International Immunopharmacology 8 (2008) 1616–1621

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Immunomodulatory pretreatment with *Kalanchoe pinnata* extract and its quercitrin flavonoid effectively protects mice against fatal anaphylactic shock

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ARTICLE INFO

Article history:
Received 16 April 2008
Received in revised form 12 June 2008
Accepted 9 July 2008

Keywords:
Anaphylaxis
Kalanchoe
Immunomodulation
Mouse

ABSTRACT

Previously, we reported the immunosuppressive action of the aqueous extract of *Kalanchoe pinnata* (Kp) in mice. In the present study, we report on the protective effect of Kp in fatal anaphylactic shock, likewise a Th2-driven immunopathology, and the identification of its active component. Mice daily treated with oral Kp during hypersensitization with ovalbumin were all protected against death when challenged with the allergen, as compared with the 100% mortality in the untreated group. A single intraperitoneal dose 3 h prior to challenge was partially effective. Oral protection was accompanied by a reduced production of OVA-specific IgE antibodies, reduced eosinophilia, and impaired production of the IL-5, IL-10 and TNF-α cytokines. *In vitro*, Kp prevented antigen-induced mast cell degranulation and histamine release. Oral treatment with the quercitrin flavonoid isolated from Kp prevented fatal anaphylaxis in 75% of the animals. These findings indicate that oral treatment with Kp effectively downmodulates pro-anaphylactic inducing immune responses. Protection achieved with quercitrin, although not maximal, suggests that this flavonoid is a critical component of Kp extract against this extreme allergic reaction.

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1. Introduction

Kalanchoe pinnata (Lam.) Pers. (Crassulaceae) is a medicinal plant largely used in the folk medicine for the treatment of gastric ulcer, pulmonary infections, and rheumatoid arthritis [1]. Its antihistaminic, anti-inflammatory, anti-ulcer, anti-tumor and hepatoprotective activities have been experimentally demonstrated [2–6]. We reported in mice the immunosuppressive and the associated therapeutic effect of the oral treatment with the aqueous leaf extract of *Kalanchoe pinnata* (Kp) in cutaneous leishmaniasis, a disease normally driven by exacerbated TH2 responses [7,8]. The main active components were later identified as fatty acids and a glycosylated flavonoid [9,10].

Like cutaneous leishmaniasis, atopic disorders, including allergen-induced asthma, rhinoconjunctivitis and anaphylaxis are also associated with TH2-type T cells. The cytokines produced by those cells induce the differentiation, the activation and the *in situ* survival of eosinophils (through IL-5), promote the production of high amounts of IgE by B lymphocytes (through IL-4 and IL-13), as well as the growth of mast cells and basophils [11]. Allergic anaphylaxis is a life-threatening systemic reaction mediated by IgE antibodies where the antigen-mediated crosslinking of FcεRI-bound IgE on mast cells or basophils stimulates systemic degranulation of these cells. This results in rapid release of histamine, inflammatory cytokines, the synthesis and secretion of platelet-activating factor (PAF) and leukotrienes that may culminate with a rapid drop in blood pressure, suffocation and death [12].

Despite the early reports on its antihistaminic activity using the experimental models of isolated guinea pig ileum contraction and vasodilatation in rats [2], and the T cell-suppressive and antileishmanial activities in mice [7,8], the therapeutic effect of Kp on allergy is still unknown. Thus, in the present work we proposed to investigate in mice the protective effect of Kp against allergen-specific anaphylaxis, and the modulation of allergy-associated factors.

2. Materials and methods

2.1. Reagents

Ovalbumin (OVA), complete Freund adjuvant, incomplete Freund adjuvant, compound 48/80, cyclosporin A, bovine serum albumin, o-phtaldialdehyde (OPT), compound 48/80, Percoll, Hanks Balanced Salt Solution (HBSS), Dulbecco-Modified Minimum Essential Medium (D-MEM), and antibiotics were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Heat-Inactivated Fetal Calf Serum (HIFCS) was purchased from Gibco Laboratories (Grand Island, NY, USA). Biotinylated rat anti-mouse IgE was purchased from R&D Systems (USA). HRP-streptavidin conjugate and tetramethylbenzidine (TMB) were purchased from ZyMax Grade (USA). Sodium heparin from Roche (São

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doi:10.1016/j.intimp.2008.07.006

Paulo, Brazil). Murine anti-DNP IgE monoclonal antibody (anti-DNP IgE Mab) and dinitrophenylated bovine serum albumin (DNP-BSA) were purchased from Calbiochem (San Diego, USA). All solutions were freshly prepared immediately before use.

2.2. Aqueous extract of Kalanchoe pinnata (Kp) and quercitrin isolation

Kalanchoe pinnata (synonymia *Bryophyllum pinnatum* or *Bryophyllum calycinum*) was collected prior to blooming from the outskirts of Rio de Janeiro, Brazil. A voucher specimen (292.697) is deposited at the Rio de Janeiro's Botanical Garden herbarium. The leaves were extensively washed and extracted with distilled water at 20% (w/v) for 30 min at 50 °C. The aqueous extract was filtered, lyophilized and stored at −20 °C [7]. The endotoxin content was always less than 10 EU/mg, as determined by the Limulus Amebocyte Lysate assay according to manufacturer instructions (Cambrex Company, USA). Kp was reconstituted to 20 mg/ml with Phosphate Buffered Saline (PBS) prior to use.

Chemical characterization of the extract was performed by High Performance Liquid Chromatography for identification and quantitation of 3 flavonoids as chemical markers. Results were expressed as % w/w of the lyophilized Kp aqueous extract: 1) quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside 0.59%; 2) quercitrin 0.02%; and 3) kaempferol 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (kapinnatoside) 0.03%, as previously described [10].

For quercitrin isolation, the extract was partitioned with dichloromethane at pH 2 and then pH 11. The residual aqueous phase was neutralized to pH 7 and partitioned with ethyl acetate. An aliquot of this organic fraction, after total evaporation of ethyl acetate, was re-suspended in distilled water and chromatographed on a RP-2 column affording 3 fractions. The flavonoid-rich fraction 2 was eluted with 3:7 MeOH/H_2O and further purified with a H_2O/MeOH gradient on a RP-18 column. Two flavonoid fractions were obtained. The first fraction (eluted with 1:9 EtOH/H_2O – elution volume: 230 ml; 30.7 mg), enriched in one compound, was chromatographed on a Sephadex G-15 column (31.0×0.8 cm; H_2O) affording quercitrin as a yellow powder: R_f 0.83; ^1H and ^{13}C-NMR (CD_3OD) as reported before [10]; $[M+Na]^+$ was determined as 471.097; purity of quercitrin was determined by HPLC as 98.1% [13].

2.3. Animals

Male BALB/c (18–20 g) mice and Lou-M rats (180–200 g) were obtained from Federal University of Rio de Janeiro Breeding (Rio de Janeiro, Brazil) and maintained using sterilized bedding, filtered water and pelleted food. They were used in accordance with the guidelines of the Committee on Use of Laboratory Animals.

2.4. Mouse hypersensitization and antigen challenge

Mice were rendered hypersensitized against OVA by two subcutaneous (*s.c.*) injections in the rump with 100 μg of OVA emulsified in Complete Freund Adjuvant and seven days later in Incomplete Freund Adjuvant. Seven days after the second dose, the animals were challenged with an intraplantar injection with 50 μg of OVA in 20 μl of PBS (2.5 mg/kg). The anaphylactic symptoms and lethality rate were recorded during 48 h after the OVA challenge. Negative controls were hypersensitized mice in which OVA was replaced by PBS as challenge.

2.5. Mouse treatment with Kp and quercitrin

During the 14-day-hypersensitization process, mice received daily doses of 400 mg/Kg of Kp or 20 mg/Kg of quercitrin by intragastric gavage from the first day on. Alternatively, mice were treated intraperitoneally (*i.p.*) as follows: a) 200 mg/Kg of Kp every other day; or b) with a single dose of 200 mg/Kg of Kp or with 12.5 mg/Kg of cyclosporin A (positive controls) 3 h before challenge on day 14. Negative controls were naive mice and untreated sensitized mice.

2.6. Eosinophil counts

Blood samples were obtained from the tail vein, prior to lethal OVA challenge, and differential analysis performed in blood smears stained with Giemsa. The results were expressed as the percentage of eosinophils in a minimum of 300 total leukocytes per smear.

2.7. OVA-specific IgE

Mice were bled by cardiac puncture and ELISA measured the OVA-specific IgE in the serum. Microtiter plates were coated with 50 μg/ml OVA and incubated overnight at 4 °C. The wells were washed 3 times with Tween 20/PBS (0.05%) prior to addition of 50 μl of serially diluted individual mouse sera. After 1 h at room temperature, plates were washed and incubated again with 50 μl of 1:5000 biotinylated rat anti-mouse IgE. The wells were washed and incubated with 100 μl of HRP-streptavidin conjugate for 20 min. Following plate washing, 100 μl of TMB was added per well and after 30 min the reaction was stopped with 50 μl of 1 N HCl. The optical density was read at 450 nm and the results expressed as anti-OVA IgE titer.

2.8. T cell proliferation

The inguinal lymph nodes were isolated from hypersensitized mice and single cell suspensions prepared in D-MEM containing 10% HIFCS and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). Viable Trypan Blue-negative cells (4×10^6 cells/ml) were stimulated with 1 mg/ml of OVA for 48 h. The proliferative response was evaluated through the incorporation of 3H-thymidine (2.5 μCi/ml) added in the last 6 h of culture. Cells were then harvested using a dot-blot device [14] and the radioactivity measured in a beta-counter.

2.9. Cytokine production

Lymph nodes cells were prepared as above for proliferation and 0.5 ml samples seeded in 24 well culture plates. The cells were restimulated with 1 mg/ml of OVA for 36 h. The levels of IL-5, IL-10 and TNF- α were determined in the supernatants by ELISA using recombinant murine cytokines and antibodies according the manufacturer instructions (R&D Systems, U.S.A.).

2.10. Mast cell degranulation in the mesentery

Mesenteric membranes were obtained from naive rats. After cutting into 1 cm×1 cm pieces, these were individually incubated for 15 min at 37 °C in 24-well culture plates with anti-OVA antiserum diluted at 1:5 in HBSS. The membranes were then washed with fresh HBSS and treated with 100 μg/ml of Kp for 30 min. After removal of Kp, they were further incubated for 10 min with 1 mg/ml of OVA, and the mast cells were stained by addition of toluidine blue dye. Mast cells were also directly stimulated by incubation of membrane pieces for 6 min with 0.5 μg/ml of compound 48/80. The number of mast cells was counted under light microscopy and the results expressed as the percentage of degranulated cells in at least 50 fields (1 mm^2/field).

2.11. Histamine release assay

Mast cells were recovered from the peritoneal cavity of naive rats and isolated to 96% purity by continuous Percoll gradient, as previously described [15]. Isolated mast cells (3×10^5/ml) were sensitized in 24-well plates with murine anti-DNP IgE mAb (0.5 μg/ml) at 37 °C for 12 h in 5% CO_2 in air. The cells were then left untreated or were

treated with 0.5 mg/ml of Kp for 1 h, when the antigen (DNP-BSA, 0.05 μg/ml) was added for 1 h at 37 °C. Cells were centrifuged at 150×g, the supernatants were collected and stored at −20 °C. For total histamine release, cells were further treated with 0.4 N perchloric acid for 1 h under agitation prior to centrifugation. Histamine release was assayed fluorimetrically [16], and expressed as percentage of total release.

2.12. Statistical analysis

The data were reported as mean±standard error of the mean (SEM) and statistically analyzed by Student's test. In the case of the lethality rate, the X^2-test (Fisher's exact test) was used. Probability values (p) of 0.05 or less were considered significant.

3. Results

3.1. Pre-treatment with Kp prevents death caused by anaphylactic shock

Death following an allergen challenge is an irrefutable evidence of anaphylactic shock. As seen in Fig. 1, the intraplantar injection of OVA (2.5 mg/kg) into actively sensitised mice elicited a severe systemic anaphylactic response with death happening within 30 min of allergen challenge. Daily treatment with 400 mg/Kg of oral Kp during the 14-day sensitization process effectively prevented fatal shock. All animals (100%) remained alive for the 48 h of follow-up. Other parameters such as piloerection, urticaria, cyanosis and dyspnea were also visibly milder as compared to untreated animals (not shown). Intraperitoneal injections with 200 mg/kg of Kp every other day during the same period also effectively enhanced survival (80%), similar to observed with 12.5 mg/Kg of i.p. cyclosporin A, an immunosuppressive drug also having anti-allergic effect [17]. Interestingly, a single i.p. dose of 200 mg/Kg of Kp 3 h prior to OVA challenge was sufficient to protect 60% of the animals, suggesting that not only immunosuppression but also modulation of acute events related to shock was critical for protection.

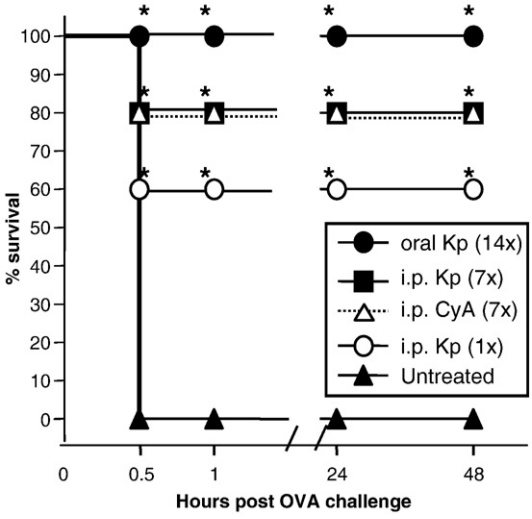

Fig. 1. Pre-treatment with Kp prevents fatal anaphylactic shock. BALB/c mice (n=5) were sensitized with OVA in adjuvant on days 1 and 7, and were challenged with 50 μg of OVA on day 14. During the sensitization period, they were treated as follows: i) by the oral route (daily doses of 400 mg/Kg of Kp for 14 days), ii) by the intraperitoneal route (7 doses of 200 mg/Kg of Kp or 12.5 mg/Kg of Cyclosporin in alternate days; or a single dose of 200 mg/Kg of Kp 3 h before OVA challenge). Controls were left untreated. Upon challenge on day 14, death events were monitored for up to 48 h and recorded as shown. The results are expressed as the percentage of surviving animals. *p<0.01 compared to untreated group.

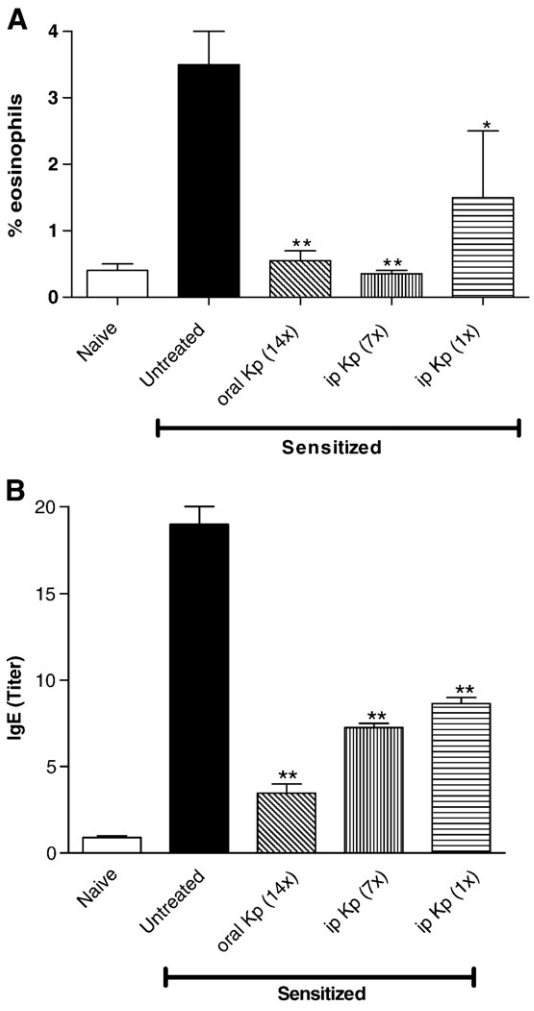

Fig. 2. Decreased eosinophilia and OVA-specific IgE levels in Kp-treated mice. Mice were sensitized and treated with Kp as for Fig. 1. A group of animals was bled 1 h before allergen challenge for the percentage of eosinophils in total leukocytes (top) and for individual assessment of the serum levels of anti-OVA IgE (bottom). Means±S.D (n=4). *p≤0.05 and **p≤0.01 in relation to untreated controls.

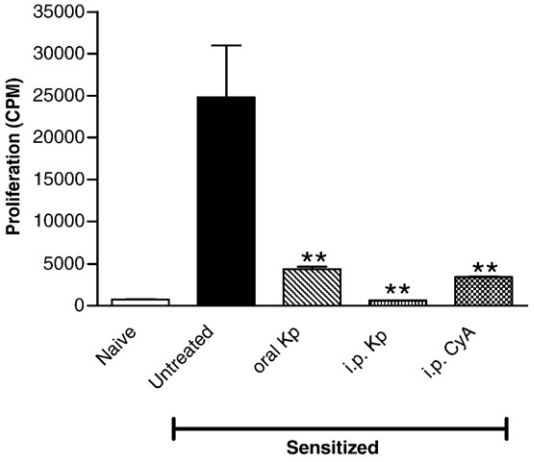

Fig. 3. Decreased T cell proliferative response in Kp-treated mice. Mice were sensitized and treated with Kp or Cyclosporin A (CyA) as for Fig. 1. One hour after allergen challenge, draining lymph nodes were collected and the cells restimulated *in vitro* with 1 mg/ml of OVA. The proliferative response was evaluated 48 h later by 3H-thymidine incorporation and expressed as counts per minutes. Means±S.D. (n=5). **p≤0.01.

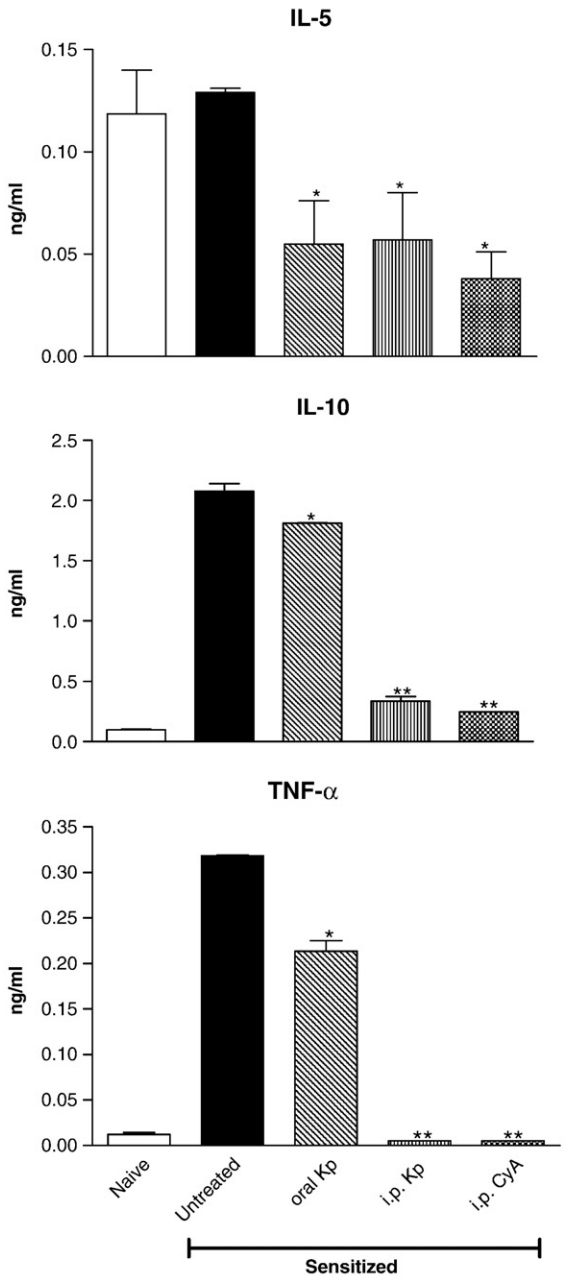

Fig. 4. Cytokine production in Kp-treated mice. Lymph nodes were obtained from mice that were sensitized and treated with Kp or Cyclosporin A (CyA) as indicated, and the cells restimulated *in vitro* with 1 mg/ml of OVA. After 48 h, the culture supernatants were collected for the determination of IL-5, IL-10 and TNF-α levels by ELISA. Means±S.D. ($n=5$). *$p\leq0.05$ and **$p\leq0.01$ in relation to untreated controls.

3.2. Kp prevents eosinophilia and OVA-specific IgE production during allergen sensitization

As parameters normally associated with systemic allergic reactions, we investigated the effect of Kp treatment on the number of circulating eosinophils and in allergen-specific IgE response. Expectedly, OVA-sensitization alone increased the numbers of eosinophils in the blood (Fig. 2A). Eosinophilia was prevented by Kp, especially in animals receiving multiple oral or i.p. doses, but a single i.p. dose of Kp was also effective. The levels of OVA-specific IgE were raised in sensitized mice, and Kp treatments, particularly when given orally, also significantly prevented this response (Fig. 2B). Altogether, these findings indicate that the anti-anaphylactic effect of Kp is accompanied by reduced mobilization of eosinophils and IgE antibody production.

3.3. Effect of Kp treatment on allergen-specific T cell proliferation and cytokine production

The correlation between the immunosuppressive action of Kp [7] and its antiallergic property was further analyzed by its capacity to suppress T cell proliferation *in vitro* and to modulate the production of allergy-associated cytokines. As seen in Fig. 3, irrespective of the route of administration, the antigen recall response of lymph node cells to OVA was abrogated in animals treated with Kp, similar to achieved with the classical immunosuppressive agent cyclosporin A. To better analyze the modulatory effect on Th2-type T cells, the IL-5 and IL-10 cytokines were measured in the cell culture supernatants. Treatment of mice with oral or *i.p.* Kp reduced the capacity of their cells to respond to OVA with IL-5 and IL-10 production (Fig. 4). The production of TNF-α was also inhibited by Kp treatment, and like IL-10, this effect was more pronounced when the *i.p.* route was used. These findings show that cytokines that contribute to allergy are down regulated by *i.p*, and to a lesser extent oral Kp treatments.

3.4. Kp directly prevents antigen-induced mast cell activation

To directly investigate the effect of Kp on mast cells, anti-OVA IgE-sensitized mesenteric rat mast cells were treated *in vitro* with 100 μg/ml of Kp prior to challenge with OVA. Fig. 5 shows that OVA triggered a marked activation of mast cells as attested by the marked increase in the number of degranulated cells as compared to non-challenged cells. Kp treatment totally prevented mast cell granule release due to OVA sensitization. In addition, we analyzed the effect of Kp on histamine release by anti-DNP IgE-sensitized mast cells challenged with DNP *in vitro*. A significant inhibition ($p<0.01$) in the levels of secreted histamine was found in cells that were pre-treated with Kp prior to DNP challenge (Fig. 6). Together, those findings show that Kp inhibits the release of histamine granules by immunologically-challenged mast cells.

3.5. Pre-treatment with the flavonoid quercitrin prevents death caused by anaphylactic shock

Flavonoids are known to have potent antiallergic activity [28], and the Kp quercitrin flavonoid was reported to be active on another Th2-mediated pathology, the murine cutaneous leishmaniasis [13]. To assess the role of quercitrin as an important antianaphylactic component of Kp, mice were daily treated with 20 mg/Kg of oral quercitrin during OVA-sensitization, a dose 5% of that used with Kp (400 mg/Kg) compatible with its content in the aqueous extract [10].

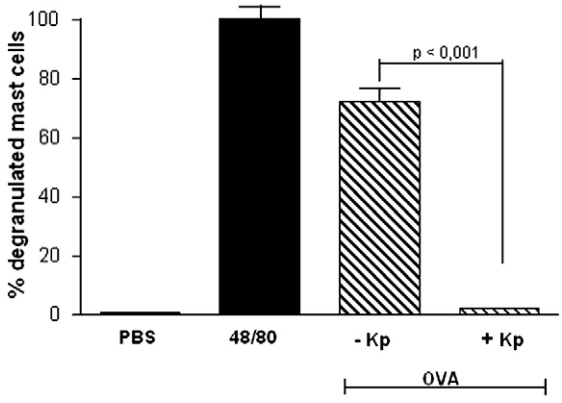

Fig. 5. Prevention of mast cell degranulation and histamine release by Kp *in vitro*. Naive rat mesenteric mast cells were sensitized *in vitro* with anti-OVA IgE, and then treated with 100 μg/ml of Kp. Cell degranulation was specifically induced with 1 mg/ml of OVA, and the granules stained with Toluidine Blue. The results are expressed as the percentage of total degranulated cells treated with 0.5 μg/ml of 48/80 compound alone. Means±S.D. ($n=5$).

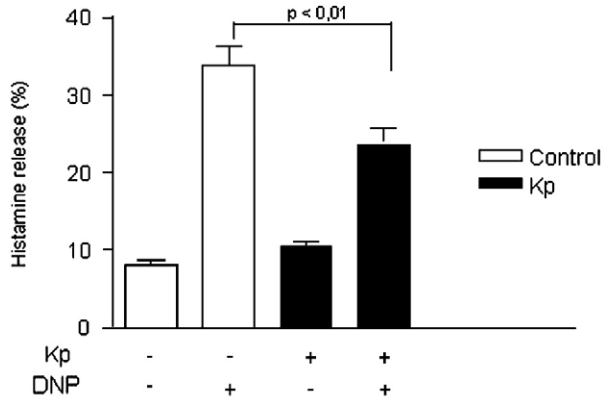

Fig. 6. Prevention of mast cell histamine release by Kp *in vitro*. Rat peritoneal mast cells were sensitized *in vitro* with anti-DNP IgE and then incubated with 500 μg/ml of Kp for 30 min prior to the 1-hour challenge with 50 μg/ml of DNP/BSA. Histamine was measured in the supernatants by a fluorimetric assay and expressed as the percentage of total histamine in cell lysates. Means ± S.D (n = 4).

We observed that oral treatment with quercitrin conferred resistance to fatal anaphylactic shock in 75% of the animals, as compared with 0% of resistance in untreated sensitized animals (Fig. 7). This finding indicates that quercitrin is an important antianaphylactic component of Kp.

4. Discussion

Anaphylactic shock is an extreme and fatal allergic reaction, and a drug capable of preventing or restraining it is normally entitled with a very potent antiallergic action. In this work we demonstrated the anti-anaphylactic and Th2-associated immunomodulatory effect of Kp using a murine model of fatal anaphylaxis.

Expression by eosinophils of several membrane receptors for immunoglobulins as well as for cytokines, chemokines and other chemotactic factors renders these cells exquisitely sensitive to a large variety of molecules and leads to the release not only of several inflammatory mediators (cationic proteins, leukotrienes, etc.) but also of immunoregulatory cytokines [18]. An important cytokine in the development of allergic inflammation is IL-5 that promotes the maturation, differentiation and recruitment of eosinophils. Its inhibition with anti-IL-5 monoclonal antibody was shown to be a feasible therapy strategy in asthma [19]. Here we showed that mice pretreated with Kp had impaired capacity to produce IL-5 when challenged with the allergen (Fig. 4), compatible with the reduced eosinophilia (Fig. 2A) and their increased resistance to anaphylactic shock (Fig. 1).

Normally, when activated by specific IgE, mast cells release histamine and lipid derivatives upon challenge with the allergen. These cells also produce cytokines and chemokines that contribute to a late phase response that begins hours later and is heralded by the onset of local inflammation with TH2 cells, eosinophils, monocytes and basophils [20]. TNF-α, a pro-inflammatory cytokine implicated in Th2-cell-mediated allergic diseases such as atopic dermatitis, rhinitis and asthma appears to exert this effect by promoting the homing of Th2 cells to the site of allergic inflammation [21]. Like IL-5, TNF-α was found to be inhibited by Kp treatment, consistent with its anti-anaphylactic effect. IL-10, a cytokine produced by Th2 cells, type 1 regulatory T cells and other cell types such as B cells and macrophages. It was shown to control allergic inflammation by suppression of Th2 cells and effector cells of allergic inflammation such as eosinophils and mast cells [22], inhibiting IgE-induced activation of human mast cells [23]. Surprisingly, we observed that the controlled anaphylaxis promoted by Kp treatment was accompanied by a reduced production of IL-10, suggesting that this cytokine was not so critical in that model of anaphylactic shock.

The allergic reaction culminates with the release of histamine and lipid derivatives from allergen-specific IgE-activated mast cells. The anti-anaphylactic activity of natural substances has been reported in *in vitro* models of mast cell degranulation or *in vivo*, in animals treated with the mast cell degranulating 48/80 compound or submitted to passive cutaneous anaphylaxis [24–27]. In the present work, we used a true model of immuno-mediated anaphylactic shock where mice collapse to death when challenged with the specific allergen. Death prevention in 60% of the challenged animals that received a single i.p. dose of Kp 3 h prior to challenge, as shown in Fig. 1, indicates a rapid and potent antiallergic action compatible with its antidegranulating/antihistaminic effect on mast cells, compatible with the results depicted in Figs. 5 and 6.

Often, a parenteral drug injection is necessary to achieve a rapid effect and revert an anaphylactic shock. However, the oral route of administration is more attractive than the parenteral route, for obvious reasons. A single oral dose with Kp prior to challenge was not as effective as the single *i.p.* dose (data not shown). Although multiple *i.p.* injections with Kp were more effective in inhibiting the production of IL-5, IL-10 and TNF-α during OVA-sensitization, orally administered Kp was more effective in preventing the IgE production and death than *i.p.* injections. It is conceivable that when orally administered, active Kp metabolites were produced and made systemically available upon passage through the digestive system. We attempted here to identify active substances responsible for the anti-anaphylactic effect of Kp. Flavonoids like quercetin glycosides were shown to possess anti-allergic activity [28–30], and are present in Kp [10]. These glycosyl flavonoids are thus strong candidates as active components of Kp The contribution of rutin (quercetin-3-rutinoside) to the anti-anaphylactic effect of Kp is unlike, as it is present in negligible amounts in the aqueous extract used here (our unpublished data). On the other hand, we demonstrate here that quercitrin in doses similar to found in Kp extract prevented acute and fatal anaphylactic shock in 75% of the challenged animals (Fig. 7). Whether or not quercitrin acts directly is not known yet. Quercitrin can be absorbed intact from the intestine and is detected as such in the plasma (our unpublished observation). However, the possibility that it was hydrolyzed to the aglycone quercetin in the liver [31], and in the intestine by

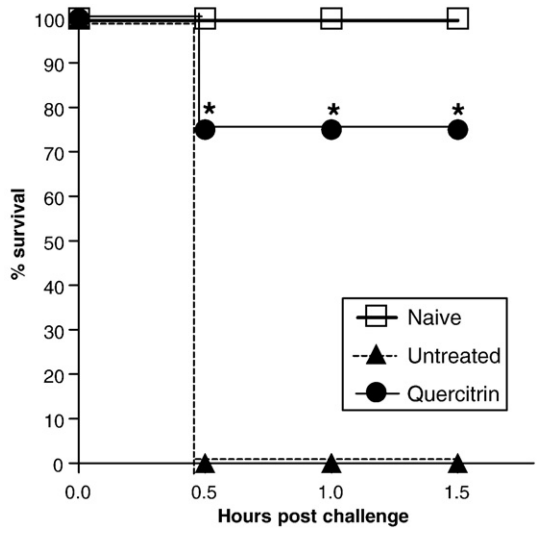

Fig. 7. Pre-treatment with quercitrin partially prevents death due to anaphylactic shock. BALB/c mice (n = 8) were sensitized as for Fig. 1. During the sensitization period, they were daily treated with 20 mg/Kg of quercitrin by the oral route, during 14 days. Controls were left untreated. Naive were untreated non-immunized mice. Upon challenge on day 14, death events were monitored for up to 90 min and recorded as shown. The results are expressed as the percentage of surviving animals. *p < 0.01 compared to untreated group.

bacterial rhamnosidases [32] cannot be ruled out. In this context, it has been demonstrated in an experimental model of rat colitis that quercitrin releases quercetin after glycoside's cleavage in the intestine, in order to perform its anti-inflammatory effect which is mediated through the inhibition of the NF-kappaB pathway [33].

The fact that the crude Kp extract can protect 100% of the animals suggests that other Kp components acts in synergy with quercitrin. The possibility that contaminating endotoxin accounted for the stronger potency Kp extract is discarded on the grounds that the extract contained negligible amounts of endotoxin (<10 EU/mg). Besides, the immunopotentiating action of endotoxin would be contradictory with the reduced capacity of Kp-treated animals to produce TNF-α.

Cutaneous leishmaniasis and allergy are normally associated with expanded Th2-type immune responses [34,35]. Interestingly, both these pathologies are benefited from the oral treatment with Kp [8, and this study]. Although blockade of histamine release may ultimately contribute to the anti-anaphylactic effect of Kp, it is conceivable that downregulation of Th2-type immune responses is more critical for the resistance phenotype. The safety of Kp to humans is substantiated by the fact that it is widely consumed in the popular medicine, and that a human case of cutaneous leishmaniasis displayed unaltered serum toxicological parameters following oral treatment [36]. For its effectiveness in mice and expected clinical safeness, quercitrin-containing Kp or quercitrin alone are potential candidates for clinical tests aiming at a prophylactic therapy for hypersensitive people under the risk of anaphylactic shock.

References

[1] Perry LM, Metzger J. Medicinal Plants of East Asia attributed properties and uses. Mit Press; 1980. p. 109–10.
[2] Nassis CZ, Haebisch EM, Giesbrecht AM. Antihistamine activity of *Bryophyllum calycinum*. Braz J Med Biol Res 1992;25:929–36.
[3] Pal S, Chaudhuri NAK. Anti-inflammatory action of *Bryophyllum pinnatum* leaf extract. Fitoterapia 1990;61:527–33.
[4] Pal S, Chaudhuri NAK. Studies on the anti-ulcer activity of a *Bryophyllum pinnatum* leaf extract in experimental animals. J Ethnopharmacol 1991;33:97–102.
[5] Supratman U, Fujita T, Akiyama K, Hayashi H, Murakami A, Sakai H, et al. Anti-tumor promoting activity of bufadienolides from *Kalanchoe pinnata* and *K. daigremontiana* × *tubiflora*. Biosci Biotechnol Biochem 2001;65:947–9.
[6] Yadav NP, Dixit VK. Hepatoprotective activity of leaves of *Kalanchoe pinnata* Pers. J Ethnopharmacol 2003;86(2–3):197–202.
[7] Rossi-Bergmann B, Costa SS, Borges MBS, Da-Silva SA, Noleto GR, Souza MLM, et al. Immunosuppressive effect of the aqueous extract of *Kalanchoe pinnata* in mice. Phytother Res 1994;8:399–402.
[8] Da-Silva SA, Costa SS, Mendonça SCF, Silva EM, Moraes VLG, Rossi-Bergmann B. Therapeutic effect of oral *Kalanchoe pinnata* leaf extract in murine leishmaniasis. Acta Tropica 1995;60:201–10.
[9] Almeida AP, Da-Silva SA, Souza ML, Lima LM, Rossi-Bergmann B, de Moraes VL, et al. Isolation and chemical analysis of a fatty acid fraction of *Kalanchoe pinnata* with a potent lymphocyte suppressive activity. Planta Medica 2000;66:134–7.
[10] Muzitano MF, Tinoco LW, Guette C, Kaiser CR, Rossi-Bergmann B, Costa SS. Assessment of antileishmanial activity of new and unusual flavonoids from *Kalanchoe pinnata*. Phytochemistry 2006;67:2071–7.
[11] Romagnani S. The role of lymphocytes in allergic disease. J Allergy Clin Immunol 2000;105:399–408.
[12] Finkelman FD. Anaphylaxis: lessons from mouse models. J All Clin Immunol 2007;120:506–15.
[13] Muzitano MF, Cruz EA, Almeida AP, et al. Quercitrin: an antileishmanial flavonoid glycoside from *Kalanchoe pinnata*. Planta Medica 2006;72:81–3.
[14] Rossi-Bergmann B, Noleto G. A low-cost and efficient procedure for harvesting DNA-labeled cells using a dot-blot apparatus. Biotechniques 1994;17:678–80.
[15] De Oliveira Barreto E, Frias Carvalho V, Diaz BL, et al. Adoptive transfer of mast cells abolishes the inflammatory refractoriness to allergen in diabetic rats. Int Arch Allergy Immunol 2003;131:212–20.
[16] Shore PA, Burkhalter A, Cohn VH. A method for the fluorimetric assay of histamine in tissue. J Pharmacol Exp Ther 1959;127:182–6.
[17] Khan LN, Kon OM, Macfarlane AJ, Meng Q, Ying S, Barnes NC, et al. Attenuation of the allergen-induced late asthmatic reaction by cyclosporin A is associated with inhibition of bronchial eosinophils, interleukin-5, granulocyte macrophage colony-stimulating factor, and eotaxin. Am J Respir Crit Care Med 2000;162: 1377–82.
[18] Dombrowicz D, Capron M. Eosinophils, allergy and parasites. Curr Opin Immunol 2001;13:716–20.
[19] Kips JC, O'Connor BJ, Langley SJ, Woodcock A, Kerstjens HA, Postma DS, et al. Effect of SCH55700, a humanized anti-human Interleukin-5 antibody, in severe persistent asthma. Am J Respir Crit Care Med 2003;167:1655–9.
[20] Chen L, Martinez O, Overbergh L, Mathieu C, Prabhakar BS, Chan LS. Early up-regulation of Th2 cytokines and late surge of Th1 cytokines in an atopic dermatitis model. Clin Exp Immunol 2004;38:375–87.
[21] Iwasaki M, Saito K, Takemura K, Sekikawa H, Fujii H, Yamada Y, et al. TNF-alpha contributes to the development of allergic rhinitis in mice. J Allergy Clin Immunol 2003;112:134–40.
[22] Wu K, Bi YT, Sun K, Wang CZ. IL-10-Producing Type 1 regulatory T cells and allergy. Cell Mol Immunol 2007;4:269–75.
[23] Royer B, Varadaradjalou S, Saas P, Guillosson JJ, Kantelip JP, Arock M. Inhibition of IgE-induced activation of human mast cells by IL-10. Clin Exp All 2001;31: 694–704.
[24] Kim SH, Kim SA, Park M, Kim SH, Park YD, Na HJ, et al. Paeonol inhibits anaphylactic reaction by regulating histamine and TNF-a. Int Immunopharmacol 2004;4:279–87.
[25] Hong SH, Jeong HJ, Kim HM. Inhibitory effects of *Xanthii fructus* extract on mast cell-mediated allergic reaction in murine model. J Ethnopharmacol 2003;88: 229–34.
[26] Ueda Y, Oku H, Iinuma M, Ishiguro K. Anti-anaphylactic and antipruritic effects of the flowers of Impatiens textori MIQ. Biol Pharm Bull 2005;28:1786–90.
[27] Nyarko AK, Addy ME. In-vitro screening for the anti-anaphylactic agent in Thonningia-sanguinea. J Ethnolpharmacol 1994;41:45–51.
[28] Kawai M, Hirano T, Higa S, Arimitsu J, Maruta M, Kuwahara Y, et al. Flavonoids and related compounds as anti-allergic substances. Allergol Int 2007;56:113–23.
[29] Lee E, Choi EJ, Cheong H, Kim YR, Ryu SY, Kim KM. Anti-allergic actions of the leaves of *Castanea crenata* and isolation of an active component responsible for the inhibition of mast cell degranulation. Arch Pharm Res 1999;22:320–3.
[30] Shaik YB, Castellani ML, Perrella A, et al. Role of quercetin (a natural herbal compound) in allergy and inflammation. J Biol Reg Homeost Ag 2006;20:47–52.
[31] Walle T. Absorption and metabolism of flavonoids. Free Radical Biol Med 2004;36: 829–37.
[32] Park SY, Kim JH, Kim DH. Purification and characterization of quercitrin-hydrolyzing alpha-L-rhamnosidase from Fusobacterium K-60, a human intestinal bacterium. J Microbiol Biotechnol 2005;15:519–24.
[33] Comalada M, Camuesco D, Sierra S, et al. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. Eur J Immunol 2005;35:584–92.
[34] Alexander J, Bryson K. T helper (h)1/Th2 and Leishmania: paradox rather than paradigm. Immunol Lett 2005;9:17–23.
[35] Ramalingam TR, Reiman RM, Wynn TA. Exploiting worm and allergy models to understand Th2 cytokine biology. Curr Op All Clin Immunol 2005;5:392–8.
[36] Torres-Santos EC, Da-Silva SAG, Santos APPT, Almeida AP, Costa SS, Rossi-Bergmann B. Toxicological analysis and effectiveness of oral *Kalanchoe pinnata* on a human case of leishmaniasis. Phytother Res 2003;17:801–3.