Gastric antisecretory and antiulcer activity of bovine hemoglobin

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Received: December 30, 2012 Revised: March 9, 2013
Accepted: April 13, 2013
Published online: June 7, 2013

Abstract

AIM: To investigate gastric antisecretory and gastro-protective activity of bovine hemoglobin (B-Hb) in rats.

METHODS: Adult Albino-Wistar rats were divided into groups of 6 animals each. B-Hb in doses of 100, 300 and 900 mg/kg body weight was tested for gastric acid secretion and antiulcer activity. Gastric secretions were measured 6 h after pylorus ligation in rats pretreated with B-Hb. The acidity was measured by titrating gastric contents against 0.01 mol/L NaOH to pH 7. Indomethacin ulcers were produced by oral administration of 30 mg/kg bw in the rats pretreated with B-Hb one hour before indomethacin. Six hours after indomethacin stomach removed and ulcer index was recorded. Ethanol ulcer were produced by 1 mL of ethanol in the rats pretreated with B-Hb 30 min before the ethanol.

RESULTS: In control rats pylorus ligation for 6 h resulted in the accumulation of 8.1 ± 0.61 mL of gastric secretion. The treatment of the rats with 100, 300 and 900 mg/kg of B-Hb produced a significant decrease in the volume of gastric secretion 5.6 ± 0.63, 5.5 ± 0.75 and 4.7 ± 0.58 mL respectively as compared to the control group (analysis of variance (ANOVA) F = 4.77, P < 0.05). The lesion area in the control group was found to be 22.4 ± 3.2 mm² six hours after the administration of indomethacin. Treatment of rats with B-Hb at doses of 100 mg/kg (24.3 ± 3.29 mm²), 300 mg/kg (16.2 ± 1.45 mm²) and 900 mg/kg (12.6 ± 1.85 mm²) produced a dose dependent decreased the lesion scores (ANOVA F = 4.50, P < 0.05). The ulcer index following one hour after 1 mL ethanol was 7.1 ± 0.31. Pretreatment of rats with B-Hb at the doses of 100 mg/kg (2.5 ± 0.42), 300 mg/kg (2.1 ± 0.4) and 900 mg/kg (0.7 ± 0.21) significantly inhibited the formation of gastric lesions (ANOVA F = 63.26, P < 0.0001). Histological examination of gastric mucosa following ethanol showed significant lesions in the form of gastric pits with detachment of the surface epithelium; vacuolation of epithelial cells and elongation of microvessels. The changes were dose-dependently attenuated by B-Hb. The treatment of rats with ethanol significantly decreased the Alcian blue binding capacity of gastric wall mucus (480 ± 25.6 μg Alcian blue/g of tissue) as compared to control rats (667 ± 25.8 μg). Pretreatment of rats with B-Hb at the doses of 100 mg/kg (516 ± 31.6 μg/g), 300 mg/kg (558 ± 28.8 μg/g) and 900 mg/kg (654 ± 33.8 μg/g) significantly attenuated ethanol induced depletion of gastric wall mucus (ANOVA F = 8.05, P < 0.005). A significant and dose dependent increase of gastric mucosal NP-SH (ANOVA F = 19.62, P < 0.001) and decrease in MPO activity (ANOVA F =
3.1, $P < 0.05$) was observed in B-Hb treated rats.

CONCLUSION: B-Hb possesses significant gastric antisecretory and gastroprotective activity against experimentally induced gastric lesion. The gastroprotective effects of B-Hb are accompanied by inhibition of neutrophils activity, reduction of oxidative stress and maintenance of mucosal integrity.

Al Asmari AK, Al Omani S, Elfaki I, Tariq M, Al Malki A, Al Asmary S. Gastric antisecretory and antiulcer activity of bovine hemoglobin. World J Gastroenterol 2013; 19(21): 3291-3299 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i21/3291.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i21.3291

INTRODUCTION

A variety of noxious factors and substances like alcohol, drugs, psychological stress, smoking and bacterial infection by Helicobacter pylori, to which the man is exposed in the present day life are known to have deleterious effect on gastric mucosa[1]. Organized at several levels the mucosal defense system comprise the pre-epithelial mucosal layer, the epithelial cell barrier, the mucosal microvasculature, the supply of the mucosa by enteric, extrinsic sensory and extrinsic autonomic neurons and mucosal immune system[2]. Maintenance and repair of gastric mucosa is a dynamic process associated with proliferation and migration of epithelial cells and connective tissue to maintain/regain mucosal architecture[3]. It involves complex host of mechanisms which work in tandem to protect gastric mucosa from damage as well as trigger the mechanism to repair mucosal defects by proliferating and migrating epithelial cells and connective tissue resulting in reconstruction of mucosal architecture[4]. Numerous studies have demonstrated the importance of gastric mucosal haemo-dynamics as a defensive factor of the gastric mucosa against injury[5-7]. Treatment with some prostaglandin derivatives such as prostaglandin E2 have been shown to increase mucosal blood flow and protect gastric mucosa against indomethacin and ethanol induced gastropathy by improving mucosal haemodynamics[8,9]. Oxygen delivery to the gastric mucosa is not only a function of blood flow but also depends on oxygen content in the arterial inflow.

A possible strategy would be to supplement oxygen using hyperbaric oxygen (HBO) to overcome ischemic injury[10]. The use of HBO in the treatment of peptic ulcer raised the efficacy of the multimodality therapy and accelerated the epithelialization of erosive and ulcerative defects[11,12]. An alternative method to improve oxygenation in critically ischemic tissue has emerged, which consists of using oxygen carriers, such as hemoglobin (Hb) or Hb based products[13]. These biomaterials have been initially developed to avoid the drawbacks of blood transfusions including immunologic reactions, blood-borne transmitted infections, limited availability and restricted storage time[14].

Hb is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates. In blood Hb carries oxygen from the respiratory organs to the rest of the body where it releases the oxygen to burn nutrients to provide energy to power the functions of the organism, and collects the resultant carbon dioxide to bring it back to the respiratory organs to be dispensed from the organism. In mammals, Hb makes up about 97% of the red blood cells dry content, and around 35% of the total content including water[15]. Hemoglobin has an oxygen binding capacity of 1.34 mL per gram of hemoglobin[16], which increases the total blood oxygen capacity seventy-fold compared to dissolved oxygen in blood. A single mammalian hemoglobin molecule can bind (carry) up to four oxygen molecules[17]. Based on these properties cell free hemoglobin (CF-Hb) products have been successfully used in a variety of ischemic/hypoxic conditions[18,19].

Bovine hemoglobin (B-Hb) has been used for a wide range of applications, including their use to enhance oxygen delivery to tissues during conditions of ischemia or hypoxia[20,21]. Strat et al[3] reported that B-Hb is much more potent than autologous red blood cells in restoring tissue oxygenation in ischemic conditions. In view of the superiority of CF-Hb compared to erythrocyte and whole blood we studied the efficiency of B-Hb against experimentally induced gastric mucosal injury.

MATERIALS AND METHODS

Adult Albino Wistar rats of either sex, weighing 150-200 g and fed on standard chow diet were used. They were divided into groups of 6 animals each. The distribution of animals into groups and the treatment allotted to each group were randomized. All the experiments were started between 8:00 and 10:00 in the morning. The protocol of the study was approved by the Institutional Research and Ethical Committee.

The aqueous solution of ulcerogens and B-Hb were freshly prepared before administration. The concentra-
Indomethacin was suspended in 1% carboxymethylcellulose in water and administered by gavage at the dose of 30 mg/kg body weight. B-Hb in the doses of 100, 300 and 900 mg/kg body weight was administered by gavage 1 h before indomethacin. The animals were sacrificed 6 h using ether after indomethacin administration. The stomachs were removed and opened along the greater curvature. After washing with saline the gastric lesions were quantified by a person unaware of the treatment protocol. The ulcer were scored according to the method of Valcavi et al. The circular ulcer induced by indomethacin were assessed on the basis of their diameter: deep circular ulcers more than 8 mm diameter = 10; 7-8 mm = 8; 6-7 mm = 7; 5-6 mm = 6; 4-5 mm = 5; 3-4 mm = 4; 2-3 mm = 3; 1-2 mm = 2; and < 1 mm = 1. Linear ulcers 10 mm or more in length were scored 6, and linear ulcers less than 10 mm in length were scored 3. The scores of each single lesion were then summed up for determination of the ulcer index.

Gastric lesions induced by ethanol (cytoprotection studies): The animals were administered (i.g) with 1 mL of absolute ethanol. B-Hb in the doses of 100, 300 and 900 mg/kg body weight was given (i.g) 30 min before the administration of ethanol. One hour after the administration of ethanol the animals were sacrificed and examined for the lesions in stomachs. The patchel lesions of stomach induced by ethanol were scored according to the method described by Schiantarelli et al using the following scale: 0 = normal mucosa; 1 = hyperemic mucosa or up to 3 small patches; 2 = from 4 to 10 small patches; 3 = more than 10 small or up to 3 medium-sized patches; 4 = from 4 to 6 medium-sized patches; 5 = more than 6 medium-sized or up to 3 large patches; 6 = from 4 to 6 large patches; 7 = from 7 to 10 large patches; 8 = more than 10 large patches or extensive necrotic zones. “small” was defined as up to 2 mm across (max diameter), “medium-sized” as between 2 and 4 mm across and “large” as more than 4 mm across.

Two separate batches of ethanol treated rats were used for biochemical and histological studies. The assay of gastric wall mucus, non-protein sulphydryl group (NP-SH), and myeloperoxidase (MPO) in the rats 1 h after ethanol exposure has been described below:

**Determination of gastric wall mucus:** Gastric wall mucus was determined according to the modified procedure of Corne et al. The glandular segment of the stomach was separated from the lumen of the stomach, weighed, and transferred immediately to 10 mL of 0.1% w/v Alcian blue solution (in 0.16 mol/L sucrose solution buffered with 0.05 mL sodium acetate at pH 5). Tissue was stained for two hours in Alcian blue, excess dye was removed by two successive rinses with 10 mL of 0.25 mol/L sucrose, first for 15 min and then for 45 min. Dye complexed with the gastric wall mucus was extracted with 10 mL of 0.5 mol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract was then vigorously shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 3600 g for 10 min and the absorbance of aqueous layer was recorded at 580 nm. The quantity of Alcian blue extracted per gram of wet glandular tissue was then calculated.

**Estimation of NP-SH:** Gastric mucosal NP-SH was measured according to the method of Sedlak and Lindsay. The glandular part of stomach was homogenized in ice-cold 0.02 mmol/L ethylenediaminetetraacetic acid. Aliquots of 5 mL of the homogenates were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloracetic acid. The tubes were shaken intermittently for 10-15 min and centrifuged at 3000 g. Two milliliters of supernatant were mixed with 4 mL of 0.4 mol/L Tris buffer at pH 8.9; 0.1 mL of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The absorbance was read within 5 min of addition of DTNB at 412 nm against a reagent blank with no homogenates.

**Determination of MPO:** MPO activity in the gastric mucosa was measured according to the methods described earlier. Preweighed tissue was homogenized (1:10 wt/vol) in 0.5% hexadeoxytrimethyl ammonium bromide in 50 mmol potassium phosphate buffer (pH 6.0) before sonication in an ice bath for 20 s. Three freeze/thaw cycles were performed followed by sonication (20 s in ice bath). The samples were centrifuged at 17000 g (5 min, 4 °C) and MPO in the supernatant was assayed by mixing of 0.1 mL of supernatant with 2.9 mL of 50 mmol potassium phosphate buffer (pH 6.0) containing 0.167 g/L O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured for 4 min using ultraviolet (UV)-visible spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan).

**Histology of ethanol-induced gastric lesions:** The stomach was opened along the greater curvature, washed with saline and fixed in 10% neutral buffered formalin for 24 h. The specimens were then processed overnight.
for dehydration and clearing steps, using an automatic tissue processor (Shandon Processor MKII; Runcorn, Cheshire, United Kingdom). The specimens were embedded in paraffin blocks and sections of 5 μm thickness were stained with hematoxylin-eosin for light microscopy observations.

**Statistical analysis**
Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Differences with $P < 0.05$ were considered as statistically significant.

**RESULTS**

**Effect of B-Hb on gastric secretion in 6 h pylorus-ligated rats**
In control rats pylorus ligation for 6 h resulted in the accumulation of 8.1 ± 0.61 mL of gastric secretion and total acid output of 498.7 ± 31.8 mEq (Table 1). The treatment of the rats with 100, 300 and 900 mg/kg of B-Hb produced a significant decrease in the volume of gastric secretion 5.6 ± 0.63, 5.5 ± 0.75 and 4.7 ± 0.58 mL respectively as compared to the control group. Treatment with B-Hb also dose dependently reduced total acid output in the rats treated with 100 mg/kg (471.1 ± 75 mEq) and 300 mg/kg (438.4 ± 65.6 mEq). However a significant reduction in total acid output was observed in a dose of 900 mg/kg (287.6 ± 24.6 mEq) of B-Hb.

**Effect of B-Hb on indomethacin-induced gastric mucosal damage**
The administration of indomethacin resulted in production of gastric lesions mainly in the glandular stomach of all the animals. The lesion area in the control group was found to be 22.4 ± 3.2 mm$^2$. Pretreatment of rats with B-Hb at doses of 100 mg/kg (24.3 ± 3.29 mm$^2$), 300 mg/kg (16.2 ± 1.45 mm$^2$) and 900 mg/kg (12.6 ± 1.85 mm$^2$) produced a dose dependent decrease of the lesion area (ANOVA $F = 4.50$, $P < 0.05$, Figure 1A).

**Effect of B-Hb on ethanol-induced gastric lesions**
The treatment of rats with absolute ethanol produced extensive gastric lesion in the glandular mucosa of the stomach. These lesions were characterized by multiple hemorrhagic red bands (patches) of different sizes along the axis of the glandular stomach. The ulcer index in the control group 1 h after ethanol administration was 7.1 ± 0.31. Pretreatment of rats with B-Hb at the doses of 100 mg/kg (ulcer index = 2.5 ± 0.42), 300 mg/kg (2.1 ± 0.4) and 900 mg/kg (0.7 ± 0.21) significantly inhibited the formation of gastric lesions (ANOVA $F = 63.26$, $P < 0.0001$, Figure 1B). Histological examination of gastric mucosa showed the appearance of these lesions in the form of gastric pits with detachment of the surface epithelium; vacuolation of epithelial cells and elongation of microvessels. Pretreatment with B-Hb dose-dependently prevented ethanol-induced mucosal damage (Figure 1).

**Effect B-Hb on ethanol-induced changes in gastric wall mucus**
The treatment of rats with ethanol significantly decreased the Alcian blue binding capacity of gastric wall mucus (480 ± 25.6 μg Alcian blue/g of tissue) as compared to control rats (667 ± 25.8 μg). Pretreatment of rats with B-Hb at the doses of 100 mg/kg (516 ± 31.6 μg/g), 300 mg/kg (558 ± 28.8 μg/g) and 900 mg/kg (654 ± 33.8 μg/g) significantly enhanced the binding capacity of Alcian blue to gastric mucosa (ANOVA $F = 8.05$, $P < 0.005$, Table 2).

**Effect of B-Hb on ethanol-induced depletion of gastric mucosal NP-SH**
The level of NP-SH in the gastric mucosa of control rats
was 4.59 ± 0.29 μmol/g of tissue, which was significantly decreased to 1.60 ± 0.12 μmol/g of tissue following the administration of ethanol. A significant and dose dependent reversal of NP-SH was observed following administration of B-Hb in low (3.11 ± 0.35 mmol/g of tissue), medium (3.73 ± 0.42 mmol/g of tissue) and high dose (5.36 ± 0.34 mmol/g of tissue) (ANOVA F = 19.62, P < 0.001, Table 2).

Effect of B-Hb on ethanol-induced changes in gastric MPO activity

The MPO activity in the normal gastric mucosa was 15.96 ± 1.9 mmol/g of wet tissue which increased significantly to 24.54 ± 1.9 mmol/g following ethanol administration. The MPO activity was slightly reduced to 24.30 ± 4.2 mmol/g in the rats treated with low dose of B-Hb prior to ethanol administration. However medium and high dose of B-Hb significantly reversed ethanol induced increase in MPO the value of MPO in these two groups being 14.25 ± 2.6 mmol/g and 17.21 ± 0.98 mmol/g respectively (ANOVA F = 5.21, P < 0.05, Table 2).

DISCUSSION

The result of this study showed a dose dependent reduction in the volume and acidity of gastric secretions following intra-gastric administration of B-Hb (Table 1). Our findings are supported by several earlier investigators who showed that gastric hemorrhage or intra-gastric administration of blood or blood products exerts inhibitory effect on gastric acid secretions[18,19,47-49]. As early as 1953, Chandler et al[53] reported that gastro-duodenal hemorrhage leads to a temporary absence of hydrochloric acid (achlorhydria) causing a significant decrease in gastric acid secretions. Fullarton et al[54] also showed that gastro-duodenal blood infusion significantly inhibits pentagastrin stimulated gastric acid and pepsin secretion. Paradoxically, intra-gastric blood/blood products including hemoglobin which constitute a protein meal instead of stimulating gastric secretions result in gastric antisecretory activity[55,56]. Gastric acid secretion is an elaborate and dynamic process that is regulated by neural (afferent and afferent), hormonal (e.g., gastrin), and paracrine (e.g., histamine, ghrelin, somatostatin) pathways as well as mechanical (e.g., distention) and chemical (e.g., amino acids) stimuli[31]. It has been suggested that intra-gastric blood induced increase in plasma gastric inhibitory polypeptide, secretin or glucogen may modulate gastric acid secretion in animals[32,33,37,38]. These mediators inhibit gastric acid secretions through somatostatin release[99-101]. However this hypothesis has been challenged by some other investigators[102].

Pretreatment of rats with B-Hb dose dependently ameliorated indomethacin-induced gastric mucosal damage (Figure 1). Protective effect of cell free hemoglobin has been reported against a variety of experimentally induced disease models including cardiac ischemic reperfusion injury[18,43,44], pancreatitis[45], renal injury[46] and neuronal injury[18,19,47-49]. The mechanism by which B-Hb exerts its protective effect against indomethacin induced gastropathy is far from clear. Indomethacin causes gastric mucosal damage via several mechanisms, including the impairment of the barrier properties of the mucosa, suppression of gastric prostaglandin synthesis, reduction of gastric mucosal blood flow and interference with the tissue repair mechanism[100]. The ability of non-steroidal anti-inflammatory drugs (NSAIDs) to reduce gastric mucosal blood flow has been recognized for several decades[9]. Prostaglandins (PGs) of the E and I series are potent vasodilators that are continuously produced by the vascular endothelium, inhibition of PG synthesis by NSAIDs lead to a reduced vascular tone and hypoxic injury[51]. Cell free hemoglobin is able to readily diffuse in the microcirculation and transport O2 to hypoxic tissue because of its high O2 affinity, low viscosity and small mean diameter as compared to red blood cells[52]. Once in microcirculation Hb offloads oxygen to the ischemic tissue, which is unlike the vasoconstrictive effect of Hb on large vessels[53,54]. Hence the mitigation of pathogen induced ischemic injury by CF-Hb may be attributed to its ability to transient oxygen delivery[13,55,57] and preservation of energy metabolism[58]. Moreover, the presence of acid in the lumen of the stomach also contributes to the pathogenesis of indomethacin -induced ulcers and bleeding, by impairing the restitution process, interfering with hemostasis and inactivating several growth factors that are important in mucosal defense and repair[13]. Our experiments using Shay rats model clearly showed an inhibition of gastric acid secretion following B-Hb administration (Table 1). Thus the reduction of acid content in stomach by B-Hb may to some extent contribute to its gastroprotective effect against indomethacin induced ulcers.

The oral administration of absolute ethanol produced significant ulcers in glandular part of gastric mucosa of rats (Figure 2). Our histopathological studies of gastric

Table 2 Effect of bovine hemoglobin on ethanol induced changes in Alcian blue binding capacity, non-protein sulphydryl groups levels and myeloperoxidase in gastric mucosa of rats

| Treatment          | Dose of B-Hb (mg/kg) | Alcian blue binding (mg/g tissue) | Non-protein sulphydryl (mmol/g tissue) | Myeloperoxidase activity (DA/g tissue) |
|--------------------|----------------------|----------------------------------|----------------------------------------|----------------------------------------|
| Control            | 0                    | 667 ± 25.8                       | 516 ± 31.6                             | 15.94 ± 1.6                            |
| EtOH alone         | 1 mL/animal          | 480 ± 25.6                       | 100                                     | 4.59 ± 0.29                            |
| EtOH ± B-Hb        | 100                  | 516 ± 31.6                       | 3.11 ± 0.35                             | 24.30 ± 4.2                            |
| EtOH ± B-Hb        | 300                  | 558 ± 28.8                       | 3.73 ± 0.42                             | 14.25 ± 2.6                            |
| EtOH ± B-Hb        | 900                  | 654 ± 33.8                       | 5.36 ± 0.34                             | 17.21 ± 0.98                           |

Values are mean ± SE. *P < 0.01 vs control; †P < 0.05, ‡P < 0.01 vs EtOH group using Dunnett’s multiple comparison test. B-Hb: Bovine hemoglobin.
mucosa showed a significant loss of glandular cells, disruption of epithelium, sub mucosal edema and infiltration of neutrophils following ethanol administration (Figure 2). A significant decrease in Alcian blue binding capacity of gastric mucosa following exposure to ethanol (Table 2) clearly suggests depletion of mucosal gel lining adhering to the gastric surface which is considered the first line of defense in stomach against endogenous and exogenous ulcerogens. Numerous mechanisms have been proposed to explain necrotizing agent induced gastropathy. Besides the direct deleterious effect of ethanol on gastric tissues, disruption of mucosal barrier also results from mucosal capillary necrosis, vascular congestion and thrombosis in the sub-epithelial microvasculature. Oral administration of ethanol results in low or no blood flow to the stomach leading to transient hypoxic mucosal damage. It is well accepted that gastric mucosal protective mechanism largely depend on appropriate microcirculation which help to orchestrate the defense mechanism at various levels of gastric mucosa. Pre-treatment of animal with B-Hb dose dependently attenuated ethanol induced gastric ulcer with almost complete protection in animal with B-Hb dose dependently attenuated ethanol induced lesion; C: Pretreatment of rats with bovine hemoglobin (B-Hb) 100 mg/kg; D: Pretreatment of rats with B-Hb 900 mg/kg.

ACKNOWLEDGMENTS
The authors are thankful to the Prince Sultan Military Medical City and the Medical Services Department of Ministry of Defense for their encouragement and support.

COMMENTS
The recent years, the treatment strategies for gastric ulcer diseases have sig-
significant changes, mirroring the revolution in the understanding of its pathogenesis. Improved oxygenation in critically ischemic gastric mucosa has emerged as a method of choice to accelerate epithelialization of erosive and ulcerative defects. Bovine hemoglobin (B-Hb) has been used in a wide range of applications including restoration of tissue oxygenation in ischemic condition.

Research frontiers
Strategies to improve oxygen delivery to ischemic tissues could potentially protect gastric mucosa and other tissues from hypoxic injury. Cell free hemoglobin is able to readily diffuse in the microcirculation and transport O₂ to hypoxic tissue because of its high O₂ affinity, low viscosity and small diameter.

Innovations and breakthroughs
Recent studies showed protective effect of cell free hemoglobin against disease models of ischemic reperfusion injury including neuronal injury, nephropathy, pancreatitis and myocardial infarction. This study first time demonstrated anti-secretory and gastric antiulcer activity of B-Hb. The gastroprotective effect could be attributed to the ability of B-Hb to improve oxygenation, reduction of oxidative stress and lowering of neutrophil activity.

Applications
The result of this study suggests that bovine hemoglobin besides having significant anti- gastric acid secretory activity protects rats against ethanol and indomethaine induced gastric ulcers. B-Hb being animal protein can be safely explored clinically for the oral treatment of hyperacidity and gastric ulcer diseases.

Terminology
Hb is the iron-containing oxygen-transport metalloprotein in red blood cells of all vertebrates. Hemoglobin is autonomous; it binds oxygen and release it without the need for any cofactor. Cell free Hb has been used for wide range of applications including enhanced oxygen delivery to ischemic tissue. Bovine hemoglobin is superior to autologous red blood cells in restoring tissue oxygen in ischemic conditions.

Peer review
This is the first study showing anti-secretory and gastric anti-ulcer activity of bovine hemoglobin. Reduction of oxidative stress and lowering of neutrophil activity might contribute to B-Hb induced gastroprotective effects. The results are valuable to explore pharmacological potential of B-Hb in a variety of ischemic/hypoxic conditions.

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