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Original article

EFFECTS OF A 1,2,4-TRIAZOLE DERIVATIVE LIPOSOME EMULSION ON THE INNATE AND ADAPTIVE IMMUNITY OF PUPPIES VACCINATED AGAINST CANINE PARVOVIRUS AND CANINE DISTEMPER

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Summary

Broshkov, M. M. & I. V. Kichun, 2017. Effects of a 1,2,4-triazole derivative liposome emulsion on the innate and adaptive immunity of puppies vaccinated against canine parvovirus and canine distemper. *Bulg. J. Vet. Med.* (online first).

The aim of the study was the evaluate the immunomodulating effect of a 1,2,4-triazole derivative liposome emulsion (Trifuzol) in puppies vaccinated against parvovirus enteritis and canine distemper. The used Trifuzol liposome emulsion led to a significant increase in the absolute number of lymphocytes and their immunoregulatory subpopulations, phagocytic activity of neutrophils. The level of antibodies against parvovirus enteritis (CPV) and canine distemper (CDV) in vaccinated and Trifuzol-treated puppies reached zero values which were statistically significantly lower than those in vaccinated-only puppies (21.0 U/mL and 27.0 U/mL, respectively). It was concluded that the tested liposome emulsion of Trifuzol had an positive effect on the humoral immunity in puppies and can be used in clinical practice as an immunomodulatory agent.

Key words: absolute lymphocyte count, neutrophil phagocytosis, puppies, specific IgG antibodies titre, vaccination

INTRODUCTION

One of the critical periods of development of dogs is the period of 2–3 months of age. At that time two- or three-fold immunisation is performed. Also, many puppies experience a physiological anaemia associated with the switch from foetal to adult haemoglobin. Blood tests performed by us in puppies showed that 65% of them had blood haemoglobin below the norm (Broshkov, 2012).

Vaccination of animals leads to changes in the immune parameters during the post-vaccination period. In a small number of newborn animals, it can result in a wide range of possible adverse effects (Strasser *et al.,* 2003). Studies of the effect of vaccination on the immune system

of puppies showed changes in the number of lymphocytes. Some authors reported lymphopaenia and an increased response of blood lymphocytes to mitogens 7 days after vaccination (Strasser *et al.,* 2003); other studies during vaccination of pups showed no response from CD4 + and IgG lymphocytes (Miyamoto *et al.,* 1995; De Cramer *et al.,* 2009). In previous studies of ours, the dynamics of immunocompetent cells in puppies before and 14 days after vaccination depended on their prevaccination counts (Broshkov & Smolyaninov, 2012).

Both adjuvants and the vaccine can cause immunosuppression (Kozhevnikova *et al.,* 2014). The most pronounced vaccine-induced effect is on the subpopulation of T-lymphocytes (Gilbert, 2012). The high number of stray animals also significantly affects the immune status of domestic dogs because they share a common environment. Therefore, there is a need for prevention of possible complications during vaccination by modulation of immunity.

Despite a fairly large arsenal of pharmacological agents that affect the immune system of the organism for ensuring its adequate response to the effects of antigens, the number of immunological dysfunctions does not decrease. Fosprenil, a natural drug produced from clean pine needles was reported as effective *in vivo* and *in vitro* immunomodulator of secondary immunodeficiencies, induced by various external factors (Broshkov, 2015).

In the 1980s, Japanese virologists discovered the alkaloid granitsidin and the immunomodulating preparation Cycloferon was developed. Subcutaneous application of cycloferon 12.5% in puppies a dose of 10 mg/kg body weight according to the scheme, prescribed by the instruction, provoked a significant T-helper activity and reduced the ability of neutrophils for phagocytosis (Broshkov & Smolyaninov, 2012).

One of the active compounds responsible for the immunomodulatory effect of many herbs are complex polysaccharides known as β-D-glucans (Ooi & Liu, 2000; Chang, 2002). Beta (1,3/1,6)-D-glucan administered at a dose of 2 mL/5 kg body weight in dogs caused, besides a marked improvement of phagocytic functions, a significant increase in CPV and antirabies antibody titres that persisted until the end of the experiment (Haladová, 2011).

The recently synthesised new veterinary drug Trifuzol, from the group of 1,2,4-triazole derivatives, has shown promising results in terms of high immunomodulatory activity, antioxidant properties, hepatoprotective properties with a pronounced pancreatoprotective effect and in particular, significant antiviral effect on some strains of viruses (Petrovova, 2010).

The active substance of the drug Trifuzol is excreted 24 hours after the last application. So, the inclusion of drugs in liposomes can significantly increase their therapeutic effectiveness. This is due to the fact that, on the one hand, the drug that is contained in the liposome, is protected by its membrane from the effects of adverse factors, and on the other – the same membrane does not allow the toxic drug to exceed the allowable concentration in body fluids (Efremenko *et al.,* 1988; Varpakhovskaya, 1999). The liposome serves as a carrier, from which the drug is released gradually, in the right doses and places and for a certain period of time.

Enough experimental data on the effectiveness of the use of drugs in the form of liposome emulsions in other animal species is accumulated. Single application

of metisazone in the form of a liposome emulsion in dogs had an antiproliferative effect on T-lymphocytes, and the relative amount of NK cells increased by 40% ($P<0.01$). Next administration of this agent led to an 3.8-fold increase in the relative number of T-suppressors ($P<0.01$) and 2.6-fold reduction of the phagocytic activity of neutrophils (Broshkov, 2017).

Considering the fact that viral antigens are the most dangerous for puppies along with the prevalence of anabolism during this period of life, the study on immune modulating agents for preventing post-vaccination complications is essential. The optimisation of the used pharmacological agents is not less imporant.

The aim of the study was to evaluate the post vaccination dynamics of specific antibodies – IgG against parvoviral enteritis and canine distemper and parameters of innate (absolute number of lymphocytes and their immunoregulatory subpopulations) and adaptive immunity (phagocytic neutrophil activity) in puppies after immunostimulation with Trifuzol liposome emulsion.

MATERIALS AND METHODS

Drugs

The active substance of the preparation Trifuzol (Kharkov State Biological Factory, Ukraine) is piperidine 2-(5-(furan-2-yl)-4-phenyl-4H-1,2,4-triazole-3-ylthio) acetate – from the group of Triazole derivatives.

Animals

Eight Labrador puppies from the same litter (1 month of age) were divided into two groups. The first (control) group of 4 puppies was subcutaneously administered a standard dose of the Nobivac Puppy DP vaccine (A139c01, Holland). The second group of 4 puppies was vaccinated using Nobivac Puppy DP (A139c01) subcutaneously in a standard dose followed by the administration of 1 mL Trifuzol liposomal emulsion subcutaneously (=10 mg active substance), twice: for the first time at the time of vaccination and for the second time after 72 hours.

Blood sampling and analysis

Fasting blood samples were collected in the morning by puncture of the ulnar vein in plastic tubes with K_2EDTA on the day of vaccination and at post vaccination day 14.

Determination of IgG antibodies to CPV and CDV. The method is based on the use of a sandwich ELISA. Researches were carried out on ELISA equipment (LabLine 022, Austria) according to the instructions of Hema reagents (Russian Federation).

The number of killer cells was counted by the method of Liddell & Weeks (1995). The absolute number of lymphocytes in 1 μL of blood was determined by counting large-plasma lymphocytes (with azurophilic granularity) from the total number of lymphocytes. The calculation was carried out using immersion oil and an immersion lens MicroOptix, Austria (eyepiece ×15, lens ×90).

Phagocytic activity of neutrophils. The reaction is carried out in 96-well plates for immunological reactions with 0.2 mL cells and a round bottom. The phagocytosis test is carried out adding to cells 0.06 mL of 0.1% suspension of baker's yeast cells, pre-killed by heating. In the blood smears, the number of phagocytic neutrophils were counted per 50 neutrophils. The neutrophil cell was considered as phagocytising when it absorbed one or more yeast cells.

Absolute number of T-lymphocytes (CD3) and their immunoregulatory populations (CD4, CD8) were quantitated using monoclonal antibodies (AbD Serotec, UK) in the immunofluorescence reaction (Vlizlo, 2012).

Relative amount of B-lymphocytes was determined by the method of rosette formation with mouse red blood cells which contain an immunoglobulin-antibody complement complex on their surface as markers (Liddell & Weeks, 1995).

Statistical analysis

Data were expressed as median [minimum-maximum] using the PRIMER software. After comparing the parameters studied and their intergroup differences, the Mann-Whitney test was used, and the result was considered significant at $P \leq 0.05$.

RESULTS

Table 1 shows the dynamics of the absolute number of lymphocytes and their immunoregulatory subpopulations. Thus, in the control group, after introduction of the vaccine, the absolute number of lymphocytes decreased from 2.75 [2.6–3.0] G/L to 2.55 [2.4–2.7] G/L. Comparing this index in the puppies of the experimental group, an increase from 2.45 [2.3–2.6] G/L to 2.75 [2.7–3.1] G/L was noted. The decrease in the control and the increase in the experimental group is established in the absolute number of T-lymphocytes and their subpopulations. However, this trend was less pronounced in T-suppressor cells and more pronounced in T-helper cells. Analysing the absolute amount of B-lymphocytes, it was found that after vaccination, these cells decreased insignificantly in the control group by 4%, and in the experimental group, they increased significantly by 7% ($P<0.05$).

A decrease in the immunoregulatory index was observed 14 days after vaccination in control animals of the group and an increase in the experimental group.

Table 1. Absolute number of lymphocytes and their immunoregulatory subpopulations in puppies vaccinated against parvovirus enteritis and canine distemper and either untreated (control group; n=4) or treated with the immunostimulatory drug Trifuzol (experimental group; n=4). Data are presented as median [minimum-maximum]

Index	Before vaccination		14 days after vaccination	
	Control group	Experimental group	Control group	Experimental group
Lymphocytes, G/L	2.75 [2.6–3.0]	2.45 [2.3–2.6]*	2.55 [2.4–2.7]	2.75 [2.7–3.1]*
T-lymphocytes (CD-3), G/L	1.6 [1.5–1.9]	1.5 [1.4–1.7]	1.5 [1.3–1.7]	1.85 [1.7–3.0]*
T-helpers (CD-4), G/L	1.25 [1.0–1.4]	1.2 [1.0–1.3]*	1.15 [0.9–1.3]	1.5 [1.3–1.7]
T-suppressors (CD-8), G/L	0.4 [0.3–0.5]	0.35 [0.3–0.5]*	0.35 [0.2–0.5]	0.3 [0.2–0.5]*
B-lymphocytes (CD-19), G/L	0.4 [0.3–0.5]	0.35 [0.3–0.4]	0.4 [0.3–0.6]	0.45 [0.4–0.5]*
Immunoregulatory index (IRI) CD4/CD8	3.8 [3.6–4.0]	3.15 [3.0–3.4]*	3.35 [3.0–3.5]	3.85 [3.8–4.2]*

* $P<0.05$ compared to the animals of the control group.

Table 2. Absolute number of phagocytic neutrophils and NK cells in puppies vaccinated against parvovirus enteritis and canine distemper and either untreated (control group; n=4) or treated with the immunostimulatory drug Trifuzol (experimental group; n=4). Data are presented as median [minimum-maximum]

Indicator	Before vaccination		14 days after vaccination	
	Control group	Experimental group	Control group	Experimental group
Phagocytosis	2.25 [2.0–2.5]	2.5 [2.3–2.8]	2.4 [2.2–2.6]	2.1 [2.0–2.4]
NK-cells	0.2 [0.1–0.3]	0.2 [0.2–0.4]	0.2 [0.2–0.3]	0.2 [0.2–0.4]

Table 3. Titres of specific antibodies (IgG) against parvoviral enteritis and canine distemper in vaccinated puppies, either untreated (control group; n=4) or treated with the immunostimulatory drug Trifuzol (experimental group; n=4). Data are presented as median [minimum-maximum]

Indicators	Before vaccination		14 days after vaccination	
	Control group	Experimental group	Control group	Experimental group
IgG titre against parvovirus enteritis, U/mL	7.3 [6.5–8.0]	6.0 [5.0–7.0]	21.0 [18.0–24.0]	0.0 [0.0–0.0]*
IgG titre against anine distemper virus, U/mL	15.0 [12.0–17.0]	6.0 [5.0–8.0]*	27.0 [20.0–32.0]	0.0 [0.0–0.0]*

* $P<0.05$ compared to the animals of the control group.

Table 2 presents data on the change in the absolute amount of phagocytic neutrophils in experimental and control puppies. In contrast to changes in lymphocytes, the phagocytic activity of neutrophils increased in control puppies from 2.25 [2.0–2.5] G/L to 2.4 [2.2–2.6] G/L (by 12%). At the same time, in the animals of the experimental group this index in the dynamics decreased significantly by 10%.

With respect to the titres of specific antibodies (IgG) against canine parvovirus enteritis and canine distemper before the introduction of the vaccine in the animals of the experimental and control groups, the titres of specific antibodies against parvoviral enteritis were relatively low and did not differ significantly. On post vaccination day 14 the puppiess of the control group showed statistically significantly higher values – the increase in IgG titre against CPV was 3.65 times ($P<0.05$), and against CDV: 2.2 times ($P<0.05$) (Table 3). In puppies of the experimental group, IgG titre against parvovirus enteritis and plague carnivorous virus in dynamics, after 14 days, decreased to zero.

DISCUSSION

Experimental data on the use of Trifuzol are reported for treatment of diseases in animals (Maslikov, 2012; Panasenko *et al.,* 2016) and for improving the meat production of poultry (Girkovy, 2016).

Changes in the immune system in ontogeny involve a decrease or increase in the activity of various immunity units in animals of different species (Tizard, 1996). The immune system is incredibly complex, therefore, to demonstrate these changes, we focused mainly on the results of laboratory studies.

Reduction of the absolute number of lymphocytes and their regulatory subpopulations in the puppies of the control group is most likely indicative of the immunosuppressive effect of the vaccine on adaptive immunity. Previous studies have shown that changes in the number of immunocompetent cells in the blood when a vaccine is administered depend on the initial number of these cells (Broshkov & Smolyaninov, 2012). In puppies with an initially high absolute lymphocyte count, immunosuppression is observed after the introduction of the polyvalent vaccine. And with the initially low level of lymphocytes, the introduction of the vaccine promotes the proliferation of cells of adaptive immunity.

It was found that in puppies of the experimental group the absolute amount of T-lymphocytes, namely CD-4, was subject to greater proliferation than B-lymphocytes. This is most likely due primarily to the fact that T-lymphocytes belong to the cellular unit of immunity and one of the first come into contact with the immunogen, in contrast to B-lymphocytes, which are attributed to humoral immunity. Similar dynamics were observed in studies of other authors (Ilyina *et al.,* 2008). There was an increase in T-helper cells and a decrease in T-suppressor lymphocytes in the blood of pups of the experimental group, injected with 1,2,4-triazole during vaccination.

When analysing the immunophysiological status of puppies, the integral indicator – immunoregulatory index (IRI) was also taken into account. The change in this indicator usually occurs with an imbalance between the immunoregulatory subpopulations of lymphocytes and is one of the risk factors for the development of immunopathological reactions. According to previous data of ours, the most adequate immune response of the organism during spontaneous viral infection was with IRI 3.0 (Broshkov, 2012). A more pronounced change in IRI in the experimental group is associated with a significant increase in CD-4.

The manifestation of the act of phagocytosis and its activity are associated with the activation of the entire biochemical system of the cytoplasm and the membrane of neutrophils. Such activation occurs as a result of adhesion to the neutrophils of the opsonised object of phagocytosis (Bahamaev & Povetkin, 2013). Reduction of phagocytic activity in the experimental group may be associated with a decrease in the number of surface receptors on neutrophil membranes due to the specific immunosuppressive action of Trifuzol on these cells.

This assumption can be confirmed by the fact that the subpopulation of NK cells, which, like neutrophils, belongs to congenital cellular immunity, tended to increase in the experimental group. An increase in the number of NK-cells can be a compensatory organism's response to a decrease of neutrophils' phagocytic activity. The lack of quantitative changes in NK cells in the control group of animals indicates that these cells do not directly react to the vaccine antigen.

The fact of the absence of specific antibodies in the puppies of the experimental group 14 days after vaccination should be checked by subsequent experiments as it is not clear whether this is a result from

the direct action of the pharmacological substance or liposome emulsion components. According to experimental data, 1,2,4-triazole at a concentration of 0.25 mg/mL contributed to the retention of the cytopathic effect of the canine distemper virus and parvovirus of dogs in cell culture. Perhaps the antiviral effect of 1,2,4-triazole contributed to a decrease of antibody formation (Ilyina *et al.,* 2008).

In conclusion, a decrease in the titre of antibodies against CDV and CPV in the blood of puppies can be considered as a consequence of the antiviral effect of 1,2,4-triazole. Trifuzol treatment of puppies before vaccination will reduce the risk if the vaccine is administered during the incubation period of the disease. Limitations of this study are the small number of experimental subjects and the brief period of treatment and observation. Therefore, long-term studies are needed to better understand the relevance of observed effects of 1,2,4-triazole on the immune system of puppies.

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