

Analysis of the cold-water restraint procedure in gastric ulceration and body temperature

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Received 3 February 2004; received in revised form 14 April 2004; accepted 28 June 2004

Abstract

Gastric mucosal injury induced by body restraint can be enhanced when combined with cold-water immersion. Based on this fact, the present study had two main purposes: (i) to examine the contribution of each of these two forms of stress on the development of gastric ulceration and regulation of body temperature and (ii) to investigate the importance of the animal's consciousness on gastric ulceration induced by the cold-water restraint. Independent groups of animals were exposed for 3 h to one of the following stressful treatments: body restraint plus cold-water (20±1 °C) immersion, body restraint alone or cold-water immersion alone. Control animals were not exposed to any form of stress. Half of the animals submitted to each of the four treatments were anesthetized with thionembutal (35 mg/kg), whereas the other half was injected with saline. Results indicated that body restraint alone was not sufficient to induce gastric ulceration or changes in body temperature. On the other hand, cold-water exposure, either alone or in conjunction with body restraint, induced the same amount of stomach erosions and hypothermia. Therefore, it appears that body restraint does not play an important role on gastric ulceration induced by the cold-water restraint procedure. Present results also indicated that conscious and anesthetized animals immersed in cold water presented robust gastric ulceration and a marked drop in body temperature. However, conscious animals developed more severe gastric damage in comparison to anesthetized animals although both groups presented the same degree of hypothermia. These findings suggest that hypothermia resulting from cold-water exposure has a deleterious effect on gastric ulceration but the animal's conscious activity during the cold-water immersion increases the severity of gastric mucosal damage. It is concluded that cold-water restraint is a useful procedure for the study of the underlying mechanisms involved in stress-induced ulceration.

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Keywords: Consciousness; Hypothermia; Body temperature; Body restraint; Gastric ulceration; Stress-induced ulceration

1. Introduction

Since its first conception [1,2] and later adaptations [3–9], physical restraint for a relatively long period of time (24 h) has been widely used as a useful procedure to induce gastric ulceration in rats [10–13]. Combination of the body restraint procedure with exposure to cold temperatures drastically increases the occurrence of gastric ulceration in a shorter period (3 h). Two different cold exposure

procedures have been used to enhance body-restraint ulceration. One involves the immobilization of the animal, generally in a supine position, and then placing the animal in a cold environment (5±1 °C) such as a refrigerator [14–17]. The other procedure consists in restraining the animal in a cylindrical tube and then immersing vertically in cold water (20±1 °C) to the level of the animal's neck [18–22]. It has been shown that both cold-exposure procedures are severe forms of stress that mobilize catecholaminergic systems in brain areas associated with behavioral responses to aversive stimuli [23–29] as well as neuroendocrine responses of the hypothalamus–pituitary–adrenal axis, including changes in corticotrophin-releasing factor (CRF),

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adrenocorticotrophic hormone (ACTH) plasma levels and adrenergic receptors in the pituitary gland [30–34].

The relationship between body restraint and these two cold-exposure methodologies has been an important question in stress ulcer disease. For example, Selye [35] suggested that combinations of different forms of stressors are better ulcerogenic stimuli when compared with each one alone. This view has been confirmed when body restraint is associated with exposure to a cold environment [14,15]. For example, Senay and Levine [15] found that restraint combined with exposure to a cold environment at 4 to 7 °C yielded a much greater incidence of ulceration than either type of stress acting separately, indicating that the two forms of stress can function synergistically to produce gastric ulceration. However, the relationship between body restraint and cold-water immersion is still unclear. Although it has been reported that cold-water restraint produces much more gastric erosions as compared to body restraint alone [18,36], no study has yet compared the amount of gastric ulceration induced by restraint versus unrestraint cold-water exposure. This is a critical comparison because it can allow an empirical test of a possible synergism between body restraint and cold-water exposure on the development of gastric ulceration as it has been reported in the cold-environment restraint procedure [14,15]. The present experiment addressed this issue, analyzing the effect of each of the two stressful procedures involved in the cold-water restraint on the occurrence of gastric ulceration. Accordingly, different groups of animals were exposed to the following treatments: body restraint plus cold-water immersion, body restraint alone and cold-water immersion alone. Control animals were not exposed to any stressful manipulation.

Another purpose of this study was to investigate whether the cold-water restraint procedure is a valid animal model of stress-induced ulceration. It has been argued that ulceration induced by cold exposure is a direct physical reaction to the cold temperature and exclusively mediated by a severe drop in core body temperature [37]. According to this view, gastric ulceration induced by cold-water restraint does not depend on the animal's higher-order systems responsible for processing the stressful stimulation. In agreement with this suggestion, it has been shown that exposure to cold-water immersion [38,39] or cold environment [40] was able to produce gastric ulceration in urethane-anesthetized animals. In contrast, Murison and Overmier [36] found that animal's consciousness was a necessary condition for the development of gastric mucosal injury induced by cold-water restraint. In this study, pentobarbital-anesthetized animals immersed in cold water did not present any gastric erosions as opposed to conscious animals exposed to the same cold-water restraint procedure. Because urethane anesthesia produces a sustained increase in pituitary–adrenal activity and can drastically raise corticosterone levels up to a point generally found in stressed rats [41–43], it is possible that the occurrence of gastric ulceration in urethane-anesthetized animals exposed to cold temperatures [38–40] might be

related to a direct effect of the anesthetic on corticosterone release. Nonetheless, Murison and Overmier [36] used a procedure in which animals were immersed in cold water for only 75 min followed by another 75-min rest period in the home cage. Therefore, an alternative explanation for the Murison and Overmier [36] results could be that 75 min of cold-water exposure might not be sufficient to induce gastric ulceration in anesthetized animals. To further investigate the importance of the animal's consciousness in the occurrence of gastric ulceration induced by a cold-water restraint procedure, half of the animals submitted to each of the four experimental treatments described above were anesthetized with thionembutal, a barbiturate with similar anesthetic characteristics to pentobarbital [44–48], whereas the other half was injected with saline.

2. Materials and methods

2.1. Subjects

Experimentally naive female Wistar rats from 3 to 4 months old and weighing between 220 to 280 g were used as subjects. They were born in the Psychology Department vivarium at the PUC-Rio and reared in groups of six. A week before the beginning of the experiment, animals were individually housed in hanging steel cages and handled daily. Animals had free access to food and water except for 24 h before the beginning of the experiment when they were food deprived but not water deprived. Vivarium temperature was controlled (23 ± 1 °C) and the light–dark cycle was maintained on a 12-h on–off cycle (0700–1900 h lights on). The experiment was conducted during the light portion of the light–dark cycle. The experimental protocol described below was in conformity with the regulations for care and use of laboratory animals of the Brazilian Society for Behavioral Neuroscience.

2.2. Procedure

Animals were randomly divided into eight independent groups according to a 4×2 factorial design. The first factor determined the four different stressful treatments that each animal was submitted to: body restraint plus cold-water immersion, body restraint alone, cold-water immersion alone or no stress. The second factor determined whether the animal was conscious or anesthetized when exposed to one of the four stressful treatments.

Rats subjected to body restraint plus cold-water immersion treatment were immobilized in a PVC tube 17.5 cm long and having an internal diameter of 5.2 cm. After restraining, the animals were immersed vertically to the level of the xiphoid process of the sternum in a 64-l tank of water maintained at 20 ± 1 °C. Animals assigned to the body-restraint-only condition had exactly the same procedure as the animals exposed to the body restraint plus cold-

water immersion procedure except that the water tank was empty. Animals subjected to the water-immersion alone treatment were simply placed into the tank of water without any body immobilization. Water level was constantly monitored so that the animal's body was always covered by the water and the head was invariably above the surface. Finally, nonstressed control animals had the same procedure as the three other groups except that they were not restrained or exposed to cold water. Instead, they were placed in a holding cage (30×30×28 cm) and left undisturbed for the entire experiment. Half of the animals submitted to each of the four experimental treatments were anesthetized with sodium thiopental (Thionembatal, Abbott Laboratórios do Brasil, São Paulo, Brazil), at the dose of 35 mg/kg ip, 15 min before the beginning of the experiment. Two animals from the unrestrained group exposed to cold water received a supplementary dose of 0.05 ml anesthesia because they started to show signs of awakening. The other half of the animals was injected with saline.

The experiment was performed in a temperature-controlled room (23±1 °C) and lasted for 3 h. After that time, animals were removed from their experimental conditions and core body temperature was measured with a lubricated rectal probe attached to a digital thermometer (Gulterm 200, Gultron do Brasil, São Paulo, Brazil). Animals were then sacrificed with a lethal dose of thionembatal and the stomachs were immediately removed. A ligature was placed around the duodenum and the esophagus and 3 ml of 10% formalin was infused into the stomach. Ten minutes later, the stomach was opened along the great curvature, rinsed with water, spread on a flat surface and fixed with 10% formalin. The stomachs were stored in formalin at least 1 week before the quantification of the gastric ulceration.

2.3. Quantification of the gastric mucosal damage

Stomachs were pinned on a flat surface and examined with a binocular dissector microscope at 8× magnification. One eyepiece was fitted with a rule permitting gastric ulceration to be quantified in terms of total area (mm²). Any discontinuity in the gastric mucosa was considered a gastric erosion and the total area was estimated by multiplying the length and the width of the lesioned area. An independent rater who was unaware of the experimental treatments examined all the stomachs.

2.4. Statistic analysis

Results of the core body temperature and gastric ulceration are graphically expressed as means plus the standard error of the mean (S.E.M.). A two-way analysis of variance (ANOVA) was used to detect overall differences followed by Fisher's least significant difference (LSD) post hoc tests to determine specific differences between groups. The level of statistical significance was $P<.05$.

3. Results

Fig. 1 presents the mean of core body temperature in anesthetized and conscious animals exposed to the four different stressful treatments. A two-way ANOVA revealed an interaction between these two factors [$F(3,64)=37.46$, $P<.001$]. Post hoc pairwise comparison indicated that body restraint alone did not induce any change in body temperature since conscious animals subjected exclusively to body restraint did not present any statistical difference in core temperature when compared with conscious nonstressed control animals ($P>.8$). Thionembatal anesthesia produced a mild hypothermia effect in nonstressed and body-restraint-only animals when compared with conscious animals exposed to the same group treatments ($P<.01$). Exposure to cold water in the presence or absence of body restraint resulted in a marked drop in body temperature both in conscious and anesthetized animals when compared with their respective anesthetized or conscious nonstressed animals (all $P_s<.05$). No statistical differences in body temperature among these four groups were found. These results indicate that conscious and anesthetized animals presented the same amount of hypothermia when exposed to cold water whether they were restrained or not.

Fig. 2 depicts the mean amount of gastric ulceration induced by the four stressful treatments across anesthetized and conscious animals. A two-way ANOVA also revealed an interaction between these two factors [$F(3,64)=3.68$; $P=.01$]. Post hoc comparison test indicated that thionembatal anesthesia did not cause any gastric ulceration since no statistical difference between conscious and anesthetized nonstressed animals was found ($P>.2$). Conscious and anesthetized animals submitted exclusively to the body restraint procedure did not present any gastric ulceration and were not statistically different from their respective anesthetized or conscious nonstressed control animals (all

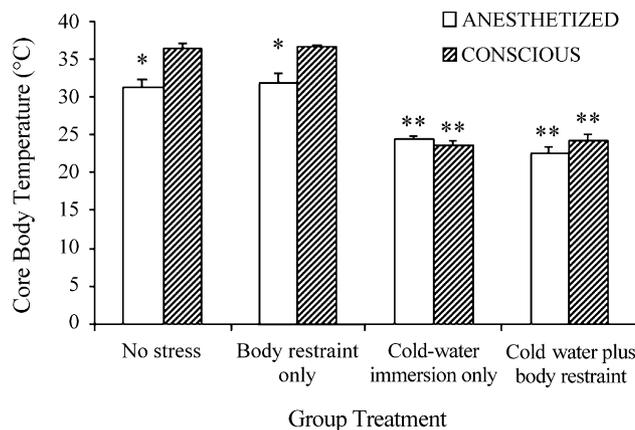


Fig. 1. Effect of the four stressful treatments across anesthetized and conscious animals on body temperature. $N=9$ /group. * $P<.01$ in comparison with conscious animals exposed to the same group treatment. ** $P<.01$ in comparison with nonstressed animals with their respective conscious or anesthetized conditions.

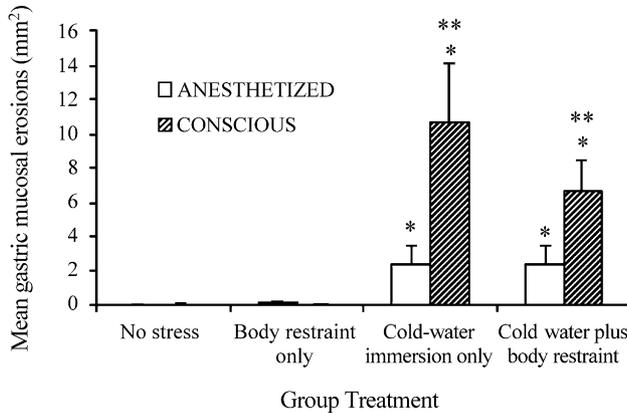


Fig. 2. Effect of the four stressful treatments across anesthetized and conscious animals on gastric mucosal erosion. $N=9/\text{group}$. $*P<.05$ in comparison with nonstressed animals with their respective conscious or anesthetized conditions. $**P<.05$ in comparison with anesthetized animals exposed to restrained or unrestrained cold-water exposure treatment.

$P_s>.1$). In contrast, cold-water immersion alone or in combination with body restraint induced clear-cut gastric erosions in the glandular portion of the stomach. Ulcerations were typically spherical or oblong and superficially covered with blood. As can be observed in Fig. 2, both conscious and anesthetized animals exposed to cold water had significantly more gastric ulceration as compared with their respective conscious and anesthetized nonstressed animals (all $P_s<.05$). Body restraint did not increase gastric ulceration induced by cold-water exposure since no statistical differences in gastric ulceration between restrained and unrestrained animals exposed to cold water was found ($P>.3$). Finally, conscious animals injected with saline and exposed to cold water, either restrained or unrestrained, presented more severe gastric erosions in relation to thionembutal-anesthetized animals exposed to the same stressful treatments (all $P_s<.05$). Therefore, although cold-water exposure was able to induce gastric ulceration in anesthetized animals, the animal's consciousness activity during the cold-water immersion procedure exacerbates the occurrence of gastric mucosal injury.

4. Discussion

Aside from the relative reduction of interest for the role of stress and psychological variables in gastric pathology in clinical settings since the discovery of the bacteria *Helicobacter pylori* [49], gastric ulceration has long been viewed as the prototypic disease of stress [50,51], and a variety of animal methodologies have been developed to investigate the underlying mechanisms involved in the occurrence of this pathology induced by a stressful situation [10–13]. Body restraint, when combined with cold-water exposure, has been used as one of these methodologies because it can induce reliable gastric ulceration in a very short time [18–22]. One of the main purposes of the present

study was to investigate the relationship between body restraint and cold-water exposure on the occurrence of gastric ulceration. Present results indicated that body restraint alone at room temperature for a 3-h period was not able to induce any gastric ulceration. This result is in accordance with previous studies, which reported that animals submitted solely to body restraint require longer periods (18–24 h) to produce a minimal amount of stomach erosion (see Refs. [11–13,50] for a review). Moreover, present results also replicated previous reports from the literature, which found that body restraint associated with cold-water exposure causes gastric ulceration with a 3-h period [18–22]. Surprisingly, restrained and unrestrained animals exposed to cold water presented the same amount of gastric ulceration, suggesting that body restraint has little effect on gastric ulceration when combined with cold-water immersion. The lack of a synergism between body restraint and cold-water immersion observed in the present experiment does not parallel other studies that found that body restraint significantly enhanced gastric ulceration induced by cold-environment exposure [14,15].

Body temperature regulation might be responsible for the differential effect of body restraint on the two cold-exposure procedures. Several reports indicate that hypothermia is a critical factor for the occurrence of gastric erosions within procedures that involves exposure to cold temperatures. For example, cold-water restraint induced more gastric ulceration and higher drop in body temperature when compared with restraint in a cold environment [52–54]. Moreover, expression of aggressive behavior among restrained animals exposed to a cold environment caused a decrease in both hypothermia and gastric ulceration [55]. Finally, increasing the time exposure to the cold temperature increased hypothermia and gastric ulceration [56,57]. In fact, occurrence of gastric erosion depends on the environment [58] or the water [36] temperature and preventing hypothermia induced by cold exposure also alleviate the development of gastric ulceration [59,60].

Based on the well-established association between hypothermia and gastric ulceration, it is possible that the synergism effect of body restraint on cold-environment exposure [13,14] but not on the cold-water immersion procedure (present experiment) might be related to differences in thermoregulatory behavior that animals displayed when exposed to cold temperatures. For example, unrestrained animals exposed to cold environments typically assume ball-like shape behavior to reduce body exposure to the cold and thus minimize heat loss [61]. Indeed, Brodie and Valitski [14], who found a synergism between body restraint and cold-environment exposure, also reported that unrestrained animals exposed to a 5–6 °C cold room did not present any drop in body temperature as opposed to restrained animals exposed to the same cold environment. On the other hand, unrestrained rats from the present experiment, when exposed to cold water, displayed some locomotor activity and rearing behavior during the first 15

to 30 min of the experiment. After that, this motor activity gradually declined and eventually disappeared, giving way to a crouching posture near the wall of the water tank. This hypoactivity probably resulted in severe hypothermia, which was not statistically different from restrained animals also exposed to cold water. Therefore, the absence of thermoregulatory behavior among unrestrained animals exposed to cold water might be responsible for the lack of the gastric ulceration synergism between body restraint and cold-water exposure generally observed in the cold-environment procedure [13,14].

The present study also demonstrated that animals exposed to cold water under thiopental anesthesia developed gastric ulceration along with a severe drop in body temperature. These results are convergent with the hypothesis that hypothermia has a deleterious effect on gastric ulceration and extend previous studies that found gastric erosions among urethane-anesthetized animal exposed to cold water [38,39] or cold environment [40]. On the other hand, these results are not consistent with the study reported by Murison and Overmier [36] who found that pentobarbital-anesthetized animals exposed to cold water did not present any disruption in the gastric mucosa. A possible explanation for the discrepancy between these two studies is that the present study used thionembutal as the anesthetic agent, whereas Murison and Overmier [36] used pentobarbital. However, thiopental and pentobarbital are both barbiturates with similar anesthetic characteristics [44–48]. Moreover, it has been shown that these two anesthetic agents have the same excitatory effect on gastric acid secretion in urethane-anesthetized rats [62]. In fact, pentobarbital-related anesthetics can produce both excitatory and inhibitory effects on gastric acid secretion. For example, pentobarbital causes a decrease in gastric acid secretion in histamine and insulin-stimulated stomachs [63,64], whereas it can induce an increase in gastric acid secretion in the perfused stomach of rats under urethane anesthesia [65–67]. Another possibility for the inconsistent results between these two studies could be that the present experiment used female rats as subjects, whereas Murison and Overmier [36] used male rats. Although there is much evidence on sex-based differences in the responses to different forms of stress, these differences have not been found in gastric ulceration induced by cold-water restraint [68–71]. Therefore, it is unlikely that the different gender of animals across the two studies might be responsible for the occurrence (present results) or the absence (Overmier and Murison [36]) of gastric ulceration in anesthetized rats.

An alternative explanation could be that Murison and Overmier [36] used a cold-water procedure in which the animals were immersed in cold water for only 75 min followed by another 75-min rest period in the home cage, whereas animals from the present experiment were continuously exposed to cold water for 3 h. It must be acknowledged that rats exposed for 75 min and then allowed an extra 75 min of rest presented greater amount

of gastric ulceration in relation to animals exposed to cold water for the full 150 min [72]. Consequently, it could be presumed that the anesthetized animals from the Murison and Overmier [36] study would not have presented any stomach ulceration even after 3 h of continuous stress. However, gastric ulceration triggered by the poststress rest period is highly modulated by the presence of stimuli associated with higher-order processing, such as danger stimuli previously associated with electrical shocks [73], which undoubtedly would not have any impact on the gastric activity among anesthetized animals. Moreover, anesthetized animals exposed to the poststress rest period presented an increase in body temperature [36], which in turn could prevent the development of gastric ulceration [59]. Therefore, methodological variations between the present study and that reported by Murison and Overmier [36] might account for the differences in gastric ulceration observed among anesthetized animals exposed to cold water.

Finally, it is clear from the present results that an animal's consciousness activity during the cold-water immersion is an important variable for the aggravation of gastric erosions independently from the hypothermia effect. Conscious animals exposed to cold water developed more severe gastric damage in comparison with anesthetized animals although both groups presented the same degree of hypothermia. These results confirm a previous study that found that conscious animals exposed to cold water developed gastric ulceration to a greater degree than urethane-anesthetized animals [26]. In another study, Murakami and colleagues [39] reported that although urethane-anesthetized animals exposed to cold water had fewer gastric erosions than conscious animals, this difference did not reach statistical significance. However, incidence of gastric ulceration among conscious and anesthetized animals was extremely high (93% among anesthetized animals and 100% among conscious animals), suggesting that the lack of statistical differences in this study might be due to a ceiling effect.

Taken together, the present results suggest that body restraint does not play an important role on gastric ulceration induced by cold-water immersion since restrained and unrestrained animals exposed to cold water presented the same amount of gastric ulceration. The absence of synergism between body restraint and cold-water immersion was probably because unrestrained animals exposed to cold water did not engage in thermoregulatory behavior, which could alleviate the hyperthermia reaction to the cold water and its negative effect on stomach mucosa. In accordance with the view that hypothermia is involved in the occurrence of gastric ulceration, it was also found that thionembutal-anesthetized animals exposed to cold water developed a marked drop in body temperature and gastric ulceration. Finally, the present study also demonstrated that conscious animals exposed to cold water developed more severe gastric damage compared with anesthetized animals

although both groups presented the same degree of hypothermia. These results confirm that hypothermia is not the only factor involved in the gastric erosions induced by cold-water restraint and show that the integrity of the systems responsible for higher-order processing among animals exposed to cold-water stress is an important variable in the process of gastric ulceration development.

Acknowledgement

Secretary for Science and Technology of Brazil grant (CNPq) 300094/95-0 supported this research. Thanks are due to Lígia Alves, Daniela Araújo, Jorge Lunz, Sandra Redner, Laura Sarmento and Pablo Velloso, who helped in this study.

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