

# Comparison of three acute stress models for simulating the pathophysiology of stress-related mucosal disease

Bhagawati Saxena<sup>1</sup>, Sanjay Singh<sup>2,\*</sup>

<sup>1</sup>Department of Pharmacology, L. J. Institute of Pharmacy, Ahmedabad, Gujarat, India;

<sup>2</sup>Division of Pharmacology and Toxicology, Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi, U.P., India.

## Summary

Stress-related mucosal disease (SRMD) is highly prevalent in intensive care patients leading to increasing treatment cost and mortality. SRMD is a disease elusive of ideal treatment. Evaluation of drugs is very pertinent for the efficient and safe treatment of SRMD. It relies mainly on in vivo screening models. There are various stress models, and till date, none of them is validated for simulating the SRMD pathophysiology. The present study aims to choose the best model, which reproduce pathophysiology of SRMD, among previously established stress models. This study evaluates ulcer index, hexosamine content, microvascular permeability, and gastric content in three acute stress models (cold-restraint, restraint, and water immersion restraint). Macroscopic pictures of the ulcerogenic stomach explain that in contrast to other models, cold-restraint stress (CRS) exposure produced marked ulcers on the fundic area of the stomach. Results of the present study depicted that each stress model significantly increased ulcer index, microvascular permeability and decreased hexosamine level, however, the maximum in the case of CRS-exposed rats. Total acidity and pH of the gastric content remains unchanged in all the stress models. On the contrary, the gastric volume significantly decreased only in case of CRS, while unchanged in other stress models. The overall results revealed that the CRS resembles the pathophysiology of SRMD closely. It is the best and feasible model among all the models to evaluate drugs for the treatment of SRMD.

**Keywords:** Mucosal barrier, microvascular permeability, gastric mucosal blood flow, restraint stress, cold-restraint stress, water immersion restraint stress

## 1. Introduction

Stress-related mucosal disease (SRMD) commonly known as stress ulcer is described as continuum conditions ranging from superficial mucosal damage to deep focal mucosal damage (1). SRMD is observed in critically ill patients during a serious illness such as surgery, trauma, sepsis, severe burns, etc., within

twenty-four hours of their admittance to intensive care unit (ICU) (2,3). SRMD is a considerable reason of morbidity in addition to mortality in critically ill patients in ICU (4). Upper gastrointestinal (GI) bleeding observed in SRMD patients places critically ill patients at a high risk of death (5,6). SRMD in the ICU patients also adds to the cost of treatment by increasing the stay of patients at the hospital (2,7). Therefore, due to the above reasons, SRMD prophylaxis has become a regular practice in ICU (8). The primary goal of clinical therapy of SRMD is to prevent bleeding. Current preventive treatment strategies use histamine-2 receptor antagonists (H<sub>2</sub>RAs), proton pump inhibitors (PPIs) and sucralfate. H<sub>2</sub>RAs and PPIs suppress acid secretion. Sucralfate provides a protective barrier against the acid in the GI tract. These drugs have their limitations (9). H<sub>2</sub>RAs,

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\*Address correspondence to:

Dr. Sanjay Singh, Division of Pharmacology and Toxicology, Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi, U.P. 221005, India.  
E-mail: sssingh.phe@iitbhu.ac.in

PPIs, and sucralfate are also commonly prescribed for the treatment of the peptic ulcer.

Peptic ulcer and SRMD are dissimilar in several aspects. Ordinary peptic ulcers are found mainly in the gastric antrum and the duodenal bulb, while SRMD is found majorly in acid producing area of the stomach like fundic mucosa and corpus (1). Primary pathological factor for peptic ulcer is mostly considered to be the increased acid output, and thus gastric mucosa is exposed to a very low intraluminal pH. Hyperacidity is not a primary pathological factor of SRMD, as normal or slightly decreased gastric acid volume is seen in affected patients. Rather, ischemia is considered as a major pathological factor of SRMD (1,10,11). It is reported that even without intragastric hydrochloric acid, ischemia followed by reperfusion caused histologic mucosal injury in the stomach (12). These findings suggest that the pathology of SRMD is probably related to a reduction in gastric mucosal blood flow, which leads to ischemia. The breakdown of the mucosal defensive barrier by ischemia and reperfusion allows offensive factors (low acid secretion) to produce gastric ulceration.

Treatment of SRMD remains a major challenge to health professionals and represents a disease elusive of ideal treatment. Therefore, evaluation of drugs leading to the efficient and safe treatment of SRMD is very pertinent. The improvement of new therapeutics for the treatment of SRMD relies mainly on *in vivo* screening models. From the experimental point of view, the selection of a model for producing SRMD in the stomach is highly required. Pyloric ligation, ethanol, aspirin, indomethacin-induced ulcer models are among several models frequently used for screening antiulcer action of drugs. However, these models are based on the aggravation of offensive factor, while the weakening of defensive barrier is a primary pathological factor of SRMD. Additionally, these are local models, whereas, the model that needs to reproduce the SRMD pathology should be central. SRMD have been developing in critically ill patients secondary to physiological stress (8). Currently, various stress models are available like restraint stress (RS), cold-restraint stress (CRS), water immersion restraint stress (WRS), food deprivation stress, activity wheel stress, chronic unpredictable stress, chronic foot shock stress, *etc.* SRMD is an acute stress condition. WRS, RS, and CRS models are acute stress models while food deprivation stress (13), activity wheel stress (14), chronic unpredictable stress (15), and chronic footshock stress (16) models are chronic stress models for ulcer formation. Till now, none of the existing acute stress models are studied for simulating the SRMD pathophysiology. Therefore, this study aims to select a model that imitates the pathological condition of SRMD, among previously established three acute stress models (CRS, RS, and WRS).

## 2. Materials and Methods

### 2.1. Materials

Evans blue and D-glucosamine hydrochloride were acquired from Sigma-Aldrich (St. Louis, MD, USA). All the other solvents and chemicals were purchased from Loba Chemie (Mumbai, Maharashtra, India).

### 2.2. Animals

Experimentations were conducted on male Wistar albino rats (180-220 g). Animals were issued from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University. The animals were kept in polypropylene cages. The temperature of  $25 \pm 10^\circ\text{C}$ , relative humidity of 45-55%, and a 12:12 h light/dark cycle was maintained. The rats were fed commercial food pellets (Doodh dhara pashu ahar, India) as well as water *ad libitum*. Institutional Ethical Committee approved all the experimental methodology. All experimental procedures were conducted as per committee for the purpose of control and supervision of experiments on animals (CPCSEA).

### 2.3. Stress models

#### 2.3.1. Cold-restraint stress

In a cold-restraint stress (CRS) model, the rats ( $n = 6$ ) fasted overnight and immobilized by tying the fore and hind limbs separately on a wooden block with an adhesive tape. Finally, all the rats were kept at  $4-7^\circ\text{C}$  for two hours (17).

#### 2.3.2. Restraint stress

In a restraint stress (RS) model, the rats ( $n = 6$ ) were immobilized for four hours in restraint cages ( $5 \times 5 \times 20 \text{ cm}^3$ ) after overnight of fasting at room temperature during the early phase of the light cycle (18).

#### 2.3.3. Water immersion restraint stress

In a water immersion restraint stress (WRS) model, rats fasted overnight and immobilized similar to RS then immersed vertically up to the xiphoid in the water bath ( $20-25^\circ\text{C}$ ). The body was wiped dry after four hours of stress, and the rats were then returned to their home cages (19).

### 2.4. Estimation of ulcer index and hexosamine content

Rats ( $n = 6$ ) were randomly assigned to control and three stress groups (CRS, RS, and WRS). Except for control group, other groups were exposed to CRS, RS, and WRS, respectively. Lastly, all the animals were

killed by cervical dislocation, and their stomachs were taken out for estimation of ulcer index and hexosamine. Hexosamine is an index of mucosal content.

#### 2.4.1. Ulcer index

Ulcer scoring was done as per Sanyal (20). The scoring as per the severity of the ulcer has been explained in Table 1. The score of the ulcers as per severity was determined by a person unaware of the experimental protocol. Ulcer index is the summation of the number of ulcers multiplied by their score per stomach. Ulcer index = 1 X (No. of ulcer of score 1) + 2 X (No. of ulcer of score 2) + 3 X (No. of ulcer of score 3) + 4 X (No. of ulcer of score 4).

#### 2.4.2. Hexosamine estimation

The content of hexosamine was estimated as per Dische (21) with slight modification. Briefly, 0.5 mL of the scrapped mucosa of the stomach was taken, and mixed with 0.5 mL of acetylacetone reagent. Then 0.1 mL of sodium carbonate (0.5 N) was added to 1 mL of the mixture. The mixture was kept on boiling water bath for 20 min and then cooled to room temperature. 1.5 mL of glacial acetic acid (90%) was added to above mixture. The absorbance was measured after 30 min by the spectrophotometer at 530 nm ( $\lambda_{max}$ ). Distilled water was used as blank. A standard curve was prepared by using D-glucosamine hydrochloride and concentration of hexosamine was expressed in mg/g of tissue.

#### 2.5. Estimation of microvascular permeability

The microvascular permeability was measured by injecting Evans blue (10 mg/kg) intravenously through the tail vein under light halothane anesthesia. Animals of all groups except control were exposed to CRS, WRS, RS, respectively. Finally, all the rats were killed by cervical dislocation. Dye from the gastric tissue was extracted, and measured as per method adopted by Katayama (22).

Briefly, the gastric content was soaked overnight in 2 mL of 3.5 N KOH. Then, 18 mL of a mixed solution of 4 N H<sub>3</sub>PO<sub>4</sub> and acetone (1.75:16.25) was added to make the volume up to 25 mL. The tube was shaken vigorously and centrifuged at 3,000 rpm for 15 min. The color intensity of the supernatant was measured at 620 nm ( $\lambda_{max}$ ) by the spectrophotometer. The amount of dye recovered from the gastric contents was expressed as  $\mu$ g/g of gastric tissue.

#### 2.6. Estimation of gastric volume, acidity and pH of gastric content of pyloric ligated rats

Rats were randomly divided into four groups (control, CRS, RS, and WRS) of six animals each. In all the

**Table 1. Score and severity of ulcer**

Score	Features
0	No ulcer
1	Pin point ulcer and changes limited to superficial layers of mucosa
2	Ulcer less than 1 mm in size
3	Ulcer more than 1 mm but less than 2 mm in sizes.
4	Ulcer more than 2 mm or perforation of ulcer.

animals, pyloric ligation was done as per Debnath (23). Briefly, animals were anesthetized by intraperitoneal injection of pentobarbitone (35 mg/kg). The abdomen of the anesthetized rat was opened, and a knot of the thread was tied around the pyloric sphincter. The stomach was put back carefully, and the abdomen wall was closed with interrupted sutures. The skin was cleaned from any blood spots and bleeding, and the collodion was applied over the wound. The rats were kept in a separate cage and allowed for recovery. All the pyloric ligated (PL) rats of all groups except control were exposed to CRS, WRS, RS, respectively.

Lastly, all the animals were killed by cervical dislocation and evaluation of acidity, volume, and pH of gastric content was done. The gastric content was collected in a centrifuge tube and centrifuged for ten mins at 1,000 rpm. The volume was noted as gastric volume. The pH of this solution was recorded with the help of a pH meter. 1 mL of supernatant was pipetted out and diluted up to 10 mL with distilled water. The solution was titrated with NaOH (0.01 N) using Topfer's agent and phenolphthalein as an indicator. Titration was done until the solution turns to pink color. It is used for detection and estimation of hydrochloric acid and total acidity in gastric fluids. The volume of NaOH was noted. Acidity ( $\mu$ Eq/mL) can be expressed as: Acidity = (Vol. of NaOH in mL  $\times$  Normality  $\times$  1,000).

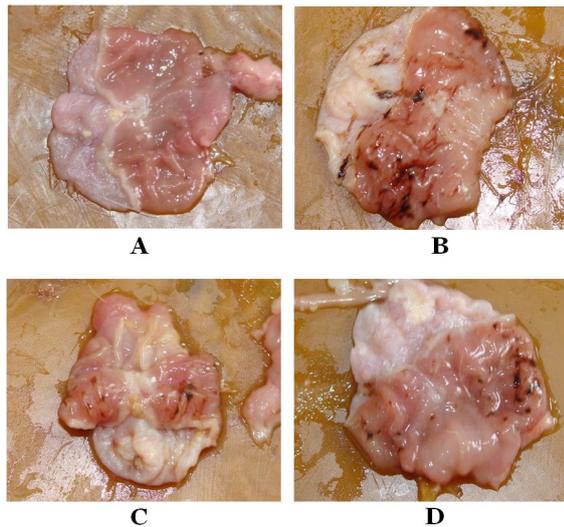
#### 2.7. Statistical analysis

The values were expressed as mean  $\pm$  SEM. GraphPad Prism 5 (San Diego, CA, USA) was used to analyze the data. Statistical significance of all the experimental data were analyzed by one-way ANOVA followed by Tukey test. A *p*-value less than 0.05 was considered to be statistically significant in all the analysis.

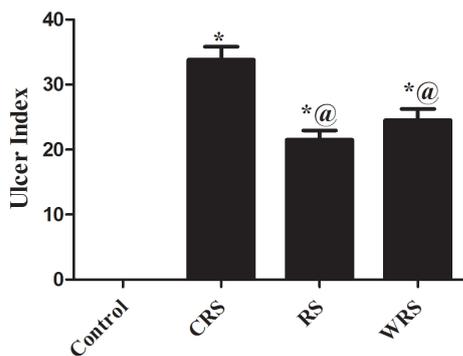
### 3. Results

#### 3.1. Effect of CRS, RS, and WRS on ulcer index and hexosamine content

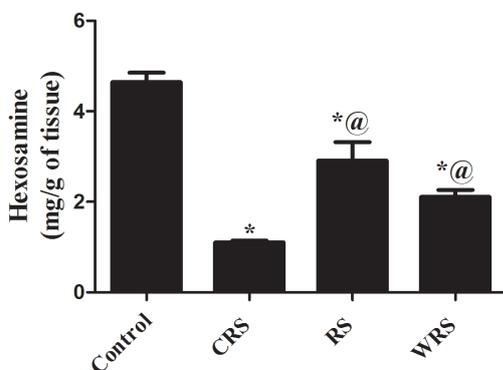
The macroscopic analysis and ulcer index in control, CRS, RS, and WRS models, respectively are represented in Figures 1 and 2. Macroscopic pictures of the ulcerogenic stomach illustrate that in contrast to other models, CRS exposure produced marked ulcers on the fundic area of the stomach. One-way ANOVA



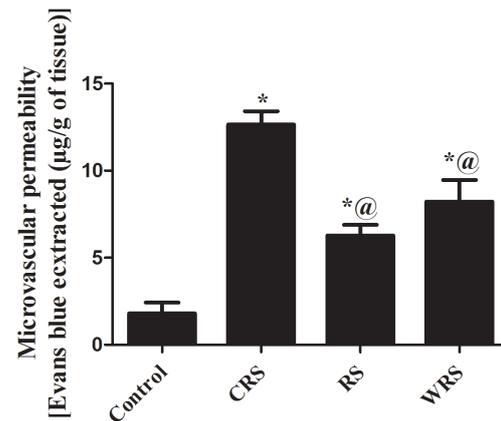
**Figure 1. Macroscopic analyses showing ulcerogenesis in the stomach of rats in different stress models.** Macroscopic pictures of stomach of (A) rat without stress and rats exposed to (B) CRS, (C) RS, and (D) WRS.



**Figure 2. Ulcer index in control, CRS, RS and WRS exposed rats.** Results are expressed in each column as the mean ± SEM ( $n = 6$ ). \* $p < 0.05$  and @ $p < 0.05$  compared to control and CRS group, respectively (one way ANOVA followed by Tukey's test).



**Figure 3. Hexosamine content in control, CRS, RS, and WRS exposed rats.** Results are expressed in each column as the mean ± SEM ( $n = 6$ ). \* $p < 0.05$  and @ $p < 0.05$  compared to control and CRS group, respectively (one way ANOVA followed by Tukey's test).



**Figure 4. Microvascular permeability in control, CRS, RS, and WRS exposed rats.** Results are expressed in each column as the mean ± SEM ( $n = 6$ ). \* $p < 0.05$  and @ $p < 0.05$  compared to control and CRS group, respectively (one way ANOVA followed by Tukey's test).

revealed significant differences in ulcer index among the groups [ $F(3, 23) = 536.7, p < 0.0001$ ]. All the stress models (CRS, RS, and WRS) increased ulcer index significantly in comparison to control. However, the ulcer index is maximum in the CRS-exposed rats.

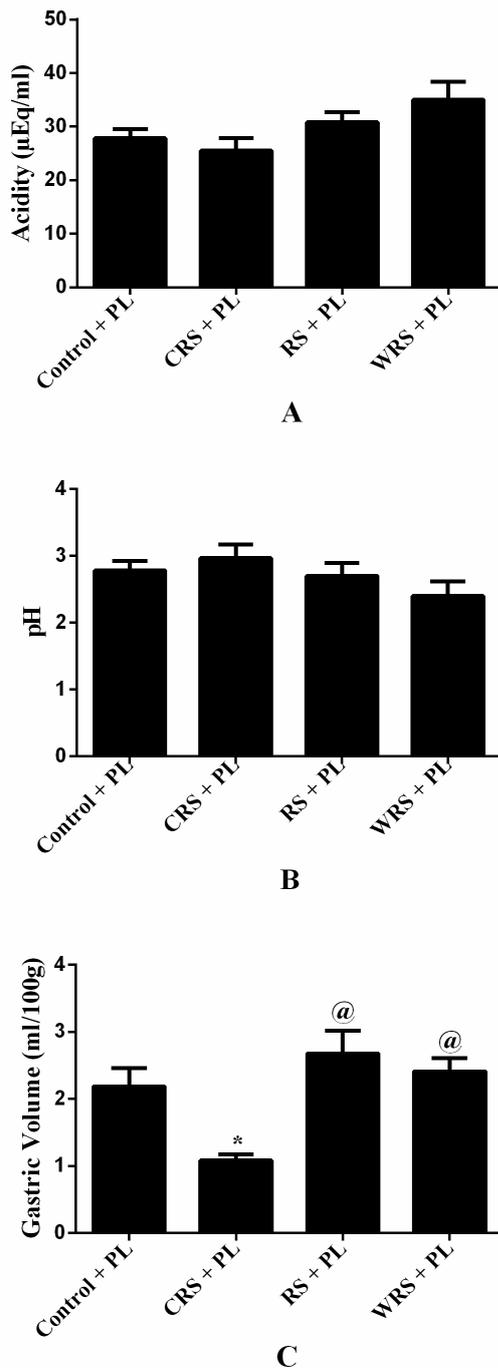
The content of hexosamine in three stress models, as well as in control is demonstrated in Figure 3. One-way ANOVA revealed significant differences in the hexosamine content among the groups [ $F(3, 23) = 39.02, p < 0.0001$ ]. All the stress models (CRS, RS, and WRS) significantly decreased hexosamine level in comparison to control. Hexosamine content decreased maximum in CRS model.

### 3.2. Effect of CRS, RS, and WRS on microvascular permeability

Figure 4 elucidates the microvascular permeability measured in all the groups. One-way ANOVA aptly demonstrated significant differences in microvascular permeability in terms of the Evans blue concentrations among the groups [ $F(3, 23) = 28.29, p < 0.0001$ ]. All the stress models (CRS, RS, and WRS) increased microvascular permeability significantly in comparison to control. Microvascular permeability increased maximum in CRS model.

### 3.3. Effect of CRS, RS, and WRS on gastric content of pyloric ligated rats

The effect of CRS, RS, and WRS on acidity, pH, and volume of gastric content in PL rats is shown in Figure 5. No significant difference in acidity and pH of gastric content among the groups were found. However, gastric volume decreased significantly only in PL rats exposed to CRS ( $p < 0.05$ ).



**Figure 5. Effect of different stress models on gastric content of pyloric ligated rats.** Bar diagram representing (A) acidity, (B) pH and (C) gastric volume of gastric content of pyloric ligated (PL) rats in different stress models (CRS, RS, and WRS). Results in each column are expressed as the mean  $\pm$  SEM ( $n = 6$ ). \* $p < 0.05$  and @ $p < 0.05$  compared to control + PL and CRS + PL group, respectively (one way ANOVA followed by Tukey's test).

#### 4. Discussion

Results of the present study demonstrated that the CRS exposure produced marked ulcers on the fundic area of the stomach, contrary to other stress models. Each of the stress models significantly increased ulcer index and microvascular permeability while decreased hexosamine

level. However, these stress-induced changes in ulcer index, microvascular permeability and hexosamine occurred to a greater extent in the case of CRS model. Total acidity and pH of the gastric content remains unchanged in all the stress models. The gastric volume significantly decreased in case of CRS while unchanged in other stress models.

Macroscopic pictures elucidate that significant ulcers were clearly visible in the fundic area of the stomach of CRS-exposed rats. The well known characteristic feature of SRMD is that the ulcers are found in the fundic area of the stomach (1). Hexosamine level declined in all the stress models. Hexosamine level decreased is the maximum in the CRS model. Hexosamine is the marker of the mucosal barrier. Thus, the mucosal barrier is considerably damaged in the CRS. Decreased mucosal blood flow results in the decreased mucosal secretion (10), which is the ultimate cause of decreased mucosal content.

Results depict that all the stress models lead to an increase in microvascular permeability. Earlier it was reported that stress decreased gastric mucosal blood flow (24). Decreased blood flow results in ischemia followed by reperfusion. Ischaemia followed by reperfusion increased gastric microvascular permeability (25). Microvascular permeability increased maximum in the case of CRS model. The gastric blood flow was markedly influenced by the exposed temperature (26). Therefore, cold exposure to immobilized rats might decrease the mucosal blood flow greater than other immobilization stress. Thus, a remarkable increase in microvascular permeability in the CRS ulcer may be the result of ischemia followed by reperfusion. As ischemia followed by reperfusion is an important pathological factor of SRMD, CRS model emulates the pathophysiology of SRMD.

In the present study, the pylorus ligated rats were exposed to CRS, RS, and WRS. Stress-exposed to PL rats results in hemorrhagic ulcers in the stomach. The gastric volume significantly decreased in case of CRS-exposed PL rats, while unchanged in other rats. However, the total acidity, as well as pH of the gastric content in all the stress models remained unchanged. The above observations represent that gastric secretion is lowered in the CRS-exposed rats without affecting the acidity and pH of released acid. The acid secretion is not a primary characteristic feature of SRMD pathology. Contrary to the patients with severe burn and head injury, in most of the critically ill patients acid secretion in the GI tract is normal or low (9). Thus, as decreased gastric volume was observed in CRS-exposed rats, it can be predicted that CRS model simulates the SRMD pathophysiology in this aspect also.

The overall results of the present study illustrated that the CRS resembles the pathophysiology of SRMD closely. It is the best and feasible model among all the models to evaluate drugs for the treatment of SRMD.

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