

◆ MANUSCRITO ORIGINAL

Effect of the treatment with monofluorophosphate on survival and tissular damage in rats with pancreatitis

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Summary

Background. Monofluorophosphate (MFP) binds to plasma alpha-macroglobulins modifying their structure and antiprotease activity. The latter is required during pancreatitis, when proteinases are released by the damaged tissue. Previously, it was demonstrated that the treatment with MFP increases the survival of rats with experimental pancreatitis. **Methods.** In this work, the pancreatic damage was quantified through a numerical score evaluating edema, fibrin deposits, neutrophils and mononuclear cells infiltration, necrosis, congestive blood vessels, hemorrhage, vascular thrombosis, and fibrosis in rats with pancreatitis and under treatment with MFP. Ten male Sprague-Dawley rats per group were used: group A: treatment with MFP 30 days before pancreatitis, control A: treatment with vehicle, 30 days before pancreatitis; group B: treatment with MFP for 14 days after pancreatitis; control B: treatment with vehicle for 14 days after pancreatitis, group Sham: rats with simulated surgery. Surviving rats were euthanized after 14 days of the induction of pancreatitis. The score was measured by light microscopy analysis and comparisons were done with One Way ANOVA. The percentage of survival was evaluated by Kaplan-Meier. The score (mean \pm SEM) and the percentage of survival were considered different with a $P < 0,05$. **Results.** Group A: score 8.6 ± 2.3 (NS vs control A), survival 70% ($P < 0.05$ vs. control A); control A: score 11.0 ± 2.2 , survival 40%; group B: score 1.7 ± 0.9 ($P < 0.05$ vs. control B), survival 40% (NS vs. control B); control B: score

7.0 ± 4.0 , survival 40%; group Sham: score 5.3 ± 1.3 , survival 100%. **Conclusions.** The treatment with MFP before pancreatitis increased survival without differences in pancreatic damage. The administration of MFP after pancreatitis decreased tissular damage without differences in survival. The treatment of rats with MFP before or after the induction of pancreatitis would improve morbi-mortality.

Key words. Monofluorophosphate, MFP, macroglobulin, pancreatitis, rats.

Efecto del tratamiento con monofluorofosfato sobre la sobrevivencia y el daño tisular en ratas con pancreatitis

Resumen

Antecedentes. El monofluorofosfato (MFP) se une a las alfa-macroglobulinas plasmáticas modificando su actividad antiproteásica. Durante la pancreatitis la liberación de proteasas requiere la acción de alfa-macroglobulinas. **Métodos.** Se cuantificó el daño pancreático a través de un score que evalúa edema, fibrina, infiltrado polimorfonuclear y mononuclear, necrosis, congestión vascular, hemorragia, trombosis y fibrosis en ratas con pancreatitis y tratamiento con MFP. Se utilizaron 10 ratas macho Sprague-Dawley por grupo: grupo A: tratamiento con MFP, 30 días antes de la pancreatitis; control A: tratamiento con vehículo, 30 días antes de la pancreatitis; grupo B: tratamiento con MFP durante 14 días posteriores a la pancreatitis; control B: tratamiento con vehículo durante 14 días posteriores a la pancreatitis; grupo Sham: ratas con cirugía simulada. Las ratas que sobrevivieron se sacrificaron a los 14 días de la pancreatitis. Se midió el score en cortes histológicos y se compararon con One Way ANOVA. El porcen-

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taje de supervivencia se evaluó por Kaplan-Meier. El score (media \pm SEM) y el porcentaje de supervivencia se consideraron diferentes con una $P < 0,05$. **Resultados.** Los valores fueron: grupo A: score $8,6\pm 2,3$ (NS vs control A), supervivencia 70% ($P < 0,05$ vs control A); control A: score $11,0\pm 2,2$, supervivencia 40%; grupo B: score $1,7\pm 0,9$ ($P < 0,05$ vs control B), supervivencia 40% (NS vs control B); control B: score $7,0\pm 4,0$, supervivencia 40%; grupo Sham: score $5,3\pm 1,3$, supervivencia 100%. **Conclusiones.** El tratamiento con MFP antes de la pancreatitis aumentó la supervivencia de los animales sin cambios significativos en el daño pancreático. La administración de MFP luego de la pancreatitis no modificó la supervivencia, pero determinó un menor daño tisular. El tratamiento con MFP antes o después de la inducción de la pancreatitis sería beneficioso ya que mejoraría su morbimortalidad.

Palabras claves. Monofluorofosfato, MFP, macroglobulina, pancreatitis, ratas.

Abbreviation

MFP: monofluorophosphate.

AM: alpha-macroglobulin.

ICDL: incomplete closed duodenal loop.

Acute pancreatitis is a necro-inflammatory disease of the pancreas that courses with damage of the gland as a consequence of an uncontrolled release and premature activation of pancreatic enzymes inside or near the pancreas.¹ The development of the inflammatory process depends on the balance between proteinases and their inhibitors. The alteration of this balance is cardinal in the pathogenesis of the disease.²

The pharmacological therapy for the prevention or treatment of acute pancreatitis has been generally unsuccessful. Studies carried out in different experimental models of acute pancreatitis with anti-proteolytic agents have shown an improvement in the outcomes. In contrast, this clinical benefit has not been observed in human beings with acute pancreatitis.^{3,4}

Alpha-macroglobulins (AM) are a family of plasma proteins whose main function is the inactivation of proteinases.^{5,6} AM bind to proteinases and undergo structural changes which determine that the complex proteinase-AM be rapidly phagocytosed and extracted from blood stream by specific receptors on several cell types, such as hepatocytes, fibroblasts

and macrophages. On the other hand, AM act as binding proteins for various growth factors, polypeptide hormones and cytokines, such as TGF β , IL-1, IL-2, IL-6, IL-8 and TNF α , which are also factors proven to act on the onset and progression of acute pancreatitis.⁷ These two functions of AM, inhibition of proteinases and binding of cytokines, suggest a moderating role of AM in the development of acute pancreatitis.⁸ The role of AM in acute attacks is undisputed,⁹ and its plasma levels decrease once pancreatitis is established.²

Monofluorophosphate (MFP, CAS 10163-15-2) is a drug used in the treatment of osteoporosis^{10,11} that binds to plasma AM, decreasing their anti-proteolytic activity and disturbing their homeostasis.¹² The mentioned phenomenon is not permanent and was confirmed in human beings under osteoporosis treatment with MFP.¹³ The MFP-AM complex is cleared from plasma with a half life of 38 min.¹⁴

It was demonstrated that the treatment with MFP in rats can modify the clinical course of acute pancreatitis.¹⁵ When MFP was orally administered to rats for 30 days, before the incomplete closed duodenal loop induced pancreatitis (ICDL), the median survival of rats (time in which 50% of animals died) was higher than in rats that did not receive MFP. The pancreatic damage and amylase levels in rats with MFP pre-treatment were lower than in control rats. Rats with MFP treatment had higher plasma levels of AM at the beginning of the illness and its concentration remained constant for at least 72 hs after ICDL. The protective effect of MFP on the development of pancreatitis can be attributed to higher plasma levels of AM at the onset of the illness. The increase of plasma levels of AM produced by chronic treatment with MFP has been reported in other experimental models.¹⁶ On the contrary, if MFP was administered simultaneously with the induction of pancreatitis, survival curves did not differ from control animals without MFP.

The aim of this work was to quantify pancreatic damage in rats with ICDL induced pancreatitis and treatment with MFP, both before and after the induction of pancreatitis by ICDL.

Methods

Animals and general procedures

The study was carried out on male Sprague

Dawley rats, in accordance with the international guidelines of animal care.¹⁷ The project was also approved by the Ethics Committee, School of Medicine, Rosario National University.

Pancreatitis was surgically induced by the incomplete closed duodenal loop (ICDL)¹⁸ in 50-day-old rats. This method allows long-term observations. Briefly, after a midline laparotomy, the duodenum was subject to two ligatures over half its circumference: beneath pylorus and 3 cm caudally. All procedures were done with aseptic techniques and under intramuscular ketamine (50 mg/kg)-xylazine (2 mg/kg) anesthesia. Before suturing, peritoneal cavity was washed with 10 ml saline solution containing 10 mg/L ceftriaxone. After that and immediately after performing the suture of the skin, rats were given 3 mg ceftriaxone/100 g body weight intramuscularly and 5 mg diclofenac/100 g body weight subcutaneously. Rats were housed in animal rooms with a 12-hour light/12-hour dark cycle, fasted for 24 hours and given water *ad libitum*. After 24 hours the rats were fed *ad libitum* with balanced food (Gepsa, Argentina). Additional doses of diclofenac were administered if the trained personnel considered it necessary.

Treatments and measurements

The rats were randomly assigned to one of the five following groups:

Group A (n=10): treated with MFP before the induction of pancreatitis. One mL of water containing 80 μ mol of MFP (Alfa Aesar, Ward Hill, MA, USA) was orally administered daily and for 30 days. At the end of this period of time rats were subject to ICDL surgery.

Control group A (n= 10): rats received 1 mL of distilled water orally, daily and for 30 days, before ICDL surgery.

Group B (n=10): treated with MFP after the induction of pancreatitis. One ml of water containing 80 μ mol of MFP was orally administered, daily and for 14 days, after ICDL surgery.

Control group B (n=10): rats received 1 mL of distilled water orally, daily and for 14 days, after ICDL surgery.

Sham operated rats (n=10): sham operation consisted only in laparotomy.

In the post ICDL surgery period, information about behaviour of rats (pain, intake of food and water, amount of feces, aggressiveness and alert state) was recorded. When pain and suffering were ex-

cessive, rats were euthanized by an intracardiac injection of 0.5 mL of saturated solution of KCl, under profound volatile anesthesia.¹⁹ Some animals died as a consequence of pancreatitis and the rest were euthanized after 14 days of the ICDL surgery. All rats were involved in the survival analysis. However, only the pancreas of rats that were euthanized after 14 days were microscopically analyzed.

After euthanasia, pancreatic and peri-pancreatic adipose tissues were removed, fixed in 10 % buffered formaldehyde (pH 7), paraffin embedded and cut at 6 μ m with conventional microtome. Samples were stained with hematoxylin and eosin for light microscopy analysis. Microscopic observations were performed by one experienced investigator masked to the specific experimental conditions (SMR).

A numerical scoring system was developed with modifications on the protocol previously described by Niederau et al.²⁰ In this system interstitial edema, fibrin, neutrophils granulocytic infiltration, fatty tissue and parenchymal necrosis, hemorrhage, vascular thrombosis and congestive blood vessels were assessed and considered as markers of "acute injury".^{21,22} They were graded independently with a qualitative scale. Number 0 was assigned if there were no such changes, 1 to minimum, 2 to moderate and 3 to severe.

Mononuclear inflammatory cells infiltration (lymphocytes, plasma cells and macrophages), nesidioblastosis and fibrosis were grouped into "chronic or reparative pancreatitis"^{21,23} and evaluated as previously described except for fibrosis which was graded in a 0-4 scale, corresponding to the number of fibrotic nodules found in the microscopic observation, since no diffuse fibrosis was detected in this model. The final score for each rat was calculated by adding the value of the score for each marker.

Statistical techniques

Survival curves, which percent survival is a function of time of death and calculation of the 95% CI for fractional survival at any particular time, were created using the method of Kaplan and Meier and comparison of two survival curves was performed using the log-rank test. Comparisons of three samples were performed with One Way ANOVA and post-hoc Bonferroni test. For better understanding of the data, they were expressed as mean \pm SEM, and analyses were done with a computer program (Graph Pad Prism 2.0. GraphPad Software, San Diego, CA USA, 1994). Differences were considered

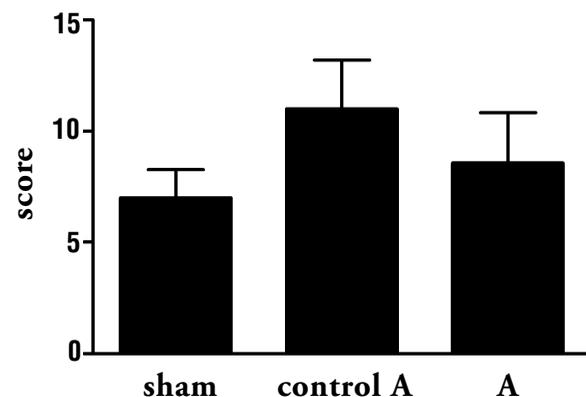
significant when the P value was lower than 0.05.

Results

This animal model of pancreatitis (ICDL) induces a mild form of disease with minimal histopathologic changes, preferably localized in peripancreatic tissues with segmental cell rich fibrosis. Necrosis of the pancreatic parenchyma is also confined to a limited area in the vicinity of inflammation with intact inner acini in the lobules. Langerhans islets also remain preserved.

As it was demonstrated in a previous work, the treatment with MFP after the ICDL induction of pancreatitis determines a higher survival of rats.¹⁵ Survival curve for group A was significantly different from control A: 70% of rats pre-treated with MFP survive after 14 days of the ICDL induced pancreatitis, while only 40% of rats that did not receive MFP survive after the induction of pancreatitis ($P < 0,05$). Although the number of rats that died as a consequence of complications of the pancreatitis was lower among rats treated with MFP, there were no differences in the score of injury (8.6 ± 2.3) when comparing them with control rats (11.0 ± 2.2) (Figure 1). The reader should note that comparison of the score only involved rats that survived after 14 days, and in control A group only 4 rats survived. In contrast, when rats received MFP after the induction of pancreatitis (group B), survival curves were not different from control B group. Forty percent of rats survived in both groups. However, the score in group B (1.7 ± 0.9) was significantly lower than that for control B (7.0 ± 4.0) ($P <$

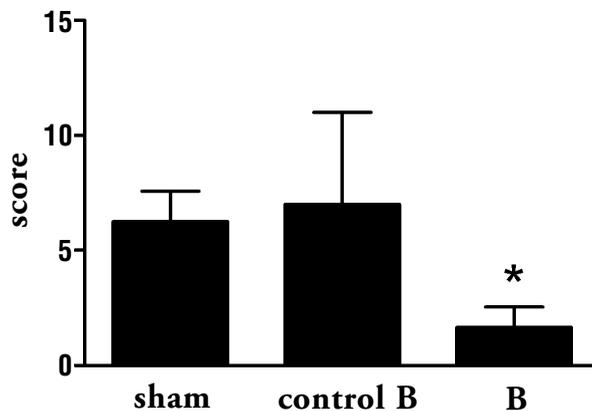
Figure 1. Score of injury for Sham operated, controls and rats treated with MFP (A) for 30 days before the induction of pancreatitis. Bars and lines indicate mean \pm SEM.



0.05) (Figure 2). One hundred percent of survival was observed in sham-operated rats.

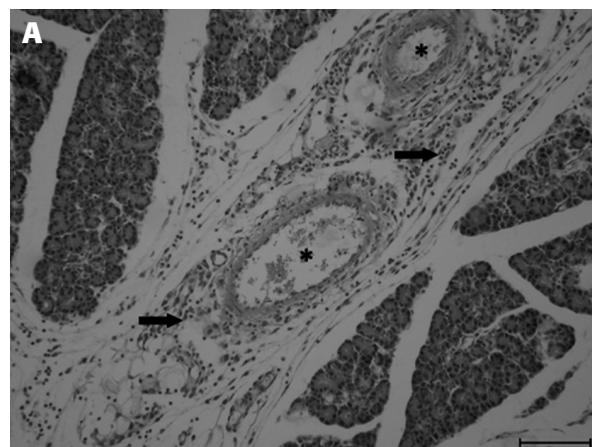
Figure 3 (A, B, C and D) displays typical changes observed in rats involved in the experiments 14 days after ICDL induced pancreatitis.

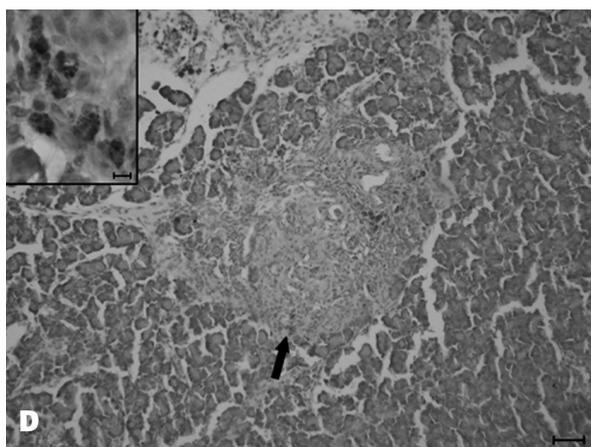
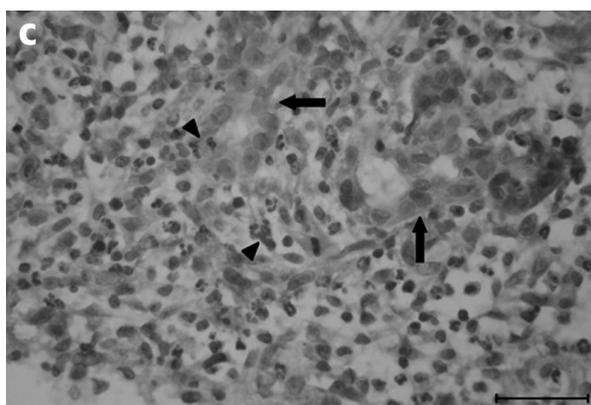
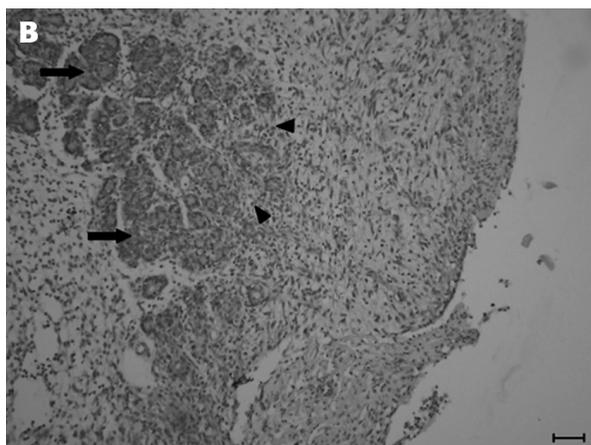
Figure 2. Score of injury for Sham operated, controls and rats treated with MFP (B) for 14 days after the induction of pancreatitis. Bars and lines indicate mean \pm SEM.



* Significant differences respect to the control B ($P < 0.05$).

Figure 3. A: Congestive blood vessels (asterisks) and edema, with mononuclear leukocytes, located in the interlobular area (arrows). Bar = 100 μ m. B: Deposits of fibrin intermingled with leukocytes seen preferentially near the outer part of the pancreas with necrosis of the adjacent parenchyma (arrowheads). The inner acinar cells remain intact (arrows). Bar = 100 μ m. C: The polymorphonuclear leukocyte infiltration predominates in the acute pancreatitis (arrowheads). Small acini with tubular changes, partially destroyed, can be recognized (arrows). Bar = 50 μ m. D: In chronic pancreatitis segmental nodular cell rich fibrosis is seen (arrows) Bar = 100 μ m. Inset: Many hemosiderophages scattered throughout the collagen bundles. Bar = 20 μ m.





Discussion

Numerous pharmacological treatments have been tried with the objective of inhibiting disseminated active enzymes or preventing from activation of zymogen produced by pancreatic cells. Antiproteinasas can potentially prevent the development of pancreatitis caused by the extracellular activation of

proteinases such as trypsin. As a consequence, the administration of protease inhibitors has commonly been employed for the therapy of acute pancreatitis.⁵

Monofluorophosphate (MFP) influences alpha-macroglobulin (AM) homeostasis both on human beings and rats.¹²⁻¹⁶ The administration to rats of 80 μmol by gastric tube modified AM levels and AM activity returned to normal after 4 hours. The mentioned dose of MFP administered to normal rats proved to have an effect on bone mass²⁴ without side effects. This effect was also verified in human beings after a therapeutic dose for the treatment of osteoporosis. The progression of acute pancreatitis was evaluated in rats that received a daily oral dose of MFP before and after the onset of the illness. It was demonstrated that MFP administered after the induction of pancreatitis did not modify the course of the illness. However, the administration of MFP for 30 days before surgery of ICDL prevented the death of the animals. After 30 days of treatment with MFP, AM-plasma levels increased when compared with animals that did not receive MFP.¹⁵ The administration of MFP has a protective action, probably through modification of AM homeostasis.

Even though an *ad-integrum* restitution of pancreatic tissue was not observed in all MFP-pre-treated animals, an undisputed increase in survival was found, concomitantly with slightly histopathological focal changes, and posterior healing with fibrosis. This is probably the cause of less morbidity, despite the presence of the ICDL inductive pancreatitis.

Rats that received MFP previous to the induction of the pancreatitis had histopathological markers of injury somewhat lower than controls, although there were not significant differences when compared with controls. On the contrary, among rats that received MFP after the induction of pancreatitis, the score of injury was significantly lower than in rats that did not receive MFP, but there were no differences in survival curves.

MFP would directly or indirectly stop signs of acute pancreatitis (vascular congestion, exit of erythrocytes from blood vessels, neutrophil infiltration) giving to the damaged parenchyma the possibility of healing with a consequent longer survival of animals. In addition, the lower levels of plasma amylase observed in MFP-pre-treated animals compared to controls suggest a protective effect of MFP on the evolution of pancreatitis.¹⁵ In this study, we suggest that AM could limit the severity of acute pancreatitis, probably not only as a proteinase inhi-

bitor but also as a potent carrier of biologically active cytokines, many of them with a pro-inflammatory effect. Experiments in this field are being carried out in order to clarify the possible mechanism of the MFP effect.

The results of this and a previous paper¹⁵ would indicate that MFP administered for the treatment of osteoporosis or the prevention of caries does not aggravate the progression of pancreatitis in rats. However, this conclusion remains to be proved in human beings. On the other hand, as MFP reduces morbi-mortality of animals, it can be used for extending the survival of animals and studying effects of pancreatitis in the chronic state.

In summary, we conclude that MFP attenuates the development of acute pancreatitis in rats, playing a beneficial role in the progression and evolution of the illness. In addition, MFP has no toxic effects at the doses employed in this research. Controlled trials must be performed in human beings in order to verify the protective action of MFP.

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