Original article

The effect of different doses of methisoprinol on the percentage of CD4+ and CD8+ T lymphocyte subpopulation and the antibody titers in pigeons immunised against PPMV-1

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Abstract

As immunosuppression in pigeons is common and results in reduced post-vaccination immunity and lower health status of the birds, studies have been taken up aimed at evaluation of the effect of three doses of methisoprinol on the percentage of CD4+ and CD8+ T lymphocyte subpopulation in peripheral blood and in the spleen and the titre of anti-NDV antibodies in the serum of pigeons in four groups (A, B, C, D), with 20 birds each. Pigeons in each group were immunised against paramyxovirosis at week 6 and 9 of life. Water for injection (group A – control) or methisoprinol at 100 mg/ kg of body weight (group B), 200 mg/kg of body weight (group C) and 600 mg/kg of body weight (group D) was administered intramuscularly for 3 days before each vaccination. The immunological analyses were carried out by flow cytometry and the ELISA test.

The findings indicate that methisoprinol administered intramuscularly at 100 and 200 mg/kg of body weight for 3 successive days before vaccination against paramyxovirosis mainly stimulates the mechanisms of non-specific humoral and cellular immunity, which is indicated by a higher percentage of the subpopulation of CD4+ T lymphocytes in peripheral blood and in the spleen and a higher titre of anti-NDV antibodies.

Key words: pigeons, methisoprinol, immunomodulation, T lymphocytes, anti-NDV antibodies

Introduction

Rapid development of sport pigeon breeding has necessitated the development of specific prevention programmes and tests to evaluate their efficacy. One of the viral diseases which has caused immense losses in pigeon flocks is paramyxovirosis. Paramyxovirus infections in pigeons (PPMV-1) are controlled by vaccinations with inactivated vaccines (Alexander et al. 1986, Stone 1989, Cross 1995); however, para-

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myxovirosis has recently been frequently detected in field conditions in pigeons which have already been vaccinated against it. This can be attributed to the fact that the birds are frequently infected with immunosuppressive viruses, such as pigeon circovirus (PiCV) (Wieliczko et al. 2005). However, immunosuppression in pigeons is obviously a complex phenomenon, which is a combination of the action of numerous pathogens and various factors related to the specific breeding conditions. The specificity of pigeon rearing (transport to the race start location, races, exhibitions and shows) is a source of intense stress for the birds. This, as well as the social stress (too many pigeons in a dovecote, birds fighting to get a place at the feeder, or a place to nest or rest) leads to stimulation of the hypothalamic-pituitary--adrenal system which, in consequence, results in intense secretion of glycocorticoids, intensification of carbohydrate and protein metabolism, disturbance of cellular and humoral immunity as well as in reduced defensive properties of connective tissue (Shot and Skinner 2008, Shini et al. 2010).

Excessive use of antibiotics, especially tetracyclines, is another immunosuppressive factor in pigeon rearing (Panigrahy et al. 1979, Rzedzicki and Kowalska 1992). The administration of illicit drugs in pigeon rearing in Poland is not monitored and makes immunosuppression increasingly frequent in pigeons, particularly by giving birds steroids which delay moulting. This manifests itself in numerous infections in early autumn, which is soon after competition races have ended.

The growing importance of immunosuppression, both in human and animal pathology, have brought about increasing interest in the use of substances which boost the reactivity of weakened immune systems or support post-vaccination immunity, i.e. immunomodulators. Immunomodulation is a new trend in clinical immunology, which includes all the agents which stimulate or inhibit the cellular and humoral defensive mechanisms. Agents which lower the immunity in birds are hardly ever administered in veterinary practice because veterinary surgeons would rather restore the proper function of the weakened immune system, which may be achieved by immunostimulation. There are reports in the literature on immunostimulators in various poultry species (Panigrahy et al. 1979, Moya et al. 1984, Singh and Dhawedkar 1993, Rumińska-Groda 2002, Stenzel et al. 2008), but there are none about the use of preparations which boost immunity in pigeons. Previous studies conducted by these authors (Stenzel et al. 2011) have shown the immunostimulating effect of levamisole, administered to pigeons at 2 mg/kg of body weight, and β-glucans – at 5 mg/kg of body weight. No such effect has been shown for methisoprinol given at 300 mg/kg of body weight. The literature data have shown that the immunostimulating effect of methisoprinol largely depends on its dose. Lower doses stimulate the immune system, whereas the effect is not observed in higher doses, which may even act as immunosuppressants (Stenzel et al. 2008).

Considering the above, a research study was undertaken to determine the effect of methisoprinol administered to pigeons at three doses – 100, 200 and 600 mg/kg of body weight – on the percentage of CD4$^+$ and CD8$^+$ T lymphocyte subpopulation in peripheral blood and the spleen, and the antibody titre following pigeon immunisation against paramyxovirosis.

Materials and Methods

The experiment with pigeons was conducted with the consent of the Local Ethical Committee for Animal Experiments. Four groups of pigeons with 20 birds in each were used in the experiment. The pigeons at the age of 5 weeks were pre-selected (to be uniform in terms of body build and mass) and randomly divided into groups. Each group contained the same number of male and female birds and the birds were subsequently immunomodulated.

Pigeons in group A used as a control, were given water for injection intramuscularly for 3 days before vaccination. The birds in the other groups were immunomodulated with different doses of 10% solution of methisoprinol (Isoprivet, VetAgro Lublin, Poland). The immunomodulator was administered by intramuscular injection for 3 successive days before each vaccination (7-9 and 35-37 days of experiment) at: 100 mg/kg of body weight (group B), 200 mg/kg of body weight (group C) and 600 mg/kg of body weight (group D). The pigeons subsequently were immunised against paramyxovirosis in accordance with the recommended vaccination schedule in weeks 6 and 9 (10 and 38 day of experiment) of their life with the PM-VAC vaccine (Biowet, Puławy) at 0.2 ml *s.c.* On day 15 (4 days after 1st vaccination), 42 (4 days after 2nd vaccination) and 63 (21 days after 2nd vaccination) of the experiment, the percentage of the subpopulation of T lymphocytes in peripheral blood and the spleen, as well as the titre of anti-NDV antibodies were determined.

Studies of the percentage of the T lymphocyte subpopulations were conducted with the use of anti-CD4$^+$ and CD8$^+$ monoclonal antibodies (Mouse anti-chicken FITC, Southern Biotech, USA) by flow cytometry on an EPICS XL apparatus (Beckmann coulter, USA). Blood was sampled directly to K$_2$EDTA-coated tubes and spleens were taken during the anatomopathological examination. Blood

samples were prepared in accordance with the procedure described by Dudek (2011); spleen leukocytes for cytometry were obtained in accordance with the procedure described by Stenzel et al. (2008). The percentage of CD4$^+$ and CD8$^+$ cells were read out in a lymphocyte gate and the results were analysed with the System II software.

The titer of specific anti-NDV antibodies was determined by the ELISA test (IDEXX, USA). The serum was diluted at 1:5. The absorbance of the solution was measured with an Elx800 spectrophotometer (Biotek) at the wavelength of 650 nm.

The results were analysed statistically with a two-factorial analysis of variance (ANOVA) and by calculating the standard deviation, the mean value and the significance of differences at $p \leq 0.05$ and $p \leq 0.01$; the coefficient of variance (CV%) was calculated for the antibody titre. The statistical analysis was performed with the STATISTICA 8,0 software using a Duncan post test.

Results

The percentage of the subpopulations of CD4$^+$ and CD8$^+$ T lymphocytes in blood and in the spleen of pigeons subjected to immunomodulation with different doses of methisoprinol are shown in Tables 1 and 2. The data in Table 1 show the existence of statistically significant differences between the percentage of the subpopulations of CD4$^+$ and CD8$^+$ T lymphocytes in blood of pigeons in different groups and between individual blood samplings. The percentage of the lymphocyte subpopulation CD4$^+$ in blood of pigeons in group C which were given methisoprinol at 200 mg/kg of body weight was much statistically significantly higher compared to the control group and compared to the birds which were given lower (100 mg/kg of body weight), and higher (600 mg/kg of body weight) doses of methisoprinol. The data in the table show that no statistically significant differences were found between the percentage of CD8$^+$ T lymphocytes in blood of pigeons in different groups and between individual blood samplings.

As the data in Table 2 show, a significantly higher percentage of the subpopulation of CD4$^+$ T lymphocytes was found in the spleens of pigeons in groups B and C than in the pigeons in groups A and D. The experiment did not reveal any highly significant differences between subpopulations of CD8$^+$ T lymphocytes, although the percentage was the

Table 1. Percentage of CD4$^+$ and CD8$^+$ T lymphocytes in peripheral blood of pigeons immunomodulated and immunised against PPMV-1.

Cell surface receptor		Group A	B	C	D	p	Sampling I	II	III	p
CD4$^+$	$\bar{x}$	9.1^A	9.1^A	13.4^B	9.5^A	0.00	9.7^A	9.3^A	12.2^B	0.00
	SD	2.4	2.6	4.3	2.3		3.1	2.8	4.0	
CD8$^+$	$\bar{x}$	8.1	9.3	10.0	8.4	0.27	9.1	8.0	10.1	0.08
	SD	2.8	3.3	1.6	3.5		3.5	1.8	3.1	

A, B – statistical differences significant at $p \leq 0.01$

Table 2. Percentage of CD4$^+$ and CD8$^+$ T lymphocytes in the spleens of pigeons immunomodulated and immunised against PPMV-1.

Cell surface receptor		Group A	B	C	D	p	Sampling I	I	III	p
CD4$^+$	$\bar{x}$	29.8^A	40.6^B	45.9^B	30.2^A	0.00	39.3	36.6	34.3^B	0.09
	SD	4.4	5.2	8.8	5.7		10.3	8.1	9.0	
CD8$^+$	$\bar{x}$	28.4	28.7	28.9	24.4	0.27	26.0	29.8	27.4	0.36
	SD	4.2	8.0	7.4	7.5		7.9	7.9	4.6	

A, B – statistical differences significant at $p \leq 0.01$

Table 3. Titre of anti-NDV antibodies (ELISA) in the serum of pigeons immunomodulated and immunised against PPMV-1

NDV titre ELISA	Group A	B	C	D	p		Sampling I	II	III	p
$\bar{x}$	1247.2	1638.1	1677.3	1464.7	0.18	$\bar{x}$	43.7^A	1271.8^B	3811.2^C	0.00
SD	1259.2	1887.5	1901.0	1469.8		SD	26.1	467.0	1402.9	
CV	100.2	115.2	113.3	100.3		CV	59.7	36.7	36.8	

A, B, C – statistical differences significant at $p \leq 0.01$

lowest in the pigeons in group D (which received methisoprinol at 600 mg/kg of body weight).

The titers of anti-NDV antibodies are shown in Table 3. It did not reveal any significant differences between the titres in serum of pigeons in different groups. The highest values were found in the pigeons which were given methisoprinol at 100 and 200 mg/kg of body weight. The antibody titre in pigeons which were given methisoprinol at 600 mg/kg of body weight was similar to that found in serum of the pigeons in the control group. Statistically highly significant differences with respect to antibody titre were shown to exist between different blood samplings. The antibody titre in each consecutive sampling was higher than in the previous one, with the CV% factor lower, which is evidence of a correct increase in post-vaccination immunity.

Discussion

The experiments were focused on the practical side of the research, hence methisoprinol was used and the recommended schedule of vaccinations of pigeons against paramyxovirosis was followed. However, it should be noted that the results of immunological tests in pigeons carried out by means of flow cytometry are illustrative rather than quantitative and they are not too accurate, which can be attributed to the antibody clones used in the tests, their species-related specificity (mouse anti-chicken) and incomplete binding of antibodies with cell receptors (Jeurissen and Janse 1998, Birdle et al. 2006). Such antibodies were used because anti-pigeon lymphocyte antibodies were unavailable. However, the results may be scientifically valuable provided the same conditions of cytometry are maintained, leukocytes are isolated from pigeons in different groups in the same way and the tests are always carried out by the same person.

This experiment has shown the highest percentage of the subpopulation of CD4$^+$ T lymphocytes in blood of pigeons which were given methisoprinol at the dose of 200 mg/kg of body weight. The results partly correspond with those obtained by Stenzel et al. (2008), who recorded a higher percentage of the CD4$^+$ T lymphocytes in peripheral blood of turkey poults hatched from eggs to which metisoprinol at the dose of 5 mg/embryo was applied on day 26 of incubation, than in the birds hatched from eggs to which the immunomodulator was injected at the dose of 20 mg/embryo.

The results of the experiment show that the percentage of the CD4$^+$ and CD8$^+$ T lymphocytes in pigeons; spleens was different than that in peripheral blood. According to the results, methisoprinol administered *i.m.* for three days before vaccination

against paramyxovirosis at doses of 100 and 200 mg/kg of body weight stimulates non-specific cellular response, which manifested itself in a higher percentage of CD4$^+$ T lymphocytes in the spleens of pigeons in groups B and C. The results only partly correspond to the results of studies conducted earlier on turkey poults, in which the percentage of the CD4$^+$ T lymphocytes was found to have increased significantly after lower doses of methisoprinol were administered, and the percentage of CD8$^+$ T lymphocytes subpopulation was found to have increased following administration of higher doses of the immunomodulator (Stenzel et al. 2008). When analysing those results, it should be noted that there are species-related differences between immune system reactions to methisoprinol. The absence of statistically significant differences in some of the discussed cases is a result of a high value of the standard deviation which, in turn, is a result of high individual variability. This is typical for birds and has been mentioned by other authors (Czekaj et al. 2005, Czekaj et al. 2008).

The titre of anti-NDV antibodies in this experiment was determined by the ELISA test because PPMV-1 is a variant of NDV and it cross-reacts with it (Stone 1989, Cross 1995). The results obtained in this experiment partly correspond with the findings of a study conducted by Rumińska-Groda (2002) on turkeys, which showed that the use of the immunomodulator at lower doses (100 mg/kg of body weight) *in ovo* on day 26 of incubation stimulated specific humoral resistance against NDV, while it brought about the effect of immunosuppression at higher doses (400 mg/kg of body weight). On the other hand, the results of this experiment do not correspond with the findings of the study conducted by Moya et al. (1984), who observed a four-fold increase of the titre of anti-NDV antibodies in comparison with the control group following administration of methisoprinol in chickens at the dose of 300 mg/kg of body weight. When juxtaposed with literature data, the results of this experiment show that the immunomodulating effect of methisoprinol may depend both on its dose (higher doses have an inhibiting effect on producing post-vaccination antibodies) and on the bird species.

The results show that there is a possibility of practical intramuscular use of methisoprinol for three successive days before vaccination at the doses of 100 and 200 mg/kg of body weight as an adjuvant for the vaccine against paramyxovirosis in pigeons.

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