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In vivo effect of EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-carboxamide) on experimental infected rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) fry with infectious pancreatic necrosis virus

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Abstract

The in vivo antiviral effect of 5-ethynyl-1-β-D-ribofuranosylimidazole-carboxamide (EICAR) was evaluated in coho salmon and rainbow trout fry, experimentally infected with infectious pancreatic necrosis virus (IPNV). Treatment consisted of a daily bath of 2 h in 0.4 μg ml^{-1} or 0.8 μg ml^{-1} of EICAR, for approximately 20 days. The behavior of the fish was studied for 45 days post-infection. The survival of the infected treated groups was compared with the survival of non-infected and infected untreated control groups. The results showed that the survival of coho salmon and rainbow trout fry in the infected group treated with both doses of EICAR was similar to the survival observed in the healthy control group (approximately 94%). While, the survival of the infected and untreated control fish was 56% for salmon and 28% for trout, there were no significant difference in the weight of coho salmon fry between those treated with EICAR and non-infected and infected untreated control groups. However, in rainbow trout there was a statistically significant weight decrease in infected untreated group. Finally, the analysis of tissue samples of the fish by reverse transcription associated with the polymerase chain reaction (RT-PCR) suggest that EICAR have decreased the viral load in infected treated fry. Consequently, the results indicate that EICAR is an effective inhibitor of IPNV replication in vivo and could be a promissory antiviral compound for the treatment of IPNV disease.

Keywords: EICAR; In vivo; Rainbow trout; Coho salmon; IPNV therapy

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1. Introduction

Infectious pancreatic necrosis virus (IPNV) has a high incidence in world aquaculture. It is known to infect over 63 marine species, including mollusks. In rainbow trout fry weighing under 1 g, it has caused up to 100% mortality. However, in salmonids of over 100 days of age, a rather low mortality rate has been observed, even though these fish are infected and become carriers of the virus, which is very serious since their transmission is both horizontal and vertical (Wolf, 1986, 1988).

The IPNV belongs to the Birnaviridae family, which has an icosahedric capsid without envelope, of approximately 60 nm in diameter. Its genome is formed by two segments of double-stranded RNA (Dobos et al., 1979).

At present, the disease is controlled principally by an early diagnosis for which, methods based on reverse transcription associated with the polymerase chain reaction (RT-PCR) have been developed (López-Lastra et al., 1994). In general, the vaccines have not had the expected effect because the fish which are more susceptible to infection (fry) have not reached a fully developed immune system (Wolf, 1988; Lillehaug, 1997). Considering this, and the success obtained by antiviral compounds in the treatment of diseases caused by human viruses, the effect of some antivirals on IPNV replication has been studied (Hirsch et al., 1996). The first study using ribavirin to inhibit IPNV replication was carried out in 1980, with good results in vitro, but not in vivo (Migus and Dobos, 1980; Savan and Dobos, 1980). More recently other compounds were assayed in our laboratory: 5-ethynyl-1-β-D-ribofuranosylimidazole-carboxamide (EICAR), a ribavirin analog, an inosine monophosphate dehydrogenase inhibitor; and 4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (pyrazofurine) an orotidine monophosphate decarboxylase inhibitor, proved to be efficient inhibitors of IPNV replication in vitro, at concentrations 100 and 40 times lower than the cytotoxic concentrations, respectively (Jashés et al., 1996). Since EICAR showed an excellent therapeutic index in vitro, its effect in vivo was studied in this work. Rainbow trout and coho salmon fry, experimentally infected with IPNV, were treated for approximately 20 days in 2-h daily baths with different EICAR concentrations.

2. Materials and methods

2.1. Virus and chemical

The IPNV (ATCC strain VR-299) was propagated in chinook salmon embryo cells (CHSE-214), titrated and stored at -70°C until used. EICAR was donated by Dr. Erik De Clercq from the Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium. The fish were donated by Juan Battaglia, Veterinarian from the Piscicultura Experimental of the Universidad de Chile located in Chiloé Island, in the south of Chile.

2.2. Experimental fish

The rainbow trout and coho salmon fry, body weight 1.5 ± 0.3 and 1.0 ± 0.2 g respectively, used in this study were distributed in groups of 50 fish each and held in 10–12°C water. Eighty percent of the water was changed daily and every 5 days it was changed totally. The fish were observed and fed (3% of their body weight) twice a day. During the 17–20 days of treatment and 30 days post-treatment, deaths or abnormal behavioral characteristics were carefully recorded.

During the one week period of acclimatization random tissue samples were collected and tested for the presence of bacteria and viruses. Samples were submitted to cultures in tryptone soya agar (TSA), kidney disease medium (KDM-2) and CHSE-214 cell cultures with and without antibiotics.

2.3. Experimental infection

The fish were exposed to the virus by placing them in 10–12°C water containing approximately 10^5 pfu ml^{-1} of IPNV (VR-299) for 2 h. Exposure to the virus coincided with feeding in order to facilitate the uptake of IPNV. Also, the air flow was increased during exposure. Control groups

were treated by the same method with cell culture medium instead of virus.

2.4. Antiviral treatment

In order to carry out the antiviral treatment, two doses with less than 1 μg ml^{-1} of EICAR were chosen, because this concentration reduces the incorporation of [methyl-^{3}H] thymidine by 50% (IC_{50}) (Jashés et al., 1996).

The fry were immersed for 2 h in a solution containing 0.4 μg ml^{-1} or 0.8 μg ml^{-1} of EICAR. Normal controls and virus controls underwent the same stress handling without the antiviral compound. This operation was performed 2 h after the virus infection was carried out, and then once a day for 20 days, for coho salmon and 17 days, for rainbow trout.

2.5. Detection of IPNV in fish tissues

IPNV identification was carried out through the reverse transcription associated with RT-PCR from the kidney and spleen of the analyzed fish according to the method previously described (López-Lastra et al., 1994). Nested PCR was performed. The first amplification was done with primers III and IV obtaining a 657 bp product. In the second amplification the primers I and II were utilized, obtaining a 228 bp product. The PCR products were visualized by silver staining, after a 12% polyacrylamide gel electrophoresis (PAGE).

3. Results

3.1. Effect of EICAR on survival of IPNV-infected coho salmon

Untreated coho salmon fry infected with IPNV (positive control) after 6 days of infection showed typical signs of infectious pancreatic necrosis disease, such as darkening of the skin, exophthalmus, abdominal distension, and erratic swimming with violent rotations. On the 8th day, mortality began in this group reaching a peak on days 10 and 11 and finally stopping around day 15. While, in both treated fish groups, either with 0.4 or 0.8 μg ml^{-1} of EICAR, several specimens showed darkening of the skin, some characteristic signs of the disease and scarce mortality around days 10 and 17 post-infection. Among the groups of infected fish (treated and untreated) a loss of appetite was observed by days 10 and 11, but they recovered from it on day 17. The treatment was stopped on day 20 post-infection due to the ceasing of mortality and the recovery of fish activity. There was follow up for up to 45 days to detect a possible new outbreak of mortality. Results are shown in Fig. 1. Accumulated mortality in untreated fish infected with IPNV (positive control) reached up to 21 fish (58% survival). In the group of infected fish treated with 0.4 and 0.8 μg ml^{-1} EICAR, 4 and three dead fish were registered respectively (92% and 94% survival respectively). Four deaths were registered in the negative control group corresponding to non-infected fish (92% survival). We believe that this mortality was due to the handling of healthy fish during the experimental work, since no pathogen was found among the samples and the fish behavior was absolutely normal.

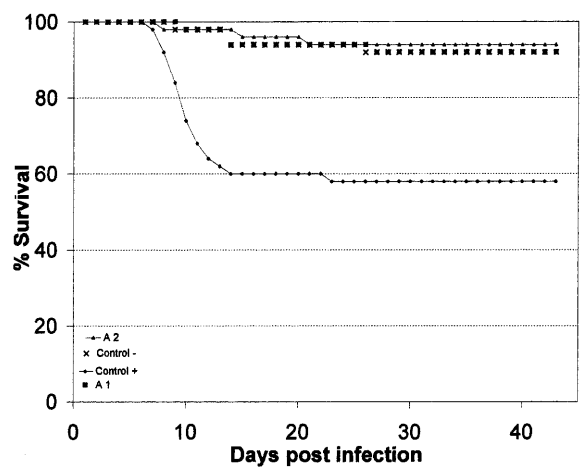

Fig. 1. Effect of EICAR treatment by immersion on the survival of coho salmon fry (body weight 1.0 ± 0.2) following infection with 10^5 pf ml^{-1} of IPNV. The treatment consists of a daily bath of 2 h for 20 days after the virus infection. (◆) positive control, untreated infected fish; (×) negative control, non-infected fish; (■) infected fish treated with 0.4 μg ml^{-1} of EICAR; (Δ) infected fish treated with 0.8 μg ml^{-1} of EICAR.

Table 1
Weight increase of coho salmon fry during the 45 days post-infection[a]

Group	Initial weight (g)	Final weight (g)	Increase weight (%)
C−	1.0 ± 0.2	2.4 ± 0.8	240
A1	1.0 ± 0.2	2.3 ± 0.7	230
A2	1.0 ± 0.2	2.3 ± 0.9	230
C+	1.0 ± 0.2	2.1 ± 0.9	210

[a] C−: negative control, non-infected fish; A1: infected fish treated with 0.4 μg ml^{-1} of antiviral EICAR; A2: infected fish treated with 0.8 μg ml^{-1} of antiviral EICAR; C+: positive control, untreated infected fish.

The untreated infected fish showed a statistically significant difference in the mortality rate with respect to the mortality observed in the treated group and healthy control group (χ^2 test, $P \leqslant 0.05$). A statistically significant difference between the EICAR treated fish and healthy control fish was not detected.

The presence of IPNV among dead fish in treated and untreated groups was verified by the RT-PCR. This technique was chosen because at present it is the most sensitive technique available and allows the detection of IPNV in fish carriers with greater certainty. It is worthy to note that at the end of the experiment, 45 days post-infection, samples were taken from the surviving fish to analyze whether they were carriers of the virus. Results of RT-PCR showed the presence of the virus in all infected fish. However, a higher viral load was observed in untreated infected fish, since in the positive control, sample products were obtained in the first and second amplification, while in the two treated groups, products were obtained only in the second amplification (data not shown). This implies a viral titer greater than 10^5 pfu ml^{-1} in untreated fish and a viral titer between 10^2 and 10^4 pfu ml^{-1} in treated fish.

Table 1 shows the mean weight registered at 45 days post-infection. A slight fall in weight increase was observed in untreated infected fish. However, the weight observed differences are not statistically significant. (ANOVA, $P > 0.5$).

3.2. The effect of EICAR on the survival of IPNV-infected rainbow trout

In the case of rainbow trout, IPNV-infected fry which were not treated (positive control) began to show characteristical signs of infectious pancreatic necrosis from the 6th day of post-infection; the first death occurred on the 7th day of post-infection, accompanied by aggravation of the sign of the disease in all the fish and ended in a high mortality on the 11th, 12th and 13th day. In infected fish treated with 0.4 μg ml^{-1} EICAR, signs of the disease were observed on day 8 and the deaths registered were 2 fish on day 10 and 1 fish on day 11. In the group treated with 0.8 μg ml^{-1} only one death was recorded on day 10 post-infection. On day 10, we also observed a darkening of the skin in both fish groups treated with EICAR but this sign began to disappear on day 13. These fish recovered their activity and appetite on day 13, while untreated infected fry reached this condition on day 15. In this case, the treatment was completed after 17 days and the fish were followed up for 45 days post-infection. The non-infected control fish showed a totally normal behavior, and it is assumed that the 2 deaths observed could be ascribed to handling, since no pathogen was found in the samples analyzed.

The results of this experiment are shown in Fig. 2. It can be observed that the accumulated mortality in untreated fish infected with IPNV (positive control) were 36 fishes of a total of 50 (28% survival). In infected fish treated with 0.4 and 0.8 μg ml^{-1} there was a very low mortality of only 3 and 1 fish repectively (94% and 98% survival respectively), whereas the survival in the negative control was 96%.

The differences were statistically significant in the mortality rate between the untreated infected fish and the treated group and healthy control group, but the differences were not statistically significant between the EICAR treated fish and healthy control fish (χ^2 test, $P \leqslant 0.05$).

Using the RT-PCR reaction the presence of IPNV was detected in all the dead fish of the infected groups (treated and untreated). Also in this case, the same occurred as in coho salmon, at

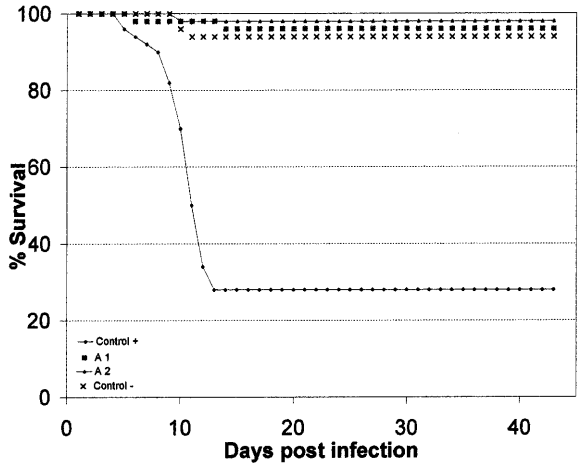

Fig. 2. Effect of EICAR treatment by immersion on the survival of rainbow trout fry (body weight 1.5 ± 0.3) following infection with 10^5 pfu ml^{-1} of IPNV. The treatment consists of a daily bath of 2 h for 17 days after the virus infection. (◆) positive control, untreated infected fish; (×) negative control, un-infected fish; (■) infected fish treated with 0.4 μg ml^{-1} of EICAR; (Δ) infected fish treated with 0.8 μg ml^{-1} of EICAR.

45 days post-infection, fish that survived in the positive control showed a higher viral load.

Table 2 shows the mean weight at 45 days post-infection. Differences were observed between treated fish in both controls. There was a smaller weight gain in untreated infected fish than in healthy fish, which showed a slightly higher weight gain. (ANOVA followed by the Duncan test for multiple comparisons, $P > 0.9$). There was no significant difference between the treated groups and the healthy control, but the untreated infected group showed a statistically significant weight decrease with respect to the other groups.

Table 2
Weight increase of rainbow trout fry during the 45 days post-infection[a]

Group	Initial weight (g)	Final weight (g)	Increase weight (%)
C−	1.5 ± 0.3	4.4 ± 1.1	293
A1	1.5 ± 0.3	3.9 ± 1.3	260
A2	1.5 ± 0.3	4.0 ± 1.1	267
C+	1.5 ± 0.3	2.8 ± 1.1	187

[a] C−: negative control, non-infected fish; A1: infected fish treated with 0.4 μg ml^{-1} of antiviral EICAR; A2: infected fish treated with 0.8 μg ml^{-1} of antiviral EICAR; C+: positive control, untreated infected fish.

4. Discussion

Results obtained in vitro cannot be extrapolated to what may occur in vivo, since most of the times the same trend is not usually observed; this was the case when ribavirin was used to inhibit IPNV replication (Migus and Dobos, 1980; Savan and Dobos, 1980). Hence, EICAR in vivo assays must be carried out.

The results of the experimental IPNV infection both in coho salmon and in rainbow trout fry of approximately 1 g, show the expected evolution of the disease, since the signs were observed at day 6 post-infection and the first deaths occurred on the 7th or 8th day. Also trout fry presented a higher outbreak of mortality than the coho salmon fry, which supports the idea that the rainbow trout is one of the most sensitive species to IPNV (Wolf, 1988). On the other hand it is worth noting that the mortality that affected the fry of both coho salmon and rainbow trout decreased significantly when treated with EICAR baths during 20 and 17 days, respectively. Both fish groups treated either with 0.4 or 0.8 μg ml^{-1} of the antiviral behaved in the same way as healthy controls, showing that EICAR is a strong inhibitor of IPNV either in vitro or in vivo.

Although a tendency to a higher survival was observed with the higher antiviral dose, that is, 94% and 98% survival with 0.8 μg ml^{-1} EICAR versus 92% and 96% with 0.4 μg ml^{-1} in coho salmon and rainbow trout respectively, the differences were not statistically significant. In preliminary studies, rainbow trout fry of approximately 0.8 g were treated for 12 days with 0.2 and 1.0 μg ml^{-1} EICAR. The survival obtained with the lower dose was 86.5%. When a higher dose was used, survival was 98%, which was the same as that of healthy controls (data not shown). This indicates that a plateau was reached in the antiviral effect of EICAR when using concentrations over 0.4 μg ml^{-1}.

The fact that there were practically no deaths during the first month of post-treatment in any of the groups under study indicates that: (a) untreated infected fish (positive controls) like in any viral disease, undergo a progressive and rapid aggravation which reaches its maximum expression. Then, the disease wanes and disappears, with a total recovery; (b) in the case of infected fish treated with EICAR, the antiviral was effective enough to diminish mortality during the acute period of the disease, which did not reappear after the antiviral was stopped. The inhibition of the IPNV replication by EICAR caused a marked decrease in the viral load so that the fry became asymptomatic carriers of the virus. This was corroborated by the analysis of tissue samples of the fish under study by RT-PCR, since in treated fish a viral titer of 10^2–10^4 pfu ml^{-1} approximately was obtained and in untreated fish control, the viral titer obtained was over 10^5 pfu ml^{-1}.

It is worth noting that one of the consequences of IPNV in infected fish is weight loss (Wolf, 1988), which was also observed in untreated infected controls of this experiment. Notwithstanding, the weight loss was much lower in fish treated with the antiviral. In the case of coho salmon, no statistically significant difference was observed between any of the groups, but in rainbow trout the weight loss of untreated infected fish was statistically significant with respect to the other groups. This is an additional advantage of the EICAR treatment.

Since EICAR is a powerful inhibitor of IPNV replication the possibility of treating the fry with this compound to diminish the viral load and evade fish mortality is an excellent instrument for increasing productivity, even though these fish become viral carriers. This EICAR treatment in any case, is better because without treatment the fish would die and those that would survive would be viral carriers too. However, it should be taken into account that the transmission of IPNV is both horizontal and vertical and therefore this treatment should be considered for increasing salmon and trout production but not for select breeders.

We can conclude that EICAR appears to be a promissory antiviral compound for the treatment of IPNV disease.

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