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Preliminary report

The role of kinin B_1 and B_2 receptors in the mouse model of oxazolone-induced atopic dermatitis

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A B S T R A C T

This study evaluated the role of kinin B_1 and B_2 receptors in the pre-clinical mouse model of oxazolone-induced atopic dermatitis. The B_1 R715 or B_2 HOE140 receptor antagonists were dosed at different schemes of treatment. After assessment of clinical lesion scores and pruritus, lesional skin samples were collected for histopathological analysis. The plasma extravasation and the expression of the metalloproteinase ADAMTS5 were also assessed. The immunopositivity for kinin receptors was evaluated in the skin, dorsal root ganglion (DRG), thoracic spinal cord and brain cortex sections. Marked upregulation of B_1 and B_2 receptors was observed in the skin of oxazolone-treated mice. The induction of atopic dermatitis led to a downregulation of both receptors in the DRG, without any alteration in the spinal cord and brain cortex. The repeated administration of HOE140 (50 nmol/kg; i.p.) partially inhibited the oxazolone-related pruritus, associated with a reduction of ADAMTS5 immunolabelling in the skin. Alternatively, R715 (438 nmol/kg; i.p.) produced a mild inhibition of plasma extravasation in oxazolone-challenged mice. Noteworthy, the repeated i.d. injection of R715 (30 nmol/site) or HOE140 (3 nmol/site) significantly reduced the histiocyte numbers, according to the histopathological analysis. Either B_1 or B_2 kinin antagonists, irrespective of the protocol of treatment, did not alter any other evaluated clinical or histological parameters. Data brings novel evidence about the role of kinin receptors in allergy-related conditions, such as atopic dermatitis. Further studies to test different protocols of treatment with kinin antagonists on in-depth cellular alterations underlying oxazolone-induced atopic dermatitis remain to be performed.

1. Introduction

Bradykinin (BK), and the related family of peptides, are a group of mediators implicated in a series of pathophysiological responses, which are produced in plasma and tissues by the action of kallikreins. Most of their effects are mediated via the activation of two G protein-coupled receptors: the housekeeping B_2 and the inducible B_1 [1,2]. The participation of BK and its receptors has been widely investigated in allergic conditions, such as rhinitis, pruritus, asthma, and anaphylaxis [3–7]. Noteworthy, the selective peptide B_2 receptor antagonist HOE140 (Icatibant) is clinically approved for the management of acute attacks of hereditary angioedema, in which BK mediates endothelial activation and edema, via histamine-independent mechanisms [8].

With respect to skin disorders, it has been demonstrated that BK application into the lesional skin of patients with atopic dermatitis elicited a marked pruritic sensation, in comparison to minor effects in healthy individuals [9]. Moreover, the administration of the selective non-peptide B_1 SSR240612 or B_2 FR173657 receptor antagonists efficiently reduced the itching sensation caused by trypsin injection into the mouse dorsal skin [4]. Liang et al. [10] showed that BK-elicited pruritus in complete Freund's adjuvant (CFA)-inflamed skin was prevented by the selective blocker of B_1 receptors des-Arg^9[Leu^8]-BK, whereas the peptide B_2 antagonist (D-Arg-[Hyp^3,D-Phe^7]-BK) enhanced the scratching bouts. Alternatively, B_2 receptor inhibition by HOE140 or FR173657 significantly reduced the pruritus caused by the dorsal injection of the bile salt deoxycholic acid in mice, without any significant effect for the B_1 antagonist des-Arg^9[Leu^8]-BK [11]. Nonetheless, the itching response secondary to the induction of contact dermatitis by diphenylcyclopropenone (DCP) was reduced by the B_1 receptor antagonist R892, whilst it was unaffected by the B_2 antagonist HOE140 [12]. Considering this controversial series of data, further studies to determine the role of kinin receptors in pruritus and chronic

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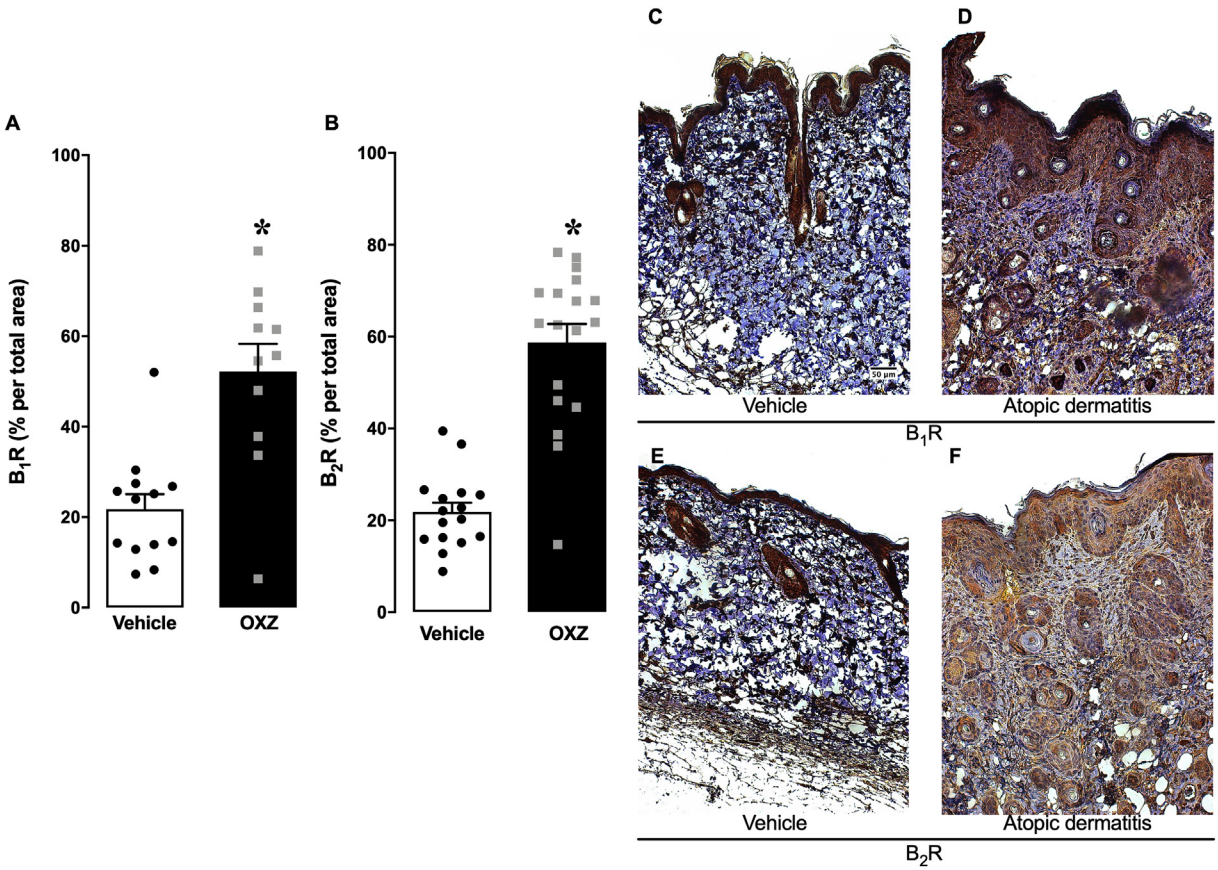

Fig. 1. Skin expression of kinin receptors in the mouse model of atopic dermatitis induced by oxazolone. Immunohistochemical analysis of kinin B_1 (A) or B_2 (B) receptors in the dorsal skin of vehicle and oxazolone-treated mice. $n = 11–18$ mice per group, $^*P < 0.05$ vs vehicle-treated group (unpaired Student *t*-test). Representative images of B_1 (C-D) and B_2 (*E*-F) receptor immunostaining in the skin. Original magnification ×200, scale bar (—) 50 µm. (OXZ; oxazolone).

skin inflammation are still required.

The present study evaluated, for the first time, the effects of selective B_1 or B_2 receptor antagonists in the clinical and microscopic alterations associated with a mouse model of atopic dermatitis elicited by oxazolone. We also evaluated whether the induction of atopic dermatitis by oxazolone might modulate the central and peripheral expression of kinin receptors, via immunohistochemistry assessment.

2. Materials and methods

2.1. Animals and reagents

The experimental protocols followed the current Brazilian guidelines for the care and use of animals for scientific and didactic procedures, from the National Council for the Control of Animal Experimentation (CONCEA, Brazil, 2014). The local Animal Ethics Committee evaluated and approved all the protocols (CEUA/PUCRS 15/00489). Male CF-1 specific-pathogen-free mice (2-month old, 18–20 g at the beginning of experiments, $N = 184$) were obtained from the Centre for Experimental Biological Models (CeMBE/PUCRS, Porto Alegre, RS, Brazil). The number of animals per group (*n*) is indicated in each legend to Figure. The mice were maintained in micro-isolator cages (4 per cage), equipped with inlet/outlet air filters, under controlled temperature (22 ± 1 °C) and humidity (50–70%), and a light-dark cycle of 12 h (lights on at 7 a.m., lights off at 7 p.m.). The cages were filled with autoclaved wood chip bedding. The animals received pelleted feed and sterile water ad libitum. The euthanasia was performed by sevoflurane inhalation.

Oxazolone was purchased from Sigma Aldrich Chemical Company (St. Louis MO, USA). The selective kinin B_1 and B_2 receptor antagonists, R715 (Ac-Lys-Arg-Pro-Pro-Gly-Phe-Ser-DβNal-Ile) and HOE140 (D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg), respectively, were purchased from Tocris Bioscience (Bristol, UK). Oxazolone was diluted in absolute acetone at the desired concentration just before using. The stock solutions of the kinin antagonists were prepared in phosphate-buffered saline (PBS) in siliconized plastic tubes, maintained at −20 °C, and diluted to the desired concentration just before use. All the other chemical reagents were of analytical grade.

2.2. Oxazolone-induced atopic dermatitis model

The protocols of sensitization and challenge with oxazolone were accomplished according to the methodology described by Tsukumo et al. [13], with minor modifications (Fig. 4 A-C). For the sensitization protocol (day −7), the nape of the neck was shaved 48 h before, and the animals received an epicutaneous application of 0.5% oxazolone solution (10 µl/site). Seven days later (day 0), the animals were shaved again, and the protocol of challenge was initiated. For this purpose, 10 µl of 0.5% oxazolone solution was applied at the same site, at 2–3-day intervals for 16 days (days 2, 4, 7, 9, 11, 14 and 16). In the control group, PBS was applied at the same region and intervals of time. After this period, the scratching behavior and the lesion scores were registered, and the animals were immediately euthanized for sample collection. Independent investigators made all the analysis, in a blinded manner.

2.3. Protocols of treatment with the kinin antagonists

To assess the acute effects of kinin receptor inhibition on the scratching behavior associated with oxazolone-induced atopic

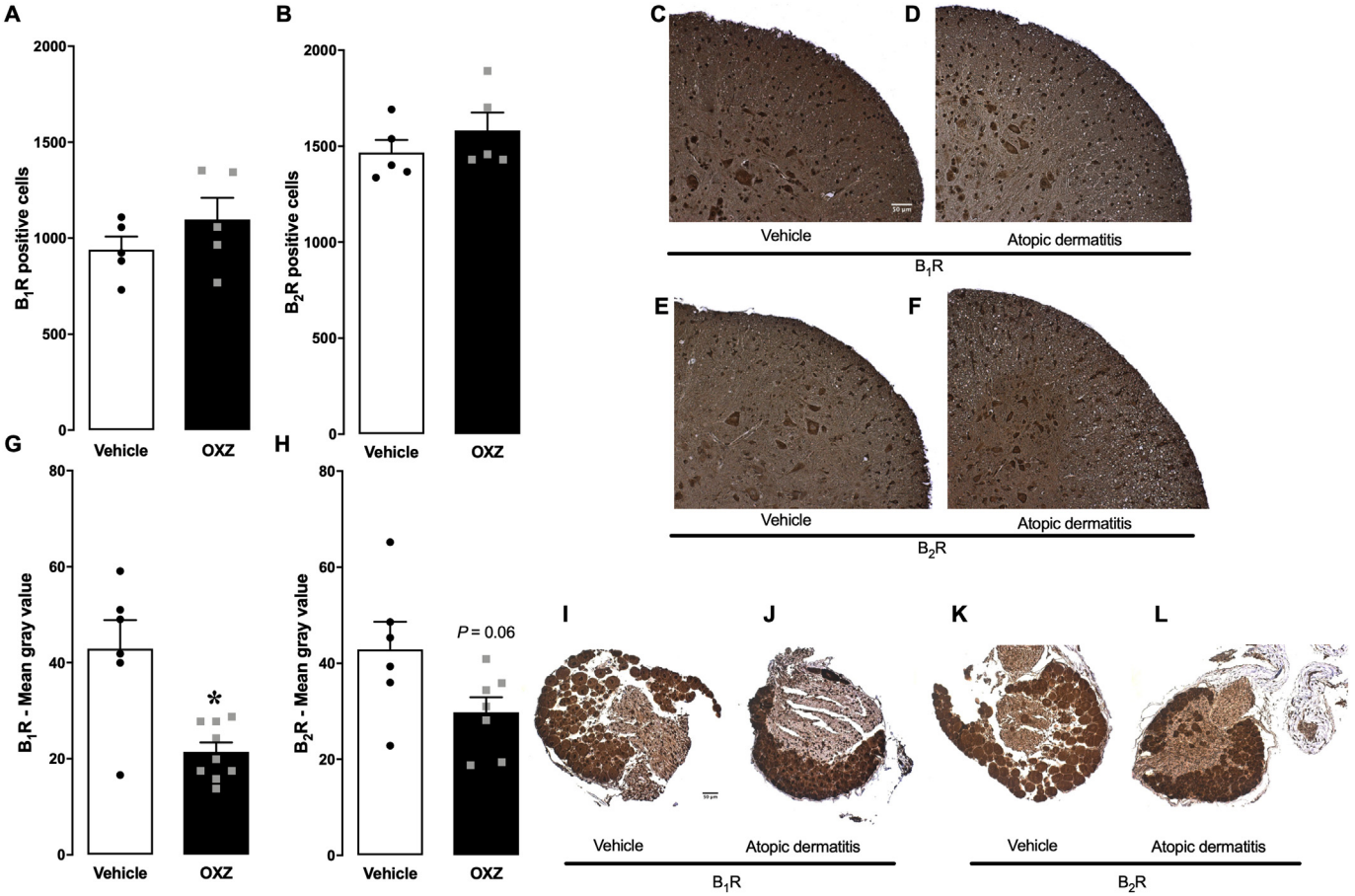

Fig. 2. Expression of kinin receptors in the thoracic spinal cord and DRG of mice submitted to the mouse model of atopic dermatitis induced by oxazolone. Immunohistochemical analysis of kinin B_1 and B_2 receptors in the thoracic spinal cord (A-B) or DRG (G-H) of the vehicle and oxazolone-treated mice. $n = 5–9$ mice per group, *$P < 0.05$ vs vehicle-treated group (unpaired Student t-test). Representative images of B_1 (C, D) and B2 (E, F) receptor immunolabelling in the thoracic spinal cord. Representative images of B_1 (I-J) and B_2 (K-L) receptor immunopositivity in the DRG. Original magnification ×200, scale bar (—) 50 µm. (OXZ; oxazolone).

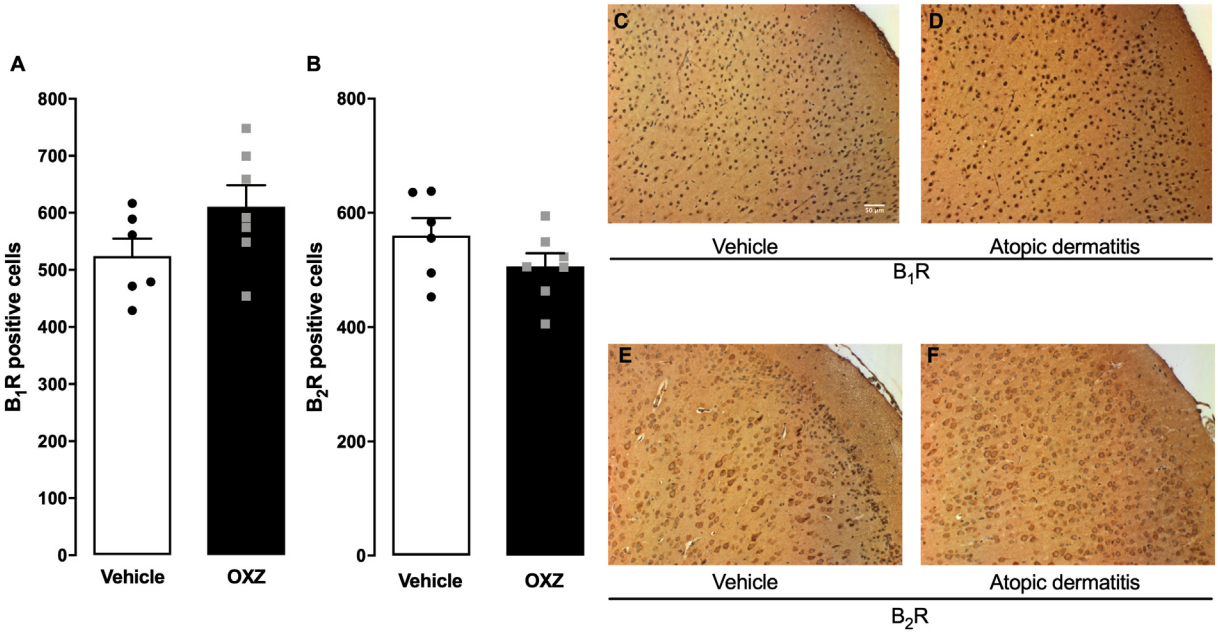

Fig. 3. Expression of kinin receptors in the brain cortex of mice submitted to the mouse model of atopic dermatitis induced by oxazolone. Immunohistochemical analysis of B_1 (A) or B_2 (B) receptors in the brain of vehicle and oxazolone-treated mice. $n = 6–7$ mice per group, $P > 0.05$ (unpaired Student *t*-test). Representative images of B_1 (C–D) or B2 (E–F) receptor immunopositivity in the brain. Original magnification ×200 scale bar (—) 50 µm. (OXZ; oxazolone).

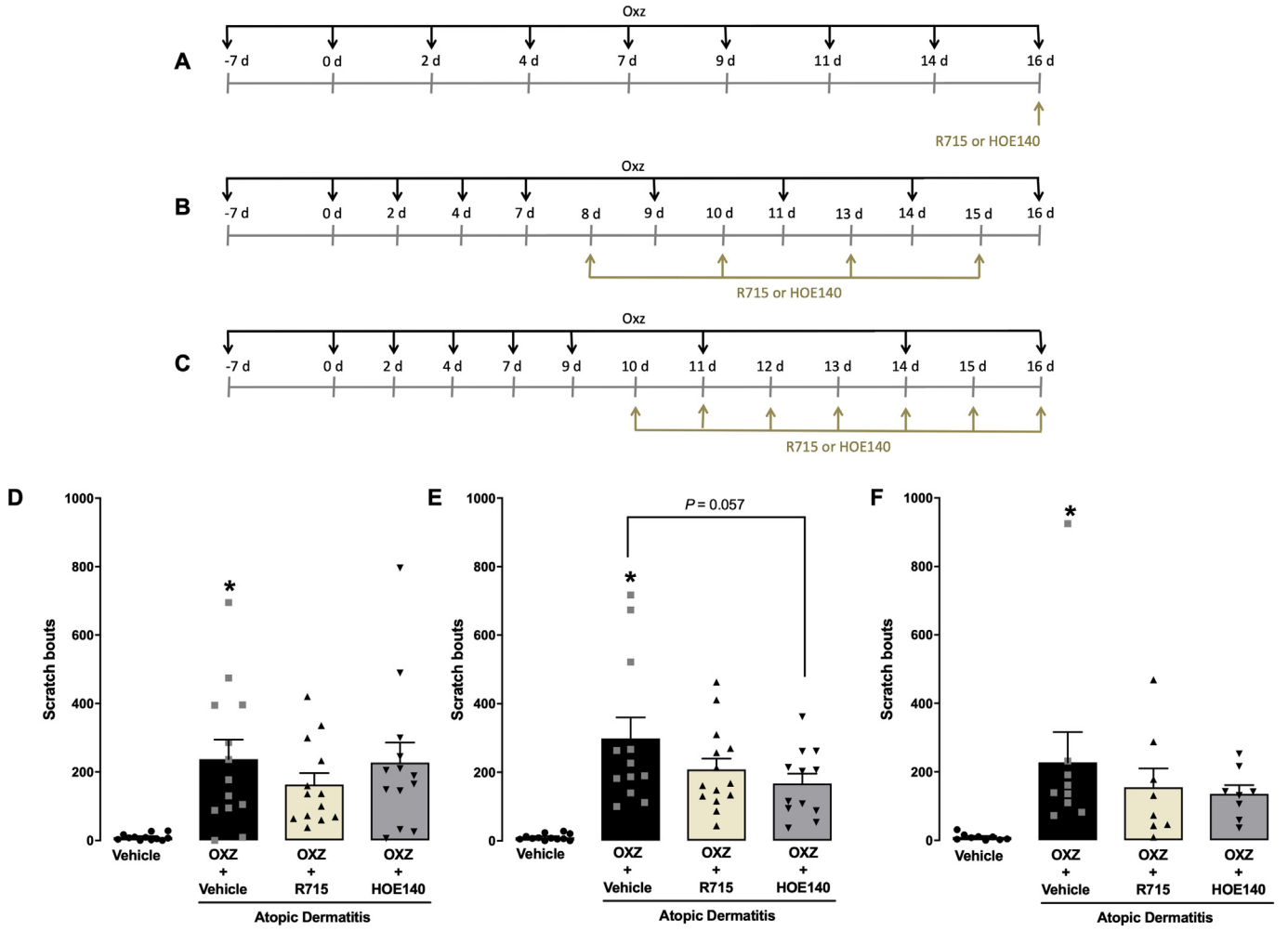

Fig. 4. Effects of kinin antagonists on the scratching behavior in the oxazolone-induced atopic dermatitis model. Schematic protocol timeline for the acute treatment (A; i.p.), or repeated treatment by intraperitoneal (B; i.p.), and by intradermal (C; i.d.) routes. (D) Effects of the acute treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given 30 min before pruritus assessment. (E) Effects of the repeated treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given on days 8, 10, 13 and 15 prior pruritus assessment. (F) Effects of the repeated local treatment with the selective B_1 R715 (30 nmol/site; i.d.) or B_2 HOE140 (3 nmol/site; i.d.) receptor antagonists, given from day 10 to 16, before pruritus assessment; the last injection was administered 5 min prior to the last oxazolone application. n = 8–14 mice per group, $^*P < 0.05$ vs vehicle-treated group (one-way ANOVA followed by Bonferroni's post hoc test). (OXZ; oxazolone).

dermatitis, separate groups of animals received the selective B_1 R715 (438 nmol/kg) or B_2 HOE140 (50 nmol/kg) receptor antagonists in a single dose, administered by intraperitoneal (i.p.) route, immediately before the last application of oxazolone (day 16; Fig. 4A). Next, to evaluate whether the repeated systemic treatment with the kinin antagonists might prevent the pruritic and the inflammatory changes elicited by oxazolone, different experimental groups received R715 (438 nmol/kg; i.p.) or HOE140 (50 nmol/kg; i.p.), dosed one day before each last four applications of oxazolone (days 8, 10, 13 and 15; Fig. 4B). Additionally, to assess the local effects of the kinin antagonists, mice received R715 (30 nmol/site) or HOE140 (3 nmol/site), injected intradermally (i.d.), near to the site of oxazolone application, every day, from day 10 to 16, after beginning the challenge phase of atopic dermatitis induction (Fig. 4C). The control groups received PBS at the same time points. The doses of kinin antagonists were chosen based on the literature data [14–17].

2.4. Scratching bouts

On day 16, the animals were individually placed into glass cylinders 20 cm in diameter, for 30 min, in order to acclimatize them to the environment. After 30 min of adaptation, the last application of 0.5% oxazolone solution or vehicle was performed. The mice were returned to the cylinders for an additional period of 30 min. Immediately after, the number of scratching bouts with both forepaws and hindpaws close to the injected site was registered during 60 min. Scratching behind the ears, but not on the face, was also counted [18]. The itching behavior was measured in animals pre-treated with the kinin antagonists, in acute or repeated schedules of administration, as described above (item 2.3.).

2.5. Clinical lesion scores

The clinical changes secondary to the induction of atopic dermatitis by oxazolone were evaluated by using the score described by Kang et al. [19]. After the registration of scratching bouts, the lesions were classified as: (0) no visible inflammatory changes; (1) mild inflammation, with dryness and scaling; (2) moderate inflammatory changes, characterized by dryness, scaling and erosion; (3) middle inflammation, with the presence of dryness, scaling, erosion and excoriation; (4) severe inflammatory response, with all the alterations described for the score 3, plus hemorrhage. The groups of animals that had been repeatedly treated with the kinin antagonists, by i.p. or i.d. routes, were included in this analysis.

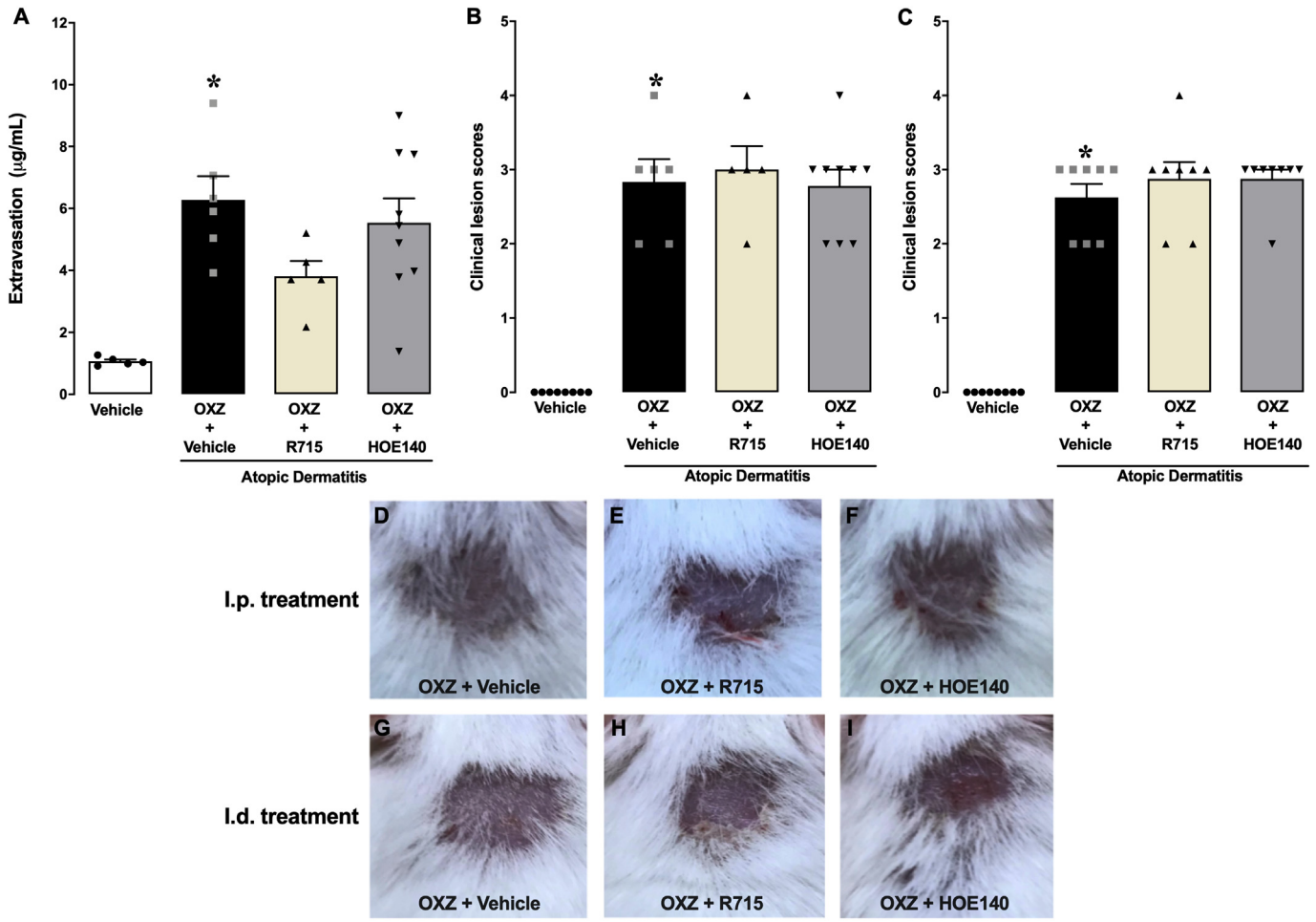

Fig. 5. Effects of kinin antagonists on plasma extravasation and skin alterations in oxazolone-induced atopic dermatitis in mice. Effects of systemic repeated treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given on days 8, 10, 13 and 15, on skin plasma extravasation (A) and lesion severity scores (B). Effects of the local repeated treatment with the selective B_1 R715 (30 nmol/site; i.d.) or B_2 HOE140 (3 nmol/site; i.d.) receptor antagonists, given on days 10 to 16, in the dermatitis score (C). $n = 5$–9 mice per group, *P $<$ 0.05 vs vehicle-treated group (one-way ANOVA followed by Bonferroni's post hoc test). (D–I) Representative images of the clinical appearance of the skin. (OXZ; oxazolone).

2.6. Plasma extravasation

The method used was similar to that described by Pereira et al. [20]. Briefly, the animals submitted to the i.p. repeated treatment with the kinin antagonists, or the respective controls, received an intravenous injection of Evans blue dye solution (25 mg/kg), one h after the last application of oxazolone or vehicle, via the infraorbital plexus, under a slight inhalatory anesthesia with sevoflurane/oxygen. After 30 min, the mice were euthanized and the dorsal skin was removed and incubated in formamide (55 °C, overnight). The dye concentration was estimated based on a standard curve (0.5 to 50 µg/ml), at 595 nm (SpectraMax M2, Molecular Devices, San Jose, CA, USA).

2.7. Skin collection and general histological procedures

For the repeated treatment groups, after the lesion scoring, the animals were euthanized and the skin region corresponding to the site of oxazolone application was collected and immediately fixed in 10% formaldehyde, for 24 h. For the histochemical procedures, consecutive transversal 4-µm-thick sections were obtained from paraffin blocks. The sections were mounted on poly-L-lysine-coated glass slides for posterior analysis.

2.8. Histopathology

The skin histological sections were stained with hematoxylin and eosin (H&E) or toluidine blue (0.04% in acetate buffer; pH 4.0) for further analysis of atopic dermatitis severity, under the repeated treatment with the kinin antagonists [21–23]. The histological images were obtained with a microscope (Axio Imager A1) coupled to an image capture system (Axio Vision Rel. 4.4 Software Multimedia), from Carl Zeiss (Hallbergmoos, Germany). The areas (in mm^2) of stratum corneum, epidermis and dermis were measured in H&E-stained slides, under ×100 magnification, by using the NIH Image J 1.50 g Software. The presence of rete ridges and the follicular inflammation was evaluated at ×200 magnification. The dermal numbers of eosinophils, lymphocytes and histiocytes were also evaluated in H&E-stained sections, at ×400 magnification. Toluidine blue-stained sections were used to determine the numbers of mast cells in dermis, at ×400 magnification.

2.9. Immunodetection of ADAMTS5

The skin immunopositivity for the metalloproteinase ADAMTS5 was evaluated according to the method described by Togni et al. [24]. The polyclonal rabbit anti-ADAMTS5 (1:200; sc-83,186, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody, and a three-step avidin-biotin complex staining procedure was carried

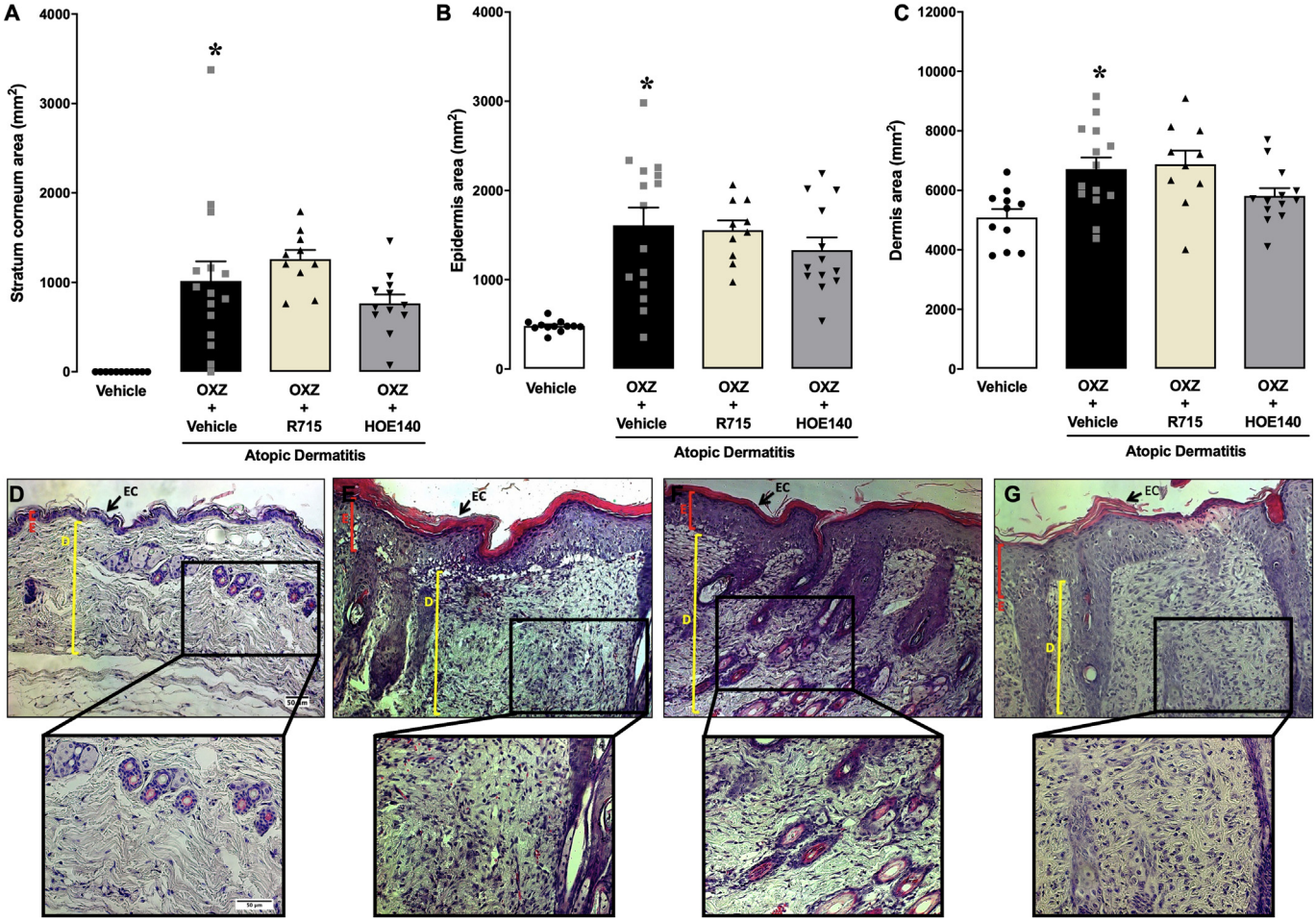

Fig. 6. Effects of kinin antagonists on the histological features in oxazolone-induced atopic dermatitis. Effects of the systemic repeated treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given on days 8, 10, 13 and 15, on the following parameters: stratum corneum area (A), epidermal area (B) or dermal area (C), measured in mm^2. $n = 10–15$ mice per group, $^*P < 0.05$ vs vehicle-treated group (one-way ANOVA followed by Bonferroni's post hoc test). Representative H&E-stained skin sections of the vehicle-treated group (D), oxazolone-treated group (E), oxazolone-treated group pre-treated with the B_1 R715 (F) or B_2 HOE140 (G) receptor antagonists. Original magnification ×200 and ×400, scale bar (—) 50 μm. EC, stratum corneum; E, epidermis; D, dermis. Arrows indicate the stratum corneum. Epidermis and dermis are indicated by red and yellow lines, respectively. (OXZ; oxazolone). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

out. The immunopositivity for ADAMTS5 was determined by using the NIH Image J 1.50 g Software, under ×200 magnification. For this purpose, a specific macro was created to quantify the positive areas based on the pixel colour from a slide of the vehicle group, as described elsewhere [25].

2.10. Immunohistochemistry analysis for kinin receptor expression

To assess the expression of kinin B_1 and B_2 receptors, the dorsal skin, the thoracic spinal cord, the dorsal root ganglion (DRG) and the brain of vehicle or oxazolone-challenged mice were collected, and fixed in 10% paraformaldehyde. Four-μm paraffin tissue sections were submitted to a standard three-step immunohistochemistry protocol, using the polyclonal rabbit anti-B_1R (1:200; ABR-011), or the polyclonal rabbit anti-B_2R (1:200 (skin, spinal cord and DRG or 1:150 (for brain samples); ABR-012), both from Alomone Labs (Jerusalem, Israel). The immunopositivity for kinin receptors was evaluated as described earlier [26], by using the NIH Image J 1.50 g Software, under ×200 magnification. For the immunohistochemistry analysis, the whole right and left DRGs of thoracic spinal cord were collected (≈8/mice) and thoroughly evaluated. For the thoracic spinal cord, the analysis was performed considering the immunolabelling of the right and left ventral horn, right and left dorsal horn, and the central canal. For the cortex, the primary and secondary motor cortices, besides the cingulate cortex, were included in the analysis [27].

2.11. Statistical analysis

Data is expressed as the mean ± SEM. Unpaired student's *t*-test or one-way analysis of variance (ANOVA) followed by the multiple comparisons Bonferroni's test were used. All of the statistical tests and the production of the graphs were performed by using GraphPad 8.0.2 Software (San Diego, CA, USA). $P < 0.05$ was considered as an indicative of significance.

3. Results

3.1. Peripheral and central kinin receptor expression in oxazolone-induced atopic dermatitis

The induction of atopic dermatitis by the repeated application of 0.5% oxazolone in the mouse dorsal skin resulted in a significant up-regulation of either B_1 or B_2 receptors, throughout the epidermis and dermis, according to the evaluation of skin sections by immunohistochemistry (Fig. 1C-F). The immunopositivity for the B_1 receptor was increased by 2.4-fold (Fig. 1A), whereas the B_2 receptor

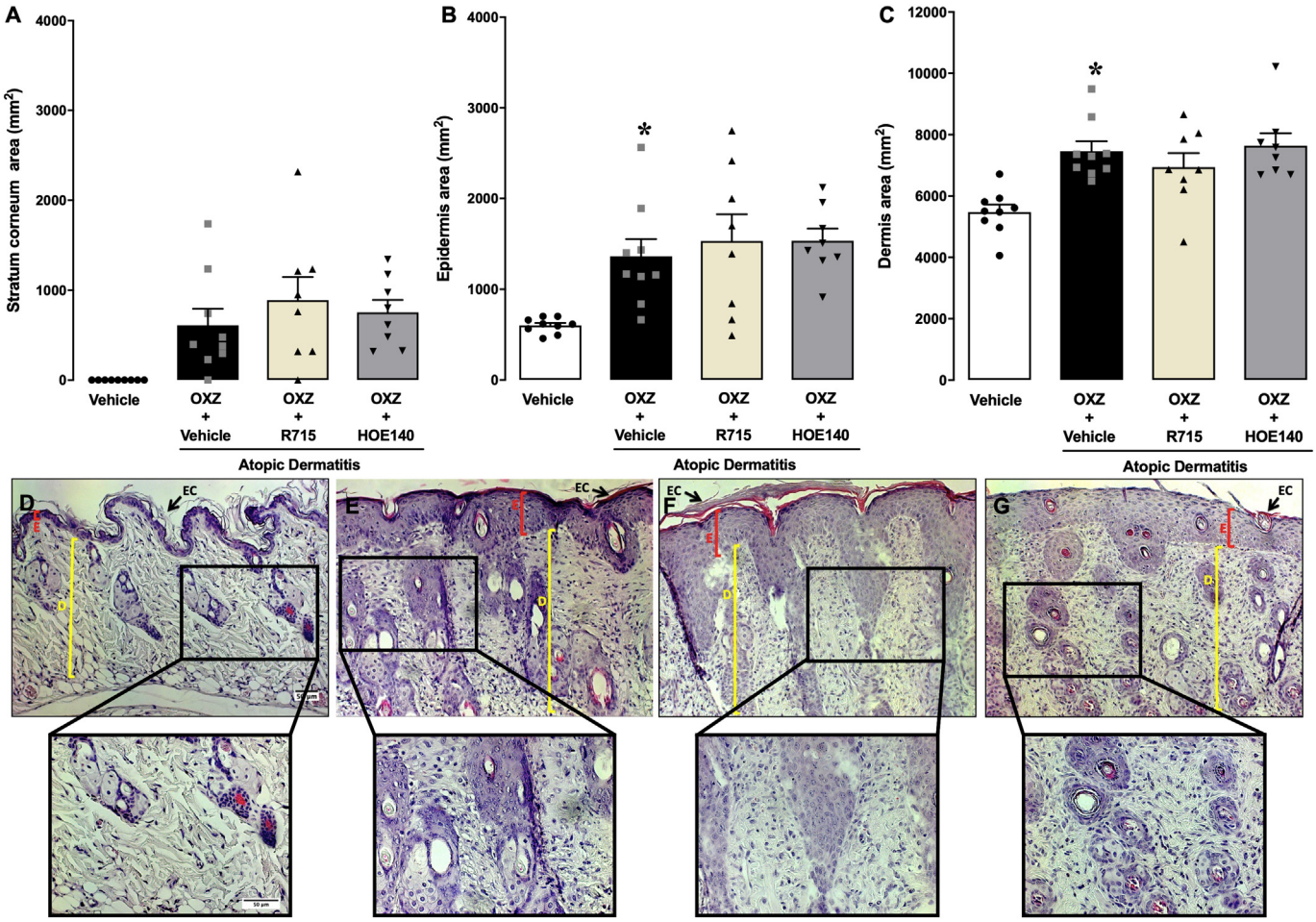

Fig. 7. Effects of kinin antagonists on the skin thickening in oxazolone-induced atopic dermatitis. Effects of the local repeated treatment with the selective B_1 R715 (30 nmol/site; i.d.) or B_2 HOE140 (3 nmol/site; i.d.) receptor antagonists, given on days 10 to 16, on the following parameters: stratum corneum area (A), epidermal area (B) or dermal area (C), measured in mm^2. n = 10–15 mice per group, *P < 0.05 vs vehicle-treated group (one-way ANOVA followed by Bonferroni's post hoc test). Representative H&E-stained skin sections of the vehicle-treated group (D), oxazolone-treated group (E), oxazolone-treated group pre-treated with the B_1 R715 (F) or B_2 HOE140 (G) receptor antagonists. Original magnification ×200 and ×400, scale bar (—) 50 μm. EC, stratum corneum; E, epidermis; D, dermis. Arrows indicate the stratum corneum. Epidermis and dermis are indicated by red and yellow lines, respectively. (OXZ; oxazolone). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunolabelling was augmented by 2.6-fold (Fig. 1B), in the skin of oxazolone-treated mice, when compared to the vehicle-treated group. The immunohistochemical analysis did not reveal any significant difference for the B_1 or B_2 receptor distribution in the thoracic spinal cord sections of mice that received oxazolone (Fig. 2A-F). However, the repeated application of oxazolone to the mouse dorsal skin elicited a down-regulation of B_1 and B_2 receptors in the DRG (Fig. 2G-L), with a significant reduction of the B_1 receptor immunopositivity in the DRG of mice with atopic dermatitis (Fig. 2G). No marked differences for B_1 or B_2 receptor immunolabelling were observed in the brain cortex, after the induction of atopic dermatitis by oxazolone, in comparison to the vehicle-treated controls (Fig. 3A-F).

3.2. Effects of kinin receptor antagonists on atopic dermatitis-related scratching behavior

The repeated application of oxazolone into the dorsal mouse skin was associated with a significant increase in the number of scratching bouts (Fig. 4D-F). The acute administration of the selective B_1 R715 (438 nmol/kg) or B_2 HOE140 (50 nmol/kg), both given i.p., failed to significantly alter the scratching behavior elicited by oxazolone (Fig. 4D). A repeated protocol of i.p. administration with R715 (438 nmol/kg), given the day before the last four applications of oxazolone, also lacked any significant effect on itching behavior elicited by oxazolone. Alternatively, the repeated treatment with HOE140 (50 nmol/kg) partially reduced the oxazolone-related pruritus (P = 0.057; Fig. 4B). Finally, the local i.d. administration of R715 (30 nmol/site) or HOE140 (3 nmol/site), dosed for the last seven days of the protocol of oxazolone-induced atopic dermatitis, did not significantly affect the scratching counts (Fig. 4F).

3.3. Skin plasma extravasation and lesion severity indexes

The induction of atopic dermatitis by oxazolone led to a marked increase of plasma extravasation and clinical lesion scores. The repeated i.p. treatment with R715 (438 nmol/kg) or HOE140 (50 nmol/kg) did not significantly change any of the evaluated parameters (Fig. 5A-B; D–F), regardless of a slight reduction of plasma extravasation by R715 (Fig. 5A). The i.d. injection of R715 (30 nmol/site) or HOE140 (3 nmol/site) was not able to modify the lesion severity, according to the clinical evaluation of the dorsal skin (Fig. 5C; G–I).

3.4. Histopathological assessment of skin lesion sites

Oxazolone-induced atopic dermatitis was associated with a significant increase of the stratum corneum, epidermis and dermis areas,

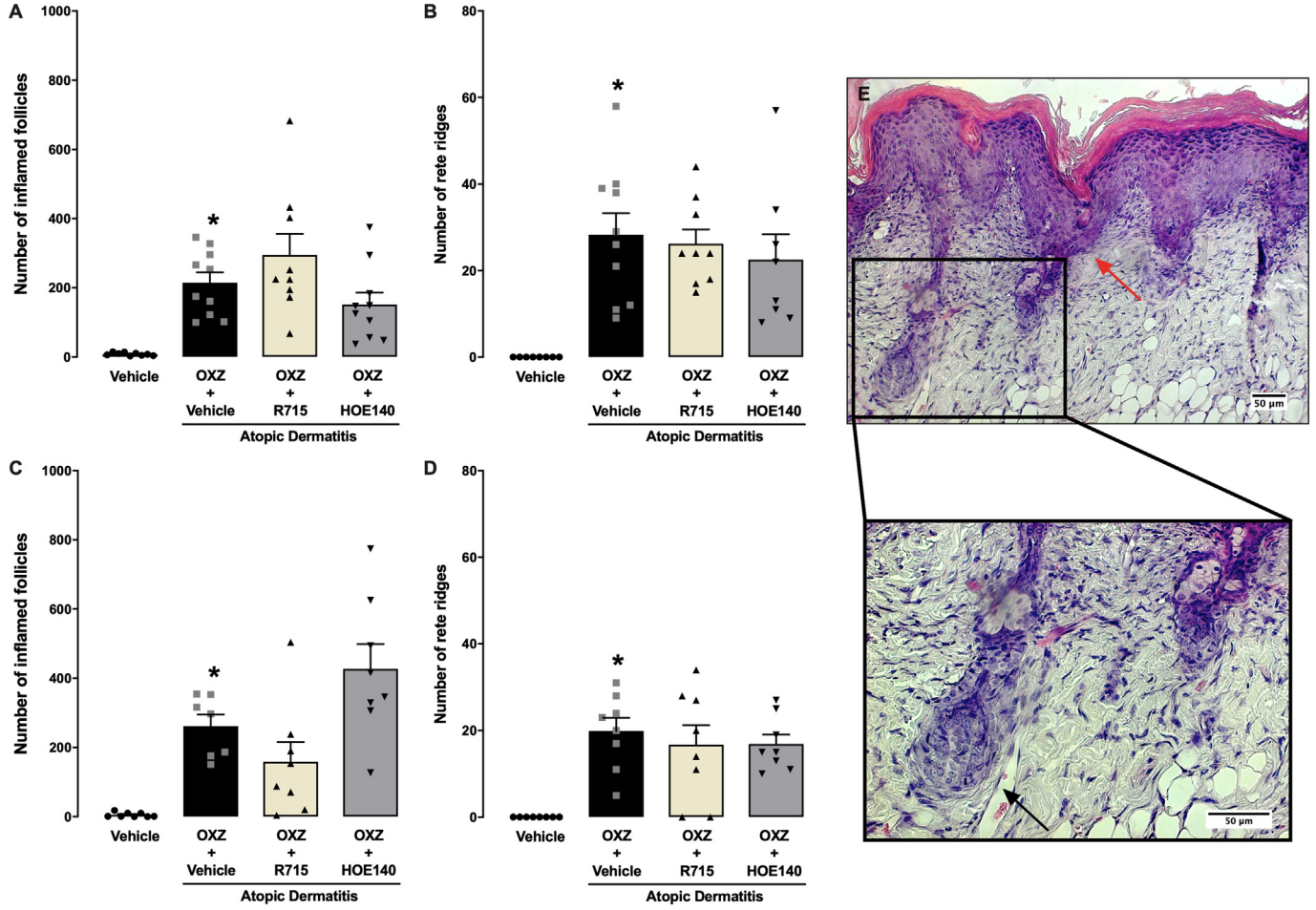

Fig. 8. Effects of kinin antagonists on the numbers of inflamed follicles and rete ridges. Effects of the systemic repeated treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given on days 8, 10, 13 and 15, in the number of inflamed follicles (A) and rete ridges (B) in the skin. Effects of the local repeated treatment with the selective B_1 R715 (30 nmol/site; i.d.) or B_2 HOE140 (3 nmol/site; i.d.) receptor antagonists, given on days 10 to 16, in the number of inflamed follicles (C) and rete ridges (D) in the skin. n = 7–10 mice per group, *P < 0.05 vs vehicle-treated group; #P < 0.05 vs control oxazolone-treated group (one-way ANOVA followed by Bonferroni's post hoc test). Red and black arrows indicate rete ridges and inflamed follicles, respectively. (OXZ; oxazolone; panel E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as an indicative of acanthosis (Figs. 6 and 7). Of note, the application of oxazolone also led to a significant elevation in the numbers of inflamed hair follicles and elongated rete ridges, which might account for the histological changes observed in atopic dermatitis (Fig. 8). The repeated administration of the selective B_1 R715 or B_2 HOE140 receptor antagonists, given i.p. (Fig. 6A-C) or i.d. (Fig. 7A-C) did not significantly alter the skin thickening parameters. The kinin antagonists, given repeatedly i.p. (Fig. 8A-B) or i.d. (Fig. 8C-D) also failed to prevent the hair follicle inflammation or the elongation of rete ridges.

3.5. Effects of kinin antagonists in atopic dermatitis cell infiltration

The evaluation of H&*E*-stained slides revealed a significant increase of the lymphocyte (Fig. 9A-D), eosinophil (Fig. 9B-E) or histiocyte (Fig. 9C-F) counts. The systemic repeated treatment with R715 (538 nmol/kg) or HOE140 (50 nmol/kg), given i.p., was not able to alter the infiltration of inflammatory cells to the skin of mice with atopic dermatitis (Fig. 9A-C). The i.d. repeated injection of R715 (30 nmol/site) or HOE140 (3 nmol/site) failed to prevent the infiltration of lymphocytes (Fig. 9D), whereas HOE140 led to a significant increase of eosinophil numbers (Fig. 9E). Noteworthy, both antagonists markedly prevented the increase of histiocytes induced by oxazolone (Fig. 9F). The percentages of inhibition for this effect were 78 ± 7% and 85 ± 4%, for R715 and HOE140, respectively.

The mast cell numbers were assessed in toluidine blue-stained slides. As expected, oxazolone triggered a significant increase in the numbers of mast cells, an effect that remained unaffected in animals that received R715 or HOE140 by i.p. (Fig. 10A-E), or i.d. (Fig. 10F-J) routes of administration.

3.6. Evaluation of the metalloproteinase ADAMTS5

Immunohistochemical analysis of the expression of ADAMTS5 in skin sections did not show any significant difference between the vehicle- and oxazolone treated mice. The systemic (Fig. 11A) or the local (Fig. 11B) administration of R715 and HOE140 also lacked any significant effect on the expression of this metalloproteinase, except by a reduction of ADAMTS5 immunopositivity in animals that had been treated with HOE140 (50 nmol/kg), by the i.p. route (Fig. 11A).

4. Discussion

Atopic dermatitis (also named atopic eczema) is a chronic inflammatory skin disorder presenting intermittent eczematous lesions and intense pruritus, which affects 1/5 of the population living in developed countries [28]. Topical ointments, corticosteroids, and calcineurin inhibitors represent the first line of treatment for atopic dermatitis, although the systemic administration of immunosuppressant

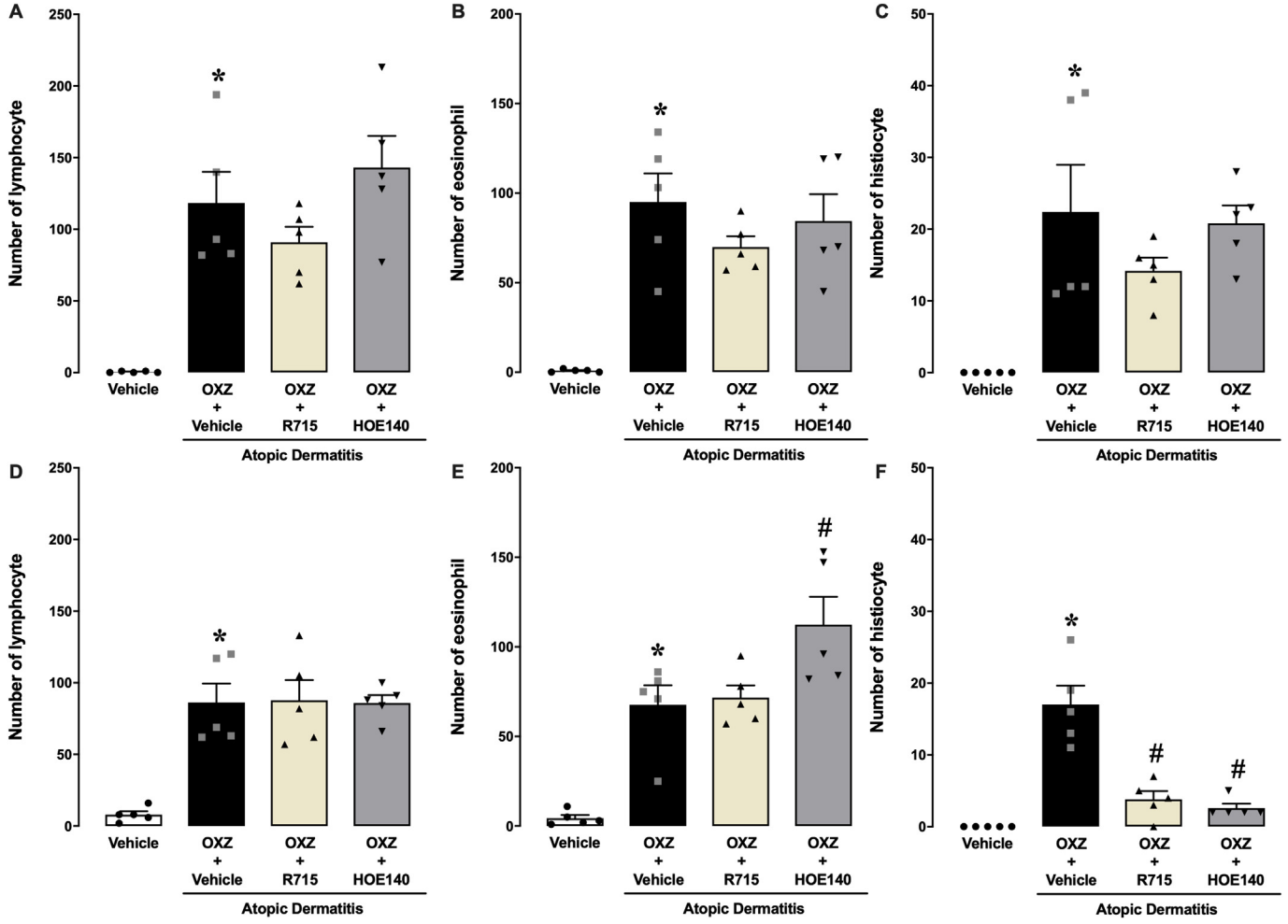

Fig. 9. Effects of kinin antagonists on the inflammatory cell infiltrate in oxazolone-induced atopic dermatitis. Effects of the systemic repeated treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given on days 8, 10, 13 and 15, in the numbers of lymphocytes (A), eosinophils (B) and histiocytes (C) in the skin. Effects of the local repeated treatment with the selective B_1 R715 (30 nmol/site; i.d.) or B_2 HOE140 (3 nmol/site; i.d.) receptor antagonists, given on days 10 to 16, in the number of lymphocytes (D), eosinophils (E) and histiocytes (F) in the skin. $n = 5$ mice per group, *P $<$ 0.05 vs vehicle-treated group; #P $<$ 0.05 vs control oxazolone-treated group (one-way ANOVA followed by Bonferroni's post hoc test). (OXZ; oxazolone).

drugs is required for refractory cases [29]. Furthermore, a series of biologic agents targeting cytokines, chemokines, IgE and the JAK-STAT pathway, among others, have demonstrated beneficial effects for the management of this condition. However, due to its intricate pathophysiology, atopic dermatitis remains without cure, markedly compromising the life quality of the affected individuals [30].

BK and its receptors have been implicated in several allergy-related conditions (for review see: Ricciardolo et al. [7]), despite the few studies investigating its possible relevance in atopic dermatitis. To our knowledge, only one study demonstrated that BK application into the lesional skin of patients with atopic dermatitis led to an intense pruritic response when compared to a mild reaction in healthy volunteers [9]. Herein, we tested the effects of selective kinin B_1 or B_2 receptor antagonists in a mouse model of atopic dermatitis evoked by the repeated administration of oxazolone to the neck dorsal skin. We have also evaluated whether the induction of atopic dermatitis might lead to central and peripheral alterations of kinin receptor expression.

As discussed above, intense itching sensation is a hallmark of atopic dermatitis, which can aggravate the skin lesions, what is related to the itch-scratch cycle [31,32]. The mechanisms underlying atopic dermatitis-related pruritus are not currently clear, but they likely involve peripheral and central mechanisms of sensitization [30]. Herein, we evaluated to what extent the induction of atopic dermatitis might alter the kinin receptor distribution in peripheral and central sites implicated in itch transmission, including the skin, spinal cord, DRG and brain cortex [27,33,34]. Our results showed that oxazolone-induced atopic dermatitis was accompanied by a marked increase in the scratching behavior in mice. Of note, the protocols of sensitization and challenge with oxazolone led to a marked upregulation of B_1 and B_2 receptors in the lesional skin, allied to downregulation of both receptors in the mouse DRG, according to the assessment by immunohistochemistry. Conversely, the immunopositivity for either kinin receptor remained unaltered in the brain cortex or the thoracic spinal cord of animals with atopic dermatitis. An upregulation of B_1 receptors in the mouse skin, more specifically at keratinocytes, has also been detected after the induction of contact dermatitis by DCP [12], supporting somewhat our data. In our study, both kinin receptors are probably upregulated in keratinocytes in the epidermis, whereas the profuse immunolabelling in the dermis does not permit to conclude that receptor expression is increased in a particular cell type. Thus, kinin receptors might be upregulated in either resident or infiltrating cells in dermis and epidermis of mice submitted to the oxazolone model, although further co-localization experiments are still necessary to confirm this proposition. Concerning the results on DRG, most literature studies demonstrate an upregulation of kinin receptors after the induction of chronic pain [35], instead of a reduction of kinin receptors, as observed by us after oxazolone application. Indeed, we are not able to explain the mechanisms underlying the downregulation of kinin receptors in DRG after

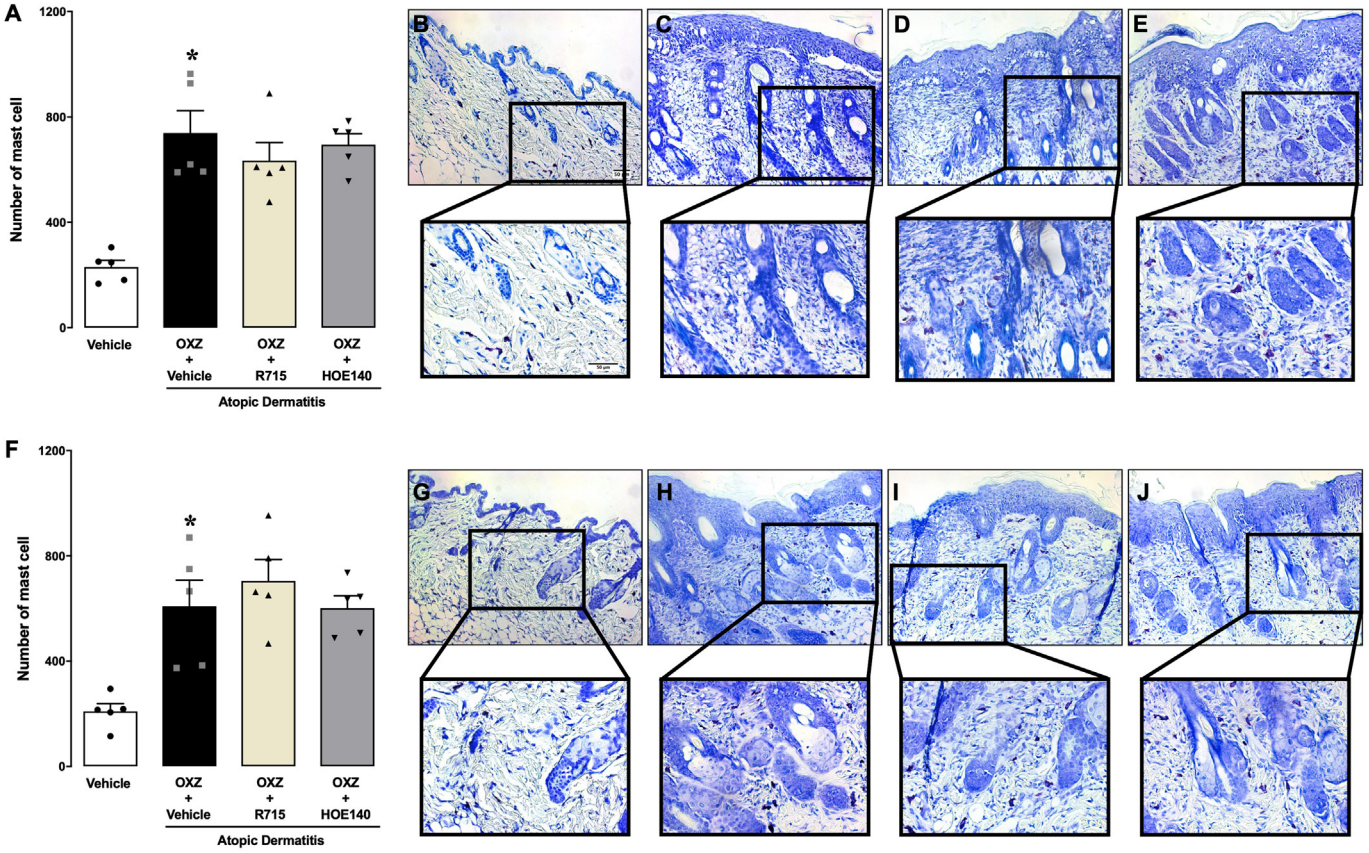

Fig. 10. Effects of kinin antagonists on mast cell numbers in oxazolone-induced atopic dermatitis in mice. (A) Effects of the systemic repeated treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given on days 8, 10, 13 and 15, in dermal mast cell numbers. Representative images of toluidine blue-stained skin sections in the vehicle-treated group (B), oxazolone-treated group (C), oxazolone-treated group pre-treated with the B_1 R715 (D) or the B_2 HOE140 (E) receptor antagonists. (F) Effects of the local repeated treatment with the selective B_1 R715 (30 nmol/site; i.d.) or B_2 HOE140 (3 nmol/site; i.d.) receptor antagonists, given on days 10 to 16, in dermal mast cell numbers. Representative images of toluidine blue-stained skin sections in the vehicle-treated group (G), oxazolone-treated group (H), oxazolone-treated group pre-treated with the B_1 R715 (I), or B_2 HOE140 (J) receptor antagonists. $n = 5$ mice per group, *$P < 0.05$ vs vehicle-treated group (one-way ANOVA followed by Bonferroni's post hoc test). Original magnification ×200 and ×400, scale bar (—) 50 μm. (OXZ; oxazolone). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induction of atopic dermatitis, and further studies on this matter are still required.

Considering the impacts of pruritus in the quality of life of patients with atopic dermatitis, the identification of novel potential tools for treating this issue is of great interest. Thus, we evaluated whether the pharmacological inhibition of kinin receptors might prevent the scratching behavior in the experimental model of oxazolone-induced atopic dermatitis. As a first approach, we tested the effects of the B_1 R715 or B_2 HOE140 receptor antagonists, given in a single i.p. dose, 30 min before the pruritus assessment. In this protocol of treatment, both antagonists failed to alter the oxazolone-induced itching. Considering the chronic pattern of this model, we also evaluated the effects of a repeated protocol of treatment with R715 or HOE140, dosed i.p. Whilst R715 was not able to modify the number of scratching bouts elicited by oxazolone, the administration of HOE140 partially reduced this behavior. Our data corroborate the previous publication by Hayashi and Majima [11] showing that B_2, but not B_1 receptor antagonists, prevented the itching attacks induced by deoxycholic acid in mice. In our experimental paradigm, the modest effects of HOE140 might be justified by the schedule administration adopted by us: the antagonists were dosed one day before the last four applications of oxazolone, but not at the day of pruritus evaluation. It is tempting to suggest that long-term protocols of treatment with the kinin antagonists might present a superior inhibition. As the first line treatment for atopic dermatitis is centered on topical agents, we also tested to what extent the i.d. repeated treatment with R715 or HOE, dosed every day, during the last seven days of the oxazolone challenge protocol. Nonetheless, this treatment approach failed to alter the scratching behavior.

To gain further insights into the relevance of kinins in atopic dermatitis, we decided to perform an additional evaluation of the macroscopic and microscopic changes in the skin of mice submitted to the oxazolone model. The sensitization and challenge with oxazolone led to various alterations featuring atopic dermatitis [28], such as middle to severe clinical lesions, besides plasma extravasation. Moreover, a detailed microscopic evaluation revealed an epidermis lichenification and edema, with the dermal infiltration of lymphocytes, eosinophils, histiocytes and mast cells, with the presence of elongated rete ridges and numerous inflamed hair follicles. In relation to the effects of repeated protocols of treatment with B_1 or B_2 receptor antagonists, the i.p. administration of R715 partially reduced the plasma extravasation, whereas the i.d. application of either R715 or HOE140 markedly reduced the number of histiocytes. A clinical study demonstrated that histiocytes are the second most predominant cell population in atopic dermatitis, according to the evaluation of the lesional skin of ten patients [36]. Thus, the reduction of this cell population by the local application of B_1 or B_2 kinin antagonists in oxazolone-treated mice deserves further investigation, by using flow cytometry and specific markers. The other evaluated parameters were not modified by R715 or HOE140, in either protocol of systemic or local repeated administration. Again, this might be an indicat ion that additional experiments using long-term treatment protocols with kinin antagonists are required. These schedules of treatment must include not only additional

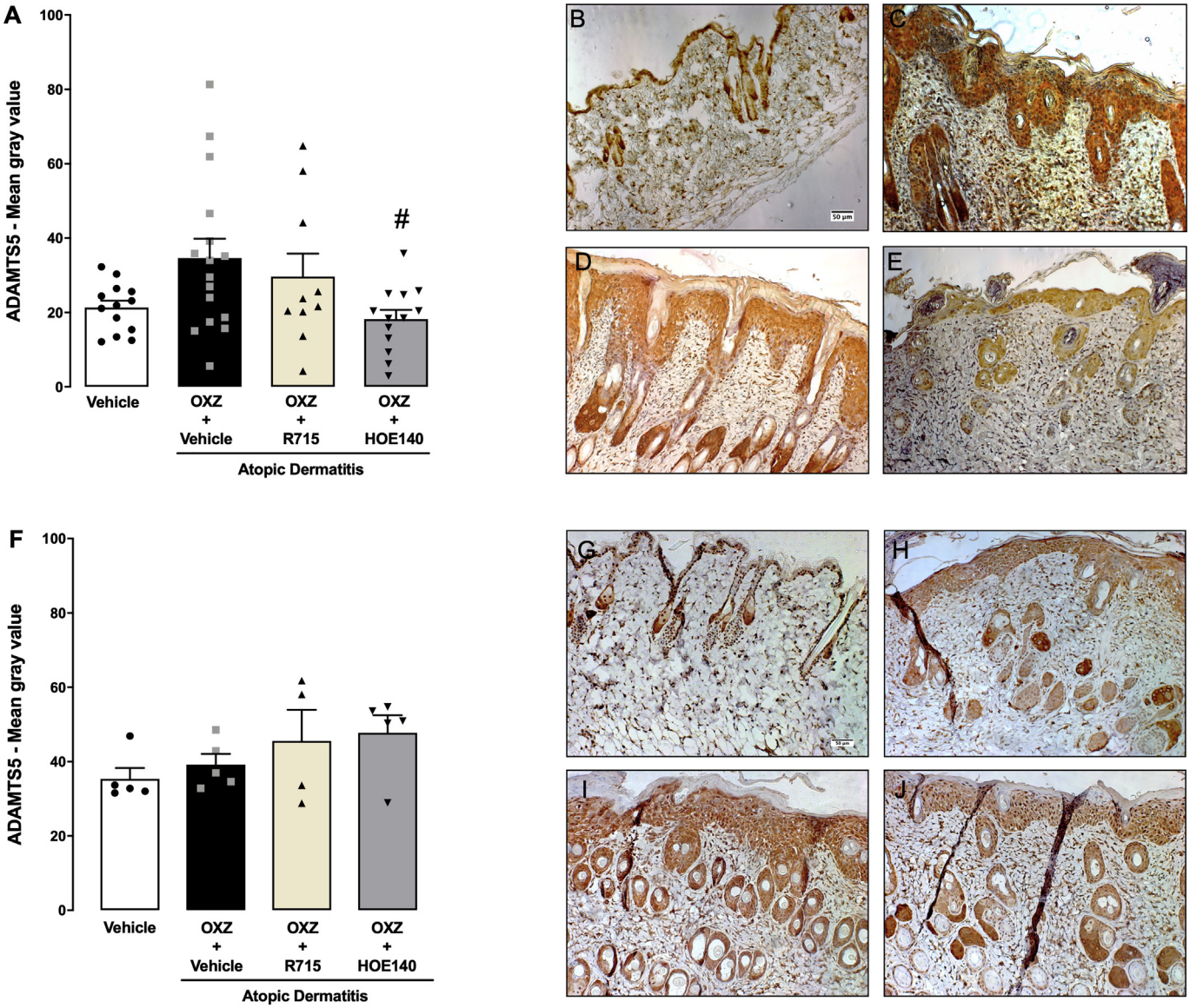

Fig. 11. Immunopositivity for the metalloproteinase ADAMTS5 in the skin of mice submitted to the model atopic dermatitis by oxazolone. (A) Effects of the systemic repeated treatment with the selective B_1 R715 (438 nmol/kg; i.p.) or B_2 HOE140 (50 nmol/kg; i.p.) receptor antagonists, given on days 8, 10, 13 and 15, in the immunolabelling for ADAMTS5 in the skin. Representative images of ADAMTS5 immunopositivity in the vehicle-treated group (B), oxazolone-treated group (C), oxazolone-treated group pre-treated with the B_1 R715 (D) or B_2 HOE140 (E) receptor antagonists (original magnification ×200). (F) Effects of the local repeated treatment with the selective B_1 R715 (30 nmol/site; i.d.) or B_2 HOE140 (3 nmol/site; i.d.) receptor antagonists, given on days 10 to 16, in the immunolabelling for ADAMTS5 in the skin. Representative images of ADAMTS5 immunopositivity in the vehicle-treated group (G), oxazolone-treated group (H), oxazolone-treated group pre-treated with the B_1 R715 (I) or B_2 HOE140 (J) receptor antagonists. Original magnification ×200, scale bar (—) 50 μm. n = 5–16 mice per group, #P < 0.05 vs oxazolone-treated group (one-way ANOVA followed by Bonferroni's post hoc test). (OXZ; oxazolone).

days of treatment along the induction of atopic dermatitis by oxazolone, but also the administration of antagonists twice a day, or even the combined treatment with B_1 and B_2 kinin receptor antagonists. Alternatively, the use of non-peptide, instead of peptide antagonists could represent an interesting strategy. An intriguing result showed a significant elevation of eosinophil numbers in oxazolone-treated mice that received the B_2 antagonist HOE140 i.d. A similar effect has been demonstrated before in a mouse model of lung inflammation elicited by ovalbumin in mice, in which the treatment with HOE140 led to an increase of eosinophils in the bronchoalveolar lavage fluid [37].

ADAMTS5 is a metalloproteinase present in the skin of humans and mice, which has been linked with the process of dermal wound healing [38]. Herein, the immunopositivity of this enzyme was slightly, but not significantly increased in the skin of oxazolone-treated mice, in comparison to the vehicle group. The hair shaving could explain the absence of great differences between these two groups. However, the i.p. repeated treatment with HOE140 promoted a significant reduction of ADAMTS5 immunolabelling in oxazolone-treated animals. One might conclude that this effect correlates with the mild anti-pruritic effects observed for HOE140 dosed at the same treatment scheme. Thus, a reduction of scratching bouts might ameliorate the eczematous lesions in oxazolone-induced atopic dermatitis, as indicated by the reduced ADAMTS5 expression in animals pre-treated with HOE140, but not R715.

Collectively, the present study shows a comprehensive characterization of the mouse model of atopic dermatitis, induced by oxazolone application into the neck dorsal skin. Additionally, we have observed an upregulation of kinin B_1 and B_2 receptors in the skin of oxazolone-treated mice, with a reduction of both receptors in the DRG. This is a clue indicating the relevance of the kinin system in atopic dermatitis

pathology. Regarding the treatment with the kinin antagonists, the effects were quite modest and distinct for B_1 or B_2 receptor antagonists, also showing different actions depending on the route of administration, i.e., dosed i.p. or i.d. Thus, it is possible to assume that B_1 and B_2 receptors might display distinct roles in atopic dermatitis and that repeated administration i.p. reaches sites of action that are not affect ed by i.d. administration, an vice-versa. An example of that is the reduction of pruritus by HOE140 given i.p. vs the marked reduction of histiocytes by the i.d. administration of either antagonist. Nevertheless, these hypotheses remain to be confirmed by additional studies, with different schemes of treatment with kinin antagonists and the investigation of in-depth mechanisms by the use of other techniques, such as flow cytometry and ELISA.

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