

## EFFECT OF *TANACETUM PARTHENIUM* ON SERUM AMYLOID A AND SIALIC ACID IN DOGS WITH *PSEUDOMONAS AERUGINOSA* SKIN INFECTION

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### ABSTRACT

The effect of *Tanacetum parthenium* (feverfew) on serum amyloid A (SAA) and free sialic acid (SA) were investigated in dogs. The animals were divided into three groups: healthy dogs (Group I; n=5), dogs infected subcutaneously with  $1 \times 10^8$  CFU/mL field *P. aeruginosa* culture (Group II; n=5) and infected dogs treated with feverfew (90 mg standardised extract, 0.7% parthenolide) (Group III; n=5). Oral phytopreparation (2 capsules daily) intake began from post infection hour 4 and lasted 6 days. SAA concentrations increased insignificantly in infected dogs (16 mg/L) while infected dogs treated with feverfew exhibited more than 100-fold increase between post infection hours 24–72 vs Group II. Substantial differences ( $p < 0.01$ ) were identified vs Groups I and II at post infection hour 4, 48 and 72, but one day after feverfew discontinuation (day 7), they were not found out. Serum SA was low in controls (1.65–2.3 mmol/L) increasing by hour 72 in Groups II and III ( $p < 0.01$ ) to 2.8 and 3.49 mmol/L respectively. Positive correlation between both studied markers was present only in infected dogs receiving feverfew (Spearman's coefficient of rank correlation=0.410,  $P=0.0086$ , n=40). *Tanacetum parthenium* is reported to have medicinal activity in our canine skin infection model.

**Key words:** dogs, *P. aeruginosa*, infection, feverfew, serum amyloid A, sialic acid

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### INTRODUCTION

Despite the variety of synthetic chemotherapeutics and antibiotics, the control of pseudomonad infections is still a challenge (Donadio *et al.*, 2010). The attempts for direct attack on Gram-negative bacterium *P. aeruginosa* with nanofibre incorporated antimicrobial substances did not show promising results (Heunis and Dicks, 2010). On that background, medicinal plants with antimicrobial effect could be alternative and efficient treatment tools (Nascimento *et al.*, 2000; Pourianezhad *et al.*, 2016). *Tanacetum parthenium* (feverfew) is an aromatic plant with antibacterial (Izadi *et al.*, 2010; Polatoğlu *et al.*, 2010; Izadi *et al.*, 2013) antiviral (Pareek *et al.*, 2011) antifungal (Farzaneh *et al.*, 2002) insecticidal (Polatoğlu *et al.*, 2011) antiprotozoal effects (Izumi *et al.*, 2008). Its wide spectrum of biological activities includes analgesic, antipyretic and anti-inflammatory properties (Pareek *et al.*, 2011; George *et al.*, 2012; Mathema *et al.*, 2012). Andonova *et al.* (2015, 2016, 2017) analyzed the effects of the medicinal herb feverfew on innate defense mechanisms in dogs with experimental *P. aeruginosa* infections, while Smolinski and Pestka (2003, 2005) used murine models. In order to elucidate pathogenetic mechanisms of *P. aeruginosa*, Alhazmi (2015) proposed an *in vitro* model although data did not correspond to clinical cases where numerous extrinsic and intrinsic factors influence the course of infection. Martin *et al.* (2008), Saewan and Jimtaisong

(2013) provided proofs for the photoprotective benefits of feverfew in human clinical studies.

*In vivo* effects of *Tanacetum parthenium* on acute phase response, an integral part of innate immunity in human and animal infections of the skin are scarce.

The production, kinetics and indication possibilities of its products – acute phase proteins (APPs) (Eckersall and Bell, 2010; Langhorn *et al.*, 2013) depend on inflammatory stimuli and the biological species (Murata *et al.*, 2004). The variety of APPs, as well as the different magnitude of occurring changes in their levels necessitates their accurate selection.

Serum amyloid A (SAA) is a major acute phase protein (APP) in many species (cattle, horses, dogs, cats) (Hulten *et al.*, 1997; Ceron *et al.*, 2005), but data on its use as a marker of inflammation are inconsistent. According to Christensen *et al.* (2014), Schmidt and Eckersall (2015) SAA has a good general diagnostic value in inflammation, infections while Jitpean *et al.* (2014) affirmed that it changed only after a severe systemic inflammation. Additional studies on the diagnostic potential of this positive APP are necessary (Christensen *et al.*, 2012, 2013, 2014) to elucidate its various immunomodulatory effects (Sack, 2018). The sialic acid (free, lipid-bound and protein-bound) which plays important role in a number of biological processes, cellular interactions, signalling pathways has also raised a number of questions (Mohebbi *et al.*, 2010). Hartnell *et al.* (2001), Cital *et al.* (2004) affirmed that the N-acetylneuraminic acid is a marker of inflammation while others (Mohebbi *et al.*, 2010; Khoshvaghti and Nazifi,

2012; Nazifi *et al.*, 2012) discussed it as an APP. This has raised our interest to changes in both APPs in dogs with skin infection, treated or not with feverfew, due to the lack of literature data.

The aim of this study was to investigate the *in vivo* effect of *Tanacetum parthenium* (feverfew) on the APPs serum amyloid A and free sialic acid as well as their correlation in healthy dogs, dogs with experimental *P. aeruginosa* skin infection and infected dogs treated with feverfew.

## MATERIALS AND METHODS

**Animals:** Experiments were approved by the Ethical Committee of National Veterinary Service in Bulgaria (permit No. 37) and performed in accordance with the animal welfare standards. Fifteen clinically healthy male mixed-breed dogs aged 2–5 years were obtained from a licensed kennel. The animals were vaccinated against canine infectious hepatitis, distemper, leptospirosis, and rabies. The treatment against ectoparasites included antiparasitic shampoo Friskies (Viale G.Richard, Milano, Italia) and permethrin and carbaryl (Tapilan-B – Dorvert, Israel), and anthelmintic treatment comprised praziquantel and abamectin (Prazimec D – Biovet Ltd, Peshtera, Bulgaria) at a dose of 1 tablet/10 kg body weight. The dogs were kept under similar housing and management conditions for acclimatization for a month and fed with a commercial diet according to the manufacture's prescriptions with free access to water.

**Experimental design:** The dogs were divided into 3 groups: Group I (n=5) were untreated (controls). Dogs from Group II (n=5) were subcutaneously infected with *P. aeruginosa* bacterial culture. A bacterial field strain culture in stationary phase with density corresponding to  $1 \times 10^8$  CFU/mL was prepared nephelometrically according to the MacFarland standard (Quinn *et al.*, 1999). Dogs from Group III (n=5) were infected with *P. aeruginosa* as those from Group II and received orally the phytopreparation feverfew (standardised extract, 90 mg, 0.7% parthenolide – Nature's Way, USA) beginning from the 4<sup>th</sup> post infection hour for 6 consecutive days at a daily dose of 2 capsules.

**Blood samples and serum analysis:** Blood for analysis was collected in all groups from v. cephalica antebrachii before the infection (0 h), and on post infection hours 4, 24, 48, 72 and days 7, 10 and 14. Sera were separated after 30-minute stay at room temperature, followed by 15-minute storage in a refrigerator and centrifugation for 10 minutes at  $3000 \times g$  and stored in plastic vials at  $-20$  to  $-80^\circ\text{C}$ . Serum concentrations of canine SAA (mg/L) were determined by ELISA kit (Cat no. TP-802) (Tridelta PhaseTM Range SAA Assay, Tridelta Development Ltd,

Kildare, Ireland) specially developed for dogs according to the manufacturer's instructions. Serum free sialic acid concentrations (mmol/L) were assayed by the spectrophotometric method of Sydow (1985), a modification of the method reported by Shamberger (1984).

**Statistical analysis:** Normal distribution of data was evaluated by the Kolmogorov-Smirnov test. As data were not normally distributed, results were presented as median and range (min-max). Differences within each group were tested by the Kruskal-Wallis test (Dunn *post hoc* test) while between-group differences – by the Mann-Whitney non-parametric test. The relationships among the different variables were examined by Spearman rank correlation analysis. All statistical tests were performed with MedCalc 10.2.0.0, Belgium) software.

## RESULTS

Data about SAA concentrations (Table 1) showed that in healthy dogs, this major APP ranges from 0 mg/L to a maximum of 7.4–7.6 mg/L. The subcutaneous injection of *P. aeruginosa* broth culture in dogs from Group II did not alter statistically significantly its levels but by post infection hours 24 and 48, the APP increased to peak levels of 15.8 mg/L and 16.0 mg/L. On the contrary, in dogs from Group III (infected and orally treated with the phytopreparation feverfew), SAA exhibited more than 100-fold increase: 156.6 mg/L on hour 24; 204.9 mg/L on hour 48 ( $p < 0.05$  vs 0 h), and 479.4 mg/L on hour 72 ( $p < 0.01$  vs 0 h). SAA levels differed significantly ( $p < 0.01$ ) vs Groups I and II by the post treatment hours 4, 48 and 72, but one day after oral administration of feverfew was discontinued, no relevant between-group differences were found out.

Free sialic acid (Table 2) in healthy control ranged between 1.26 mmol/L and 2.42 mmol/L. In infected untreated dogs (Group II), its concentration increased only by the 72<sup>nd</sup> hour to a maximum of 3.38 mmol/L, but no statistically significant intra- and between group differences were proved. Infected dogs treated orally with feverfew reacted with substantially increased sialic acid concentration by post infection hour 48 ( $p < 0.05$  vs Group I and II) and hour 72 ( $p < 0.01$  vs Group I and II) when the peak value of 4.4 mmol/L was attained.

Spearman rank correlation analysis showed no statistically significant relationship between SAA and SA in controls ( $\rho = 0.0883$ ,  $P = 0.5881$ ,  $n = 40$ ) and in infected only dogs ( $\rho = -0.00729$ ,  $P = 0.9644$ ,  $n = 40$ ), while in the feverfew-treated infected group, a strong positive correlation between both parameters was found ( $\rho = 0.410$ ,  $P = 0.0086$ ,  $n = 40$ ).

**Table 1. Dynamics of serum amyloid A concentrations (mg/L) in control dogs (Group I), dogs with experimental skin *P. aeruginosa* infection (Group II) and dogs with experimental skin *P. aeruginosa* infection treated with feverfew (Group III). All data are presented as median [min-max range]; n=5.**

Post infection period	Controls Group I	Skin infection Group II	Infection+feverfew Group III	P between groups
0h	0 [0–0]	0.1 [0–0.7]	1.3 [0.2–3.0]	
4h	0 [0–1.3]	0.2 [0–0.4]	1.1 [0.3–5.3]	P <sub>I-III</sub> <0.01
24h	0 [0–7.4]	4.2 [3.6–15.8]	156.6 [34.3–300.9]	P <sub>II-III</sub> <0.01
48h	0 [0–0.6]	7.7 [0–16.0]	204.9* [98.7–680.0]	P <sub>I-II</sub> <0.01 P <sub>I-III</sub> <0.01 P <sub>II-III</sub> <0.01
72h	0 [0–0.7]	0.5 [0–0]	479.4** [233.0–661.6]	P <sub>I-III</sub> <0.01 P <sub>II-III</sub> <0.05
7d	0 [0–2.5]	0 [0–0]	2.2 [0.8–14.7]	
10d	0 [0–0]	0 [0–0.2]	3.0 [1.3–3.9]	P <sub>II-III</sub> <0.01
14d	0 [0–7.6]	0 [0–0]	2.7 [1.1–3.6]	P <sub>II-III</sub> <0.05

\*P&lt;0.05; \*\* P&lt;0.01 vs 0h

**Table 2. Dynamics of serum free sialic acid concentrations (mmol/L) in control dogs (Group I), dogs with experimental skin *P. aeruginosa* infection (Group II) and dogs with experimental skin *P. aeruginosa* infection treated with feverfew (Group III). All data are presented as median [min-max range]; n=5.**

Post infection period	Controls Group I	Skin infection Group II	Infection+feverfew Group III	P between groups
0h	1.9 [1.44–2.42]	1.76 [1.53–2.0]	1.52 [1.26–2.00]	
4h	2.3 [1.44–2.55]	1.48 [1.03–1.77]	2.24 [1.18–3.76]	
24h	2.0 [1.38–2.31]	1.41 [1.23–1.57]	1.55 [1.38–2.18]	
48h	1.7 [1.4–1.92]	1.31 [1.15–1.53]	1.20 [0.71–2.08]	P <sub>I-III</sub> <0.05 P <sub>II-III</sub> <0.05
72h	1.79 [1.45–2.0]	2.80 [2.68–3.38]	3.49 [2.33–4.40]	P <sub>I-III</sub> <0.01 P <sub>II-III</sub> <0.01
7d	1.86 [1.26–2.42]	1.64 [1.45–2.83]	1.71 [1.43–2.02]	
10d	1.65 [1.5–2.0]	1.74 [1.23–1.94]	1.69 [0.82–2.79]	
14d	1.75 [1.29–2.2]	1.76 [1.59–2.39]	1.74 [1.45–3.59]	

## DISCUSSION

The information for infectious agent, its virulence and site of host penetration is essential for adequate decision-making in infection control. The course of infection, however, largely depends on systemic defense potential and compensatory mechanisms. In our *in vivo* model of skin *P. aeruginosa* infection, many of environmental conditions influencing the infection are controlled. The analysis of results is however difficult, as the microbial pathogen attacks the host using a rich arsenal of virulence factors (He *et al.*, 2004; Fu *et al.*, 2010; Cabot *et al.*, 2012; Alhazmi *et al.*, 2015). The multicomponent defense system whose task is to combat and eradicate the pathogen, its products and disorders it may have caused mobilises numerous intricately interrelated cellular and humoral elements, which are controlled by local, neuroreflectory, humoral and

hormonal mechanisms (Lavoie *et al.*, 2011). That is why, parameters indicating occurring homeostasis disturbances that would permit to differentiate the extent of damage and to evaluate systemic defense potential, is essential. The fact that in healthy individuals APPs values are low but in homeostatic disorders their concentrations rapidly changes, allowed researchers recommending their use in clinical practice (Cray *et al.*, 2009; Jain *et al.*, 2011; Kjelgaard-Hansen and Jacobsen, 2011). In our experiment, the major canine APP serum amyloid A had low values in healthy controls throughout the study: from 0 to 1.3 mg/L (min 0; max 7.6 mg/L). Under physiological conditions, Kim *et al.* (2013) also established very low APPs levels. Hadžimusić and Hrković (2018) using a commercial ELISA, reported normal SAA concentration of 4.87±0.73 mg/L in dogs. In most available reports, SAA has been assayed via automated latex bead based immunoturbidometric assay (LZ-SAA, Eiken Chemical Co.) (Yamamoto *et al.*, 1993; Christensen *et al.*, 2012).

The existing methodological difficulties, presence of multiple isoforms of SAA (Sellar *et al.*, 1991), and numerous dog breeds impede the specification of SAA reference ranges and limit the use of this APP in routine veterinary clinical biochemistry (Petersen *et al.*, 2004; Kjelgaard-Hansen and Jacobsen, 2011).

In dogs from Group II, SAA concentrations increased insignificantly to a peak value of 16 mg/L by post infection hour 48. This findings did not correspond to numerous reports outlining this APP as a marker of inflammation and infection (Eckersall and Bell, 2010; Langhorn *et al.*, 2013), but agreed with other studies, e.g. in dogs with dental problems (Buttke *et al.*, 2005) and dogs with cystitis, anal sacculitis, chronic dermatitis (Christensen *et al.*, 2013). Considering that skin immune protection is potent, it could be hypothesised that the local cascade of mediators, cytokines and enzymes released from activated skin cells, although responsible for inflammation signs (swelling, pain, redness, heat) did not provide a sufficiently strong inflammatory impulse for intensive APPs production. That is why, although SAA is a major positive APPs in dogs, its alterations in *P. aeruginosa* infection of the skin and soft tissues are not relevant in this species.

In infected dogs treated with feverfew (Group III) however, serum amyloid A increased drastically between post infection hours 24 and 72, with more than 100-fold higher values than those in Group II. It is therefore clear that differences between the two groups were rather a result from the application of the phytopreparation that has started from the post infection hour 4 to post infection day 6. It could be presumed that SAA elevation in Group III could be due to its intensive liver synthesis or extrahepatic production (Upragarin *et al.*, 2005), but this would imply the presence of a potent inflammatory stimulus raised by proinflammatory cytokines and chemokines. Feverfew is a phytopreparation with anti-inflammatory effect due to blockade of nuclear factor kappa-beta (NF- $\kappa$ B) reported earlier in this model (Andonova *et al.*, 2015). Uluçkan *et al.* (2015) outlined the role of activator protein-1 (AP-1), suggesting that SAA expression could result from transcriptional activation of AP-1. The six day phytopreparation intake by infected dogs exerted a direct effect on the intestinal mucosa. Meek and Benditt (1986) demonstrated amyloid A gene family expression in epithelial cells of the small and large intestine. Chu *et al.* (2018) showed a group of 3 innate lymphoid cells in gut associated lymphoid tissues, taking part in innate mucosal immune defense that being activated, release a substantial amount of proinflammatory cytokines. Yet, the molecular mechanisms underlying the distribution, regulation, functions of this defense cell population of gastrointestinal lymphoid tissue and its interactions with other cells elements and intestinal microbiota are unclear (Sawa *et al.*, 2011). In their studies, Chen and Cheng (2009) identified 400

genes that are consistently regulated by feverfew extracts and hypothesised that feverfew components mediated metabolism, cell migration and cytokine production in human monocyte/ macrophages. Smolinski and Pestka (2005) found out 1.4 times enhanced IL-6 expression in the spleen than in the liver of mice although they were treated with the combination LPS+parthenolide (the primary active component of *Tanacetum parthenium*). The belief that the strong inflammatory stimulus for extrahepatic SAA synthesis was due to cytokine release following cellular elements destruction by feverfew-induced attacks of their membrane lipids seems inconsistent because SAA concentrations did not persist high during the 6 day phytopreparation intake but increased considerably only to post infection hour 72 – time of acute phase response. As the latter is a dynamic homeostatic event aimed at neutralisation of the challenging agent, support of body recovery and normalisation of systemic functions, it could be speculated that increased SAA concentrations were an *in vivo* effect of feverfew, associated with its antibacterial activity as SAA blocks cellular receptors and thus, the influence of bacteria and their products on them (Burgess *et al.*, 2018). Also, it is an opsonin and retinol-binding protein (Shah *et al.*, 2006; Liu *et al.*, 2016). Further studies are however needed to support this hypothesis.

Serum free sialic acid concentrations in controls ranged between 1.65–2.3 mmol/L and increased statistically significantly ( $p < 0.01$ ) in dogs from Group II and III by post infection hour 72 with ranges of 2.68–3.38 mmol/L and 2.33–4.4 mmol/L respectively. The recorded elevation was not attributed to infectious agent specifics, as in rats infected with *Trichinella spiralis* Ivanov *et al.* (2014) observed similar changes. The findings about increased free sialic acid blood levels in dogs with *P. aeruginosa* skin and soft tissue infection, treated or not with feverfew at post infection hours 48 and 72 are in line with many other reports (Mohebbi *et al.*, 2010; Khoshvaghti and Nazifi, 2012; Nazifi *et al.*, 2012) interpreting sialic acid as an APPs. Ghoshal *et al.* (2010) commented the correlation between sialic acid and nitric oxide, while in this experimental model, SAA and SA concentrations were strongly associated only in the infected and feverfew treated group ( $\rho = 0.410$ ,  $P = 0.0086$ ,  $n = 40$ ).

In conclusion, the changes of SAA and SA levels in dogs subcutaneously infected with *P. aeruginosa* were specific for acute phase response, but the concentrations of both APPs were not indicative for bacterial skin and soft tissue infections. The administration of feverfew to infected animals increased markedly the blood levels of both, in particular those of SAA, which reflected one of *in vivo* effects of this phytopreparation in this canine skin infection model.

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