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Tolerance of Biopronil Spot on® after repeated single- or multiple-dose topical treatments in dogs

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Abstract: A variety of toxic effects of fipronil (FIP), the active substance of Biopronil Spot on®**,** on animals and humans has been reported and raises the need to investigate the FIP toxic effects. The objectives of the study were the evaluation of the local and systemic tolerance of Biopronil Spot on® and the assessment of its influence on haematological and biochemical blood parameters after single and multiple topical treatment in dogs. Thirty-two mixed breed dogs were included in the study assessing the local and general tolerance of Biopronil Spot on® following single, triple and fivefold dose after spot-on multiple applications in dogs (on days 0, +28 and +56) at a dosage 134 mg for a dog weighing 10–20 kg and 268 mg for a dog weighing 21–40 kg. A physical examination and biochemical and haematological analyses were performed on the days of the study as follows: –14, –5, +3, +31, +59, +70. No visible pathological changes on the skin were observed. The biochemical and haematological indicators rarely exceeded the reference values. No influence of Biopronil Spot on® administered in single, triple and fivefold repeated doses on the assessed clinical, haematological and biochemical parameters in dogs was found under the conditions described in the study.

Keywords: adverse effects; biochemical indicators; fipronil; haematological indicators; substance-related disorders

Fipronil [5-amino-3-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-fluoromethylsulfinyl pyrazole] (FIP) is a second-generation phenylpyrazole insecticide used in veterinary medicine for

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the control of ectoparasites (Tingle et al. 2003). FIP blocks the gamma-aminobutyric acid (GABA)-gated chloride channels of neurons in the central nervous system, resulting in the hyper-excitation of the central nervous system and death (Koslowski et al. 2020). GABA-gated chloride channels are expressed in the central nervous system of both vertebrates and invertebrates, nevertheless, the specificity of FIP for insect GABA chloride channels is 700 to 1 300 times greater than in mammals and permits the selective control of invertebrate ectoparasitic hosts on the target species, such as dogs and cats (Zhao et al. 2003; Zhao et al. 2004). Glutamate-activated chloride channels are also the target for FIP and are present in invertebrates, such as insects, but not in mammals, which explains the stronger influence of FIP on invertebrates than vertebrates (Simon-Delso et al. 2015). However, FIP is known to have side effects on mammals, especially in the kidneys, liver, thyroid and has an influence on the reproductive function (De Oliveira et al. 2012; Khan et al. 2015) in non-target organisms (Tingle et al. 2003; Badgujar et al. 2015; Badgujar et al. 2016).

Both haematological and biochemical indicators appear to be good biological markers of the health conditions and outcome from pesticide-induced toxicity within organisms (Abouelghar et al. 2020). However, the changes are non-specific to a wide scope of substances (Prashanth and David 2006). Biochemical indicators, such as serum aminotransferase activities, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), are considered the most useful measures of liver cell injury (Schomaker et al. 2013). An increase in the activity of the serum AST, ALT, and ALP indicates loss of biochemical and basic consistency of the liver. Badgujar et al. (2015) reported that FIP causes significant increases in the activities of the serum aminotransferases, AST and ALT, with a connected increase in the absolute and relative weight of the liver.

Tolerance/safety evaluations to detect toxic or adverse events that may potentially occur following single and multiple topical treatments with FIP in dogs is an essential aspect of implementation procedures when approved for use as an antiparasitic agent for animals. Inflammatory reactions and systemic effects after spot-on applications are some of the potential safety concerns investigated in toxicity studies. Hence, the aim of this study was the evaluation of the tolerance of the new fipronil product, Biopronil Spot on®, after topical treatments in dogs. The present study was designed as a parallel, blinded, randomised trial assessing the local and general tolerance of Biopronil Spot on® following single, triple and fivefold dose spot-on multiple applications in dogs [on days (D) 0, +28 and +56].

MATERIAL AND METHODS

Ethics

The Ethical Committee of Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv approved the protocol of the study and the use of animals in the experiment with Resolution No. 8/2019 of June 3, 2019. Informed consent was obtained for the client-owned animals included in this study.

Animals

Mixed breed dogs with an average body weight of 20.93 kg (from 10.8 kg to 38 kg), an average age of 4.39 years (from 1 to 8 years) and varying hair length took part in the experiment. In total, 32 dogs (16 male and 16 female) were included in the study. The dogs belonged to clients of the Veterinary Clinic of the Department of Surgery at the Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Lviv. The dogs remained in their familiar home environments during the clinical trial.

The owners were required to bring the animals to the research unit for the clinical examinations, drug applications and blood collection. The animals were fed with commercial Purina Dog Chow® adult food (over 1 year old) adapted to the needs of adult dogs. Food and water were available without restriction. Each animal was marked with a collar with a unique number, unchangeable during the experiment.

The design of the experiment was guided by the principles contained in the Guideline on target animal safety for veterinary pharmaceutical products, London, September 22, 2008 (Doc. Ref. EMEA/CVMP/VICH/393388/2006). However, the present study was performed under field conditions (dogs were kept at their owner's homes). The

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study included healthy animals, representative of the population in which the product will be most commonly used (aged 1 to 8 years). As the tests were conducted in field conditions, the selection of the animals in this age range guaranteed more reliable and objective results in terms of the haematological and biochemical parameters – the animals had lived with their owners for some time and their health status was well known and regularly monitored by a veterinarian, which excluded the risk of introducing newly purchased puppies/dogs with an unknown health status to the experiment. No particularly sensitive target sub-population was identified for the tested product.

Drug

Biopronil Spot on® containing 134 mg or 268 mg of FIP and excipients: butylhydroxytoluene (E-320), butylhydroxyanisole (E-321), povidone, isopropanol, diethylene glycol monoethyl ether (serial Nos. 020517 and 010517) manufactured by Drwalewskie Zakłady Przemysłu Bioweterynaryjnego S.A. (Drwalew, Poland).

Inclusion criteria

All the animals included were clinically healthy and did not receive antibiotics or other veterinary medicinal products prior to the experiment. They had not been subjected to agents containing FIP or other pesticides before the experiment or agents that may affect the study results.

Exclusion criteria

The occurrence of adverse reactions, serious adverse reactions, severe unexpected adverse reactions or a treatment which affects the experimental period were reasons for the exclusion of an animal from the experiment.

Study design

The dogs were randomly assigned to three study groups (eight animals in each group) and one control group (eight animals) and marked with a numbered collar. The animal allocation into groups is presented in Table 1.

Table 1. Study design

Group	Number of dogs	Treatment schedule
Control	8	control product administered on days 0, +28, +56
I	8	single dose of the tested product on days 0, +28, +56
II	8	triple dose of the tested product administered on days 0, +28, +56
III	8	fivefold dose of the tested product administered on days 0, +28, +56

Thirty-two dogs were admitted to the experiment 14 days prior to the Biopronil Spot on® administration (D-14) and were subjected to a clinical examination which included the assessment of the:

1. Condition and behaviour;
2. Skin condition, especially where the product was to be applied;
3. Body temperature;
4. Respiratory system condition;
5. Gastrointestinal condition: appetite, faeces;
6. Condition of mucous membranes and lymph nodes.

On day D 0, the animals underwent a clinical examination was repeated. A physical examination was also performed on days +1 to +3, +28 to +31, +55, +56 to +59, +70 and included the assessment of the: behaviour, application site and assessment of skin, mucous membranes, lymph nodes, basic clinical parameters (rectal temperature, resting heart rate, resting respiratory rate, appetite). The test results, observations and animal's weight were recorded for each dog separately. The animals' body weights were recorded on days D –14, D 0, D +1, D +2, D +28, D +29, D +30, D +56, D +57, D +58 and D +70.

The doses of Biopronil Spot on® in the study groups and the control product in the control group were calculated for each animal in accordance with the manufacturer's instructions: 134 mg for a dog weighing 10–20 kg, 268 mg for a dog weighing 21–40 kg. The product was given to the dogs directly on the skin (after parting the fur) in a spot-on form, once in the neck area, between the shoulder blades. Group I received the drug in a single dose, group II in a triple dose and group III in a fivefold

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Table 2. Scoring system of the local tolerance evaluation

Erythema	Oedema	Scores
No erythema	no oedema	0
Very slight (barely perceptible)	very slight (barely perceptible)	1
Well-defined	slight (edges of area well defined by definite raising)	2
Moderate to severe	moderate (raised approx. 1 mm)	3
Severe and tendency to eschar formation	severe (raised above 1 mm)	4
Fur/hair condition	visual fur/hair condition	
No fur/hair loss	no perceptible lesions	0
Slight	greasy/clumped	1
Significant	solidification in the ends	2
Eye irritation	skin	
No irritation	no irritation	0
Very slight redness	pruritus	1
Eye pruritus	slight desquamation	2
Tearing	large squamae (> 2 × 2 mm)	3
Purulent discharge	alopecia	4
Eyes closed with oedema	discoloration	5
Corneal opacity	severe desquamation	6

dose. The dogs from the control group received the control product at a dose corresponding to the volume of the preparation tested in group III: dogs weighing 10–20 kg received the control preparation in the amount of 6.70 ml, while dogs weighing 21–40 kg received 13.40 ml of the control product. The tested and control product (the same ingredients, but without fipronil) were administered three times in each group at the required doses on day 0, +28, +56.

The general health of the dogs was monitored by observing the application site every hour for at least 4 h (i.e., four times after the application) and then 24, 48 and 72 h after each application. The local tolerance (oedema, erythema, coat condition, eye and skin irritation) after administration of the preparations was assessed based on a scoring system (see Table 2).

Blood samples were collected from the cephalic vein on days: D –14, D –5, D +3, D +31, D +59, D +70. The blood was intended for the determination of the following haematological and biochemical parameters: red blood cell (RBC), haemoglobin (HGB), haematocrit (HCT), average red blood cell size (MCV), haemoglobin amount per red blood cell (MCH), platelet (PLT), red cell distribution width (RDW), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine (CREA), blood urea (UREA), glucose (GLU).

The physiological range of the haematological and biochemical parameters in the dogs is presented in Table 3 (Winnicka 2004).

Haematological and biochemical blood analyses

The haematological and biochemical blood analyses were carried out on certified analysers: A URIT-2900 PLUS veterinary automated haematology analyser (Urit Medical Electronic Co, Ltd., Guilin, P.R. China) and an EMP-168 chemistry analyser (Shenzhen Emperor Electronic Technology Co., Ltd., Shenzen, P.R. China) in an external laboratory (VIBa-MED in Lublin, Poland).

Table 3. Physiological values of the haematological and biochemical indicators in the dogs

Indicator	Physiological values	Unit
ASPAT	0.02–0.75	µkat/l
ALAT	0.05–1.00	µkat/l
ALP	0.33–2.57	µkat/l
CREA	79.58–150.31	µmol/l
UREA	4.65–10.60	mmol/l
GLU	3.89–6.66	mmol/l
RBC	5.5–8.5	10^{12}/l
HCT	0.37–0.55	fraction
HGB	7.45–11.27	mmol/l
MCV	60–77	µm3
MCH	1.18–1.49	fmol
PLT	200–580	10^{9}/l
RDW	0.14–0.19	proportion of 1.0

ALAT = alanine aminotransferase; ALP = alkaline phosphatase; ASPAT = aspartate aminotransferase; CREA = creatinine; GLU = glucose; HCT = haematocrit; HGB = haemoglobin; MCH = haemoglobin amount per red blood cell; MCV = average red blood cell size; PLT = platelet; RBC = red blood cell; RDW = red cell distribution width; UREA = blood urea

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Statistical analysis

The obtained data were subjected to the Shapiro-Wilk test for normality and the Levene's test for equality of variances. The differences between the means (control vs. experimental) were tested for statistical significance by Student's *t*-test. All the data were analysed with the use of Statistica software v13.0 (Statsoft, Poland). The significance level was set at $\alpha = 0.05$ and a *P*-value of < 0.05 was considered statistically significant. As the study had a safety-in-use character with regards to the preparation in the dogs, the values obtained in the study groups were compared to the control group. The mean values, standard deviation and number of observations in a given group (*n*) were determined during the study. In addition, the minimum and maximum (range) of the investigated parameters were obtained (data not shown).

RESULTS

Assessment of the local and systemic tolerance

No swelling, erythema, eye or skin irritation were observed in any of the dogs from the control group. The visual condition of the coat/hair was also correct. No undesirable disturbances in the behaviour or functioning of the digestive system were observed. The condition of the mucous membranes, lymph nodes and internal heat were in the physiological norm.

No dogs in the study groups showed changes in body weight or rectal temperature during the experiment. No changes in the appearance of the mucous membranes or lymph nodes and digestive system were observed after the application of the preparation in any of the dogs.

No swelling, erythema, eye or skin irritation were observed in any of the dogs from group I after the administration of the test product in a single dose and the visual condition of the coat/hair was also correct. A slight solidification in the ends of the coat/hair was observed in two dogs from group II after the application of the tested preparation at a triple dose on D +28 and D +56. Solidification in the ends of the coat/hair was observed in three dogs from group III after the application of the test product at a fivefold dose on D +28 and D +56. Minimal erythema was observed in one dog from group II after the application of the test preparation in a triple dose on D +28 and in two dogs on D +56. Minimal erythema also occurred in two dogs from group III after the application of the test preparation at a fivefold dose on D +28 and in three dogs on D +56.

A greasy/clumped appearance of the coat/hair was found in two dogs from group II after application of the product at a triple dose on D +28 and D +56 and in two dogs from group III after application at the fivefold dose on D +28 and on two dogs on day D +56.

All the revealed symptoms were considered to be cosmetic changes, no visible pathological changes on the skin were observed. Appetite disorders were not observed during the study.

Assessment of the haematological indicators

The mean (± SD) values of the haematological profile after the single, triple and fivefold dosage of Biopronil Spot on® and in the control groups are presented in Table 4. The analysis of the statistical differences found in the study (Table 4) in relation to the selected indicators between the study groups and the control group on the individual days of the experiment indicates their short-term nature.

Moreover, there is no correlation between the value of the indicators and the dose of the preparation used.

Assessment of the biochemical indicators

The mean (± SD) values of the biochemical indicators after the single, triple and fivefold dosage of Biopronil Spot on® and in the control group are presented in Table 5.

As in the case of the differences in the haematological parameters, the statistical differences presented in Table 5 in relation to the selected biochemical indicators between the study groups and the control group on the individual days of the experiment indicates their short-term nature. As before, no correlation between the value of the indicators and the dose of the preparation used was found.

Table 4. Effect of the single, triple and fivefold dose of Biopronil Spot on® on the haematological indicators of the dogs

Indicator	Group								*P*-value		
	control		I		II		III		control vs. I	control vs. II	control vs. III
	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max			
D –14											
RBC (10^{12}/l)	6.97 ± 0.56	6.03–7.66	7.05 ± 0.77	5.90–8.17	6.54 ± 1.17	4.36–8.23	6.82 ± 1.17	4.72–8.60	0.82	0.36	0.75
HCT (fraction)	0.44 ± 0.06	0.38–0.52	0.46 ± 0.04	0.40–0.51	0.41 ± 0.07	0.29–0.50	0.42 ± 0.08	0.31–0.51	0.63	0.31	0.47
HGB (mmol/l)	10.71 ± 1.79	7.14–12.60	10.98 ± 1.13	9.12–12.47	10.04 ± 1.45	7.14–11.98	10.61 ± 1.21	8.56–12.10	0.72	0.42	0.89
MCV (μm^3)	66.36 ± 2.73	60.90–69.90	65.61 ± 5.75	56.20–72.60	63.26 ± 3.30	59.40–67.30	62.98 ± 3.44	59.30–69.40	0.74	0.06	0.05
MCH (fmol)	**1.53 ± 0.15**	1.20–1.65	**1.58 ± 0.08**	1.45–1.71	1.49 ± 0.13	1.24–1.63	1.49 ± 0.11	1.33–1.69	0.47	0.51	0.51
PLT (10^9/l)	239.25 ± 29.61	213.00–291.00	277.25 ± 74.98	181.00–402.00	267.38 ± 97.71	85.00–367.00	275.25 ± 148.74	97.00–554.00	0.20	0.45	0.51
RDW (proportion of 1.0)	0.15 ± 0.01	0.14–0.17	0.15 ± 0.01	0.14–0.16	0.15 ± 0.01	0.14–0.16	0.15 ± 0.00	0.14–0.16	0.24	0.74	0.83
D –5											
RBC (10^{12}/l)	6.81 ± 0.58	5.87–7.60	7.03 ± 0.70	6.22–8.15	7.24 ± 0.90	5.29–8.22	7.07 ± 0.53	5.16–8.22	0.52	0.28	0.53
HCT (fraction)	0.43 ± 0.05	0.39–0.51	0.43 ± 0.03	0.36–0.47	0.45 ± 0.06	0.31–0.57	0.42 ± 0.06	0.33–0.48	0.97	0.61	0.67
HGB (mmol/l)	10.40 ± 1.69	6.89–12.35	10.53 ± 0.78	9.56–11.67	10.88 ± 0.34	7.51–12.16	10.36 ± 1.36	8.44–11.85	0.84	0.55	0.97
MCV (μm^3)	65.81 ± 3.43	59.90–69.80	**59.00 ± 11.22**	32.20–65.80	62.51 ± 3.51	58.20–66.50	62.08 ± 3.93	53.20–66.30	0.12	0.08	0.06
MCH (fmol)	**1.53 ± 0.13**	1.29–1.65	**1.53 ± 0.08**	1.43–1.66	1.45 ± 0.10	1.32–1.60	**1.56 ± 0.14**	**1.42–1.87**	0.93	0.20	0.74
PLT (10^9/l)	237.38 ± 45.33	177.00–303.00	292.88 ± 85.48	211.00–491.00	260.00 ± 101.00	112.00–471.00	239.50 ± 82.52	88.00–321.00	0.13	0.60	0.95
RDW (proportion of 1.0)	0.15 ± 0.01	0.14–0.16	0.15 ± 0.01	0.15–0.17	0.15 ± 0.00	0.15–0.16	0.15 ± 0.01	0.14–0.17	0.01*	0.06	0.11
D +3											
RBC (10^{12}/l)	6.76 ± 0.71	5.85–7.76	7.00 ± 0.78	6.19–8.66	7.38 ± 0.62	6.32–8.18	7.16 ± 0.27	6.32–8.18	0.52	0.08	0.27
HCT (fraction)	0.44 ± 0.04	0.39–0.50	0.45 ± 0.06	0.40–0.54	0.48 ± 0.04	0.41–0.52	0.44 ± 0.06	0.41–0.52	0.57	0.11	0.89
HGB (mmol/l)	10.64 ± 1.28	8.38–12.04	10.62 ± 1.51	8.50–12.78	11.38 ± 0.15	9.56–13.16	10.21 ± 1.81	9.56–13.16	0.97	0.24	0.59
MCV (μm^3)	62.99 ± 7.31	46.20–69.70	64.74 ± 4.35	59.60–72.50	64.09 ± 4.73	52.70–67.20	59.73 ± 5.95	52.70–67.20	0.57	0.73	0.34
MCH (fmol)	**1.53 ± 0.13**	1.63–1.63	1.47 ± 0.21	0.99–1.68	**1.52 ± 0.07**	1.37–1.61	1.49 ± 1.48	1.37–1.61	0.55	0.94	0.38
PLT (10^9/l)	235.50 ± 44.01	182.00–302.00	428.75 ± 308.77	223.00–1 160.00	298.38 ± 105.86	202.00–503.00	216.88 ± 77.66	202.00–503.00	0.10	0.14	0.56
RDW (proportion of 1.0)	0.15 ± 0.01	0.14–0.16	0.16 ± 0.02	0.14–0.21	0.15 ± 0.01	0.14–0.16	0.16 ± 0.01	0.14–0.16	0.46	0.7	0.18

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Table 4 to be continued

Indicator	Group								*P*-value		
	control		I		II		III		control vs. I	control vs. II	control vs. III
	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max			
D +31											
RBC (10^{12}/l)	6.64 ± 0.97	4.77–7.74	8.02 ± 1.18	6.01–9.53	7.14 ± 0.77	6.34–8.30	6.98 ± 0.54	4.88–8.47	0.02*	0.27	0.54
HCT (fraction)	0.46 ± 0.04	0.40–0.52	0.53 ± 0.09	0.36–0.69	0.42 ± 0.06	0.31–0.49	0.46 ± 0.06	0.40–0.57	0.06	0.18	0.95
HGB (mmol/l)	10.15 ± 1.07	9.06–12.54	**11.79 ± 1.81**	**9.12–13.96**	10.44 ± 0.42	7.07–12.47	10.65 ± 0.96	9.62–12.04	0.05	0.67	0.34
MCV (μm^3)	61.31 ± 6.73	46.70–68.50	63.41 ± 3.11	60.20–68.90	64.06 ± 2.11	61.30–66.70	63.21 ± 3.09	59.30–67.80	0.44	0.29	0.48
MCH (fmol)	**1.54 ± 0.15**	1.19–1.64	**1.52 ± 0.09**	1.42–1.68	**1.53 ± 0.06**	1.41–1.59	**1.54 ± 0.06**	**1.46–1.62**	0.82	0.93	0.95
PLT (10^9/l)	216.50 ± 64.57	87.00–292.00	240.88 ± 55.19	186.00–325.00	259.88 ± 65.00	165.00–338.00	244.25 ± 36.04	190.00–287.00	0.43	0.20	0.31
RDW (proportion of 1.0)	0.14 ± 0.00	0.14–0.15	0.15 ± 0.00	0.14–0.15	0.15 ± 0.01	0.14–0.17	0.15 ± 0.01	0.14–0.17	0.50	0.03*	0.14
D +56											
RBC (10^{12}/l)	6.33 ± 0.59	5.31–7.11	7.41 ± 0.81	5.90–8.32	7.63 ± 0.73	7.02–9.06	7.16 ± 0.03	5.62–8.26	0.36	0.17	0.12
HCT (fraction)	0.43 ± 0.07	0.32–0.52	0.49 ± 0.06	0.40–0.57	0.47 ± 0.05	0.40–0.58	0.46 ± 0.03	0.42–0.52	0.08	0.19	0.25
HGB (mmol/l)	9.75 ± 1.08	7.94–11.54	12.19 ± 1.21	10.67–13.84	**11.54 ± 0.01**	**9.81–13.65**	10.20 ± 1.61	7.32–13.09	0.00	0.01	0.52
MCV (μm^3)	62.20 ± 5.70	50.20–67.00	65.73 ± 5.31	53.60–71.90	63.41 ± 3.85	56.30–67.40	61.54 ± 4.52	55.30–66.40	0.22	0.63	0.80
MCH (fmol)	**1.55 ± 0.08**	1.42–1.67	**1.54 ± 0.12**	1.31–1.71	**1.58 ± 0.11**	1.44–1.78	**1.53 ± 0.09**	1.42–1.68	0.83	0.55	0.72
PLT (10^9/l)	238.50 ± 69.51	90.00–319.00	255.24 ± 58.49	149.00–316.00	251.50 ± 66.66	163.00–350.00	**198.38 ± 31.49**	131.00–238.00	0.61	0.71	0.16
RDW (proportion of 1.0)	0.15 ± 0.01	0.14–0.17	0.15 ± 0.01	0.14–0.16	0.15 ± 0.01	0.14–0.16	0.15 ± 0.01	0.15–0.16	0.74	0.96	0.72
D +70											
RBC (10^{12}/l)	6.83 ± 0.76	5.65–7.81	7.74 ± 1.05	6.38–9.53	7.49 ± 0.78	6.69–8.80	6.47 ± 0.38	5.58–8.30	0.07	0.11	0.38
HCT (fraction)	0.43 ± 0.07	0.29–0.49	0.49 ± 0.07	0.40–0.59	0.47 ± 0.04	0.40–0.52	0.41 ± 0.05	0.34–0.48	0.11	0.17	0.6
HGB (mmol/l)	9.92 ± 1.29	7.76–11.42	**11.70 ± 0.82**	10.67–12.66	**11.53 ± 0.01**	10.67–12.35	10.27 ± 1.07	9.12–11.73	0.03*	0.01*	0.56
MCV (μm^3)	62.26 ± 6.28	48.80–68.20	65.65 ± 3.33	62.60–72.40	65.71 ± 1.30	63.10–67.30	62.70 ± 4.32	56.90–69.10	0.20	0.15	0.87
MCH (fmol)	1.49 ± 0.11	1.24–1.60	**1.57 ± 0.08**	1.45–1.74	**1.54 ± 0.05**	1.46–1.62	**1.50 ± 0.09**	1.38–1.61	0.12	0.23	0.72
PLT (10^9/l)	260.13 ± 70.78	121.00–343.00	219.63 ± 52.41	166.00–288.00	274.88 ± 65.72	204.00–403.00	235.50 ± 56.54	199.00–344.00	0.21	0.67	0.46
RDW (proportion of 1.0)	0.15 ± 0.01	0,.14–0.17	0.15 ± 0.01	0.13–0.16	0.15 ± 0.01	0.14–0.15	0.15 ± 0.01	0.14–0.16	0.52	0.78	0.41

Values represent means ± SD; *Values significantly different from control ($P < 0.05$); Mean values outside the reference range are in bold font

HCT = haematocrit; HGB = haemoglobin; MCH = haemoglobin amount per red blood cell; MCV = average red blood cell size; PLT = platelet; RBC = red blood cell; RDW = red cell distribution width

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Table 5. Effect of the single, triple and fivefold dose of Biopronil Spot on® on the biochemical indicators in the dogs

Indicator	Group								*P*-value		
	control		I		II		III		control vs. I	control vs. II	control vs. III
	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max			
D –14											
ASPAT (µkat/l)	0.48 ± 0.19	0.23–0.80	0.56 ± 0.20	0.23–0.89	0.49 ± 0.23	0.11–0.74	0.68 ± 0.30	0.19–1.19	0.43	0.98	0.26
ALAT (µkat/l)	0.68 ± 0.10	0.53–0.82	**1.25 ± 0.92**	0.42–2.82	0.61 ± 0.37	0.10–1.21	0.63 ± 0.23	0.26–0.97	0.11	0.61	0.57
ALP (µkat/l)	0.74 ± 0.27	0.42–1.26	0.78 ± 0.46	0.32–1.59	0.92 ± 0.48	0.19–1.60	1.02 ± 0.90	0.40–3.08	0.81	0.37	0.41
CREA (µmol/l)	94.61 ± 17.68	70.74–127.32	101.68 ± 8.84	91.07–119.37	93.73 ± 11.49	72.50–108.76	101.68 ± 21.22	72.50–145.01	0.36	0.90	0.48
UREA (mmol/l)	5.90 ± 1.40	4.00–7.40	4.92 ± 2.03	2.75–8.78	**3.68 ± 1.97**	1.78–5.78	5.32 ± 1.88	3.25–7.32	0.28	0.02	0.49
GLU (mmol/l)	5.45 ± 0.68	4.20–6.11	5.11 ± 1.52	3.71–7.99	5.01 ± 0.82	3.90–6.13	5.18 ± 0.84	3.64–6.58	0.57	0.26	0.50
D –5											
ASPAT (µkat/l)	0.56 ± 0.10	0.36–0.65	0.65 ± 0.26	0.39–1.10	0.50 ± 0.20	0.14–0.76	0.68 ± 0.30	0.45–1.10	0.34	0.49	0.26
ALAT (µkat/l)	0.67 ± 0.06	0.58–0.75	0.92 ± 0.75	0.29–2.16	0.60 ± 0.31	0.16–1.08	0.67 ± 0.22	0.29–1.02	0.36	0.53	0.98
ALP (µkat/l)	0.73 ± 0.29	0.44–1.18	0.81 ± 0.51	0.40–1.84	0.90 ± 0.43	0.41–1.66	1.22 ± 1.30	0.46–4.30	0.70	0.36	0.32
CREA (µmol/l)	81.35 ± 11.49	68.08–97.26	111.41 ± 15.92	86.65–129.09	92.84 ± 18.57	69.85–125.56	90.19 ± 15.03	69.85–115.83	0.00*	0.17	0.21
UREA (mmol/l)	6.13 ± 1.56	3.28–8.29	5.94 ± 1.93	2.71–7.83	**3.71 ± 1.28**	2.40–5.68	5.27 ± 2.30	2.08–8.67	0.83	0.00*	0.40
GLU (mmol/l)	5.52 ± 0.60	4.47–6.55	5.19 ± 0.86	4.11–6.77	5.35 ± 0.89	4.41–6.63	5.09 ± 0.84	3.90–6.07	0.39	0.65	0.26
D +3											
ASPAT (µkat/l)	0.48 ± 0.12	0.32–0.67	0.58 ± 0.21	0.40–1.03	0.50 ± 0.16	0.21–0.67	0.60 ± 0.03	0.43–1.04	0.28	0.80	0.20
ALAT (µkat/l)	0.72 ± 0.11	0.56–0.92	0.89 ± 0.74	0.19–2.27	0.60 ± 0.35	0.29–1.26	0.74 ± 0.19	0.38–0.97	0.51	0.82	0.77
ALP (µkat/l)	0.78 ± 0.24	0.49–1.26	0.88 ± 0.96	0.31–3.19	0.93 ± 0.27	0.47–1.29	1.14 ± 0.85	0.34–2.88	0.77	0.23	0.26
CREA (µmol/l)	87.54 ± 18.57	54.82–115.83	107.87 ± 22.11	59.24–129.09	97.26 ± 9.73	86.65–112.29	121.14 ± 38.90	93.73–213.09	0.07	0.20	0.04*
UREA (mmol/l)	5.76 ± 1.51	3.35–7.69	5.07 ± 1.60	2.51–7.59	**3.89 ± 1.54**	1.65–6.81	6.39 ± 2.48	2.80–10.01	0.39	0.03*	0.55
GLU (mmol/l)	5.10 ± 0.39	4.33–5.47	5.30 ± 1.07	4.11–7.60	5.02 ± 0.33	4.50–5.36	5.52 ± 1.28	3.70–7.27	0.63	0.65	0.39
D +31											
ASPAT (µkat/l)	0.62 ± 0.23	0.32–1.02	0.79 ± 0.25	0.58–1.22	0.56 ± 0.13	0.35–0.80	**0.95 ± 0.72**	0.40–2.64	0.17	0.53	0.01*
ALAT (µkat/l)	0.72 ± 0.08	0.64–0.82	**1.05 ± 0.83**	0.21–2.59	0.87 ± 0.44	0.21–1.63	0.58 ± 0.18	0.32–0.82	0.29	0.38	0.05
ALP (µkat/l)	0.83 ± 0.33	0.43–1.36	1.18 ± 0.74	0.46–2.83	1.17 ± 0.67	0.55–2.15	0.91 ± 0.48	0.47–1.88	0.24	0.21	0.69
CREA (µmol/l)	98.15 ± 10.61	88.42–120.25	104.34 ± 13.26	80.46–127.32	89.30 ± 27.41	37.14–115.83	**188.33 ± 269.68**	77.81–854.14	0.30	0.41	0.36
UREA (mmol/l)	6.69 ± 1.78	4.63–9.82	4.66 ± 2.11	1.50–7.66	**3.62 ± 1.23**	2.06–6.29	4.69 ± 1.90	2.81–8.49	0.06	0.00*	0.05
GLU (mmol/l)	5.51 ± 0.42	4.99–6.41	5.30 ± 0.83	4.33–6.44	5.04 ± 0.49	4.44–5.67	5.48 ± 0.87	4.13–6.94	0.53	0.06	0.93
D +56											
ASPAT (µkat/l)	0.60 ± 25	0.31–0.96	0.53 ± 0.08	0.42–0.67	0.54 ± 0.12	0.32–0.69	**0.86 ± 0.60**	0.34–2.12	0.47	0.52	0.04*
ALAT (µkat/l)	0.61 ± 0.11	0.42–0.75	0.83 ± 0.49	0.37–1.65	0.76 ± 0.18	0.53–1.08	0.58 ± 0.20	0.32–0.88	0.24	0.07	0.69
ALP (µkat/l)	0.79 ± 0.30	0.36–1.10	0.87 ± 0.44	0.44–1.72	1.23 ± 0.83	0.31–2.81	0.94 ± 0.61	0.31–2.11	0.65	0.18	0.52

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Table 5 to be continued

Indicator	Group								*P*-value		
	control		I		II		III		control vs. I	control vs. II	control vs. III
	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max	mean ± SD	min–max			
CREA (µmol/l)	88.42 ± 16.80	52.17–107.87	101.68 ± 13.26	85.77–122.90	91.96 ± 10.61	71.62–101.68	101.68 ± 17.68	87.54–144.12	0.1	0.57	0.14
UREA (mmol/l)	6.38 ± 1.52	3.83–8.77	4.74 ± 1.61	2.70–7.19	**3.62 ± 1.23**	2.26–4.70	4.88 ± 0.92	3.81–6.59	0.06	0.07	0.07
GLU (mmol/l)	5.53 ± 0.38	4.97–6.33	5.56 ± 0.59	4.38–6.50	4.64 ± 1.13	3.46–7.30	5.45 ± 0.93	4.13–6.94	0.91	0.05	0.82
D +70											
ASPAT (µkat/l)	0.60 ± 0.30	0.33–1.27	0.64 ± 0.15	0.51–0.98	0.57 ± 0.14	0.39–0.72	0.68 ± 0.21	0.39–0.98	0.77	0.77	0.36
ALAT (µkat/l)	0.86 ± 0.69	0.43–2.54	0.97 ± 0.49	0.54–1.94	0.66 ± 0.18	0.38–0.94	0.64 ± 0.17	0.39–0.83	0.71	0.46	0.41
ALP (µkat/l)	0.75 ± 0.25	0.37–1.10	0.87 ± 0.72	0.26–2.26	1.49 ± 0.80	0.44–2.82	0.82 ± 0.65	0.42–2.09	0.66	0.03*	0.77
CREA (µmol/l)	93.55 ± 21.22	66.32–133.51	107.87 ± 11.49	87.54–129.09	92.84 ± 26.53	57.47–150.31	105.22 ± 23.87	70.74–144.12	0.12	0.95	0.32
UREA (mmol/l)	5.37 ± 2.10	3.25–18.98	**4.52 ± 1.77**	2.56–8.66	5.07 ± 1.00	2.88–8.09	5.37 ± 2.10	4.08–7.16	0.19	0.08	0.12
GLU (mmol/l)	5.61 ± 0.40	4.99–6.19	5.37 ± 0.95	3.87–7.05	5.76 ± 1.15	4.67–7.40	5.49 ± 0.87	4.10–6.79	0.52	0.74	0.71

Values represent means ± SD; *Values significantly different from control ($P < 0.05$); Mean values outside the reference range are in bold font

ALAT = alanine aminotransferase; ALP = alkaline phosphatase; ASPAT = aspartate aminotransferase; CREA = creatinine; GLU = glucose; UREA = blood urea

DISCUSSION

Although FIP induces lower toxicity in mammals than in insects, it was documented to have numerous adverse effects on various animals (i.e., rats, birds, dogs) and humans (Rohdich et al. 2014; Wang et al. 2016; Meadows et al. 2017). In addition, application of the veterinary spot-on products can cause skin irritation or hair loss at the site of application (Gupta 2007).

Rohdich et al. (2014) observed alopecia and crusts in the dorsal lumbo-sacral area in three dogs (1.7%) and intense pruritus in one dog (0.6%) in 178 FIP-treated dogs. In our study, no alterations in the skin or coat appearance were observed in any of the dogs from group I, treated three times with a single dose of the tested product. In group II, treated three times with a triple dose, the slight solidification in the ends of the coat/hair was observed in two dogs, while in group III, treated three times with a fivefold dose, it was observed in three dogs. A greasy/clumped appearance of the coat/hair was noted in four dogs: two in group II and two in group III. The above-mentioned symptoms are considered to be not clinically significant. Minimal erythema was observed only in one dog in group II and in two dogs from group III, exposed to the highest dosage of FIP, however, it disappeared in a few days after application.

As mentioned earlier, FIP and fipronil sulfone (FIP main metabolite) act as inhibitors of insect GABA receptors and prevent GABA binding to its receptor, blocking the inhibitory function of GABA in the central nervous system and, thus, leading, at low doses, to neuronal hyperexcitation and, at high doses, to the paralysis and death of insects (Koslowski et al. 2020). Fipronil has a notably higher affinity for insect GABA receptors than for mammalian GABA receptors (Hainzl et al. 1998). Both FIP and fipronil sulfone are able to cross the blood–brain barrier in rats and mice (Hainzl et al. 1998; Cravedi et al. 2013). A recent study, evaluating the long-term low dose administration of FIP in mice and its relation to cognitive deficiencies showed that a 48-week-FIP treatment leads to behavioural perturbations, indicating an accumulative effect of sustained exposure to low doses of FIP (Koslowski et al. 2020). In the present study, no significant symptoms specific to the nervous system were observed over the whole experiment. Apathy was observed only in one dog from group I, where

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a single dose of the tested product was administered, suggesting the lack of a dose-dependent relationship and may be a result of other factors, not connected with the Biopronil Spot on® application.

There are also reports that FIP can induce gastrointestinal signs included vomiting and diarrhoea. Meadows et al. (2017) conducted an experiment, where vomiting was the most frequent event in dogs, affecting 6.0% of the fipronil-methoprene treated dogs. Diarrhoea was reported in 11.0% of the fipronil-methoprene treated dogs. FIP was applied once every 28 days in three doses in a group of 100 dogs and the product remained in its commercial packaging containing volumes of 0.67, 1.34, 2.68 or 4.02 ml (Meadows et al. 2017). In contrast, in our study, no gastrointestinal disorders were observed in any of the dogs after the application of the tested product in the single, triple and fivefold multiple doses (3 times every 28 days).

A recent study conducted on mice demonstrated that FIP induces alterations in the haematological parameters via oral sub-acute toxicity exposure (Abouelghar et al. 2020). In the above-mentioned study, the MCV, MCH, HGB, RBC, HCT and PLT levels of the mice were significantly decreased in the FIP exposed groups as compared to the controls. With regard to these parameters, the levels of decreasement were dose-dependent. Available data concerning the evaluation of the impact of FIP on canine haematological parameters are scarce. Ziliotto et al. (2017) showed that a single exposure to this pesticide (6.7 mg/kg) does not induce DNA damage in the peripheral blood cells in dogs. In the present study, the MCH was slightly above normal [reference range presented in Table 3 (Winnicka 2004)] in all four groups at various stages. There was also a slight increase in the HGB in group 1 on D +31 and D +70 and in group 3 on D +56 and D +70, probably due to long storage time which is associated with the increase in the HGB (Arif et al. 2017) The increase in the HGB during storage is directly related to the significant modifications of the MCH. The observed differences in the HGB level concerned, in particular, group I, and did not appear (in group III) or were limited (in group II), which indicates that the observed changes were probably not related to the administration of the investigated product. These differences may also result from differences in the amount of water consumed by the animals. Other values (PLT in group 3 on D +56 and MCV in group I on D –5) were marginally outside the reference range and revealed the individual, short-term nature of the deviations and does not indicate a cause-effect relationship with the tested product. After repeated doses during the 70-day-long present study and exposure to Biopronil Spot on®, the erythrocyte indices of the dogs were only slightly altered, even in the group treated with the fivefold dose of Biopronil Spot on®.

Moreover, the differences in the number of erythrocytes found in all the treated groups were transient and no correlation was found between the dose of Biopronil Spot on® and the changes in the erythrocyte indices. The obtained results suggest that the changes observed should not be associated with the tested product.

The liver and kidneys play a major role in the biotransformation of pesticides and are the most sensitive and main target organs of pesticide toxicity and damage (Mansour and Mossa 2010), which may be observed in alterations to the biochemical indicators. Previous studies demonstrated that pesticides can change the enzymatic and non-enzymatic antioxidant and induce oxidative stress in animals that were investigated as a potential mechanism of pesticide toxicity (Mohamed et al. 2004; Mansour and Mossa 2009). Sub-chronic exposure to the FIP on the liver and kidneys of male rats at three concentrations 0.1, 1 and 10 mg/l in drinking water for 45 days revealed significantly increased values of the ASPAT, ALAT, uric acid, creatinine and histopathological alterations in FIP-treated groups (Mossa et al. 2015). In the present study, UREA was below the reference value in group II to D +56, although at D +70 it was normal. The CREA concentration was above the reference range on D +31 in group III, nevertheless, it may result in measurement bias (high SD) and was normal on D +56 and D +70 in this group. The differences in the CREA concentration in groups I and II were transient and no correlation was found between the dose and the change in the concentration of this indicator. The observed changes are, therefore, not associated with the administration of the tested product. The ALT was slightly increased in group I on D –14 and D +31. In group I, the UREA was slightly below the reference range on D +70. The deviations from the ref-erence values regarding ASPAT, ALAT, CREA were also transient, individual, of a short-term nature and were probably not associated with the tested prepara-

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tion, as in the case of the deviations in the haematological indicators.

In conclusion, it could be stated that Biopronil Spot on®, containing FIP, administered in a single, triple and fivefold repeated dosage was found to have no influence on the clinical, haematological and biochemical indicators in dogs. An analysis of the results indicates that the changes in the skin hair/coat appearance, behaviour and biochemical and haematological indicators observed in the present study were not likely to be a result of the Biopronil Spot on® administration as there was no correlation between the observed deviations of the investigated parameters and the tested dose of Biopronil Spot on®. In conclusion, the tested product was considered safe after being used in animals under the conditions described in the experiment.

Conflict of interest

The authors declare no conflict of interest.

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