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Original article

Intestinal inflammation enhances the development of egg white-induced anaphylaxis in Balb/c mice

L'inflammation intestinale favorise le développement de l'anaphylaxie induite par le blanc d'œuf chez la souris Balb/c

I. Bouchikhi^{1,*}, H. Grar², M. Guendouz³, C.E. Brahimi⁴, O. Kheroua⁵, D. Saidi⁶, H. Kaddouri⁷

Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria

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Abstract

Objective. – To date, there has been insufficient research on the role of intestinal inflammation in the development of food allergy. The present study investigated whether intestinal inflammation may elicit the development of systemic and intestinal hypersensitivity in egg white-sensitized mice.

Material and methods. – Female Balb/c mice were divided into four groups as follows: controls, treated with 4% dextran sulfate sodium (DSS) for 4 days, orally sensitized with egg white (EW), treated with 4% DSS and orally sensitized with EW. Ovalbumin (OVA)-specific and ovomucoid (OVM)-specific IgG and IgE were determined in mouse serum by enzyme-linked immunosorbent assay (ELISA). Anaphylactic symptom scores and body temperature were determined *in vivo* after oral challenge to EW. Secretory responses to OVA and OVM challenge were assessed *ex vivo* in jejunal tissue mounted in Ussing chambers by measuring short-circuit current and epithelial conductance. Intestinal inflammation was assessed by histopathologic examination.

Results. – Oral sensitization of DSS-treated mice resulted in high production of anti-OVA and anti-OVM ($P < 0.001$) specific IgG and IgE, marked anaphylactic reactions with a decrease in body temperature and a clear decrease in villus length ($P < 0.001$). Interestingly, we found that oral sensitization of DSS-treated mice promoted certain intestinal dysfunctions, as illustrated by increased secretory response and increased epithelial conductance ($P < 0.001$).

Conclusion. – These findings show that intestinal inflammation enhanced allergic sensitization to food allergens by inducing marked local and systemic hypersensitivity.

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Keywords: Dextran sulfate sodium; Ovalbumin; Ovomucoid; Anaphylactic response; Ussing chamber

* Corresponding author.

E-mail address: ibtissembouchikhi@gmail.com (I. Bouchikhi).

¹ I Bouchikhi is a Ph.D. student at Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria.

² G Hadria is a Post-doctoral at Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria.

³ M Guendouz is a Ph.D. student at Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria.

⁴ CE Brahimi is a Ph.D. student at Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria.

⁵ O Kheroua is a Head of Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria.

⁶ D Saidi is a Head Associate of the Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria.

⁷ H Kaddouri is a Head Associate of the Laboratory of the Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran 1 Ahmed Ben Bella, 31000 Oran, Algeria.

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Résumé

Objectif À ce jour, les recherches sur le rôle de l'inflammation intestinale dans le développement de l'allergie alimentaire sont insuffisantes. Cette étude examine le rôle de l'inflammation intestinale dans le développement d'une hypersensibilité systémique et intestinale chez des souris sensibilisées au blanc d'œuf.

Matériels et méthodes. – Des souris Balb/c traitées au dextran sulfate sodium à 4 % (DSS), sensibilisées par voie orale au blanc d'œuf (EW), traitées au DSS et sensibilisées au blanc d'œuf ont été étudiées. Les IgG et IgE spécifiques à l'ovalbumine (OVA) et à l'ovomucoïde (OVM) ont été déterminées par la méthode immunoenzymatique ELISA. Les symptômes anaphylactiques ont été déterminés *in vivo* après challenge oral au blanc d'œuf. La réponse sécrétoire à l'OVA ou l'OVM a été évaluée *ex vivo* sur des tissus jéjunaux montés en chambre de Ussing en mesurant le courant de court-circuit et la conductance épithéliale. L'inflammation intestinale a été évaluée par un examen histopathologique.

Résultats. – La sensibilisation orale des souris traitées au DSS entraîne une production élevée d'IgG et d'IgE spécifiques anti-OVA et anti-OVM ($p < 0,001$), des réactions anaphylactiques importantes associées à une diminution de la température corporelle ainsi qu'une diminution marquée de la hauteur des villosités ($p < 0,001$). De plus, la sensibilisation des souris traitées au DSS favorise le dysfonctionnement intestinal, comme le montre l'augmentation de la réponse sécrétoire et la conductance épithéliale ($p < 0,001$).

Conclusion. – Ces résultats indiquent que l'inflammation intestinale augmente la sensibilisation allergique aux allergènes alimentaires en induisant une hypersensibilité locale et systémique importante.

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Mots clés : Dextran sulfate sodium ; Ovalbumine ; Ovomucoïde ; Réponse anaphylactique ; Chambre de Ussing

1. Introduction

In recent decades, food allergies have appeared to increase at a dizzying rate, sparking a search for environmental factors possibly underlying this increase [1]. Symptoms of food allergy range from mild responses to life-threatening reactions, including anaphylaxis [2].

The development of food allergy is complex and multifactorial, with factors including ethnicity, genetics, microbial exposure (improved hygiene, antibiotic use, dog exposure), allergen exposure (timing and route of exposure, antacid use) [3].

Unfortunately, the contribution of barrier defects that contribute to the risk of developing food allergies has not been widely assessed. It is recognized that a primary defect of the intestinal barrier is not without consequences for the organism and can lead to excessive entry of dietary or microbe-derived macromolecules.

Several studies have shown that pro-inflammatory cytokines such as IL-1 β , TNF- α , and IFN- γ secreted during inflammatory response may cause an increase in intestinal tight-junction permeability, allowing increased tissue penetration of luminal antigens in inflammatory bowel disease (IBD) patients [4,5]. In some IBD patients, an increase in epithelial uptake of protein antigens, whether into endocytic cell compartments or into the cytosol via rapid antigen uptake into cytosol enterocytes (RACE) [6] or via endosomal uptake into ileal enterocytes, was found to be mediated by tumor necrosis factor TNF- α [7].

All of these studies suggested that intestinal inflammation is a causal factor for the development of food allergy. Indeed, the degree of overlap between allergic disorders and IBD is striking; in particular, both disorders feature histamine release and IgE overexpression [8].

Furthermore, Th2 immune response was also a common factor in both disorders. It has been shown that the immune phenotypes of IBD include Th2 lymphocyte activation, which plays

a key role in inflammation of the colonic mucosa [9]. On the other hand, activation of Th2 lymphocyte response was found to be necessary for the sensitization phase of food allergy [10]. However, there are conflicting results regarding the association of allergic diseases with intestinal inflammation. Some studies have reported a negative correlation [11], whereas others have noted a positive correlation [12,13], but direct evidence has not been fully demonstrated.

Based on the above information, we hypothesize that intestinal inflammation may promote the passage of antigens into the circulation and may lead to an allergic reaction characterized by allergen-specific IgE production and further anaphylactic reactions.

The present study was therefore designed to investigate whether intestinal inflammation could initiate allergic sensitization. We used Balb/c mice as a murine model of intestinal anaphylaxis to examine the potential effect of dextran sulfate sodium (DSS)-induced intestinal inflammation on food allergy development in mice orally sensitized with egg white.

2. Materials and methods

2.1. Chemicals

Egg white (EW), ovalbumin, ovomucoid, dextran sulfate sodium (40,000 kDa), biotinylated anti-mouse IgG, ExtrAvidin peroxidase, diamine-orthophenylene (OPD), Tween 20, potassium phosphate, di-potassium hydrogen phosphate, magnesium chloride, poly(vinyl alcohol) and H₂O₂ were purchased from Sigma (France). Calcium chloride, sulfuric acid and disodium hydrogen phosphate were purchased from Merck (France). Potassium chloride and sodium chloride were purchased from Prolabo (France). Monoclonal anti-IgE antibody was obtained from (BD Pharmingen, Europe).

All Ussing chamber equipment and materials were purchased from Physiologic Instruments (CA, USA).

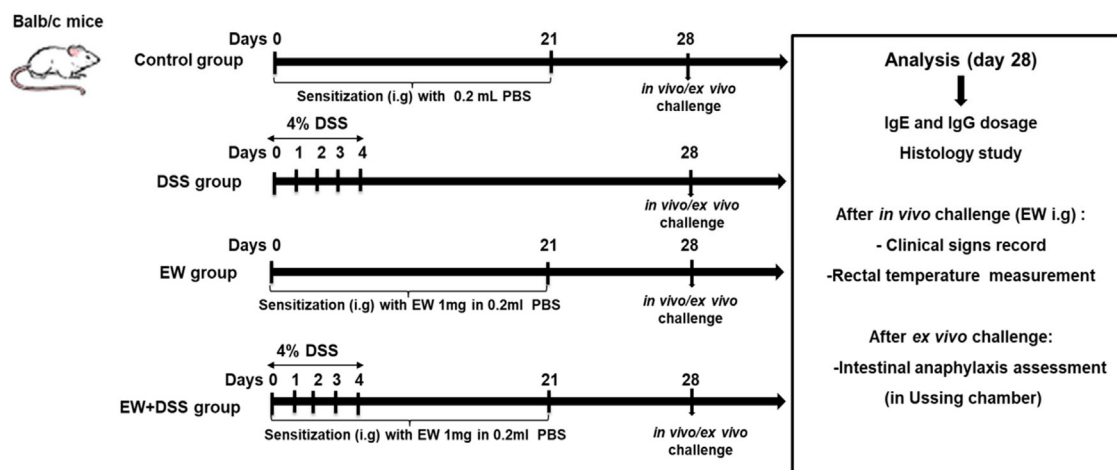


Fig. 1. Experimental inflammation induction, EW sensitization and challenge design.

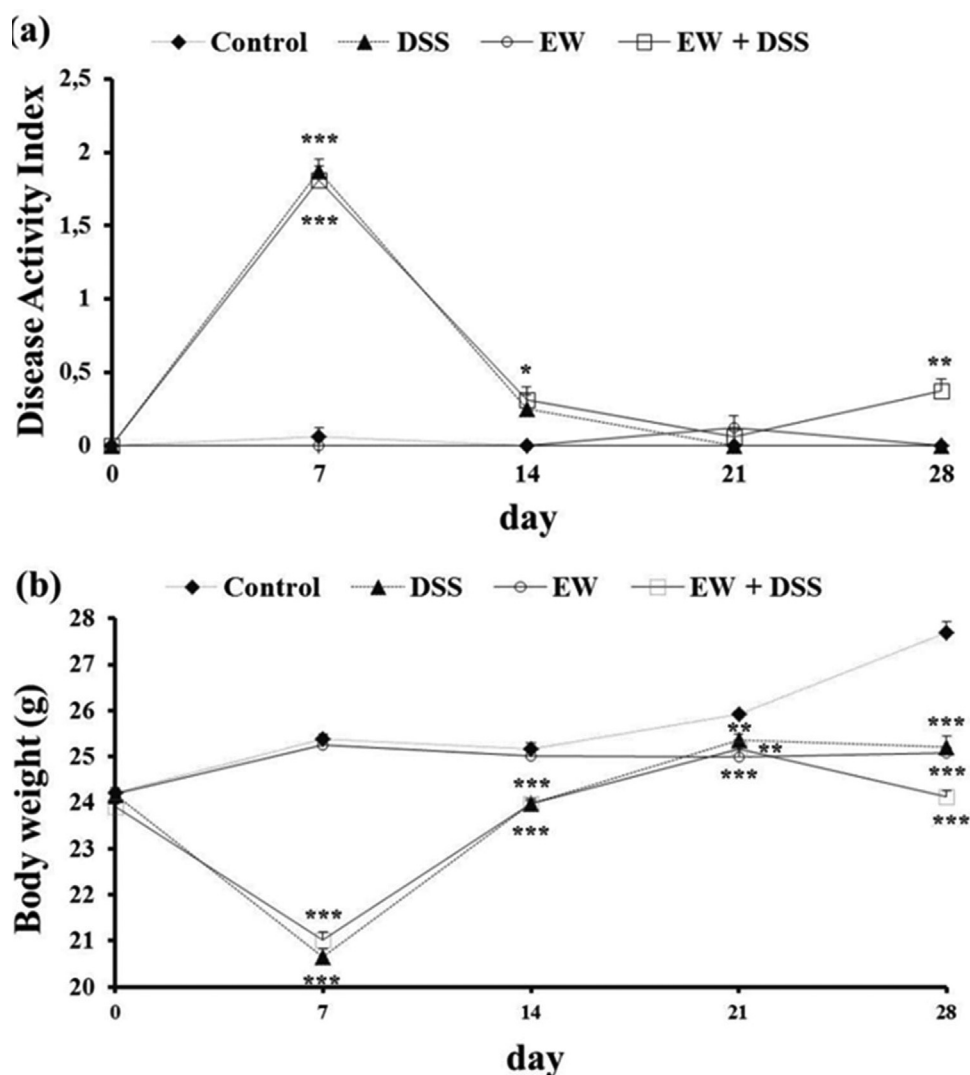


Fig. 2. Disease activity index (a) and body weight changes (b) in the different groups. Data are reported as mean $\pm$ SE (standard error) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control group; $n = 8$ per group).

2.2. Animals

Female Balb/c mice, 6–7 week old, weighing 24 ± 0.2 g, were purchased from the Pasteur Institute of Algiers in Algeria. The mice were acclimatized to the housing environment for one week prior to the study. The animals were kept in a controlled environment at 22°C with adequate humidity, and with 12-hour light/dark cycles.

All animals were handled in accordance with current Algerian legislation governing animal welfare.

2.3. Intestinal inflammation induction and sensitization protocol

Chemical-induced models of gut inflammation are the most commonly used and best described models of IBD [14]. In our study, induction of intestinal inflammation in mice via administration of dextran sulfate sodium (DSS) in drinking water was based on previous studies [15].

The mice were randomly divided into 4 groups of 12 animals: Group 1 (Control) received 0.2 ml PBS orally for 21 days; Group 2 (DSS) was provided with a solution of filtered water containing 4% DSS (w/v) *ad libitum* over a 4-day period, after which DSS was replaced by normal drinking water until the end of the experiment; Group 3 (EW) was sensitized orally with 1 mg of egg white in 0.2 ml PBS for 21 days; Group 4 (EW + DSS) was provided with a solution of filtered water containing 4% (DSS) over a 4-day period, and were sensitized orally with 1 mg of egg white in 0.2 ml PBS for 21 days (Fig. 1).

One week after the end of the experimental period (day 28), clinical signs were assessed in one subgroup of mice which were challenged orally with 10 mg egg-white/mouse.

The other subgroup was used to evaluate systemic antibodies, intestinal anaphylactic response in an Ussing chamber, and morphological intestinal changes by histological examination.

Blood samples were collected from the retro-orbital venous plexus and sera were stored at -20°C pending analysis.

2.4. Disease activity index (DAI)

Intestinal inflammatory reaction was characterized in all mice by daily assessment of: morbidity, body weight, stool consistency and presence of blood in stools with blinded evaluation using a previously reported scoring system [16].

2.5. Determination of anti-ovalbumin and anti-ovomucoid IgG and IgE by ELISA

The titer of mouse serum anti-ovalbumin (anti-OVA) and anti-ovomucoid (anti-OVM) IgG and IgE antibodies was determined by Enzyme-linked Immunosorbent Assay (ELISA). The ELISA protocol was that described in [17]. The microtiter plates were coated with OVA or OVM.

2.6. In vivo study: oral challenge to egg white

Anaphylactic symptoms were evaluated by analysis of the clinical signs obtained 30 min after oral challenge with 10 mg of egg white/mouse.

Shock severity was assessed by measuring rectal temperature with a rectal probe before and 30 min after challenge [18]. Symptoms were quantified using a previously-reported scoring system [19].

2.7. Ex vivo study: intestinal challenge to egg white with Ussing chamber assay

The effect of specific sensitization to EW on the intestinal response of mice treated with DSS was analyzed *ex vivo* by Ussing chamber assay. Measurement of mucosal electrophysiological parameters was performed essentially as described [20].

After a stabilization phase, the short-circuit current (I_{sc} , mA/cm^2 ; a measure of active ion transport) and conductance (G , mmho/cm^2 ; a measure of passive ion permeability) response to OVA or OVM was measured as the peak increase in I_{sc} or G within 10 min after addition of $60\text{ }\mu\text{g}/\text{mL}$ of OVA or OVM to the serosal buffer (in certain cases β -lactoglobulin was added as a non-specific antigen).

In some experiments, tissue was exposed to $10\text{ }\mu\text{M}$ of furosemide, an inhibitor of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. Furosemide was added to the serosal side 10 min before challenge with OVA or OVM. In order to evaluate tissue viability, glucose was added to the luminal bathing medium at a concentration of 10 mM at the end of each experiment.

2.8. Histology study

Segments of jejunum adjacent to those mounted in the Ussing chambers were processed for histological analysis.

Tissues were fixed in 10% formalin solution buffered at pH 7.2 and embedded in paraffin. Sections were cut into $5\text{-}\mu\text{m}$ slices and stained with hematoxylin. Villus length was measured using an optical microscope equipped with a micrometer.

2.9. Statistical analysis

The statistical differences between groups were determined using analysis of variance (ANOVA) or unpaired Student's *t*-test as appropriate. For non-parametric data, a Mann–Whitney test was used. Data are presented as mean $\pm$ standard error of mean (SEM). A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Disease activity index (DAI)

The DSS-treated group (DSS) and the egg white-sensitized group treated with DSS (EW + DSS) exhibited a marked increase in DAI score (Fig. 2a), characterized by progressive body weight

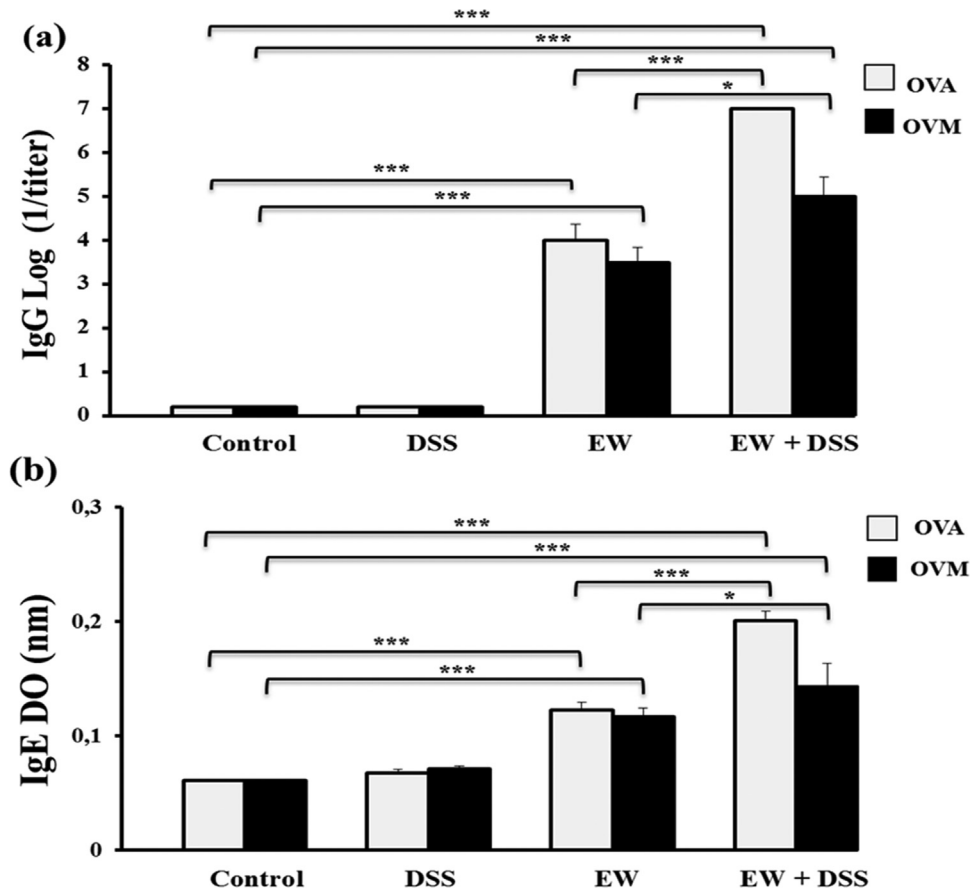


Fig. 3. Serum anti-OVA and anti-OVM IgG (a) and IgE (b) of the different groups. Data are reported as mean $\pm$ SE (standard error); $n = 6$ per group (* $P < 0.05$; *** $P < 0.001$).

loss (Fig. 2b), diarrhea from the second day, and severe diarrhea with blood from the third day of treatment.

The DAI scores peaked at day 6 of the study and then began to decrease. Body weight loss peaked at day 8 following the study, but by day 16 body weight had been regained.

At the end of the study, body weight loss and diarrhea were observed in the (EW + DSS) group.

None of the mice in the control group or sensitized (EW) group showed severe diarrhea or bloody stools at any time in the study.

3.2. Antibody response

Our results showed that anti-OVA and anti-OVM IgG (Fig. 3a) and IgE (Fig. 3b) were undetectable in the control group and the DSS-treated (DSS) group.

Compared to control mice, a clear increase in serum anti-OVA and anti-OVM IgG and IgE titers was detected in the (EW) and (EW + DSS) groups ($p < 0.001$).

However, this response was greater in the (EW + DSS) group (anti-OVA IgG $P < 0.001$; anti-OVM IgG $P < 0.05$), (anti-OVA IgE $P < 0.01$; anti-OVM IgE $P < 0.05$) compared with the (EW) group.

3.3. Anaphylaxis induction by oral egg white challenge

3.3.1. Clinical signs and body temperature

Clinical scores were severe, ranging from stage 3 to stage 4 in the (EW + DSS) group (Fig. 4a). However, the (EW) group showed less severe symptoms (from stage 1 to stage 2). No clinical signs were observed following EW challenge in the control or DSS groups.

Only the sensitized (EW) mice, whether or not treated with DSS (EW + DSS), showed a decline in body temperature 30 minutes after the oral challenge.

It should be noted that the drop in body temperature was more severe in the (EW + DSS) group (Fig. 4b). However, the control and DSS groups showed only a marginal decrease or even an increase in body temperature.

3.4. Ex vivo study of intestinal anaphylaxis: secretory responses to OVA or OVM challenge

In the Ussing chamber, stimulation with 60 $\mu\text{g/mL}$ of OVA or OVM added to the serosal buffer induced an increase in short-circuit current (I_{sc} , mA/cm^2 ; a measure of active ion transport) and conductance (G , mmho/cm^2 ; a measure of passive ion permeability) (Fig. 5a) and G (Fig. 5b) values in the jejunum of EW-sensitized mice (EW) (after serosal exposure to OVA ($\Delta I_{sc} = 12.37 \pm 1.93 \mu\text{A/cm}^2$

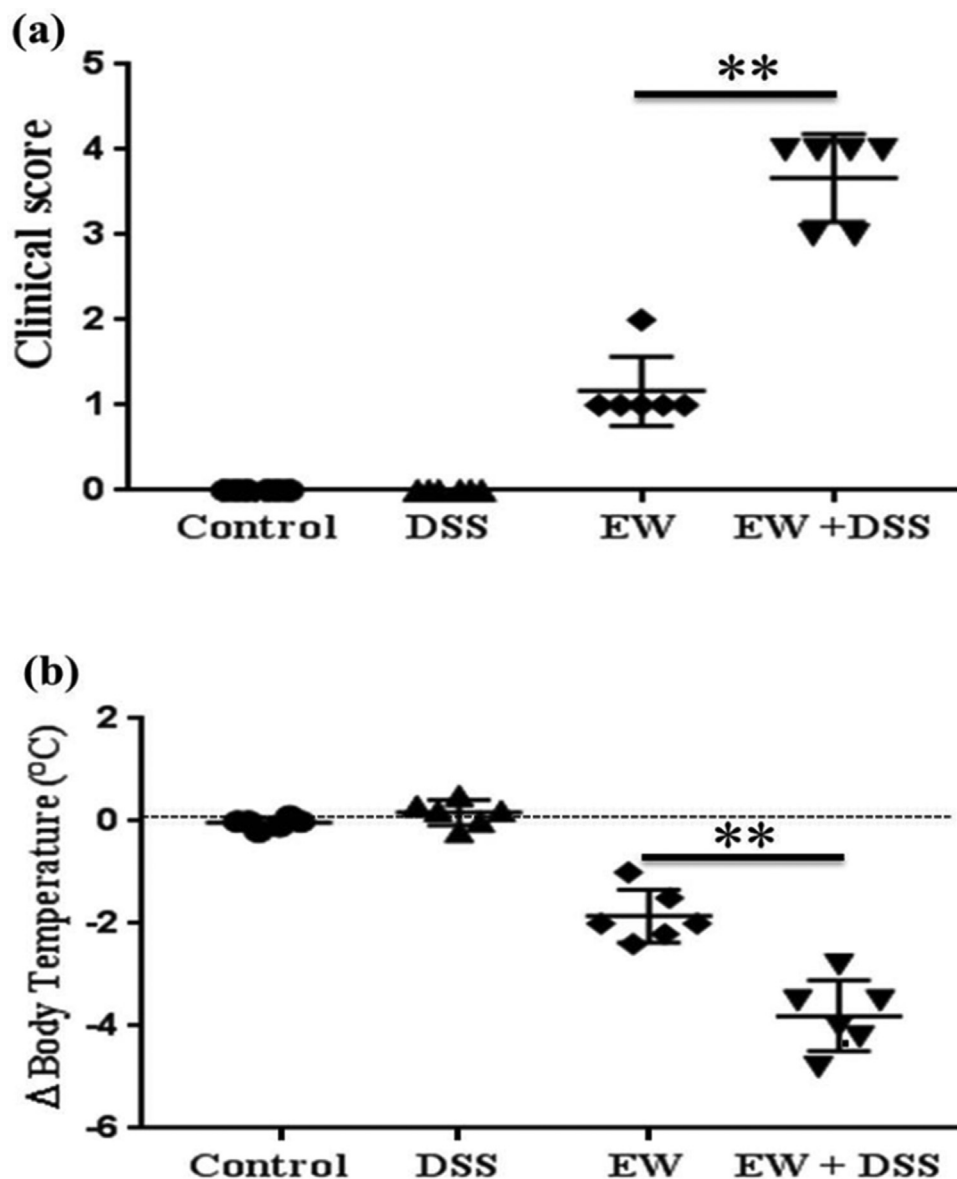


Fig. 4. Clinical scores (a) and body temperatures (b) in mice after EW oral challenge. Data are reported as mean $\pm$ SE (standard error) (***) $P < 0.001$ vs. control group; $n = 6$ per group).

$P < 0.001$, $\Delta G = 7.24 \pm 0.97$ mmho/cm² $P < 0.001$; after serosal exposure to OVM $\Delta I_{sc} = 10.78 \pm 0.84$ μ A/cm² $P < 0.001$, $\Delta G = 4.07 \pm 1.21$ mmho/cm² $P < 0.05$) compared to the control group.

These I_{sc} and G responses were greater in EW-sensitized mice treated with DSS (EW+DSS) (after addition of OVA $\Delta I_{sc} = 40.21 \pm 4.96$ mA/cm² $P < 0.001$, $\Delta G = 27.54 \pm 4.29$ mmho/cm² $P < 0.001$; after addition of OVM $\Delta I_{sc} = 34.99 \pm 4.73$ μ A/cm² $P < 0.001$, $\Delta G = 13.17 \pm 3.24$ mmho/cm² $P < 0.01$) on the serosal side.

The increase in I_{sc} induced by OVA (Fig. 6a) or OVM (Fig. 6b) challenge in jejunum was inhibited when tissue was pre-treated with furosemide. These results indicate that the increase in I_{sc} is due to electrogenic Cl^- secretion.

The introduction of glucose led to clear activation of I_{sc} , indicating that furosemide had no toxic effect on jejunum (Fig. 6).

To verify OVA-specific or OVM-specific response, the tissue was challenged with β -lactoglobulin, but no changes in I_{sc} values were observed (data not shown).

A slight but statistically significant increase in conductance was observed in DSS-treated mice (DSS) after addition of OVA ($\Delta G = 2.1 \pm 0.15$ mmho/cm², $P < 0.001$) or OVM ($\Delta G = 2.31 \pm 0.23$ mmho/cm², $P < 0.01$) compared to control mice (control).

3.5. Morphology

The microscopic images revealed clear villus atrophy and an increase in inflammatory infiltrate in the lamina propria in the (DSS) (Fig. 7b) and (EW) groups (Fig. 7c).

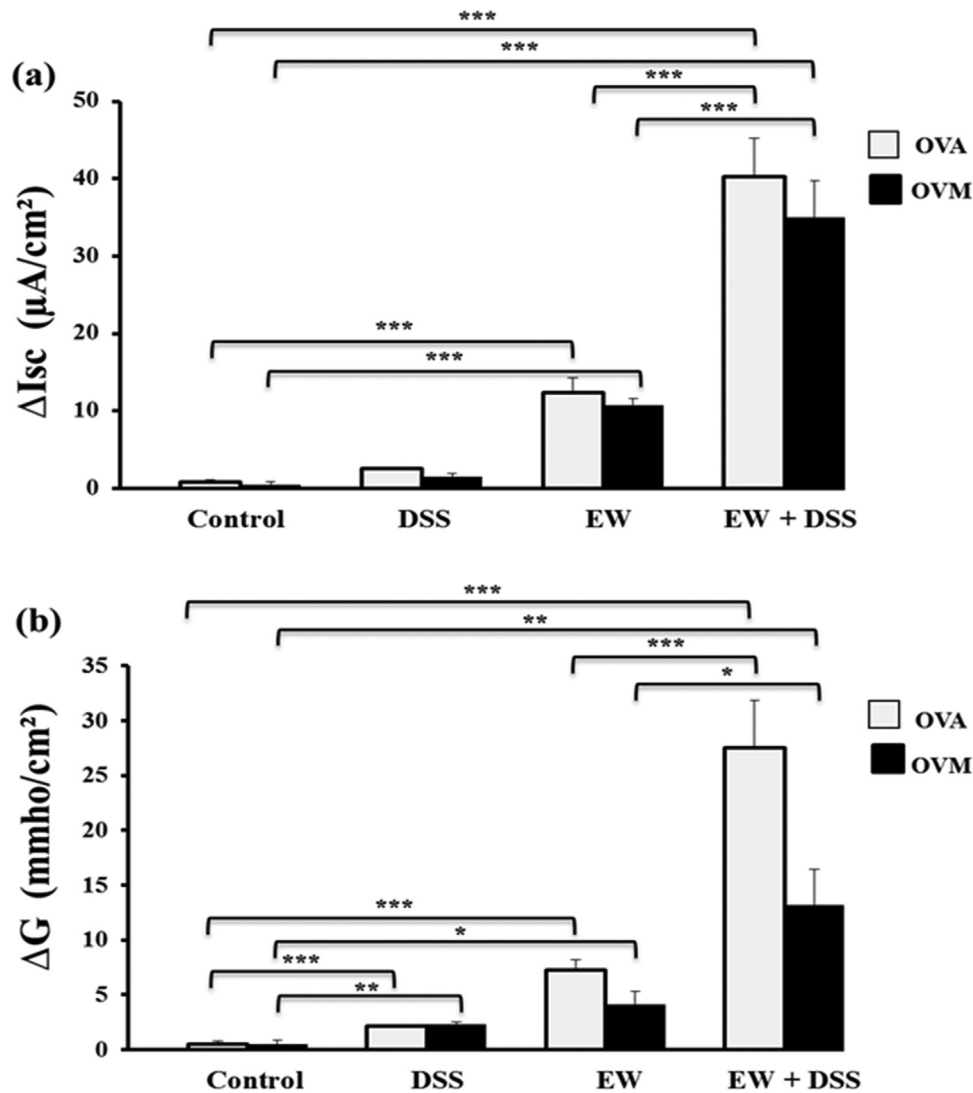


Fig. 5. Changes in I_{sc} (a) and conductance (b) values of jejuna mucosa of mice after *ex vivo* OVA or OVM challenge in an Ussing chamber. The increase in I_{sc} (ΔI_{sc}) and G (ΔG) is the difference between the peak value after OVA or OVM challenge and the baseline value. Data are reported as mean $\pm$ SE (standard error); $n = 6$ per group (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Interestingly, in the (EW + DSS) group (Fig. 7d), the intestinal mucosa presented major villus atrophy with severe intraepithelial lymphocytes infiltration and marked crypt hyperplasia.

The effects of DSS treatment and/or EW sensitization on villus length are shown in Fig. 8.

Villus length was significantly reduced in the (DSS) group ($P < 0.05$) and the (EW) group ($P < 0.01$) vs. controls. Additionally, sensitization of DSS-treated mice (EW + DSS) resulted in significantly lower villus length ($P < 0.001$) vs controls.

DSS-treatment of mice induced clear villus atrophy at day 8 with partial recovery at day 28 (data not shown).

4. Discussion

This study tested the hypothesis that intestinal inflammation may increase allergic sensitization to egg white proteins in Balb/c mice commonly used as an egg allergy model [18,21].

To directly test this hypothesis, we used egg white, which is considered an important source of allergens. The main allergic components found in egg white are ovalbumin (OVA or Gal d 2) and ovomucoid (OVM or Gal d 1) [22]. To mimic natural human exposure to food allergens, we used oral administration without adjuvants.

In this study, body weight and disease activity index score were used to assess intestinal inflammation.

The results showed a marked increase in DAI score characterized by gradual bodyweight loss in both DSS-treated mice (DSS) and EW-sensitized mice treated with DSS (EW + DSS), which peaked at day 8 and decreased thereafter. Similar results were reported in several previous studies [15,16].

Moreover, on the last day of study, we have noted body-weight loss associated with diarrhea in the (EW + DSS) group. Indeed, typical responses of the intestinal epithelium to allergens include an increase in watery diarrhea [23], which can result in significant weight loss.

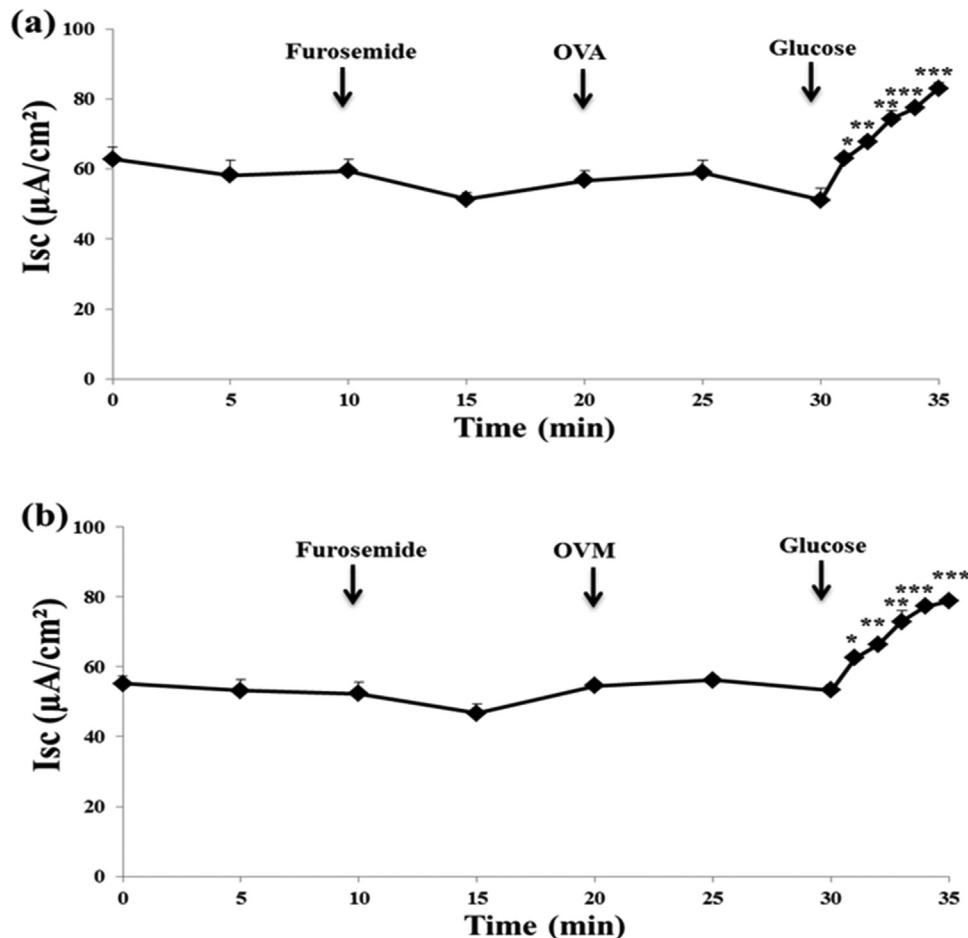


Fig. 6. Effect of furosemide on Isc values. Data are reported as mean $\pm$ SE (standard error). (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, where the P value represents the comparison before and after glucose addition; $n = 4$ per group).

In our study, allergic response was assessed by measuring serum anti-OVA- and OVM-specific IgG and IgE produced in EW-sensitized mice. The same increase in serum levels of IgG and IgE specific to OVA and OVM was observed in other reports in Balb/c mice orally sensitized to EW in the presence of adjuvant [21]. This response is probably due to the high resistance of OVA and OVM to the action of pepsin and pancreatic enzyme [24].

In the present study, antibody production in mice orally sensitized to EW and treated with DSS was greater than that of mice sensitized but not treated. Our results concur with those reported in other studies [12,13] in which elevated serum IgE levels and a higher frequency of positive reactions to food allergens were observed in IBD patients.

In animal models, the diagnosis and characterization of food allergy should reproduce both the specificity of the IgE response and the anaphylactic symptoms observed in allergic humans upon challenge testing [25].

In this study, the *in vivo* oral challenge with egg white induced significant clinical symptoms and hypothermia in the (EW) and (DSS + EW) groups.

In the (DSS + EW) group, diarrhea was also visually detected (data not shown). Rupa et al. (2014) reported that oral

administration of 20 mg EW induced clinical signs in EW/cholera toxin-sensitized mice [21].

The body temperature decrease noted in our study reflects the presence of clinical symptoms of shock accompanied by blood centralization. The same result was obtained by Ahrens et al. (2012) after allergen challenge in sensitized mice [18].

In the intestine, antigen cross-links IgE antibodies bound to mast cells releasing mediators that act on epithelial receptors to enhance permeability of the paracellular pathway and induce secretion of ions such as Na^+ and Cl^- into epithelium, leading to diarrhea [18,23].

The most well-established method for measuring electrolyte transport across epithelial tissue and assessing intestinal barrier function is the Ussing chamber technique [26]. Short-circuit current (Isc), transepithelial voltage potential (V_t) and conductance (G) measurements in Ussing chamber studies have shown that electrogenic Cl^- secretion is not only important in normal digestive physiology, but that it is also a marker of enterotoxic and inflammation-mediated secretory diarrhea [26].

Our results showed that stimulation of the jejunum with 60 μg OVA or OVM induced a significant increase in short-circuit current (a measure of active ion transport).

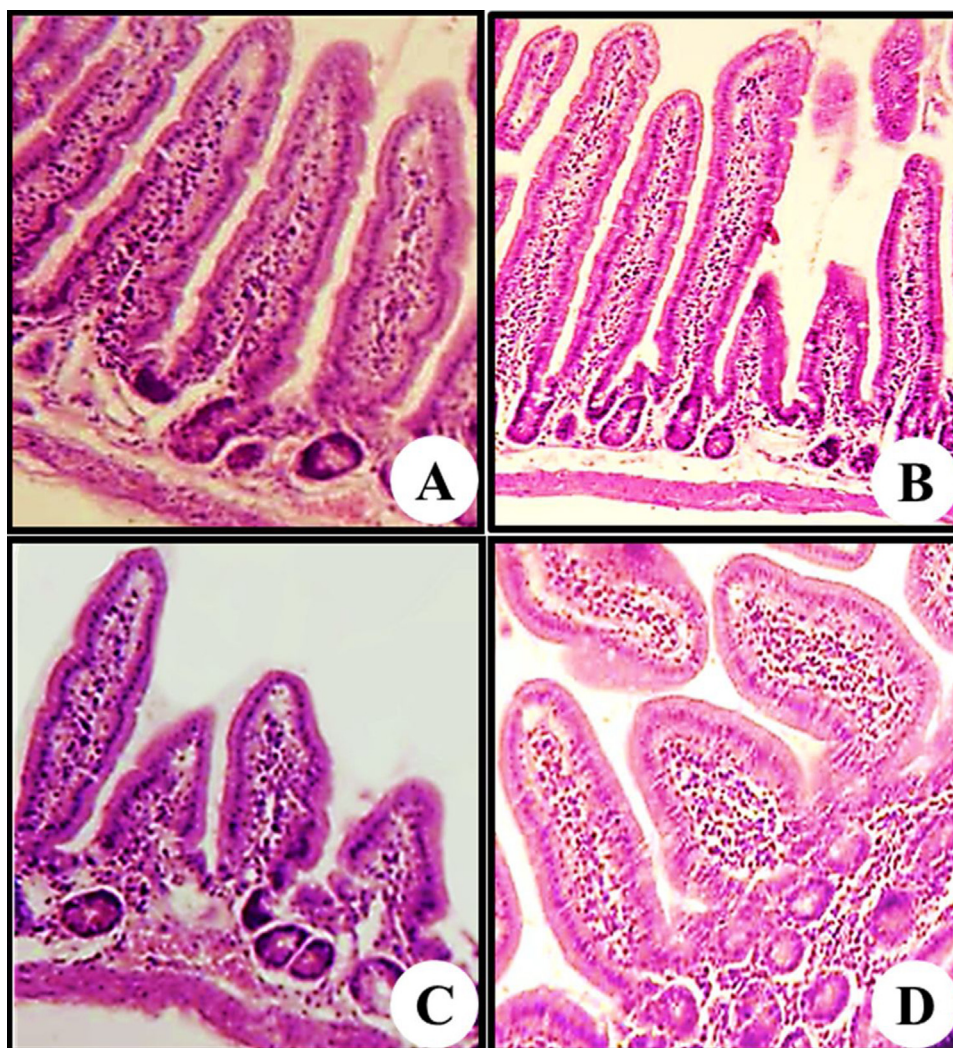


Fig. 7. Light microscopy ($G \times 40$) showing intestinal villi stained with hematoxylin-eosin. Jejunal tissue was obtained from unsensitized mice (controls) (a), mice treated with DSS (DSS) (b), egg white-sensitized mice (EW) (c) and egg white-sensitized mice treated with DSS (EW + DSS) (d).

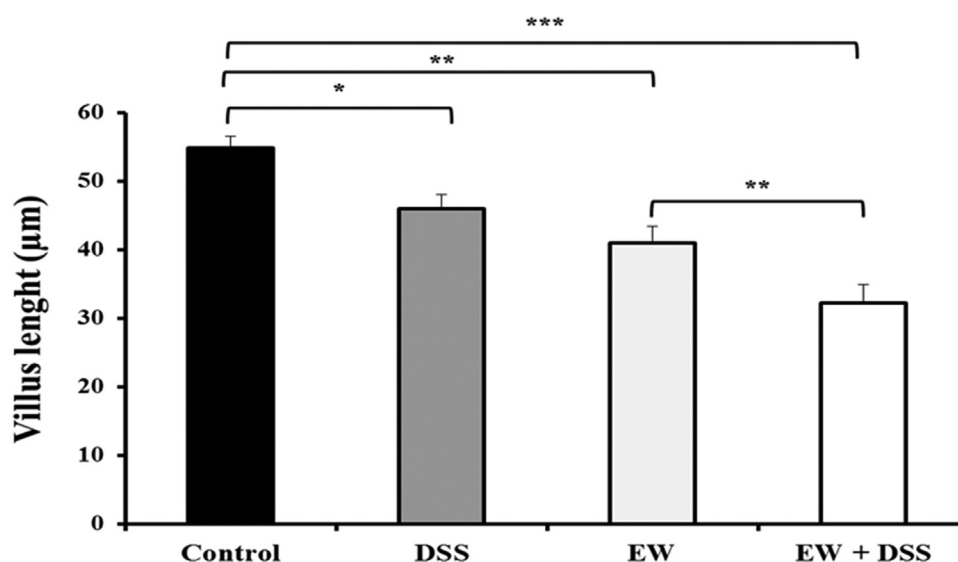


Fig. 8. Evaluation of villus length of jejunum fragments for the different groups. Data are reported as mean $\pm$ SE (standard error); $n=6$ per group (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

This increase in *I*_{sc} was totally inhibited by furosemide, which acts as an inhibitor of Na⁺/K⁺/2Cl[−] cotransporter, causing a decrease in Cl[−] secretion [27].

These findings suggest that the variation in *I*_{sc} values was essentially due to Cl[−] secretion induced by histamine release.

Our results are concordant with those reported in the literature and show a significant increase in *I*_{sc} following contact of a sensitizing antigen with intestinal fragments in an Ussing chamber [20,28].

The increase in *I*_{sc} response was more pronounced in the (EW + DSS) group, suggesting marked anaphylactic response, probably due to extensive secretory response.

Our results show that the increase in *I*_{sc} was correlated with a significant increase in tissue conductance (a measure of passive ion permeability), implying the occurrence of a local anaphylactic reaction on interaction between OVA or OVM and the intestinal immune system of EW orally-sensitized mice and indicating that sensitization affects the tight junction and increases intestinal epithelial conductance.

Interestingly, the (DSS + EW) group showed greater increase in conductance vs. the (EW) group.

These data are probably due to marked impairment of tight junctions due to extensive local anaphylactic reaction and DSS treatment.

Histological analysis showed partial morphological changes (villous atrophy and an increase in inflammatory infiltrate) in epithelium for the (DSS) group. The mechanism by which DSS induced jejunum inflammation could not be explained in the present study. However, it was shown that DSS-induced damage is not restricted to the colon but also extends to the small intestine [29].

The morphological changes observed in the epithelium of sensitized mice were probably due to immunologic reaction. Hiraide et al. (2017) reported similar results for mice sensitized with egg white [30].

Moreover, the intestinal mucosa of the (DSS + EW) group presented major villus atrophy with severe intraepithelial lymphocytic infiltration and marked crypt hyperplasia. This result confirmed the presence of extensive inflammation, probably due to DSS treatment, associated with strong allergic response to EW.

5. Conclusion

Our study shows for the first time that intestinal inflammation enhances development of immune response to food allergens by inducing marked production of specific IgE, strong intestinal anaphylactic response and enteropathy. Consequently, measurement of antigen permeability (uptake) *in vivo* and study of the cellular and molecular mechanisms involved in the allergic response would be of great interest.

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Disclosure statement

The authors declare that they have no competing interest.

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