Maintenance of lung myeloperoxidase activity in proestrus females after trauma-hemorrhage: upregulation of heme oxygenase-1

Huang-Ping Yu,1,2 Shaolong Yang,1 Ya-Ching Hsieh,1 Mashkoor A. Choudhry,1 Kirby I. Bland,1 and Irshad H. Chaudry1

1Center for Surgical Research and Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama; and 2Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taoyuan, Taiwan

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YU, HUANG-PING, SHAOULONG YANG, YA-CHING HSIEH, MASHKOOR A. CHOUDHRY, KIRBY I. BLAND, AND IRSHAD H. CHAUDRY. Maintenance of lung myeloperoxidase activity in proestrus females after trauma-hemorrhage: upregulation of heme oxygenase-1. Am J Physiol Lung Cell Mol Physiol 291: L400–L406, 2006. First published March 23, 2006; doi:10.1152/ajplung.00537.2005—Previous studies showed that females in the proestrus stage of the reproductive cycle maintain organ functions after trauma-hemorrhage. However, it remains unknown whether the female reproductive cycle is an important variable in the regulation of lung injury after trauma-hemorrhage and, if so, whether the effect is mediated via upregulation of heme oxygenase (HO)-1. To examine this, female Sprague-Dawley rats during diestrus, proestrus, estrus, and metestrus phases of the reproductive cycle or 14 days after ovariectomy underwent soft tissue trauma and then hemorrhage (mean blood pressure 40 mmHg for 90 min followed by fluid resuscitation). At 2 h after trauma-hemorrhage or sham operation, lung myeloperoxidase (MPO) activity and intercellular adhesion molecule (ICAM)-1, cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-3, and HO-1 protein levels were measured. Plasma 17β-estradiol concentration was also determined. The results indicated that trauma-hemorrhage increased lung MPO activity and ICAM-1, CINC-1, and CINC-3 levels in ovariectomized females. These parameters were found to be similar to sham-operated animals in proestrus female rats subjected to trauma-hemorrhage. Lung HO-1 protein level in proestrus females was increased significantly compared with female rats subjected to trauma-hemorrhage during diestrus, estrus, and metestrus phases of the reproductive cycle and ovariectomized rats. Furthermore, plasma 17β-estradiol level was highest in proestrus females. Administration of the HO inhibitor chromium mesoporphyrin (CrMP) prevented the attenuation of shock-induced lung damage in proestrus females. Thus these findings suggest that the female reproductive cycle is an important variable in the regulation of lung injury following trauma-hemorrhage and that the protective effect in proestrus females is likely mediated via upregulation of HO-1.

hemorrhagic shock; cytokine-induced neutrophil chemoattractant-1; cytokine-induced neutrophil chemoattractant-3; intercellular adhesion molecule-1

HEMORRHAGIC SHOCK is a major contributor to the development of acute respiratory distress syndrome in patients sustaining major mechanical trauma (18). Patients who survive the initial traumatic insult remain susceptible to sepsis, septic shock, and multiple organ failure, which are the major causes of death in the injured patient (4, 23, 34). Cellular dysfunction following hemorrhagic shock occurs in many organs, including the lung, and such alteration persists despite fluid resuscitation for a prolonged period of time (12, 26).

A large number of studies have demonstrated that the enhanced secretion of proinflammatory cytokines by mast cells, dendritic cells, and macrophages is an important factor in the initiation and perpetuation of lung inflammation (9). These cytokines recruit other immune cells including neutrophils, thereby increasing leukocyte trafficking and lung permeability (20). Neutrophils can release mediators, which diffuse across the endothelium and injure parenchymal cells, or, alternatively, neutrophils can leave the microcirculation and migrate to and adhere to matrix proteins or other cells (19). Intercellular adhesion molecule (ICAM)-1 is known to play a major role in the firm adhesion of neutrophils to the vascular endothelium. ICAM-1 is constitutively present on the surface of endothelial cells and is markedly upregulated after trauma-hemorrhagic shock (8). In addition to adhesion molecules, chemokines such as cytokine-induced neutrophil chemoattractant (CINC)-1, and CINC-3, members of the CXC chemokine family, are also potent chemotactic factors for neutrophils (35).

Previous studies have demonstrated that upregulation of heme oxygenase (HO)-1 causes a reduction in adhesion molecules and neutrophil chemoattractant (25, 35). Furthermore, estrogen can reduce neutrophil accumulation in the lung, and this effect is mediated via the estrogen receptor (7). In addition to reduction of neutrophil accumulation, estrogen administration in males after trauma-hemorrhage is also known to upregulate HO-1 expression and protects the organs against dysfunction and injury (29). However, administration of the HO inhibitor chromium mesoporphyrin (CrMP) prevented the 17β-estradiol-induced attenuation of shock-induced organ dysfunction and damage (29).

The gonadal steroids, androgen and estrogen, play a major role in the regulation of cardiovascular and immune function after trauma-hemorrhage (3, 14). Both immune and cardiac functions are depressed in males after trauma-hemorrhage (3, 36). In contrast, these functions are maintained in proestrus females under those conditions (2, 6). It has also been demonstrated that the proestrus state of the female rodent has the highest plasma concentration of estradiol (13). Studies of Slimmer and Blair (24) showed that female rats in the proestrus stage of the reproductive cycle exhibit a more vigorous volume restitution response than either estrus females or males after simple hemorrhage. Likewise, Kuebler et al. (16) showed that differences in the regulation of plasma and tissue volumes exist...
between males and proestrus females during and after hemorrhage and that proestrus females have increased circulating blood volume compared with males after resuscitation. Furthermore, Jarra et al. (13) showed that female rats subjected to hemorrhage during the proestrus stage have enhanced cardiac and hepatic functions as opposed to decreased responses in males.

Although our previous findings (13) suggested that the female reproductive cycle is an important variable with regard to cardiac and hepatic functions, it remains unknown whether the alterations in estrogen levels in different female reproductive cycle phases are associated with lung neutrophil infiltration following trauma-hemorrhage and, if so, whether the effect is via upregulation of an HO-1 pathway. To examine this, lung tissues from different female reproductive cycle phases after trauma-hemorrhage were examined for neutrophil infiltration and HO-1 expression in the present study.

MATERIALS AND METHODS

Animals. Adult female (200–250 g) Sprague-Dawley rats (Charles River Laboratory Services, Wilmington, MA) were used in this study. All experiments were performed in adherence to the National Institutes of Health Guidelines for the Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Ovariectomy procedures. Rats were ovariectomized 14 days before the experiment as described previously (15). Briefly, after initiation of general anesthesia with isoflurane (Attane; Minrad, Bethlehem, PA), a small incision was made in the skin on the back of the animal midway between the last rib and the hip. A second incision was made through the muscle layer ~1 cm lateral to the spinal muscle into the peritoneal cavity. The ovaries were separated and tied off with a silk ligature (3-0 surgical silk; Deknatel, Fall River, MA). The ovaries were then removed, and the incisions were closed with 4-0 nonabsorbable nylon monofilament sutures (Ethilon; Ethicon, Somerville, NJ).

Experimental procedures. We used a nonheparinized model of trauma-hemorrhage and resuscitation in the rat as described previously (13), with minor modifications. Briefly, female Sprague-Dawley rats were fasted overnight before the experiment but were allowed water ad libitum. The stage of the female reproductive cycle was determined by regular examination of vaginal smears as described previously (13). Experiments were performed only after at least one complete estrus cycle had been documented. The cycle phase was determined from the cytology of vaginal smears obtained daily at 0700–0830. The hemorrhage procedure was started between 0900 and 1000. The animals were anesthetized with 1.5% isoflurane and 100% oxygen inhalation and underwent a 5-cm ventral midline laparotomy to induce soft tissue trauma before the onset of hemorrhage. The abdomen was then closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (polyethylene [PE-50] tubing; Becton-Dickinson, Sparks, MD). After catheterization of the first femoral artery, ~600 μl of blood was withdrawn as described below (see Plasma collection and storage). The wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to minimize postoperative pain. The rats were then allowed to awaken, after which they were rapidly bled to a mean blood pressure (MBP) of 35–40 mmHg within 10 min. The time at which the animals could no longer maintain a MBP of 35–40 mmHg without fluid infusion was defined as maximum bleed-out volume. After that point, the MBP was maintained at 40 mmHg by infusion of Ringer lactate (RL) intraveneously in 0.2-ml bolus increments until 40% of the shed blood volume was returned in that form. The sham-operated animals underwent the same surgical procedure but were neither bled nor resuscitated. The time required for maximum bleed-out was ~45 min, the volume of maximum bleed-out was ~60% of the calculated circulating blood volume (33), and the total hemorrhage time was ~90 min. The animals were then resuscitated with four times the volume of the shed blood over 60 min with RL. After the rats were resuscitated, the catheters were removed, the vessels were ligated, and the skin incisions closed with sutures. The animals were returned to their cages and were allowed food and water ad libitum until death. In some experiments, a group of sham-operated or proestrus hemorrhaged females was treated with the specific HO inhibitor CrMP (2.5 mg/kg ip; Frontier Scientific, Logan, UT) 30 min before the procedure of sham operation or trauma-hemorrhage. The dose of CrMP was selected from our previously published study (29). The animals were killed at 2 h after the end of resuscitation.

Preparation of lung samples. Immediately after the rats were anesthetized, the chest was opened and the left side of the lung was obtained. Excess blood was blotted, and the tissue section was stored at ~80°C until being analyzed.

Measurement of myeloperoxidase activity. Myeloperoxidase (MPO) activity in homogenates of whole lung was determined as described previously (21, 35). All reagents were purchased from Sigma (St. Louis, MO). Briefly, equal weights (100 mg wet wt) of lung from various groups were suspended in 1 ml of buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) and sonicated at 30 cycles twice for 30 s on ice. Homogenates were cleared by centrifuging at 12,000 rpm at 4°C, and the supernatants were stored at ~80°C. Protein content in the samples was determined with a Bio-Rad (Hercules, CA) assay kit. The samples were incubated with the substrate o-dianisidine dihydrochloride. This reaction was carried out in a 96-well plate by adding 290 μl of 50 mM phosphate buffer, 3 μl of substrate solution (containing 20 mg/ml o-dianisidine dihydrochloride), and 3 μl of H2O2 (20 mM). Sample (10 μl) was added to each well to start the reaction. Standard MPO (Sigma) was used in parallel to determine MPO activity in the sample. The reaction was stopped by adding 3 μl of sodium azide (30%). Light absorbance at 460 nm was read. MPO activity was determined by using the curve obtained from the standard MPO.

Determination of CINC-1, CINC-3, and ICAM-1 levels. Lung CINC-1, CINC-3, and ICAM-1 levels were determined with ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, the samples were homogenized in PBS (1:10 wt/vol; pH 7.4) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Boehringer Mannheim, Germany). The homogenates were centrifuged at 12,000 g for 20 min at 4°C, and the supernatant was assayed for CINC-1, CINC-3, and ICAM-1 levels. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC protein assay).

Western blot assay. Rat lung tissues were homogenized in a buffer containing (mM) 10 Tris (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 50 NaF, 0.5 phenylmethylsulfonyl fluoride, and 1 sodium vanadate, with 1% Triton X-100, 0.5% Nonidet P-40, and 1 μg/ml aprotinin. The homogenates were centrifuged at 12,000 g for 15 min at 4°C. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC protein assay). Protein aliquots were mixed with 4× sample buffer and were electrophoresed on 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred electrophoretically onto nitrocellulose transfer membranes (Invitrogen). The membranes were incubated with anti-HO-1 (1:100) in Tris-buffered saline-Tween buffer (TBST) overnight at 4°C and then washed with TBST. The membranes were later incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000 dilution in TBST for HO-1) for 1.5 h at room temperature and washed with TBST. The blots were immersed for 5 min in Super Signal West Pico detection reagent and then exposed to film. Signals were quantified with ChemiImager 5500 imaging software (Alpha Innotech).

Plasma collection and storage. At the start of the experiments, ~600 μl of heparinized whole blood for the determination of baseline
17β-estradiol levels was withdrawn (13). At 2 h after resuscitation, heparinized whole blood was obtained. Blood was placed in micro-centrifuge tubes and then centrifuged at 15,000 rpm for 15 min at 4°C. Plasma and serum were separated, placed in pyrogen-free micro-centrifuge tubes, immediately frozen, and stored (−80°C) until assay.

**Determination of plasma 17β-estradiol level.** Plasma 17β-estradiol concentration was determined with the use of a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

**Statistical analysis.** Results are presented as means ± SE (n = 5 or 6 rats/group). The data were analyzed with one-way analysis of variance and Tukey’s test, and differences were considered significant at a P value of ≤0.05.

**RESULTS**

**Effects of trauma-hemorrhage on lung MPO activity.** Lung MPO activity in sham-operated and trauma-hemorrhaged animals during different phases of the reproductive cycle is shown in Fig. 1. There was a significant increase in lung MPO activity in the diestrus, estrus, and ovariectomized animals after trauma-hemorrhage. However, in proestrus females, there was no difference in lung MPO activity after trauma-hemorrhage compared with sham-operated animals. Furthermore, the increase in lung MPO activity in ovariectomized females was found to be higher compared with female rats during the diestrus, estrus, and metestrus phases of the reproductive cycle.

**Lung CINC-1, CINC-3, and ICAM-1 levels.** Trauma-hemorrhage significantly increased CINC-1, CINC-3, and ICAM-1 expression in the lung in the diestrus, estrus, and ovariectomized animals (Fig. 2); however, proestrus females did not exhibit an increase in CINC-1, CINC-3, and ICAM-1 expression after trauma-hemorrhage compared with sham-operated animals. Consistent with MPO activity, the levels of CINC-1, CINC-3, and ICAM-1 were also significantly higher in ovariectomized females compared with female rats during the diestrus, estrus, and metestrus phases of the reproductive cycle.

**HO-1 protein expression in lung.** Trauma-hemorrhage induced a significant increase in lung HO-1 protein expression in all groups compared with sham-operated animals (Fig. 3). Female rats during the diestrus, proestrus, estrus, and metestrus phases of the reproductive cycle after trauma-hemorrhage demonstrated a further significant increase in lung HO-1 protein levels compared with trauma-hemorrhage ovariectomized female rats. However, proestrus females after trauma-hemorrhage demonstrated the highest increase in HO-1 expression compared with the other groups subjected to trauma-hemorrhage.

**Plasma estradiol levels.** Plasma levels of estradiol were found to be highest in proestrus female rats (Fig. 4) and were lowest in ovariectomized rats at the start of the experiments. At 2 h after trauma-hemorrhage and resuscitation, plasma estradiol levels remained significantly higher in proestrus females compared with the other hemorrhaged groups (Fig. 4).

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**Fig. 1.** Lung tissue myeloperoxidase (MPO) levels in rats at 2 h after sham operation (Sham) or trauma-hemorrhage and resuscitation (T-H). Data are shown as means ± SE of 6 rats in each group. *P < 0.05 compared with Sham; #P < 0.05 compared with Sham, T-H + DE, TH + PE, TH + E, and T-H + ME.

**Fig. 2.** Cytokine-induced neutrophil chemoattractant (CINC)-1 (A), CINC-3 (B), and intercellular adhesion molecule (ICAM)-1 (C) levels in rats after sham operation or trauma-hemorrhage and resuscitation. Data are shown as means ± SE of 6 rats in each group. *P < 0.05 compared with Sham; #P < 0.05 compared with Sham, T-H + DE, TH + PE, TH + E, and T-H + ME; @P < 0.05 compared with Sham, TH + PE, TH + E, T-H + ME, and T-H + OVX.
HO-1 and lung MPO activity. To determine the role of HO-1 in proestrus phase-mediated attenuation of trauma-hemorrhage-induced increase in lung MPO activity, sham-operated or proestrus trauma-hemorrhaged rats were pretreated with the HO inhibitor CrMP. The results as shown in Fig. 5 suggest that although the administration of HO inhibitor did not influence lung MPO activity in sham-operated animals, similar treatment of trauma-hemorrhage rats resulted in a significant increase in lung MPO activity after trauma-hemorrhage.

**HO-1 and lung CINC-1, CINC-3, and ICAM-1 levels.** As shown in Fig. 6, there was no significant difference in CINC-1, CINC-3, and ICAM-1 levels in sham-operated animals treated with either vehicle or the HO inhibitor CrMP. However, administration of CrMP resulted in significant increase in CINC-1, CINC-3, and ICAM-1 expression in the trauma-hemorrhage group (Fig. 6).

**DISCUSSION**

Our results indicate that at 2 h after trauma-hemorrhage lung MPO activity and CINC-1, CINC-3, and ICAM-1 levels are markedly increased in female diestrus, estrus, and postestrus and ovariectomized hemorrhaged rats. The above parameters after trauma-hemorrhage in proestrus females, which have the highest estradiol levels, were not significantly different from those in sham-operated animals. Furthermore, the level of HO-1 expression was also found to be highest in proestrus females after trauma-hemorrhage. The treatment of proestrus females undergoing trauma-hemorrhage with the HO-1 inhibitor CrMP resulted in an increase in lung MPO activity and levels of CINC-1, CINC-3, and ICAM-1 similar to those observed in non-proestrus and ovariectomized females. These findings therefore collectively suggest that the lack of alteration in lung MPO activity and CINC-1, CINC-3, and ICAM-1 levels in proestrus females after trauma-hemorrhage appears to be due to the upregulation of HO-1.

The lung is considered a critical organ in the development of the delayed organ dysfunction in patients suffering from traumatic injuries and severe blood loss (1). Multiple organ failure or dysfunction secondary to a systemic inflammatory response remains the major cause of mortality and morbidity after trauma (34). Neutrophils are the principal cells involved in host defense against acute bacterial and fungal infections (17), and thus these cells have a protective effect. However, under conditions such as those described in this study the infiltration of these cells can cause tissue damage (9, 20). Furthermore, neutrophil movement and migration are mediated by multiple adhesion molecules on neutrophils and endothelial cell surfaces and chemotactic factors. Initially, neutrophils interact...
CINC-1 levels correlate with tissue MPO activity, a marker of neutrophil content.

A growing body of evidence indicates that HO-1 expression is upregulated after hemorrhagic shock and that the HO product carbon monoxide plays a central role in the preservation of tissue microcirculation under such conditions (30, 35). In addition, induction of HO-1 has been shown not only to improve local tissue circulation but also to attenuate lung injury following hemorrhagic shock (11). It has also been reported that HO-1 can reduce the expression of adhesion molecules and may therefore also prevent subsequent leukocyte-endothelial cell interactions (25). Furthermore, our recent study (29) showed that inhibition of HO-1 prevents estrogen-induced improvement in organ function after trauma-hemorrhage. These findings therefore suggest that HO-1 plays an important role in improving organ functions after trauma-hemorrhage.

A previous study from our laboratory (13) also showed that females in the proestrus stage of the estrus cycle maintain cardiac and hepatic function after trauma-hemorrhage. In the present study, proestrus females were found to have the highest induction of HO-1 enzyme expression in the lung after trauma-hemorrhage. This upregulation in HO-1 was associated with attenuation of lung injury under these conditions. A number of studies have also shown that HO-1 is induced after various stressful conditions (27, 29, 35). In line with these findings, our results indicate a significant increase in HO-1 expression in diestrus, estrus, and metestrus phases of the reproductive cycle and in ovariectomized rats after trauma-hemorrhage compared with sham-operated rats. Studies have also indicated that the markedly increased HO-1 expression found in proestrus females improves liver function, and blockade of HO abolished these protective effects after trauma-hemorrhage (31). Thus the higher levels of HO-1 in proestrus females compared with the other trauma-hemorrhage groups were effective in protecting the host under these conditions. Our results also showed that increased lung CINC-1, CINC-3, and ICAM-1 levels and MPO activity were observed in all groups except the proestrus group after trauma-hemorrhage. However, treatment of animals with the HO inhibitor CrMP abolished the above effects in proestrus females after trauma-hemorrhage. These findings suggest that the protective effects in the proestrus rats were mediated via upregulation of HO-1 expression in the lung. Recent studies from our laboratory (29, 35) showed that estrogen administration after trauma-hemorrhage upregulates HO-1 expression and that treatment of animals with HO-1 inhibitor prevents estradiol- and flutamide-mediated salutary effects on organ function after trauma-hemorrhage. Consistent with these findings, we observed overexpression of HO-1 in females after trauma-hemorrhage. These findings therefore suggest that HO-1 plays an important role in improving organ functions after trauma-hemorrhage.

Studies have also shown that cardiac output and cardiac index are improved in proestrus females, but not in females during other phases of the reproductive cycle, after trauma-hemorrhage (13). Furthermore, the reduction of neutrophil accumulation after hemorrhagic shock correlated with the improvement of cardiac function (28). It is therefore possible that with endothelial selectins, resulting in neutrophils rolling along the endothelial surface. This rolling process appears to allow neutrophils to become activated (“primed”) by chemokines and other mediators secreted by the endothelium, resulting in their firm adhesion to endothelial adhesion molecules (10). Among adhesion molecules, ICAM-1 is an important mediator in the firm adhesion of neutrophils to the vascular endothelium and is strongly upregulated after trauma-hemorrhagic shock (35). With regard to chemokines, rat CINC-1 and CINC-3 are members of the IL-8 family and are potent chemotactic factors for neutrophils (9, 35). Chemotaxis of neutrophils is an important functional response to chemokines and is a key event in the recruitment of neutrophils at the site of inflammation. With the use of CINC antibodies, it was demonstrated that CINC-1 and CINC-3 contribute significantly to the influx of neutrophils in rat inflammation models including lung injury (21). Our previous study (32) also indicated that after trauma-hemorrhage.
the protective effect found in proestrus females may also be due to the improvement in cardiac function. Nonetheless, because proestrus females after trauma-hemorrhage had markedly upregulated lung HO-1 expression, it is also possible that the proestrus phase, i.e., estrogen, also has a direct protective effect on the lung. Additional studies are, however, needed to precisely elucidate the mechanism by which the proestrus phase attenuates lung injury after trauma-hemorrhage.

It could be argued that because the present study used measurement at a single time point, i.e., at 2 h after trauma-hemorrhage, it remains unclear whether similar effects of the estrus cycle are maintained for longer periods of time, i.e., beyond 2 h after trauma-hemorrhage. In this regard, our previous studies (6) showed that if improvement in organ functions by any pharmacological agent is evident early after treatment, those salutary effects are sustained for prolonged intervals and they also improve the survival of animals. Thus, although a time point other than 2 h was not examined in this study, on the basis of our previous studies it would appear that the salutary effects of the proestrus phase would be evident even if those effects were measured at another time point after trauma-hemorrhage and resuscitation.

It can also be argued that we should have examined the female reproductive cycle in sham-operated animals. In this regard, our previous study (13) showed that different female reproductive cycle phases did not produce any difference on organ function in sham-operated animals. Because the female reproductive cycle did not influence organ function in sham-operated animals, the female reproductive cycle in sham-operated animals was not examined in the present study.

In summary, our results indicate that the high estradiol level in the proestrus reproductive cycle phase is likely to upregulate HO-1 expression and attenuates lung injury after trauma-hemorrhage. Although the precise mechanism of the salutary effects of the highest estradiol level in proestrus females on organ functions and the contribution of HO pathways in reducing organ injuries following trauma-hemorrhage remain unclear, our study provides evidence that upregulation of HO-1 serves as a significant effector mechanism in the reduction of lung injury following trauma-hemorrhage. Furthermore, because high estradiol levels appear to be beneficial for lung function after trauma-hemorrhage, increases in estradiol plasma concentration in estrus females may be novel approaches for improving organ functions under such conditions.

GRANTS

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