Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Pirfenidone prevents rat esophageal stricture formation

J. Orozco-Perez, PhD,[a] O. Aguirre-Jauregui, MD,[a] A.M. Salazar-Montes, PhD,[b] A.A. Sobrevilla-Navarro, PhD,[b] M.S. Lucano-Landeros, MD,[b] and J. Armendáriz-Borunda, MS, PhD[b,c,*]

[a] Department of Pediatric Surgery, OPD Hospital Civil de Guadalajara FAA, Guadalajara, Jalisco, México
[b] Department of Molecular Biology and Genomics, Institute for Molecular Biology and Gene Therapy, University of Guadalajara, Guadalajara, México
[c] Research Department, INNOVARE, Guadalajara, Jalisco, México

ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form
26 September 2014
Accepted 7 November 2014
Available online 15 November 2014

Keywords:
Pirfenidone
Caustic esophageal stenosis
RT-PCR
Fibrogenic esophageal stenosis
Sirius-red stain

ABSTRACT

Background: Accidental ingestion of caustic substances induces esophageal injuries and stenosis formation. The main aim for acute phase treatment is to prevent esophageal stenosis. Pirfenidone (PFD) is a pyridone with antifibrotic and anti-inflammatory effects. Esophagus stenosis takes place after a strong inflammation process where proinflammatory and profibrogenic cytokines play an important role. The present study investigates the efficacy of PFD on the prevention of stricture development after esophageal caustic injuries in a rat model.

Material and methods: Caustic esophageal burn was produced by application of 32% of NaOH to the distal esophagus of healthy rats. PFD in the form of 8% gel was administered at a dose of 200 mg/kg/d. Animals were divided in three experimental groups as follows: healthy rats, animals injured with NaOH without PFD treatment, and rats injured with NaOH and treated with PFD. Efficacy of the treatment was assessed by measuring image esophagoscopy and esophagography with contrast barium at the 21st d. Histology staining with Sirius-red was performed to evaluate collagen deposition and stenosis area. Gene expression of transforming growth factor β1, collagen-1, plasminogen activator inhibitor-1, connective tissue growth factor, and matrix metalloproteinase 2 were measured by quantitative reverse transcription polymerase chain reaction.

Results: There was significant difference in means of stenosis by esophagoscopy and esophagogram. Collagen deposition in the damaged area increased significantly when rats were burned with NaOH, and decreased notably in PFD treated group. Profibrogenic key molecules transforming growth factor β1, collagen 1, plasminogen activator inhibitor-1 and connective tissue growth factor expression were significantly lower respect to control group without PFD treatment where matrix metalloproteinase 2 expression was no different in all groups.

Conclusions: This study suggests that PFD reduces stenosis on caustic esophageal burn by decreasing profibrogenic genes expression and ameliorates fibrosis significantly in the chronic phase.

* *Corresponding author*. Department of Molecular Biology and Genomics, CUCS, University of Guadalajara, Sierra Mojada # 950, Guadalajara, Jalisco 44281, Mexico. Tel.: +(52) 33 10585317; fax: +(52) 33 10585318.
E-mail address: armdbo@gmail.com (J. Armendáriz-Borunda).
0022-4804/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2014.11.009

1. Introduction

Accidental ingestion of caustic substances induces esophageal injuries and stenosis formation. Children aged ≤5 y are especially vulnerable for accidental ingestion of toxic substances and represent a significant problem [1]. In Mexico, lack of socially oriented programs to prevent this type of household accidents, along with opportune and timely medical attention to minimize gastrointestinal damage, worsens the clinical outcome [2].

The main aim for acute phase treatment of caustic esophageal burn injury is to prevent stricture formation. Stricture onset or esophagus stenosis will take place after the inflammatory phase where proinflammatory and profibrogenic cytokines perform their deleterious function. Although several treatment protocols have been devised and used to accomplish anti-inflammatory and antifibrogenic actions, the benefit and standardization of these treatment methods are still controversial [3]. Nowadays, the effects of many therapeutic agents in preventing esophageal stricture formation continue to be investigated in experimental caustic esophageal burn injury models [4,5].

Pirfenidone (PFD) is an orally available pyridone derivative that has antifibrotic and anti-inflammatory effects. Since its discovery as an antifibrotic agent in a hamster model of bleomycin-induced pulmonary fibrosis [6], PFD has been tested in a variety of cellular and animal models of inflammation and fibrosis and has been shown to have anti-inflammatory, antioxidant, and antiproliferative properties [7,8]. Beneficial effects have been shown for PFD in the treatment of fibrotic diseases, including renal, liver, and pulmonary fibrosis, multiple sclerosis, and hypertrophic scars conditions. All these diseases share most of the cellular and molecular mechanisms involved in the pathology of abnormal deposition of collagen, which is determinant for a poor clinical outcome [9]. In these fibrosis-related diseases, the amount of collagen deposited in the tissue is controlled by the balance between synthesis (regulated at the transcriptional and translational level) and degradation of different types of collagen components of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs), which, at the same time, are regulated by tissue inhibitors of metalloproteinases [10]. In fibrosis, the positive balance for fueling collagen synthesis is influenced mostly by production of transforming growth factor β1 (TGF-β1), connective tissue growth factor (CTGF), interleukin-1, interleukin-6, platelet derived growth factor (PDGF), among other growth factors, which can be downregulated by PFD [11]. On the other hand, it has been clearly demonstrated by several groups, ours included, that PFD is able to upregulate MMPs-gene expression rendering an equilibrium of the tissue collagen amount.

Therefore, this study was aimed to evaluate the effects of PFD on wound healing, inflammation, and stricture formation and gene expression in the rat esophagus after caustic injury. Our results shown here suggest that PFD could be a suitable candidate for evaluation on esophageal healing in a given clinical scenario.

2. Material and methods

2.1. Materials

PFD was obtained from Cell Therapy and Technology (Mexico City, Mexico), in the form of an 8% crystalline gel. Ketamine was purchased from Virbac Inc (Carros, France). Primers and probes to perform real-time polymerase chain reaction were acquired from Applied Biosystems (Hammonton, NJ).

2.1.1. Animals

Male Wistar rats used in this study were obtained from Charles Rivers Inc (Boston, MA) weighing between 350 and 450 g were housed according to the principles and procedures outlined in the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Rats were divided into three groups of six each.

2.2. Surgical procedure

After 12 h of fasting, rats were anesthetized with 50 mg/kg of ketamine hydrochloride given intraperitoneally. Two of the groups were subjected to caustic esophageal injury by following indications to reproduce the experimental model of Gehanno and Guedon [12]. Briefly, after a median laparotomy, 1-cm segment of the distal esophagus was exposed. A catheter was placed through puncturing a stomach anterior wall into the distal esophagus. Catheters were secured, and an isolated segment of distal esophagus was obtained. The distal catheter was clamped, and the isolated segment was distended by administering 1 mL of 32% NaOH under intraluminal pressure for 90 s until slight translucency of the esophageal wall and branching of the vessels was noted. Afterward, the distal catheter was unclamped and the esophagus rinsed with 10 mL of distilled water. The laparotomy was closed subsequently. All animals were kept on a standard rodent pellet diet with tap water *ad libitum* after surgery. One experimental group ($n = 6$) received treatment of 200 mg/kg of PFD 8% gel by gavage once daily for 21 consecutive days after caustic injury. A parallel group ($n = 6$) was treated in the exact surgical manner but rats were not administered with 8% PFD gel. The healthy group ($n = 6$) was not subjected to surgical procedure, not exposed to caustic injury, and did not receive PFD treatment. Contrast esophagograms and esophageal endoscopy were performed 21 d after caustic ingestion, and all animals were sacrificed with a high-dose of phenobarbital, esophageal tissue biopsy was obtained, and histology and molecular studies were performed.

2.3. Esophageal esophagogram

Esophageal contrast radiological study with barium was carried out after 21 d of treatment in all groups of rats. Percentage of stenosis was determined by measuring transversal diameter in both proximal area (nondamaged tissue) and stenosed and/or strictured distal (NaOH-damaged area) found in the same esophagus of each rat. Quantitative determination made by a computerized ImageJ program from National

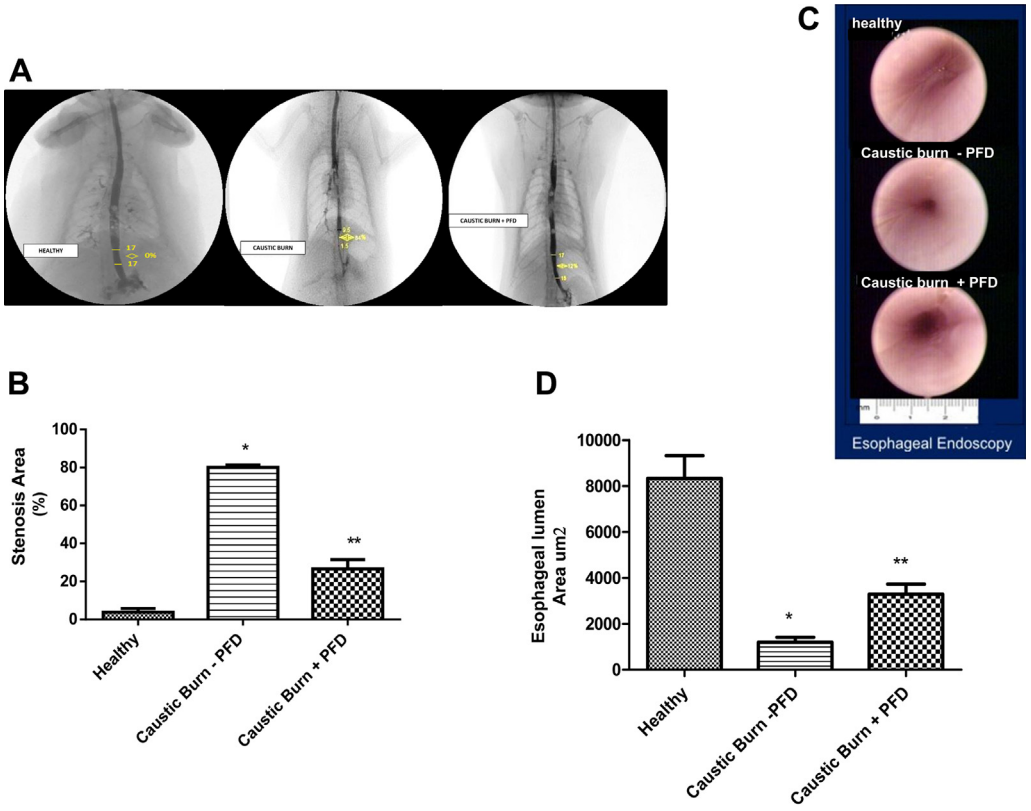

Fig. 1 – Esophagogram. PFD treatment decreases stenosis esophageal area. Esophageal contrast radiological study with barium was carried out after 21 d of treatment in all groups of rats. Percentage of stenosis was determined by measuring transversal diameter in both proximal area (nondamaged tissue) and stenosed and/or strictured distal (NaOH-damaged area) found in the same esophagus of each rat (A). Quantitative determination by a computerized ImageJ program was performed for the different groups. Graph demonstrates significantly less stenosis esophageal in rat group treated with caustic burn + PFD respect to caustic burn group (B) ($P < 0.05$). Esophageal endoscopy. This study was performed with an Endoscope Storz 2.7 mm × 110 mm 0, 3 wk after esophageal burn injured (C). Lumen area was quantitatively evaluated and compared in all groups with the ImageJ software from NIH and reported like lumen area. Graphs demonstrate a significant larger lumen area in rat group treated with caustic burn + PFD respect to caustic burn group without treatment (D) ($P < 0.05$). (Color version of figure is available online.)

Institute of Health (NIH) for different groups were compared and analyzed.

2.4. *Esophageal endoscopy*

Esophageal endoscopy study was performed with an Endoscope Storz 2.7 mm × 110 mm 0, 3 weeks after esophageal burn injured. Lumen area of the three different groups was quantitatively evaluated and compared among them using the image J software from the NIH [13].

2.5. *Esophageal histopathology*

Esophageal stenosis distal sections (0.5 cm) were obtained 3 wk after injury with 32% of NaOH in two groups. Likewise, healthy control rats underwent the same procedure. Esophageal specimens were fixed in formalin, embedded in paraffin, and 5-μ sections were stained with Sirius-red stain. Collagen deposition and area of stenosis were analyzed with ImageJ software from NIH, and results of the three groups were compared.

2.6. *Real-time polymerase chain reaction*

A portion of 0.5 cm of distal esophageal tissue from each sacrificed rat was frozen at −70°C for real-time reverse transcription/polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from tissue according to the Chomzynsky and Sacchi [14] modified technique. RT-PCR was performed according to our previous report [8] for the follows genes: collagen (COL)-1α, TGF-β1, CTGF, plasminogen activator inhibitor-1 (PAI-1), tissue inhibitor of metalloproteinase 1, and MMP2. For each sample, glyceraldehyde 3-phosphate dehydrogenase as a house-keeping gene was included. TaqMan probes were acquired from Applied Biosystems, and data were analyzed according to the 2D DCT methodology [15,16].

2.7. *Statistical analysis*

Results are expressed as mean ± standard deviation. Kruskal–Wallis test was used for the analysis. $P < 0.05$ was considered to indicate a significant difference between

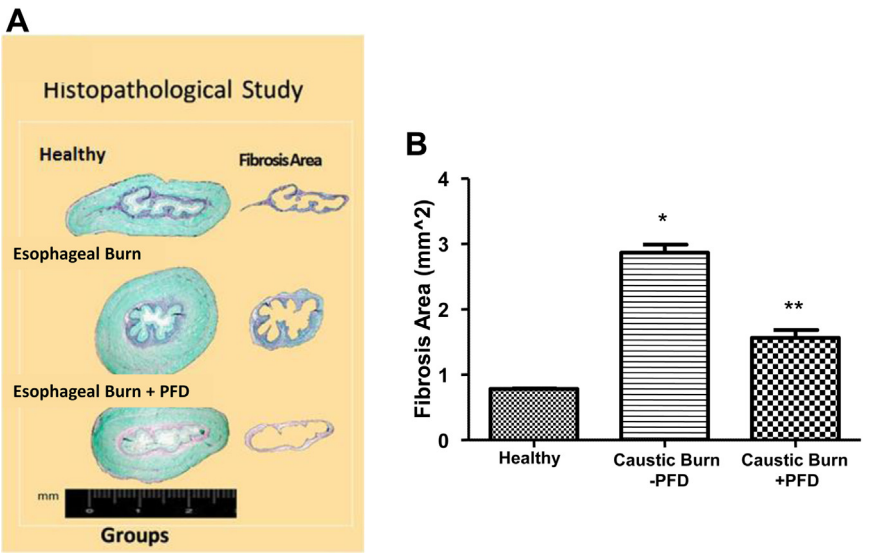

Fig. 2 – Fibrosis determination by histology. Percentage of fibrosis was determined in Sirius-red stained slides of all groups (A). Collagen deposition was 50% significantly higher in the group burned with NaOH compared with that in the group burned with NaOH plus PFD treatment (B) (*P < 0.01; **P = 0.027). (Color version of figure is available online.)

groups. Statistical analysis was performed using Prism software (GraphPad Prism, San Diego, CA).

3. Results

3.1. *PFD prevents esophagus stenosis in NaOH-burned rats*

To determine stenosis in the esophagus of rats burned with 32% of NaOH, we carried out esophageal esophagograms in all animals 21 d after treatment. The control group represented by healthy rats showed a negligible stenosis, as the esophagus lumen was clearly unhindered. Animals that underwent NaOH damage and not treated with PFD presented significant stricture formation represented by a stenosis column of 84% in the analyzed area with only a 16% of unaffected esophagus. Notably, animals burned with NaOH and concomitantly treated with PFD presented a stenosis column of only 26% in the injured area with a significant difference of 58% ($P = 0.005$). This experimental finding clearly suggests the preventive role of PFD in the inflammatory and fibrogenic phases induced by the caustic (Fig. 1A and B).

3.2. *Effect of PFD on stenosis lumen area*

Stenosis lumen area was determined by esophageal endoscopy and radiologic contrast barium study, which is shown in Figure 1C. Stenosis percentage in the control group was set as 4% considering this value as basal or normal. Stenosis increased to 80% in the burned group with NaOH ($P = 0.001$). The lumen area was significantly larger in the burned group with NaOH plus 3 wk of PFD treatment compared with that in the burned group without PFD ($P = 0.008$) finding a stenosis of only 25%. Figure 1D shows the mean of lumen area of each animal group expressed in squared millimeter. These findings suggest that PFD prevents stricture formation in esophagus when it is administrated concomitantly with the causal agent in esophageal burn.

3.3. *Effect of PFD on fibrosis*

To determinate collagen deposition on damaged esophagus, slides of all groups stained with Sirius-red were analyzed to determinate collagen deposition (Fig. 2A). Accumulation of collagen was observed in the submucosal layer. This deposition analyzed by densitometry was 50% significantly higher in the burned group with NaOH compared with that in the burned group with NaOH plus PFD treatment ($P = 0.027$; Fig. 2B) indicating that fibrosis is being inhibited by PFD. This histopathologic data represent one of the main contributions of this work because inhibition on collagen synthesis could be an excellent measure to prevent esophagus obstruction posterior to a burn.

3.4. *PFD decreases gene expression of profibrogenic genes*

Quantitative RT-PCR was used to detect gene expression of key molecules involved in fibrosis progression such as TGF-β1, COL-1, PAI-1, and CTGF. Concurrent PFD treatment for 3 wk significantly suppressed the profibrogenic genes TGF-β1, PAI-1, and CTGF and suppressed overexpression of COL-1 detected by quantitative RT-PCR. We found that these genes increased significantly their expression in the group burned with NaOH compared with that in the healthy rats group. The increase was fifteen times for TGF-β1 ($P = 0.001$), thirty-five times for COL-1, seven times for PAI-1A, and 1200 times for CTGF. The expression of all these genes decreased to normal values in the burned rats when they were treated with PFD. On

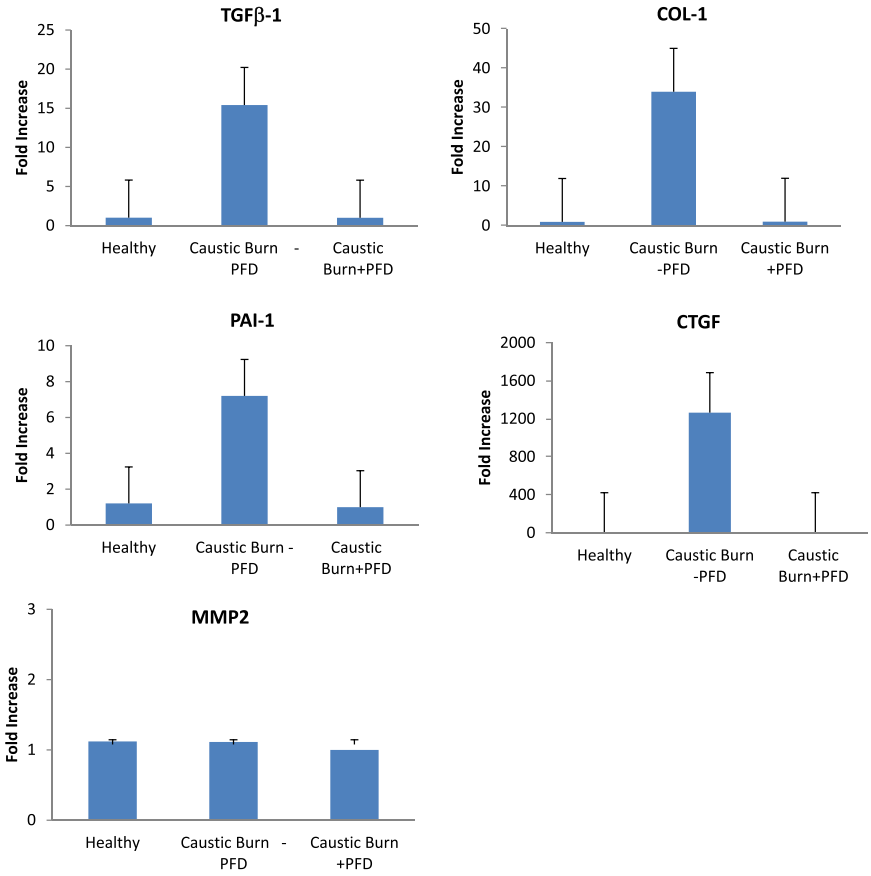

Fig. 3 – Genes expression analysis. Gene expression of TGF-β1, COL-1, PAI-1, CTGF, and MMP2 from esophageal tissue was performed 21 d after treatment with PFD. Messenger RNA levels were analyzed by RT-PCR in all groups showing significantly decreased profibrogenic gene expression in PFD group compared with that in caustic burn group without PFD treatment ($P < 0.05$). No differences were observed in MMP2 expression between the three groups. (Color version of figure is available online.)

the other hand, MMP2 gene expression did not show differences in all groups presenting similar expression to healthy rats (Fig. 3).

4. Conclusions

An important number of children are damaged daily by esophageal burn; for this reason, caustic burn of the esophagus still constitutes a serious health problem both in immediate and subsequent late complications. These complications require long periods of treatment due to esophageal strictures [17–19].

Many agents have been used experimentally to prevent stricture formation inhibiting development of fibrosis; only a few of them have gained clinical application. Steroids are the most clinically used with well-known associated side effects and controversial results. In general, most agents have been administered systemically in either clinical or experimental use [12,20,21] and used to inhibit new collagen formation directly or indirectly.

In a study of esophageal strictures in children, a single local application of mitomycin C was reported to be successful in treating four children with refractory benign esophageal strictures. All the children remained asymptomatic and none of them required additional dilation [18]. In another study in rats, topical mitomycin C treatment in caustic esophageal stricture presented significantly lower stenosis index and hydroxyproline values respect to the control group without treatment [22].

In this context, PFD is an antifibrotic molecule, ECM degradation modulator, which downregulates profibrogenic cytokines along with its ability to upregulate MMPs [23]. Also, PFD can control collagen alterations in pathologic burn scars.

The aim of this study was to evaluate PFD effectiveness in the esophageal caustic burns in rats. Esophageal burns represent one of the most common causes of esophageal strictures in children. Despite efforts, prevention of pediatric caustic ingestion injuries continues to impose a significant burden on psychological illness, family and health social care [17].

It is already known that the second week after injury is the setting for fibroblast proliferation and collagen deposition representing the pathophysiologic pathway of stricture formation. Therefore, the aim of any pharmacologic treatment is to prevent this process and the application of a drug could be

useful before the end of third week after injury [24]. In the present study, application of 1 mL of 32% of NaOH for 90 s into the distal esophagus in adult rats resulted in standard caustic esophageal burns with stricture formation, which was significantly prevented in the group concomitantly treated with PFD.

These results agree with previous studies realized by our group and others in different animal models of fibroproliferative disorders such as hepatic, kidney, and lung fibrosis, as well as human breast capsular contracture where deposition of ECM and profibrogenic genes expression were inhibited with PFD treatment [23,25–27]. This significant reduction could be effected through the inhibition of profibrogenic genes expression (TGF-β1, COL-1, and CTGF).

In this context, we consider that PFD is an effective and safe drug for treatment of fibroproliferative pathologic events such as abnormal scars caused by burns and liver fibrosis. This statement is supported by several publications where PFD has been shown to be safe presenting only minimal adverse events such as rash, local erythema, and no change in any laboratory tests such as aspartate aminotransferase and alanine aminotransferase for topical burned patients [9] and abdominal distention and nausea without changes in blood count and blood chemistry [28].

Specifically, our group has published a number of articles that together with other researchers' publications indicate the high safety profile of PFD. This drug has shown to be safe and efficient when administered topically to pediatric patients affected with abnormal hypertrophic scars as a consequence of extensive burns [9]. Data shown in there clearly demonstrated that no effect induced hepatic functional tests alterations during the entire study. Additional studies of our own when using oral prolonged-released-PFD tablets in the treatment of women with capsular contracture after mammary implants surgery demonstrated no alterations in hepatic functional tests [27]. More recently, we have published a study of efficacy of PFD tablets in a cohort of hepatitis C-infected patients extending and confirming our data on the safety of the drug and detecting only minor side effects [28].

Our group has conducted rigorous biopharmacokinetic studies with patients and topical PFD (data not published). The very same 8% PFD gel has been used in this study, and our belief is that its effect is due to a direct mucosal penetration at the time of swallowing. Nonetheless, some absorbed drug could eventually penetrate in the digestive system.

Although the results shown here are promising, additional research is required to investigate the effect of PFD in ameliorating-established esophageal strictures in children and to constitute a safe protocol for its use in caustic esophageal injuries.

Acknowledgment

Authors' contributions: O.-P.J. is the principal author. O.-P.J., S.-M.A.M., and A.-B.J. did the article writing. O.-P.J. and S.-N.A.A. did the sample processing, analysis of results, and statistics. O.-P.J. contributed to the laboratory studies. A.-J.O. and L.-L.M.S. did the protocol administration and clinical evaluation. S.-M.A.M. did the methodological analysis. A.-B.J. was the principal investigator who did the supervision.

Disclosure

The authors do not have anything to disclose regarding conflict of interest for this article.

REFERENCES

[1] Bronstein AC, Spyker DA, Cantilena LR, Rumack BH Jr, Dart RC. 2011 Annual report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 29th annual report. Clin Toxicol 2012;50:911.
[2] Sánchez-Ramírez CA, Larrosa-Haro A, Vásquez-Garibay EM, Macías-Rosales R. Socio-demographic factors associated with caustic substance ingestion in children and adolescents. Int J Pediatr Otorhinolaryngol 2012;76:253.
[3] Turkyilmaz Z, Sönmez K, Demirtola A, et al. Mitomycin C prevents strictures in caustic esophageal burns in rats. J Surg Res 2005;123:182.
[4] Herek O, Karabul M, Yenisey C, Erkuş M. Protective effects of ibuprofen against caustic esophageal burn injury in rats. Pediatr Surg Int 2010;26:721.
[5] Larios-Arceo F, Ortiz GG, Huerta M, et al. Protective effects of melatonin against caustic esophageal burn injury in rats. J Pineal Res 2008;45:219.
[6] Iyer SN, Wild JS, Schiedt MJ, Hyde DM, Margolin SB, Giri SN. Dietary intake of pirfenidone ameliorates bleomycin-induced lung fibrosis in hamsters. J Lab Clin Med 1995;125:779.
[7] Peng ZZ, Hu G, Shen H, et al. Fluorofenidone attenuates collagen I and transforming growth factor-beta1 expression through a nicotinamide adenine dinucleotide phosphate oxidase-dependent way in NRK-52E cells. Nephrology (Carlton) 2009;14:565.
[8] Salazar-Montes A, Ruiz-Corro L, López-Reyes A, Castrejón-Gómez E, Armendáriz-Borunda J. Potent antioxidant role of pirfenidone in experimental cirrhosis. Eur J Pharmacol 2008;595:69.
[9] Armendariz-Borunda J, Lyra-Gonzalez I, Medina-Preciado D, et al. A controlled clinical trial with pirfenidone in the treatment of pathological skin scarring caused by burns in pediatric patients. Ann Plast Surg 2012;68:22.
[10] Tian XL, Yao W, Guo ZJ, Gu L, Zhu YJ. Low dose pirfenidone suppresses transforming growth factor beta-1 and tissue inhibitor of metalloproteinase-1, and protects rats from lung fibrosis induced by bleomycina. Chin Med Sci J 2006;21:145.
[11] Choi K, Kihwang L, Seung-Wook R, Minju I, Koung Hoon -K, Chulhee C. Pirfenidone inhibits transforming growth factor-beta1-induced fibrogenesis by blocking nuclear translocation of Smads in human retinal pigment epithelial cell line ARPE-19. Mol Vis 2012;18:1010.
[12] Gehanno P, Guedon C. Inhibition of experimental esophageal lye strictures by penicillamine. Arch Otolaryngol 1981;107:145.
[13] Vardar E, Vardar R, Yükselen V, et al. Image-based assessment of esophageal stricture in experimental corrosive esophagitis in animals: an objective, adjunct diagnostic tool. Turk J Gastroenterol 2009;20:3.
[14] Chomzynsky P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156.
[15] User Bulletin #02 ABI PRISM 7700 sequence detection system December 11, 1997. Available from: http://dna-.intmed.

uiowa.edu/RealtimePCRdocs/Bulletin%202%20Applied%20Byosistem. Accessed January 2012.
[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402.
[17] Johnson CM, Brigger MT. The public health impact of pediatric caustic ingestion injuries. Arch Otolaryngol Head Neck Surg 2012;138:1111.
[18] Chang CF, Kuo SP, Lin HC, et al. Endoscopic balloon dilatation for esophageal strictures in children younger than 6 years: experience in a medical center. Pediatr Neonatol 2011;52:196.
[19] Zhang C, Zhou X, Yu L, Ding J, Shi R. Endoscopic therapy in the treatment of caustic esophageal stricture: a retrospective case series study. Dig Endosc 2013;25:490.
[20] Butler C, Madden JW, Davis WM, Peacock EE Jr. Morphologic aspects of experimental esophageal lye strictures. II. Effects of steroid hormones, bougienage and induced lathyrism on acute lye burns. Surgery 1977;81:431.
[21] Gunel E, Cxaglayan F, Cxaglayan O, Canbilen A, Tosun M. Effect of antioxidant therapy on collagen synthesis in corrosive esophageal burns. Pediatr Surg Int 2002;18:24.
[22] Uhlen S, Fayoux P, Vachin F, et al. Mitomycin C: an alternative conservative, treatment for refractory esophageal stricture in children? Endoscopy 2006;38:404.
[23] García L, Hernández I, Sandoval A, et al. Pirfenidone effectively reverses experimental liver fibrosis. J Hepatol 2002;37:797.
[24] Yeming W, Somme S, Chenren S, Huiming J, Ming Z, Liu DC. Balloon catheter dilatation in children with congenital and acquired esophageal anomalies. J Pediatr Surg 2002;37:398.
[25] Shimizu T, Kuroda T, Hata S, et al. Pirfenidone improves renal function and fibrosis in the post-obstructed kidney. Kidney Int 1998;54:99.
[26] Ragha G, Johnson WC, Lockhart D, et al. Treatment of idiopathic pulmonary fibrosis with a new antifibrotic agent, pirfenidone. Am J RespiR Crit Care Med 1999;159:1061.
[27] Veras-Castillo EV, Cardenas-Camarena L, Lyra-Gonzalez I, et al. Controlled clinical. Ann Plast Surg 2011;70:16.
[28] Flores-Contreras L, Sandoval-Rodriguez AS, Mena-Enriquez MG, et al. Treatment with pirfenidone for two years decreases fibrosis, cytokine levels and enhances CB2 gene expression in patients with chronic hepatitis C. BMC Gastroenterol 2014;14:131.