The Possible Protective Effect of Platelet Rich Plasma on Aspirin Induced Gastric Ulcer in Adult Male Albino Rat

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ABSTRACT

Background: High morbidity and mortality due to gastric ulcer made its management a medical challenge. Platelet rich plasma (PRP) had been used for treatment of skin ulcers, some hair and bone problems as PRP contains many growth factors which make it suitable for management of gastric ulcer. Objectives: The current work was aimed to study the protective role of PRP on gastric ulcer induced by aspirin. Materials and Methods: Forty adult male albino rats were divided into four groups. Group I; sham control, group II received PRP, group III received aspirin for induction of gastric ulcer and group IV was provided with PRP then received aspirin. All rats were sacrificed then the fundic mucosa was processed for light and scanning electron microscopic study. Paraffin sections were stained by hematoxylin and eosin, mallory trichrome and combined periodic acid Schiff (PAS) and alcin blue (AB) stains. Immunohistochemical staining for iNOS and EGFR were also performed. Results: Aspirin had induced mucosal destruction in the form of ulceration, epithelial surface desquamation, hemorrhage, vacuolation and inflammatory cellular infiltration. Scanning electron microscopic examination of aspirin group showed decreased mucus, widening of gastric pits and cavitation of mucosal folds. PRP produced a great preservation of gastric structure in protected group (IV). Conclusion: It could be concluded that aspirin caused ulcerating changes in gastric mucosa and PRP had ameliorating effects on these changes.

Keywords: Aspirin, Gastric ulcer, PRP, Scanning electron microscopy.

INTRODUCTION

Gastric ulcer has the most prevalence of gastrointestinal disorder ever known. Gastric ulcer is a major health hazard in terms of mortality and morbidity (1). Jafer et al. (2) mentioned that complicated gastric ulcer may induce upper gastrointestinal bleeding. Around 15 mortality from gastric ulcer has been reported out of the world's 15,000 complications annually.

Gastric ulcer is caused by imbalance between defensive and destructive factors of the mucosa. Destructive factors against gastric mucosa include pepsin, hydrochloric acid, Helicobacter Pylori and consumption of non-steroidal anti-inflammatory drugs (NSAIDs). The local defensive factors include mucus secretion, bicarbonate, maintenance of normal blood flow, cellular regeneration which is maintained by growth factors especially prostaglandins (PG) and epidermal growth factor (2,3). Although various methods which suppress one or more processes of gastric ulcer pathogenesis are under evaluation, a perfect curative method for this disease isn’t established (2).

Platelet-rich plasma (PRP) has a great concern to doctors who are involved in wound healing and regenerative medicine. Growth factors of PRP are available, cost-effective and stable (4). The aim of the current work was to study the protective role of PRP on aspirin induced gastric ulcer in adult male rats.

MATERIALS AND METHODS

Forty male albino rats of average body weight 200-250 g were used in this study. They were housed at room temperature and had free access of diet and tap water. Rats were fasted 8 hours before ulcer induction.

Ethical approval: Strict care and hygiene were provided to keep them in normal and healthy conditions under the guidelines recommended by ethical committee for animal research, Faculty of medicine Menoufia University.

Experimental substances:

A. Acetyl salicylic acid powder: was provided from Sigma-Aldrich chemical Co., St. Louis. Mo. (USA).

B. Inducible Nitric Oxide synthase (iNOS) (5), a rabbit polyclonal antibody (Medico trade company, Giza, Egypt) 1:100.

C. Epidermal growth factor receptor (EGFR) antibody: a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA).

Preparation of platelet rich plasma:

Selected ten rats (170–200 g) were fed on normal chaw and tap water. They were held for one week before the start of the experiments for 12 hours of light/12hours of dark conditions. The research procedure followed the Faculty of Medicine, Menoufia University, Egypt's recommendations for animal care.

We anesthetized the rats with ether. Under aseptic technique, 2 ml of blood was collected from the retro-orbital plexus via capillary tube that was initially dipped in 3.2% sodium citrate, then the blood was collected into tubes containing 0.5 mL of acid citrate dextrose. The blood was subjected to double centrifugation process, firstly the tubes were centrifuged at 1600 revolutions per minute (rpm) for ten minutes.

Three different density layers were formed: the inferior one layer containing red blood cells, the middle containing buffy coat of white blood cells, and the superior one containing plasma. We pipetted the plasma and the portion just above buffy coat without disturbance of the buffy coat. In the second centrifugation, the plasma was centrifuged again for ten minutes but at 2000 rpm. This resulted in 2 compartments: the superior...
compartment consisted of platelet-poor plasma (PPP) and the inferior one consisted of the platelet button. The lower Part of the PPP was remained in the tube along with platelet button which then gently agitated to promote platelets suspension, but the upper part of PPP had been removed. Production of PRP was done by this procedure. For conformation, the PRP sample was counted in an automatic apparatus to verify that the platelet count was greater than 1,000,000/μL. This procedure was conducted at Theodor Bilharz Research Institute, Cairo, Egypt.

Experimental design:
- The rats were divided into four groups, ten rats each.
  - **Group I (control group):** They were subdivided into 2 equal subgroups: Ia (Negative control): included five rats. They were kept without treatment and served as control for all the experimental groups and Ib (Sham control): included five rats. Each received 0.6 ml of 1% carboxymethylcellulose (the solvent used to dissolve acetyl salicylic acid) orally by gastric tube.
  - **Group II (PRP treated group):** They were received 0.5ml/kg body weight of PRP two days per week for three weeks subcutaneously.
  - **Group III (Aspirin induced gastric ulcer group):** Each rat received a single dose of aspirin (300 mg/kg body weight) dissolved in 0.6 ml of 1% carboxymethylcellulose orally by gastric tube.
  - **Group IV (Aspirin induced gastric ulcer pretreated with PRP group):** Each rat received a dose of 0.5ml/kg body weight of PRP, two days per week for 3 weeks. One hour after administration of the last dose of PRP, a single dose of aspirin (300 mg/kg body weight) was administrated orally by gastric tube. Five hours later rats were sacrificed.

At the end of experiment the rats were starved for 12 h. All animals then were killed by cervical dislocation, and abdomen was opened. We dissected out the stomach, opened it along the greater curvature and washed it gently with saline to release any debris. We divided each specimen into two parts: one for light microscopic study, so we fixed it in 10% neutral buffered formalin and the other part was prepared for electron microscopic study by fixing it in 2.5% glutaraldehyde in phosphate buffer. The collected data were presented as mean ± SD. We performed data analysis by using SPSS (Inc., Chicago, IL, USA) version 23 on IBM compatible computer. The obtained data were analyzed by using one-way-ANOVA then post hoc Bonferroni test. Statistically, the results were considered significant and nonsignificant when the p-values were <0.05 and >0.05, respectively.

**RESULTS**

All outcomes of this study showed non-significant differences between subgroup I (a) (rats kept without medication) and subgroup I (b) (rats given carboxymethylcellulose). Therefore, these two subgroups were pooled in one control group.

**Gross morphology:** studying the stomach grossly showed normal bright mucosa with rugae in control and PRP groups. Group III showed multiple hemorrhagic areas and ulceration but we noticed only mucosal congestion as a redness of mucosa in group IV Fig. 1.
Histological results:
A) Hematoxylin and eosin staining:

The fundus of control and PRP treated rats was characterized by numerous fundic glands occupying all the thickness of the mucosa. These glands opened in the bottom of gastric pits. They were long straight tubular glands parallel to each other and perpendicular to the surface. They were differentiated into 3 parts; from inner to outer isthmus, neck and base. Lamina propria and submucosa were composed of loose connective tissue containing blood vessels. The isthmus was occupied by surface columnar cells which are tall columnar cells with basal rod shaped nuclei. The neck was lined by mucous neck cells which were cubical with basal flattened nuclei. The body of the glands was lined by parietal cells which were large polyhydral cells with central rounded vesicular nuclei and acidophilic cytoplasm. The base contained both Parital and chief cells with basal rounded nuclei and deeply basophilic cytoplasm (Fig.2A-C).

Group III (Aspirin- induced ulcer group) showed ulceration of mucosa with discontinuity and exfoliation of the surface epithelial cells. Some areas showed vacuolation and cystic dilatation. Parietal cells had vacuolated cytoplasm with deeply stained pyknotic nuclei and hemorrhage appeared between the cells (Fig.3A-C).

Group IV (protected group) the mucosa was nearly similar to that of control group. However, some areas of cystic dilatation, hemorrhage and parietal cell vacuolation were occasionally observed (Fig.4A-C).

Fig. (1): Gross morphology
A photograph of rat stomach of all experimental groups, (A): showing normal bright rugae (red arrow head) of gastric mucosa of group I and II (B): group III showing multiple hemorrhagic areas (yellow arrows) and ulceration (green arrow) (c): group IV showed only mucosal congestion (redness of mucosa).

Fig. (2): A photomicrograph of rat stomach of control rats (A): showing mucosa (M) with long, straight, packed gastric glands differentiated into isthmus (I), neck (N) and base (B). Gastric glands (G) appear circular in cross section. Lamina propria contained normal lymphocytic infiltration (yellow arrow), gastric pits (brown arrows), muscularis mucosa (MM) and submucosa (SM) were also showed (H&E X100) (B);Higher magnification at upper part showed normal cells lining gastric glands; surface columnar cells (blue arrow), mucous neck cells (black arrows) and parietal cells (red arrows) (H&E X400) (c): Higher magnification at lower part showed normal parietal cells (red arrow) and normal chief cells (green arrow head) (H&E X400)
Fig. (3): A photograph micrograph of group III (A): showing ulcer (U) exfoliation (green arrow), vacuolation (V) and massive hemorrhage (H) (H&E X100) (B): showing destruction of superficial layer and its exfoliation (green arrow), minimal ulceration (red arrow), massive lymphocytic infiltration (yellow arrow), vacuolation (V) and minimal hemorrhage (blue arrow) (H&E X100) (C): higher magnification at lower part showed ulcer (U) and hemorrhage (blue arrow) parietal cells with vacuolated cytoplasm (green arrow), others with deeply stained nuclei (red arrows) and destructed chief cells (black arrow) were also appeared (H&E X400).

Fig. (4): A photograph micrograph of rat stomach of group IV (A): showing nearly normal architecture of fundic glandular tissue except for some small hemorrhagic areas (blue arrows) (H&E X100) (B): higher magnification of upper part showing nearly normal cells lining gastric glands; surface columnar cells (red arrow), mucous neck cells (black arrow) and parietal cells (blue arrows) (H&E X400) (C): higher magnification of lower part showed nearly normal parietal cells (red arrows) and chief cells (blue arrows) but there was some cystic dilation of glands (black arrows) (H&E X400).

B) Combined PAS & Alcian blue staining:
In control group the mucosal surface was covered by a thin film of PAS positive mucous coat which extended to fill gastric pits. The mucous neck cells were found to contain AB (alcian blue) positive mucous (Fig. 5A). PRP administration in group II resulted in appearance of a thick film of PAS stained mucous on the surface with (P<0.001) when compared to control rats (Fig. 5B).

Group III showed that the surface epithelium lacked the PAS positive mucous coat but PAS positive reaction was still present in the pits of these glands. Mucous, neck cells contained AB positive mucous (Fig. 5C). The superficial cells in group IV as well as the basal cells of the glands showed PAS positive reaction. The mucous neck cells contained AB positive mucous (Fig. 5D).
Fig. (5): PAS & alcian blue X 200

A photograph micrograph of rat stomach of all experimental groups, (A): control rats showed PAS positive mucous coat in the surface (black arrow) and on the bases of the glands (blue arrow) mucous neck cells contain alcian blue positive mucous (red arrow) (B): Thick layer of PAS positive mucous (black arrow) and bases of the glands (blue arrow) appeared in group II mucous neck cells contain also alcian blue stained mucous (red arrow) (C): group III showing ulcer (U), absent PAS positive on the surface (black arrow) but still present in the bases of the glands (blue arrow) alcian blue stained mucous neck cells still present (red arrow) (D): group IV showed PAS positive reaction as a surface mucous coat (black arrow) and at the bases of the glands (blue arrow) alcian blue stained mucous was minimal (red arrow) Graph (1) showing mean area of PAS positive reaction in different studied groups.

N.B

** Significant increase in PRP group mean area (p1 < 0.001) as compared to the control group.
** Significant decrease (p1 < 0.001) in mucous layer in aspirin group as compared to the control group.
♦♦ Significant increase (p 2 < 0.001) in protected group as compared to Aspirin group.

C) Mallory trichrome staining:

Few collagenous fibers were seen within the core of glands as well as within the submucosa. Some collagenous fibers extended in between the basal part of the glands in control and PRP treated rats (Fig. 6A). Collagen fiber deposition was significantly increased (P1 < 0.001) in group III around blood vessels (Fig. 6B).

Group IV (protected group), on the other hand, showed a significant decrease of collagen fiber deposition (P2 < 0.05) when compared to aspirin group (Fig. 6C).
Fig. (6): Mallory trichrome X 200
A photomicrograph of rat stomach of all experimental groups, (A): showing few amount of collagenous fibers in both submucosa (blue arrow) and around the bases of the gland (black arrow) of group I and II (B): group III showing increased amount of collagenous fibers around blood vessels in submucosa (blue arrow) and around the bases of the gland (black arrow) (c): group IV increased collagenous fibers in all submucosa (blue arrow), around the bases of the gland (black arrow) and in between gastric glands (red arrow) when compared to control group. Graph (2) showing mean area of collagen fiber deposition in different studied groups
** Significant increase (p1 <0.001) in collagen fibers in aspirin group as compared to the control group.
♦ Significant decrease (p2<0.05) in protected group as compared to aspirin group.

D) Immunohistochemical results:
Immunohistochemical analysis showed that iNOS immunoreactivity was significantly increased in Group III (P<0.001) when compared to Control group. This increase was significantly decreased by PRP administration (P<0.001) (Fig.7A-C). On the other hand Aspirin group showed weak positive EGF immunoreactivity that was insignificant as compared to control group. PRP administration resulted in upward regulation on EGF expression (P<0.001) (Fig.7D-F)

Fig. (7): A photomicrograph of immunostaining of fundic mucosa : iNOS immunoreaction was dramatically increased in Aspirin group(A,B) and this increase was significantly diminished by PRP administration(C).insignificant weak increase in EGFR immunoreactivity in aspirin group than control group(D,E),but PRP administration cause significant up regulation of EGFR (F) immunostaining X400 graphs (3,4) showing mean area of iNOS, EGFR immunostaining respectively.
E) Scanning Electron Microscope (SEM) study:

Control as well as PRP treated rats showed normal surface cells (forming mucosal folds) with gastric pits in between. Mucous appeared as sheets extending between gastric pits. Mucous also covered surface cells or pits so lost well demarcation between glands (Figs. 8A&B). Aspirin group showed gastric ulceration, surface cell cavitation and destruction of gastric pits (Figs. 8C&D). Honey comb appearance appeared due to mucosal folds cavitation. Gastric ulcer reached the muscularis mucosa (Figs. 9&10). Group IV (protected group) showed widening of gastric pits. There is normal mucosal folds but cavitation also appeared (Figs. 8 E&F).

![Fig. (8): Scanning electron photomicrograph of rat stomach of all experimental groups. control group showed normal mucosal folds (green arrow) and pits(red arrow) of gastric glands with mucous sheet( blue arrow)appeared. (A,B). Group III showed gastric ulceration (brown arrow), surface cell cavitation (yellow arrows) (C,D). Group IV showed some normal mucosal folds (green arrow) but cavitation also appeared (yellow arrow) minimal affection to surface contiuity between 2 brownish arrows .(E). At higher magnification of group IV there was widening of gastric pit (red arrows) but nearly normal gastric pit are also present (red arrow head). Few surface cell cavitation also appeared (yellow arrows)(F).](https://ejhm.journals.ekb.eg/)
Fig. (9): Scanning electron photomicrograph of the stomach of group III (aspirin induced gastric ulcer group) showing gastric ulcer (brown arrow) reaching muscularis mucosa. Surface cells cavitation appeared (yellow arrow) giving the appearance of honey comb. (mag X 100)

Fig. (10): Scanning electron photomicrograph of the stomach of group III (aspirin induced ulcer group) showed gastric ulcer (brown arrow). Surface cells cavitation appear (yellow arrow) giving the appearance of honey comb(yellow arrow heads). (mag X 100)
Morphometric study and statistical analysis

Area percentage of periodic acid schiff stained sections

The periodic acid schiff (PAS) area percentage is maximum in group II with a mean value of 43.60±2.87518 and minimum in group III (with a mean value of 15.70±3.71334 (P1<0.001) as compared to control rat in which PAS area percentage was 31.20±1.9321 and increased in group IV with a mean value of 25.60±1.42984 (P2<0.001) when compared to group III. Histogram (1) Fig.(5).

Area percentage of collagen fibers in Mallory trichrome stained sections in submucosa, and around the bases of the glands

Significant increase in percentage of collagen fibers surface area was observed in group III (P1<0.001) with a mean value of 58.40±3.62706 compared with group I with a mean value of 25.70±1.63639.

Significant difference in percentage of collagenous fibers surface area in group III (P2<0.05) with a mean value of 58.40±3.62706 compared with group IV with a mean value of 52.20±2.29976 Histogram (2) Fig.(6)

Area percentage of iNOS immune stained section in mucosa

The maximum percentage of iNOS immunoreactivity was in group III with a mean value of 38.00±1.82574, when compared with group I with a mean value of 9.40±2.01108 (P1<0.001). The increase in iNOS immunoreactivity was protected in group IV (P2<0.001) with a mean value of 17.70±1.88856 as compared with group III. Histogram (3) Fig(7)

Area percentage of EGFR immune stained section in mucosa

Significant increase in percentage of EGF immunoreactivity surface area in group III (P1<0.001) with a mean value of 19.80±1.93 as compared with group I with a mean value of 9.50±1.58114.

Significant increase in percentage of EGF immunoreactivity surface area in group IV (P2<0.001) with a mean value of 29.40±2.06559 as compared with group III with a mean value of 19.80±1.93218. Histogram (4) Fig (7)

DISCUSSION

Aspirin is a potent anti-inflammatory drug that is commonly used in prevention of cardiovascular thrombotic diseases and treatment of rheumatoid arthritis and its related diseases (13). Aspirin administration resulted in gastric ulceration by increasing antiangiogenic factors like endostatin and decrease proangiogenic growth factors (14).

Platelets contain many growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor (TGF), vascular endothelial growth factor(VEGF) and platelet-derived growth factor (PDGF) (4,15). These growth factors cause proliferation and differentiation of cells (16). PRP was used in treatment of skin ulcer, hair problems ,orthopedic injuries and diabetes due to anti-inflammatory and antiangiogenic properties (6).others test the role of PRP in treatment of gastric ulcer (48, 17) so, we decided to detect its possible protective effect on gastric ulcer.

In this work, Aspirin induced ulcer group revealed ulceration, desquamation, and exfoliation of surface cells (3,7).

Surface epithelial cells are interconnected with tight junctions that form a "barrier" preventing back diffusion of pepsin and acid. These cells secrete mucous, prostaglandins, and bicarbonate. The presence of phospholipids in surface cells made them hydrophobic, preventing acid and water soluble damaging agents from their invasion. Epithelial loss means loss of an important line of defense mechanism (18).

Loss of mucosal barrier explained cellular infiltration where the mucosa was exposed to acidity and enzymes with subsequent bacterial invasion. This bacterial invasion by its role released chemotactic factors that attracted neutrophils ,macrophages and inflammatory cells to the site of the lesion (19).

Parietal cells appeared vacuolated as previously observed by Sistani et al. (20). They explained that the release of lysosomal enzymes that accumulate in parietal cells may result in these vacuoles Cellular vaculation may occur due to disturbed cellular membrane permeability, caused by oxygen-free radicals that enhance transport of electrolytes and water into the cells causing cells swelling and destruction of organelles causing cytoplasmic vaculation (21).

Also, scanning electron microscopy of group III showed ulceration ,loss of mucous sheets and cavitation of cells as appeared with Zaki and Mohamed (22).

In this study, PAS & alcian blue (AB) stained sections of control rats demonstrated presence of PAS positive reaction (pink) on surface cells of the mucosal glands and AB positive reaction (blue) in the mucous neck cells as surface cells secrete neutral mucin that stained magenta by PAS stain but mucous neck cells contain acidic mucin that stained blue by AB .This was in agreement with El Husseiny et al. (3).

Although our results did not show any histological changes between control and PRP groups, there was significant increase in mucus secretion in PRP group detected in PAS- AB. This may be due to the ability of PRP to induce proliferation of cells by its growth factors (14).

Specimens from the ulcer group, on the other hand, showed depletion of PAS positive cells with...
absent reaction on mucosal surface cells with increased cells stained with AB in the neck of the fundic glands. Depletion of surface mucous may be due to surface cell lysis or degeneration either insitu or after extrusion. It may be due to failure of gastric adaption (23). This was in accordance with Abdelatif et al. (8).

The ulcer group showed significant upregulation of iNOS expression of gastric mucosa. This goes in line with the findings of Wang et al. (24) who determined that this increase in iNOS was due to increased oxidative stress in gastric tissue.

This study revealed that there is significant increase in collagenous fibers in ulcer group which may occur as a response to inflammatory response of tissue to aspirin. This was supported by Hafez and Ramadan (25) who mentioned that when aspirin administrated to pregnant rats as multiple dosing from gestational day (GD) 6 to GD 17 the rat stomach showed thickened submucosal blood vessels with excess collagen fiber deposition. This increase is protected in group IV as PRP increase protective factors causing less destruction and less fibrosis.

Treatment with PRP prior to ulcer induction resulted in great improvement in the architecture of gastric mucosa which became similar to control group with no areas of ulceration or desquamation except for a few areas with cystic dilatation of some fundic glands.

This improvement might be due to receiving PRP early before aspirin exposure that lead to creation of antioxidant enzymes, decrease lipid peroxidation and increase immunity response (26).

Group IV showed preservation of mucosal folds as PRP have many growth factors which promote regeneration of cells this is in agreement with Alves and Grimalt (27) who stated that, the bioactive molecules and the GFs present in PRP promote angiogenesis, migration proliferation and cell differentiation.

EGF plays significant role in proliferation, differentiation and repair of gastrointestinal tract mucosal cells of including stomach. Other potent biological effects on mucosa involve suppression of gastric acid secretion, protection against several ulcerogenic factors and stimulation of gastric mucous production (23). In this work we found up regulation of EGFR .This was in agreement with RagHAVENDRAN et al. (28) that concluded that EGFR activate re-epithelization with migration of epithelial cells to restore mucosal epithelial continuity.

Jeong et al. (29) used endoscopic application of PRP as an effective treatment of gastric ulcer resulted from endoscopic submucosal dissection. He showed decrease gastric ulcer size by measuring by endoscopic ruler. There weren’t adverse effects for application of PRP in gastrin ulcer.

Wallace et al. (17) determined that platelet modulate gastric ulcer healing through increase angiogenic factors like vascular endothelial growth factor(VEGF) and (EGF) and decrease antiangiogenic factors like endostatin(14,17). Although two studies Luzo et al. (26) and Ramos-Torrecillas et al. (30) were intravascular, Wallace et al. (17) study used oral administration of intact platelet that accelerate healing of gastric ulcer. Administration of growth factors or serum or platelet poor plasma or lysed platelet didn’t affect healing of gastric ulcer. We used heterogeneous application from rat to another rat. This was tested in treatment of diabetes mellitus (6) This will be available in case of thrombocytopenia.

Oral administration of growth factors didn't accelerate ulcer healing may be due to their destruction by HCL or ulcer may promote more release of growth factors. Growth factors released from platelet induce proliferation and migration of cells in site of ulcer promoting angiogenesis and ulcer healing (17).

These all studies use PRP for treatment of gastric ulcer, but our study determine that PRP may have available role for protection from gastric ulcer. We used subcutaneous injection as an available route for PRP administration. Also use of heterogeneous PRP is important in cases of thrombocytopenia as ulcer indeed stimulate platelet to release its anegogic factors (17), so in case of thrombocytopenia it is a good way for protection.

CONCLUSION
Aspirin caused ulcerating changes in gastric mucosa and PRP had ameliorating effects on these changes.

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