (MDA) and nitric oxide (NOx): The MDA levels, an index of lipid peroxidation were measured by double heating method of Draper and Hadley (1990). The assay is based on spectrophotometric measurement of the purple colour generated by the reaction of thiobarbituric acid (TBA) with MDA. The concentration of MDA was calculated on the basis of absorbance coefficient ($1.56 \times 10^5 \text{ cm}^{-1}$) of the TBA-MDA complex. The nitrite and nitrate (NOx) in plasma was measured by nitrate reduction on vanadium chloride followed by colour

Present address: ¹Scientist (Senior Scale) (ujjwalde@gmail.com), ²Principal Scientist (reenam1992@gmail.com), ⁵Head, Division of Medicine (udimriivri@gmail.com), ³Principal Scientist, (snandi03@yahoo.com) CADRAD, ⁴Principal Scientist and In-Charge, Swine Production Farm, ⁶Head (vermaak62@gmail.com), Division of Animal Nutrition.

development with Griess reagent as per the method described earlier (Yucel *et al.* 2012).

Assay for total antioxidant activity, superoxide dismutase (SOD) and catalase: Total antioxidant, superoxide dismutase (SOD) and catalase activity in blood serum were estimated by antioxidant assay kit, SOD assay kit and catalase assay kit respectively as per manufacturer's instruction.

Assay for high mobility group box-1 (HMGB1) protein, haptoglobin (Hp) and ceruloplasmin (Cp): Before analysis, all the plasma samples were diluted into 1: 10 and HMGB1 protein in diluted serum was estimated using HMGB1 ELISA kit according to the manufacturers' instructions. Similarly, Hp was measured in diluted plasma samples (1: 40,000) using Assay swine haptoglobin ELISA kit as per the manufacturers' instructions. All ELISA measurements were performed in duplicates, and measured at 450 nm with the Multiskan RS Microplate Reader. The Cp level in serum was determined by measuring paraphenylene diamine (PPD) oxidase activity as per Sunderman and Nomoto (1970).

Statistical analysis: The data were analyzed statistically using Student's t-test ($P < 0.05$) to determine the significance between the mean values of the two groups (Snedecor and Cochran 1994).

Oxidative stress is manifested either due to inadequate availability of antioxidant enzymes or uncontrolled production of free radicals in the body. Free radicals and lipid peroxidation exert harmful effect on tissue due to alterations in the integrity of cell membrane (Lobo *et al.* 2010). MDA, derived from lipid peroxidation is an important indicator to decide the degree of oxidative damage of cell membrane. It reacts with cellular membrane elements and results to increase cellular permeability and enzyme activities. NO is considered as an important component of the host defense against invading pathogens. This molecule, a principal endothelial-derived relaxing factor, reacts with superoxide anion (O_2^-) to yield peroxynitrite ($ONOO^-$), which is a powerful oxidant and nitrosating agent. It triggers a cascade of events leading to the generation of highly reactive and damaging radicals and oxidative species (Soneja *et al.* 2005). Body counteracts the ill effects of oxidative damage by neutralizing the free radicals via antioxidant defense system that comprises antioxidant enzymes, like superoxide dismutase (SOD) and catalase (CAT). In the present study, serum MDA (7.523 ± 0.451 nmol/ml vs 2.455 ± 0.182 nmol/ml) and NOx (4.004 ± 0.224 μ M/ml vs 2.104 ± 0.173 μ M/ml) were significantly ($p < 0.05$) increased and total antioxidant activity, (1.766 ± 0.160 Trolox mM/ml vs 3.856 ± 0.112 Trolox mM/ml), SOD (143.251 ± 19.498 U/ml vs 336.547 ± 9.462 U/ml) and CAT (6.195 ± 0.532 formaldehyde nmol/min/ml vs 16.430 ± 2.155 formaldehyde nmol/min/ml) level were decreased ($p < 0.05$) in diarrheic piglets as compared to control animals (Table I). It might be due to excess production of free radicals and poor antioxidant reserve in piglets suffering from acute diarrhoea. Authors reported that enhanced activity of

oxidative stress markers in pigs experimentally infected with ETEC *E. coli*, *Cryptosporidium parvum* and other enteric pathogens (Argenzio and Rhoads 1997, Keel and Songer 2006, Zadrozny *et al.* 2006, Daudelin *et al.* 2011). The present finding warrants the need of antioxidant supplementation with standard treatment for better therapeutic response in acute undifferentiated piglet diarrhea.

The HMGB1 is released from necrotic cells during tissue damage as a consequence of infection and leads to leakage of epithelial-cell barriers because of down regulation of expression of some cell-surface proteins responsible for the tight adhesion between adjacent epithelial cells (Kono and Rock 2008). This cellular mechanism is also responsible for the systemic and organ-specific toxicity of HMGB. In the present study, significantly ($p < 0.05$) higher levels of serum HMGB1 (6.629 ± 0.367 ng/ml vs 2.350 ± 0.065 ng/ml) in diarrheic piglets as compared to control animals (Table I) might be due to excessive damage of intestinal epithelial cells in piglets suffering from acute enteritis. Increased HMGB1 activity has been reported in acute gastroenteritis induced by *E. coli*, *Shigella dysenteriae* in piglet model and in children with inflammatory bowel disease (Jeong *et al.* 2010, Splichalova *et al.* 2011, Vitali *et al.* 2011). However, it has been reported that the lethal effect caused by HMGB1 protein in gut could be neutralized using HMGB1-specific antibodies (Yang *et al.* 2004). There is no published report on HMGB1 protein in natural cases of piglet diarrhea. From the present study, we hypothesize that serum HMGB1 could be used as a reliable clinical evaluation tool of the severity of enteric infections in piglets.

The acute phase response is a nonspecific reaction to tissue damage by infection, inflammation, neoplasia and immunological disease (Chen *et al.* 2003, Petersen *et al.* 2004). It triggers the production of acute-phase proteins (APPs) (such as α -1 acidglycoprotein, haptoglobin and ceruloplasmin) and involves local and systemic effects (Green and Adams 1992). Moreover, the APPs are also the

Table 1. Comparison of parameters of oxidative stress markers, high mobility group box-1 protein and acute phase response in crossbred piglets affected with acute undifferentiated diarrhoea and healthy piglets

Parameters	Healthy piglets (n=15)	Diarrheic piglets (n=15)
Antioxidant activity (trolox mM/ml)	3.856 $\pm$ 0.112	1.766 $\pm$ 0.160*
Catalase (Formaldehyde nmol/min/ml)	16.430 $\pm$ 2.155	6.195 $\pm$ 0.532*
SOD (U/ml)	336.547 $\pm$ 9.462	143.251 $\pm$ 19.498*
MDA (nmolml ⁻¹)	2.455 $\pm$ 0.182	7.523 $\pm$ 0.451*
NOx (μ M/ml)	2.104 $\pm$ 0.173	4.004 $\pm$ 0.224*
HMGB1 (ng/ml)	2.350 $\pm$ 0.065	6.629 $\pm$ 0.367*
Hp (ng/ml)	0.455 $\pm$ 0.064	2.415 $\pm$ 0.103*
Ceruloplasmin (g/litre)	0.213 $\pm$ 0.019	0.605 $\pm$ 0.039*

*Statistically significant at $P < 0.05$.

components of innate immunity mediated by cytokines (Suffredini *et al.* 1999). In the present study, serum Hp (2.415 ± 0.103 ng/ml vs 0.455 ± 0.064 ng/ml) and ceruloplasmin (0.605 ± 0.039 g/litre vs 0.213 ± 0.019 g/litre) level were significantly ($P < 0.05$) higher in piglets suffering from acute diarrhea as compared to healthy piglets. Over the past few years, intestinal epithelial cells have been shown to play a central role in the inflammatory response of the intestine in response to enteric pathogen (Stadnyk and Waterhouse 1997). Researchers observed that genes of acute phase proteins are expressed and regulated by multiple cytokines and cAMP in intestinal epithelial cells (Pelletier *et al.* 1998). The enteric pathogens stimulate the synthesis of pro and anti-inflammatory cytokines and activity of cAMP in intestinal epithelial cells during infection (Moen *et al.* 2010, Shea-Donohue *et al.* 2010). Significant elevation of Hp and ceruloplasmin in diarrheic piglets as compared to healthy animals in the present study could be due to inflammation and intestinal tissue injury caused by enteric pathogens.

From the present findings, it can be concluded that that oxidative stress, HMGB1 and acute phase response play a definite role in the pathogenesis of piglet diarrhoea, suggesting a possible beneficial role for HMGB1 specific antibody and supplementation of antioxidants along with standard treatment in piglet diarrhoea.

SUMMARY

The objective of the present study was to investigate the status of high mobility group box-1 (HMGB1) protein, oxidative stress and acute phase proteins in natural cases of acute undifferentiated diarrhoea in piglets aged 1–15 days old. The study was conducted on 30 crossbred (Landrace $\times$ indigenous) piglets; fifteen suffering from acute enteritis (group 1) and fifteen healthy piglets as control (group 2). The diarrhoea was diagnosed on the basis of clinical symptoms. From the results of the study, it is concluded that HMGB1 protein, markers of oxidative stress and acute phase proteins might play important roles in the pathophysiology of piglet diarrhoea and that these may be targets for supportive therapy.

ACKNOWLEDGEMENTS

We acknowledge Director IVRI, for financial support and facilities under the institute funded project. We thank staff of the farm for technical support to carry out this work.

REFERENCES

- Argenzio R A and Rhoads J M. 1997. Reactive oxygen metabolites in piglet cryptosporidiosis. *Pediatric Research* **41**: 521–26.
- Chen H H, Lin J H, Fung H P, Ho L L, Yang P C, Lee W C, Lee Y P and Chu R M. 2003. Serum acute phase proteins and swine health status. *The Canadian Journal of Veterinary Research* **67**: 283–90.
- Dai S, Sodhi C, Cetin S, Richardson W, Branca M, Neal M D, Prindle T, Ma C, Shapiro R A, Li B, Wang J H and Hackam D J. 2010. Extracellular high mobility group box-1 (HMGB1) inhibits enterocyte migration via activation of Toll-like receptor-4 and increased cell-matrix adhesiveness. *Journal of Biological Chemistry* **285**: 4995–5002.
- Daudelin J F, Lessard M, Beaudoin F, Nadeau É, Bissonnette N, Boutin Y, Brousseau J P, Lauzon K, Fairbrother J M. 2011. Administration of probiotics influences F4 (K88)-positive enterotoxigenic *Escherichia coli* attachment and intestinal cytokine expression in weaned pigs. *Veterinary Research* **42**: 69.
- Draper H H and Hadley M. 1990. Malondialdehyde determination as index of lipid peroxidation. *Methods in Enzymology* **186**: 421–31.
- Grau-Roma L, Heegaard P M, Hjulsgaard C K, Sibila M, Kristensen C S, Allepuz A, Piñeiro M, Larsen L E, Segalés J and Fraile L. 2009. Pig-major acute phase protein and haptoglobin serum concentrations correlate with PCV2 viremia and the clinical course of postweaning multisystemic wasting syndrome. *Veterinary Microbiology* **138**: 53–61.
- Green E M and Adams H R. 1992. New perspectives in circulatory shock: pathophysiologic mediators of the mammalian response to endotoxemia and sepsis. *Journal of the American Medical Association* **200**: 1834–41.
- Jeong K I, Zhang Q, Nunnari J and Tzipori S. 2010. A piglet model of acute gastroenteritis induced by Shigella dysenteriae Type 1. *Journal of Infectious Diseases* **201**: 903–11.
- Katsuda K, Kohmoto M, Kawashima K and Tsunemitsu H. 2006. Frequency of enteropathogen detection in suckling and weaned pigs with diarrhea in Japan. *Journal of Veterinary Diagnostic Investigation* **18**: 350–54.
- Keel M K and Songer J G. 2006. The Comparative Pathology of Clostridium difficile -associated Disease. *Veterinary Pathology* **43**: 225–40.
- Kim Y J, Kim E H and Hahm K B. 2012. Oxidative stress in inflammation-based gastrointestinal tract diseases: challenges and opportunities. *Journal of Gastroenterology and Hepatology* **27**: 1004–10.
- Kono H and Rock K L. 2008. How dying cells alert the immune system to danger. *Nature Reviews Immunology* **8**: 279–89.
- Lobo V, Patil A, Phatak A and Chandra N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Review* **4**: 118–26.
- Luan Z G, Zhang H, Ma X C, Zhang C and Guo R X. 2010. Role of high-mobility group box 1 protein in the pathogenesis of intestinal barrier injury in rats with severe acute pancreatitis. *Pancreas* **39**: 216–23.
- Lubos E, Handy D E and Loscalzo J. 2008. Role of oxidative stress and nitric oxide in atherothrombosis. *Frontiers in Bioscience* **13**: 5323–44.
- Moen S T, Blumentritt C A, Slater T M, Patel S D, Tutt C B, Estrella-Jimenez M E, Pawlik J, Sower L, Popov V L, Schein C H, Gilbertson S R, Peterson J W and Torres A G. 2010. Testing the Efficacy and Toxicity of Adenylyl Cyclase Inhibitors against Enteric Pathogens Using In Vitro and In Vivo Models of Infection. *Infection and Immunity* **78**: 1740–49.
- Pelletier N, Boudreau F, Yu S J, Zannoni S, Boulanger V and Asselin C. 1998. Activation of haptoglobin gene expression by cAMP involves CCAAT/enhancer-binding protein isoforms in intestinal epithelial cells. *FEBS Letters* **439**: 275–80.
- Petersen H H, Nielsen J P and Heegaard P M H. 2004. Application of acute phase protein measurements in veterinary clinical chemistry. *Veterinary Research* **35**: 163–87.
- Ranjan R, Naresh R, Patra R C and Swarup D. 2006. Erythrocyte lipid peroxides and blood zinc and copper concentrations in acute undifferentiated diarrhoea in calves. *Veterinary Research*

Communication **30**: 249–54.

- Shea-Donohue T, Fasano A, Smith A and Zhao A. 2010. Enteric pathogens and gut function: Role of cytokines and STATs. *Gut Microbes* **1**: 5: 316–24.
- Snedecor G W and Cochran W G. 1994. Statistical methods, (Iowa State University Press, Ames, Iowa, USA).
- Soneja A, Drews M and Malinski T. 2005. Role of nitric oxide, nitroxidative and oxidative stress in wound healing. *Pharmacological Reports* **57** Suppl: 108–19.
- Splichalova A, Splichal I, Chmelarova P and Trebichavsky I. 2011. Alarmin HMGB1 is released in the small intestine of gnotobiotic piglets infected with enteric pathogens and its level in plasma reflects severity of sepsis. *Journal of Clinical Immunology* **31**: 488–97.
- Stadnyk A W and Waterhouse C C M. 1997. Epithelial cytokines in intestinal inflammation and mucosal immunity *Current Opinion in Gastroenterology* **13**: 510–17.
- Suffredini A F, Fantuzzi G, Badolato R, Oppenheim J J and O'grady N P. 1999. New insights into the biology of the acute phase response. *Journal of Clinical Immunology* **19**: 203–14.
- Sunderman S W Jr and Nomoto S. 1970. Measurement of human serum ceruloplasmin by its *p*-phenylenediamine oxidase activity. *Clinical Chemistry* **16**: 903–10.
- Vitali R, Stronati L, Negroni A, Di Nardo G, Pierdomenico M, del Giudice E, Rossi P and Cucchiara S. 2011. Fecal HMGB1 is a novel marker of intestinal mucosal inflammation in pediatric inflammatory bowel disease. *American Journal of Gastroenterology* **106**: 2029–40.
- Wang H, Ward M F, Fan X G, Sama A E and Li W. 2006. Potential role of high mobility group box 1 in viral infectious diseases. *Viral Immunology* **19**: 3–9.
- Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris H E, Susarla S M, Ulloa L, Wang H, DiRaimo R, Czura C J, Wang H, Roth J, Warren H S, Fink M P, Fenton M J, Andersson U and Tracey K J. 2004. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proceedings of National Academy of Science USA* **101**, 296–30.
- Yucel A A, Gulen S, Dincer S, Yucel A E and Yetkin G I. 2012. Comparison of two different applications of the Griess method for nitric oxide measurement. *Journal of Experimental and Integrative Medicine* **2**: 167–71.
- Zadrozny L M, Stauffer S H, Armstrong M U, Jones S L and Gookin J L. 2006. Neutrophils Do Not Mediate the Pathophysiological Sequelae of *Cryptosporidium parvum* Infection in Neonatal Piglets. *Infection and Immunity* **74**: 5497–505.
- Zyczko K and Zyczko G M. 2005. Effects of transferrin and ceruloplasmin on the resistance of sucking piglets to *Escherichia coli* strains causing diarrhea. *Medycyna Weterynaryjna* **61**: 426–29.