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Influence of methisoprinol on the course of an experimental infection with PPMV-1 in pigeons

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Summary

Aim of the study: In view of the frequent occurrence of immunosuppression in pigeons, which is a consequence of viral infections, studies with the use of a synthetic immunomodulator (methisoprinol) were undertaken to evaluate its impact on the course of an experimental infection with PPMV-1. The aim of the study was to determine the usefulness of methisoprinol for the treatment or prophylaxis of viral infections in pigeons.

Materials and Methods: Three groups of 5-week-old pigeons, with 15 birds in each, were used in the experiment. Before the experiment, the birds were tested for the presence of antibodies specific to paramyxovirus, and their sensitivity to PPMV-1 infection was evaluated. The virus had been cultured earlier on SPF chicken embryos of 9-12 days.

The pigeons from all three groups were infected intravenously with a paramyxovirus suspension (strain APMV-1/pigeon/Poland/AR3/95 obtained from the Veterinary Institute in Puławy) at a concentration of LD50 10-7 in 0.1 ml at a dose of 0.1 ml/pigeon. The birds in the experimental groups (B1 and B2) were immunomodulated with methisoprinol administered at a dose of 200 mg/1 kg of body weight. The immunomodulator was administered by intramuscular injection for 3 successive days before (group B1) or three successive days after (group B2) the experimental infection. Pigeons in group K (used as a control) were given water by intramuscular injection for 3 days before and 3 days after infection. On days 4, 8, and 12 after infection, 5 birds from each group were euthanized, and sections of internal organs (lung, kidney, brain), as well as cloacal swabs, were collected to detect viral RNA by the RT-PCR method.

Results and discussion: Symptoms were recorded from the first day after infection. Neurological symptoms occurred in birds from all groups: in 100% of pigeons from groups B1 and K, and in 80% of pigeons from group B2. Deaths of birds occurred from day 5 after infection in group B1. In the other groups, deaths were observed from day 6 after infection. The total mortality of the infected birds ranged from 70% (group B2) to 100% (groups B1 and K). The resolution of symptoms was observed from day 6 after infection in pigeons from group B2.

During molecular examination, it was noted that the highest number of positive samples (presence of PPMV-1) on each day of the investigation was obtained from brain samples and cloacal swabs. The highest number of positive results in kidney samples was obtained from groups B1 and K on day 4 after infection. On the successive days of the investigation the percentage of positive samples increased to 100 in birds from all groups except group B2.

Based On the basis of the results of the present study, it can be concluded that methisoprinol, used at a dose of 200 mg/kg of body weight after infection, has antiviral activity, manifested by a slower development of paramyxovirosis in pigeons infected intravenously with PPMV-1. Therefore, the administration of methisoprinol to naturally infected and diseased birds may be useful in the treatment of viral diseases.

Keywords: pigeons, methisoprinol, PPMV-1, RT-PCR

The increased breeding of carrier pigeons and their participation in flying competitions has significantly facilitated the spread of infectious diseases in pigeon populations around the world. This problem has been compounded by the fact that international trade in carrier pigeons is also expanding. Among the infectious diseases of pigeons, the greatest losses are caused by viral infections, which have resulted in decreased race performance and an increased number of deaths. One of the viral diseases generating large losses in pigeons every year is paramyxovirosis. The first cases of this disease were recorded in 1977, and it has very quickly

spread across the globe. The disease is caused by an antigenic variant of Newcastle Disease Virus (NDV), which is highly pathogenic for poultry and is defined as a Pigeon Paramyxovirus Type 1 (PPMV-1). Since paramyxoviruses occurring in pigeons greatly differ in pathogenicity, the course of the disease may vary widely (2, 5-7, 11). Infection with this virus spreads rapidly in the flock (3). The vaccines used in the immunoprophylaxis of paramyxovirosis play an important role in the fight against this disease and in proper veterinary care of pigeons. However, because vaccination against paramyxovirosis is not mandatory, the disease continues to cause significant losses, especially among pigeons in the first year of life, in autumn and winter. Moreover, in recent years, many cases of broken post-vaccination immunity have been noted. This is partly due to improper vaccination techniques and the incompleteness of the vaccination program. The widespread prevalence of immunosuppressive Pigeon Circovirus (PiCV) infection has also had a significant impact on the situation (15).

A useful tool in the fight against immunosuppression is immunomodulation. In the available literature there are several reports on the use of immunostimulants in birds, including pigeons (12, 16). However, there is no work on the use of immunomodulators in pigeons infected with a virus.

Therefore, studies with a synthetic immunomodulator (methisoprinol) were undertaken to evaluate its impact on the course of an experimental infection with PPMV-1. The aim of the study was to determine the usefulness of methisoprinol for the treatment or prophylaxis of viral infections in pigeons.

Material and methods

The investigation was conducted with the consent of the local ethics committee (number 7/2007, valid through 31.12.2007).

Three groups of 15 pigeons 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 divided randomly into groups. Each group contained the same number of male and female birds.

Before the experiment, the birds were tested for the presence of antibodies specific to paramyxovirus, and their sensitivity to PPMV-1 infection was evaluated. The virus had been cultured earlier on SPF chicken embryos of 9-12 days.

Pigeons in group K (used as a control) were given water by intramuscular injection for 3 days before and 3 days after the experimental infection. The birds in the experimental groups (B1 and B2) were immunomodulated with methisoprinol administered at a dose of 200 mg/1 kg of body weight. The immunomodulator was administered by intramuscular injection for 3 successive days before (group B1) or three successive days after (group B2) experimental infection. The pigeons were infected intravenously with a paramyxovirus suspension (strain APMV-1/pigeon/Poland/AR3/95 obtained from the Veterinary Institute in Puławy) at a concentration of LD_{50} 10^{-7} in 0.1 ml at a dose of 0.1 ml/pigeon. On days 4, 8, and 12 after the experimental infection, 5 birds from each group were euthanized. From the euthanized or dead pigeons, sections of internal organs (lung, kidney, brain) and cloacal swabs were collected to detect viral RNA by the RT-PCR method. Before attempting to isolate the RNA, the cloacal swab samples were incubated in a 500 µL phosphate buffered saline solution (PBS) at room temperature for 30 min. The internal organ samples were collected in an amount of 50 mg and were then homogenized in 500 µL PBS with a Tissulyser II (Qiagen, Germany). The remaining incubation fluid from the cloacal swabs and organ homogenate in a volume of 200 µL for each sample was used in later stages of the RNA isolation process. RNA was extracted with a ready-made RNeasykit (Qiagen, Germany) in accordance with the manufacturer's instructions.

RT-PCR was conducted in a Mastercykler II (Eppendorf, Germany) thermocycler with a ready-to-use Qiagen one--step RT-PCR Kit (Qiagen, Germany) and the specific primer pairs: NDV-NF: 5'GACTGCRTATGAGACWGCAGAT3' and NDV-NR: 5'ATGCTACTYCCYTGAGCCTGA3' developed by Śmietanka (17). The reaction conditions were as follows: reverse transcription 50°C/30 min., initial denaturation 95°C/15 min; followed by 40 cycles: denaturation 94°C/30 s, annealing 63°C/ 30 s, chain elongation 72°C/30 s; final elongation after the last cycle 72°C/10 minutes.

An 8 µL volume of amplification products was electrophoresed on a 2% agarose gel (Promega, E.U.) in the presence of ethidium bromide (Sigma Aldrich, Germany) in a 1% TAE buffer (MBI Fermentas, Lithuania) under 60 V for 60 min. A Gene Ruler 100 bp DNA Ladder Plus (MBI Fermentas, Lituania) was used as a PCR-weight size marker. The electrophoresis results were read by means of a GelDoc gel imaging system (Biorad, Italy) and saved as digital photographs.

Results and discussion

Due to the high number of deaths of birds in all experimental groups, the tabulated data are incomplete. The results of the experimental PPMV-1 infection are summarized in Table 1. Symptoms were recorded from the first day after the experimental infection. Green diarrhoea occurred in birds from all groups 24 hours after infection. Polyuria appeared in all pigeons on the second day after infection and, except for those from group B2, and lasted until day 7 after infection. After this time, this symptom was noted in a smaller number of birds. Swelling of the eyelids and tearing was noted in pigeons from groups B1 and K. Neurological symptoms occurred in birds from all groups. These symptoms appeared in 100% of pigeons from groups B1 and K and in 80% of pigeons from group B2. Deaths occurred from day 5 after infection in group B1. In the other groups, deaths were observed from day 6 after infection. The total mortality of the infected birds ranged from 70% (group B2) to 100% (groups B1 and K). The resolution of symptoms was observed from day 6 after infection in pigeons from group B2.

The results of RT-PCR are listed in Table 2 and in Figure 1. As shown in Figure 1, the amplification of eluted RNA with specific primers gave a product of 518 base pair (bp) size.

The data presented in Table 2 shows that the highest number of positive samples (presence of PPMV-1) on each day of the investigation was obtained from the brain samples and cloacal swabs. The highest number of positive results in kidney samples was obtained from birds from groups B1 and K on day 4 after infection. The percentage of positive results was lower on each subsequent day of the investigation in kidney samples obtained from groups B1 and K. However, in the kidney samples of pigeons from group B2, the percentage of positive results was higher on each subsequent day of the study. The lowest number of positive samples on each day of the study was found in kidney samples from group B2.

For lung samples, the highest number of positive results on day 4 after infection was obtained from the pigeons of groups B1 and K. On subsequent days, the percentage of positive tests increased to 100 in all groups, except for B2.

Tab. 1. The course of experimental infection with PPMV-1

Character of symptoms	Group					
	B1		K		B2	
	The day after infection	%	The day after infection	%	The day after infection	%
Green diarrhea	1	100	1	100	1	100
Poliuria	2-7	100	2-7	100	2-5 6-8	100 80
Swelling of the eyelids, tearing	5	10	5	20	–	–
Apathy	2 3 4 5-7	7 13 80 100	2 3 4 5-7	27 27 60 100	2 3 4 5 6-9	0 0 10 60 80
Paralysis of the limbs	5 6 7	40 40 20	5 6 7	40 30 30	5 6 7 8	30 20 20 10
Death	5 6 7 8	10 30 30 30	5 6 7 8	0 50 30 20	5 6 7 8 11	0 20 20 20 10
Relief of symptoms	–	–	–	–	6 7-12	40 20

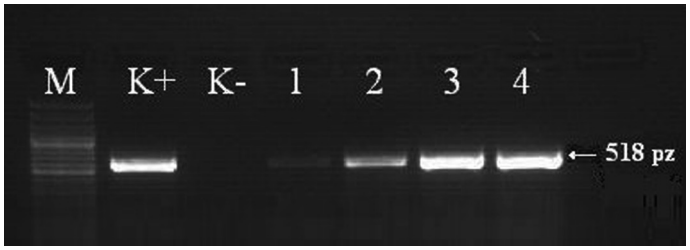

Fig. 1. The agarose gel electrophoresis of RT-PCR products obtained from selected samples from group B2 on day 4 after the experimental infection. From the left: M – DNA size marker (Gene Ruler 100 bp, Fermentas), K+ – positive control (RNA isolated from the virus used for experimental infection), K – negative control (without RNA), 1 – RNA isolated from the kidney sample, 2 – RNA isolated from the lung sample, 3 – RNA isolated from the brain sample, 4 – RNA isolated from the cloacal swab

Because of the significant pigeon health hazard caused by immunosuppressive viruses and infections with opportunistic pathogens, studies were undertaken to verify the literature data regarding the potential antiviral activity of methisoprinol (8-10, 12-14). PPMV-1 was used for the experimental infection because of the scale of the epizootic problem caused by this virus and because of its availability.

For a reliable assessment of the anti-viral impact of methisoprinol, it was necessary to cause a severe course of paramyxovirosis in pigeons. Therefore, prior to the experiment, a control infection was performed to determine the response of the pigeons to the virus administered by various routes (intravenously, oculonasally). Strong clinical symptoms and deaths of the pigeons were observed only after infection by the intravenous route. Hence this method of infection was selected for the experiment. In the study, the effects of methisoprinol given at a dose of 200 mg/kg of body weight on the course of a PPMV-1 infection were recorded. The investigation was conducted in

Tab. 2. The results of the amplification of PPMV-1 genetic material by the RT-PCR method in the samples of selected organs from pigeons infected with PPMV-1 and treated with an immunomodulator. The "ne" abbreviation means "not examined"

Group	The presence of PPMV-1 genetic material in organs examined (%)											
	Kidney (days after infection)			Lung (days after infection)			Brain (days after infection)			Cloacal swab (days after infection)		
	4	6-8	10-12	4	6-8	10-12	4	6-8	10-12	4	6-8	10-12
B1	80	20	ne	80	100	ne	100	80	ne	100	66.6	ne
K	80	40	ne	100	100	ne	100	100	ne	66.6	100	ne
B2	20	40	66.6	40	60	66.6	100	100	100	100	100	100

two experimental systems: therapeutic (the use of an immunomodulator after infection) and prophylactic (the use of an immunomodulator before infection).

The results observed in the course of the experimental infection with PPMV-1 are summarized in Table 1. As is clear from the data, the first symptoms appeared on the second day after infection, which does not correspond with the literature, according to which, the symptoms should have appeared at least 4 days after infection (4). This is strongly related to the route of virus administration. In the case of infection by natural routes, the disease develops more slowly, and the symptoms appear much later.

As can be seen from the data presented in Table 1, the experimental infection did not proceed evenly in pigeons from all examined groups. A less severe disease, involving the regression of symptoms and a lower mortality rate, was observed in birds receiving methisoprinol after infection (group B2). In some birds of this group, the relief of symptoms was already recorded on day 6 after infection. In addition, in group B2 the survival rate was the highest (30%), and the disease had a milder course, which was probably related to the antiviral activity of methisoprinol. These results correspond to those obtained by Moya et al. (1984), who showed that the disease developed more slowly (12) in chickens infected with the ND virus and then treated with methisoprinol.

This study also sought to determine the effect of an immunomodulator on the tissue distribution of paramyxovirus in the course of an experimental infection of pigeons. The development and application of the molecular diagnostics of viral diseases also greatly facilitated their fast recognition in pigeons (1-4, 7, 15, 18). In the present study, a one-step RT-PCR method was used, which had been validated for detecting PPMV-1 by Śmietanka (17). After electrophoresis in a 2% agarose gel, 518 bp was obtained – which is consistent with the literature on this method (Figure 1) (17).

This study showed the presence of viral genetic material in kidney samples by the 4th day after infection, which coincided with the appearance of symptoms (Table 1). These results correspond with the data in the available literature (4). The highest percentage of birds, in which the renal samples showed the presence of PPMV-1 RNA, was found in the group receiving methisoprinol for 3 consecutive days prior to infection (group B1) and in the control group (group C), whereas the lowest percentage of such birds was found in the group receiving methisoprinol for three days after infection (the group B2). In groups B1 and K, the number of birds in whose samples the presence of viral genetic material was detected decreased with the passage of time from the experimental infection. This was probably related to the normal course of the disease. In group B2, there was an increasing proportion of birds with viral genetic material present in the kidneys during the experiment, which coincided with the end of methisoprinol administration. This indicates the inhibition of PPMV-1 replication in the kidneys by methisoprinol administered after infection (Table 2). A similar pattern was observed in the case of lung samples, in which the percentage of birds with viral genetic material increased with each subsequent examination in all groups. The presence of PPMV-1 genetic material was found in almost all brain samples and cloacal swabs, regardless of the experimental group or time after infection. These results correspond to those obtained by Barbezange and Jestin (2003), who found that PPMV-1 RNA was present in the lungs of pigeons on day 2 after inoculation, and 100% of the birds were positive on day 4 after infection. Barbezange and Jestin found a similar pattern in the gastrointestinal tract, whereas in the brain the virus was detected as late as day 4 after infection. The percentage of birds in which viral genetic material was found in the brain was the highest within 7 days after inoculation (4). The differences in the results obtained by these authors could be related to the different route of infection (oculonasal).

The presence of pigeon paramyxovirus genetic material in brain samples and cloacal swabs from almost 100% of the pigeons examined indicates the viscero- and neurotrophic properties of the strain (APMV-1/pigeon/Poland/AR3/95) used in the study. The presence of viral genetic material in brain samples and cloacal swabs from the birds of group B2 indicates that methisoprinol may not have penetrated through the blood-brain barrier and the intestinal wall, which resulted in strong diarrhoea and neurological symptoms (Table 1). However, the fact that these symptoms disappeared, despite the continued presence of PPMV-1 genetic material in the organs of the infected pigeons, suggests that methisoprinol administered at 200 mg/kg of body weight for 3 days after infection had some antiviral activity.

To summarize, it should be noted that the classical RT-PCR used in the present study does not provide a complete picture of the course of infection. The fact that the symptoms disappeared, despite the presence of viral genetic material in the organs of pigeons from group B2, suggests that virus replication was slower, and the number of viral particles was smaller under the influence of the methisoprinol. However, with the classical RT-PCR it was impossible to determine whether the number of viral particles was the same in organs obtained from the pigeons of all groups. Therefore, in this case, the real-time RT-PCR method (qRT-PCR) would have been more appropriate for a proper interpretation of the results.

On the basis of the results of the presented study, it can be concluded that methisoprinol used at a dose of 200 mg/kg of body weight after infection has antiviral activity, manifested by a slower development of paramyxovirosis in pigeons infected intravenously with PPMV-1. Therefore, the administration of methiso-

prinol to naturally infected and diseased birds may be useful in the treatment of viral diseases.

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