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Research paper

Single blinded, randomized, placebo-controlled study on the effects of ciclosporin on cutaneous barrier function and immunological response in atopic beagles

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ABSTRACT

Ciclosporin (CsA) is a common treatment for canine atopic dermatitis (cAD). cAD is a very common skin disease with a multifactorial pathogenesis due to complex interactions between the host and the environment. The purpose of this study was to describe the physical and immunological effects of CsA in cAD using a canine model of AD. Fourteen beagles were enrolled; seven received CsA orally every 24 h for 28 days, and seven received placebo. All dogs were exposed to relevant allergens, house dust mite solution, one day prior to treatment and once weekly thereafter for 28 consecutive days. Canine atopic dermatitis extent and severity index-03 (CADESI-03) and skin biopsies were performed on day 0, 14, and 28. Quantitative RT-PCR was used to determine levels of cutaneous cytokines and barrier function markers. Indirect immunofluorescence was used to determine protein expression and distribution of nuclear messengers, barrier function and inflammatory [thymic stromal lymphopoietin (TSLP)] markers. The data were tested for normality and then the upaired two samples Student's *t*-test and the repeated measurements ANOVA, followed by the Dunnett's Multiple Comparison Test as post-hoc analysis, were performed. A P value of < 0.05 was considered statistically significant. A significant decrease in CADESI-03 occurred for the treatment group compared to placebo ($p = 0.023$) on day 28. On day 14, a significant increase in TSLP protein expression [$p = 0.019$ (placebo); $p = 0.02$ (CsA)] and a significant decrease in *Transforming Growth Factor (TGF)-β* mRNA [$p = 0.01$ (placebo); $p = 0.015$ (CsA)] were noted in both groups compared to baseline. On day 28, a significant increase in *canine beta defensin (cBD)103* [$p = 0.012$ (placebo)] and *cBD3-like* mRNAs [$p = 0.044$ (placebo)], and filaggrin [$p = 0.035$ (CsA)] and TSLP protein expressions [$p = 0.0092$ (CsA)] were seen compared to baseline. In contrast, a significant decrease in mRNA of *Tumor Necrosis factor (TNF)-α* [$p = 0.013$ (CsA)], *Interleukin (IL)-10* [$p = 0.038$ (CsA)], *TGF-β* [$p = 0.017$ (CsA)], and *caspase 14* [$p = 0.014$ (CsA)] was seen on day 28 compared to baseline. Comparison of the groups revealed no significant effect on skin immunologic milieu or barrier markers despite evident improvement of physical signs in the treatment group. Although this study confirmed the usefulness of CsA for the treatment of cAD, a clear involvement of CsA on some of the currently known immunological alterations present in cAD was not determined. However, it is important to note that there was no measurable exacerbation of skin barrier dysfunction secondary to CsA administration in this model.

1. Introduction

Current therapeutic options for canine atopic dermatitis (cAD) are varied and differ in mechanism of action, efficacy, and side effects. Ciclosporin (CsA) is a therapeutic option that has been under intense investigation in the recent years. Ciclosporin interferes with the activation and proliferation of T cells via inhibition of calcineurin, resulting in a modification of the cell-mediated immune response and decreased pro-inflammatory cytokine production (Forsythe and Paterson, 2014). Due to its mechanism of action and clinical efficacy, it has been approved for treatment of cAD.

In both human and veterinary medicine, much is known about the systemic pharmacodynamics of CsA; however, only few studies have assessed its direct effects on the cutaneous immunological milieu and barrier function (Grzanka et al., 2012; López-Flores et al., 2011). Impaired skin barrier function has been demonstrated to play a role in the pathogenesis of both human and canine AD (Miller et al., 2013). The most studied components of the cutaneous skin barrier are filaggrin

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(FLG) and antimicrobial peptides (AMPs) along with lipid components of the stratum corneum (Palmer et al., 2006; Santoro et al., 2011b; Smith et al., 2006). In particular, in people it has been demonstrated that loss of function mutations of epidermal proteins, such as FLG, and altered expression of AMPs and protease-activated receptors (PARs) are associated with AD development (Palmer et al., 2006; Santoro et al., 2011b; Smith et al., 2006); however, such abnormalities can be exacerbated by local inflammation. Indeed, inflammatory cytokines are able to decrease *FLG* mRNA expression through down-regulation of caspase-14, an enzyme crucial in the FLG degradation process (Hvid et al., 2011). Conversely, pro-inflammatory cytokines are associated with increased AMP production in human and canine keratinocytes (Nomura et al., 2003; Santoro et al., 2015a). The global cutaneous effects of CsA are currently unknown in dogs, and no study has evaluated its effects on cytokine milieu or barrier function in the skin of atopic dogs.

The objective of this study was to describe the clinical and immunological effects of CsA using a canine model of AD. Two hypotheses were tested: 1) there will be an amelioration of the physical signs of AD as well as the markers for barrier function and a decrease of inflammatory cytokines in the skin of atopic beagles after oral CsA administration, and 2) there will be a decrease in canine atopic dermatitis extent and severity index (CADESI-03) scores (clinical score) as relates to cytokine expression and markers for barrier function in atopic beagles after oral CsA.

2. Materials and methods

2.1. Animal population selection

This was a single blinded, randomized, placebo-controlled study using a validated canine model of AD (Marsella and Girolomoni, 2009; Marsella et al., 2006). This model allows the evaluation of the immunological alterations present in cAD in a controlled environment, and it is able to reduce confounding factors and facilitate the possibility to have significant results with a very small number of dogs (Santoro and Marsella, 2014). The canine model is composed of beagle dogs that have been previously epicutaneously sensitized to house dust mite (HDM) and have high immunoglobulin E for HDM. These dogs are able to produce physical and histologic lesions identical to that of naturally-occurring atopic dermatitis when challenged with topical HDM solution (Marsella et al., 2006).

Fourteen age- and sex-matched beagles were randomly assigned to two groups using an online random number generator (www.randomizer.org). All investigators (AW, DS, and RM) were blinded to the treatment being administered, except for the one who was responsible for medication administration (KA). The study was conducted and documented in accordance with procedures approved by the University of Florida Institutional Animal Care and Use Committee.

2.2. Treatment dosing and allergen challenge

The treatment group of seven beagles received 5 mg/kg CsA (Atopica®, Novartis Animal Health, Inc., Greensboro, NC, USA) orally once every 24 h for 28 days, and seven beagles received a placebo capsule once every 24 h for 28 days. All dogs were stimulated with HDM as previously described (Marsella et al., 2006). Briefly, HDM was prepared from a culture (natural *D. farinae*, Greer Laboratories Inc, Lenoir, NC, USA), and mixed with filtered (0.2 μm syringe filter) PBS (pH = 7.2) to obtain a final concentration of 31 mg/ml. The solution was vortexed and rocked for 30 min prior to use. Dogs had 1.6 ml (total of 50 mg HDM/challenge) of the solution smeared on the ventral aspect of their chest and abdominal area. All dogs were monitored for 30 min to ensure solution had sufficient contact time without disruption via the animal or environment. The environmental challenge was performed one day prior to the first dose of CsA and every seven days afterwards (days −1, 6, 13, 20, and 27) until the end of the study to keep the lesions active and mimic a chronic allergen exposure. The concentration and volume of HDM used was selected based on previous studies (Marsella et al., 2006).

2.3. CADESI-03 scoring protocol

Clinical evaluation was performed using the CADESI-03 scoring system (Olivry et al., 2007). This is a validated clinical scoring system for assessment of cAD disease severity in clinical trials and research studies. The scoring system combines the evaluation of five degrees of severity (none [0], mild [1], moderate [2–3], and severe [4–5]) for each of five cardinal signs of cAD (erythema, papules, macules, excoriations, and self-induced alopecia) at 62 different body areas. The maximal achievable CADESI-03 is 1240 (Olivry et al., 2007). All dogs were scored by the same blinded investigator (RM) on days −1, 7, 14, 21, and 28. Scoring was performed before and 6 and 24 h after each allergen challenge.

2.4. Sample collection

Two 8-mm-punch biopsies from the abdomen were taken on days 0, 14, and 28 at 6 h after allergen exposure. The abdomen was chosen for the skin biopsy as it is an easily accessible area with low level of hair density and because it is an area commonly involved in AD. The skin was locally anesthetized using 1 ml of buffered lidocaine 2% (Hospira, Inc., Lake Forest, IL, USA) per biopsy site. Each sample was immediately divided in two halves. One half was fixed in 10% buffered formalin for no more than 48 h before being stored in phosphate buffer solution (PBS) until processing for immunofluorescence (IF). The other half was further divided in two quarters, placed in 1.5 ml microfuge tubes, and immediately flash-frozen in liquid nitrogen. These latter samples were stored at −80 °C until processed for molecular biology evaluation.

2.5. Measurement of inflammatory mediators and markers of barrier function: PCR technique

Total RNA was isolated from skin biopsies. Briefly, one quarter of the 8 mm biopsy punch was homogenized using a PowerGen 125 (Fisher Scientific, Pittsburgh, PA, USA) and then processed into RNA using the 5 PRIME PerfectPure™ RNA fibrous tissue kit (5 PRIME, Inc.; Gaithersberg, MD, USA) according to manufacturer's protocol including the DNAse treatment. The total RNA concentrations were determined at 260 nm using UV spectrophotometry (ND-800C NanoDrop®; Thermo Fisher Scientific, Inc.; Wilmington, DE, USA).

Canine mRNA was quantified using reverse transcriptase (RT)-PCR. Total RNA (0.5 μg) was converted to complementary DNA (cDNA) by reverse transcription of mRNA using SuperScript™ II Reverse Transcriptase First-Strand Synthesis System® (Invitrogen, Carlsbad, CA, USA). All mRNA levels were quantified using ABI (Applied Biosystems Inc, Foster City, CA) qRT-PCR methodology. All samples were analyzed in triplicate 25 μl RT reactions in an ABI 7500 FAST Real Time PCR System (Applied Biosystems Inc, Foster City, CA, USA). PCR amplifications were carried out using either the SYBR green [*canine beta defensin (cBD)1-like, cBD3-like, cBD103, canine cathelicidin (cCath), s100A8, FLG,* and *RPLO*] or the Taqman [*Interleukin (IL)-2, IL-4, IL-10, IL-13, Interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, Transforming Growth Factor (TGF)-β,* and *caspase (CASP) 14*] methodology. For the SYBR green methodology, an initial cycle of 50 °C for 20 s and 95 °C for 10 min was followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min followed by dissociation (melting) curves to ensure specificity of the primers. For the FAST Taqman™ methodology, an initial cycle of 50 °C for 2 min and 95 °C for 10 min was followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The results were analyzed using the comparative C_T (cycle threshold) method (placebo *versus* treated), and the