Assessment of the protective effects of oral tocotrienols in arginine chronic-like pancreatitis

Ana María González, Tania García, Esther Samper, Mariana Rickmann, Eva Cristina Vaquero, and Xavier Molero

1Grup de Recerca en Patologia Pancreàtica Exocrina, Hospital Universitari Vall d’Hebron, Institut de Recerca (VHIR), Universitat Autònoma de Barcelona, CIBER-EHD, Barcelona; 2Servei de Gastroenterologia, Institut de Malalties Digestives i Metabòliques, Hospital Clinic (CIBER-EHD), Barcelona, Spain

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González AM, García T, Samper E, Rickmann M, Vaquero EC, Molero X. Assessment of the protective effects of oral tocotrienols in arginine chronic-like pancreatitis. Am J Physiol Gastrointest Liver Physiol 301: G846–G855, 2011.—Tocotrienols exhibit anti-inflammatory properties over macrophages and promote cytotoxicity in activated pancreatic stellate cells, suggesting that they may limit chronic pancreatitis progression. We aimed to quantitate the effect of oral tocotrienols on a rat model of chronic pancreatic injury. Chronic-like pancreatitis was induced by repeated arginine pancreatitis. Palm oil tocotrienol-rich fraction (TRF) was given by gavage before and after pancreatitis inductions. Amylase and hydroxyproline were determined in pancreatic homogenates; collagen, fibronectin, α-smooth muscle actin (SMA), glial fibrillary acidic protein (GFAP), and phosphorylated Smad3 were assessed by Western blotting. Transforming growth factor (TGF)-β1 was measured in plasma. Morphological assessment included light microscopy, fibrosis area fraction, and collagen network fractal analysis. Arginine pancreatitis induced pancreatic atrophy and increased hydroxyproline that ameliorated after TRF. Arginine increased TGF-β1 (185 ± 40 vs. 15 ± 2 ng/ml; P < 0.01) that was blunted by TRF (53 ± 19; P < 0.01). TRF reduced protease and Smad3 activation, collagen, and fibronectin. α-SMA increased and GFAP diminished in arginine pancreatitis, consistent with long-term stellate cell activation, and TRF reversed these changes to basal. Arginine pancreatitis increased fibrosis area fraction (4.5 ± 0.3% vs. 0.2 ± 0.2%), collagen network complexity (fractal dimension 1.52 ± 0.03 vs. 1.42 ± 0.01; P < 0.001), and inhomogeneity (lacunarity 0.63 ± 0.03 vs. 0.40 ± 0.02; P < 0.001), which were all reduced by TRF (1.3 ± 0.4%, 1.43 ± 0.02%, and 0.51 ± 0.03%, respectively; P < 0.01). Best correlation coefficients were obtained when comparing fibrosis area fraction with lacunarity (r = 0.88) and both parameters with pancreatic weight (r = −0.91 and −0.79, respectively). TRF administered only before pancreatitis best, but not fully, recapitulated the beneficial effects of TRF. Tocotrienols improve quantitative measures of chronic pancreatic damage. They may be of benefit in human chronic pancreatitis.

vitamin E; fibrosis

CHRONIC PANCREATITIS OFTEN PRESENTS AS A PROGRESSIVELY DISABLING DISEASE CARRYING A POOR QUALITY OF LIFE. IT MAY CAUSE ABDOMINAL PAIN, MALNUTRITION, DIARRHEA, AND DIABETES MELLITUS (42, 51). OCCASIONALLY IT EXHIBITS AN INDOLENT COURSE, BUT CHRONIC PANCREATITIS IS PROGRESSIVE IN NATURE AND MAY FAVOR THE RISE OF ONE OF THE MOST DELETERIOUS FORMS OF CANCER (2, 36).

Except for particular forms of pancreatitis, no therapeutic strategy has been shown to halt the progression of the disease. There is an urgent need for therapies that could mitigate disease progression and, perhaps, cancer development. Current understanding of the etiopathology of chronic pancreatitis indicates that continuous or intermittent damaging insults of variable intensity account for initiation and progression of typical forms of chronic pancreatitis (51). Therefore, therapies intended to both protect and revert tissue damage may well have a role in future treatment strategies.

A distinctive feature of chronic pancreatitis is increased extracellular matrix (ECM) deposition that leads to fibrosis. Fibrosis alters pancreatic normal architecture, and it is usually accompanied by atrophy of acinar lobules (often replaced by adipose tissue) and by islet destruction, eventually leading to exocrine and endocrine insufficiency (42, 51). Other pathological features include variable inflammatory cell infiltration, abnormal and metaplastic ducts, and neural hypertrophy (6, 21, 42).

The pancreatic stellate cell (PSC) is thought to be the main source of ECM in the pancreas. In the normal tissue, PSCs reside in the interstitial space interacting with neighboring cells through cytoplasmic junctions. PSC functions in the quiescent state are unknown. Upon injury to pancreatic cells, PSCs are activated, proliferate, adopt a myofibroblastic phenotype expressing α-smooth muscle actin (α-SMA), and produce newly expressed proteins including ECM constituents, cytokines, chemokines, growth factors, and proteases and their inhibitors (2, 18, 49, 51). PSC activation, proliferation, migration, and adoption of a pro-fibrogenic phenotype are regulated by a number of stimuli, including transforming growth factor (TGF)-β1 (30). It is believed that PSCs play a major role in the pancreatic response to injury that leads to full scarless regeneration (49). However, sustained PSC activity may perpetuate ECM production and initiate the path toward development of chronic pancreatitis. Selective removal of activated PSCs may help to avoid further ECM deposition and even facilitate ECM resorption (4, 17). We have previously shown that tocotrienols induce activated PSC death in vitro but preserve quiescent PSCs and resident acinar cells (38). This ability, in conjunction with their known anti-inflammatory properties (52), signals tocotrienols as potential new therapeutic agents worth examining in vivo models of chronic pancreatitis.

Natural tocotrienols are an integral part of vitamin E. Natural vitamin E comprises eight distinct molecules: α-, β-, γ-, or δ-tocopherol; and α-, β-, γ-, or δ-tocotrienol. Most experimental work on vitamin E has been performed on α-tocopherol, paying little attention to possible divergent effects of tocotrienol-
ols (40). Both tocopherols and tocotrienols are potent antioxidants, are well absorbed from dietary nutrients, and concentrate in cellular membranes in a dose- and time-dependent manner (40). However, tocotrienols show a higher potency than tocopherols in inducing apoptosis in transformed cells in vitro, particularly in cancer cells (33), and inhibit colon, prostate, mammary, and lung carcinogenesis (19). Intriguingly, α-tocopherol may counteract tocotrienol-induced cytotoxicity in cancer cells (41).

Experimental models of chronic pancreatitis suitable for evaluating therapeutic approaches are not readily accessible. We decided to test the potential benefits of tocotrienols on chronic pancreatitis in the arginine-induced rat model because it induces persistent pancreatic lesions reminiscent of human chronic pancreatitis (11, 50), it does not require surgical manipulations, and arginine does not seem to affect any organs other than the pancreas.

Besides selecting an appropriate animal model, it is important to decide reliable and meaningful outcome measures capable of discriminating treatment-related effects. Histological examination can generate semiquantitative scores for fibrosis and pancreatic atrophy (14). Atrophy can also be quantitated by measuring pancreatic weight and amylase content. The amount of fibrosis can be inferred from semiquantitative evaluation of ECM proteins in pancreatic immunoblots. Biochemical determination of hydroxyproline in tissue samples is widely used as a surrogate for total collagen. However, hydroxyproline may also be the result of increased collagenase activity, as it occurs in tissue remodeling (35, 53). An indirect method to estimate chronic pancreatic damage is the determination of elements actively involved in fibrogenesis, such as cytokines and growth factors (TGF-β, PDFG, and monocyte chemoattractant protein) and cytofilaments overexpressed by activated PSCs (α-SMA). Quantitative ECM assessment by morphometry has gained credit to evaluate fibrosis burden because it may better reflect ECM deposition than other parameters (5). In addition, the increased complexity of collagen in fibrosis can be quantitated by fractal analysis (12, 25).

In this work we have given tocotrienol-rich fraction (TRF) from palm oil at a nontoxic dose to rats with arginine-induced pancreatic damage. Here we show that TRF supplementation mitigates arginine-induced chronic pancreatic damage. TRF reduces ECM deposition (assessed both by biochemical and histological approaches), the degree of pancreatic atrophy, TGF-β signal activity, and myofibroblast activation and improves collagen network arrangement.

**MATERIAL AND METHODS**

**Animals.** All experiments were performed in 350 g male Wistar rats supplied by Charles River Laboratories (Santa Perpetua de la Mogoda, Spain) housed in rack-mounted cages under conditions of constant temperature and 12-h:12-h light/dark cycle. Rats were fed with Teklad Global 2014 diet (Harlan-Teklad, Castellar del Vallés, Spain) containing 100 mg/kg of α-tocopherol. Procedures were approved by the Animal Experimentation Committee of the Institut de Recerca Hospital Vall d’Hebron.

**Induction of chronic pancreatitis.** Persistent pancreatic damage was induced by repeated arginine pancreatitis following previously described methods with minor modifications (11). Briefly, fasted rats received two intraperitoneal injections of 20% L-arginine hydrochloride in normal saline, pH 7.2 (250 mg/100 g body wt) 1 h apart on day 1, followed by additional injections on days 4, 7, and 10 (Fig. 1). In ancillary experiments, some rats (n = 5) on a normal unsupplemented diet underwent pancreatitis inductions. Samples from these rats were used in histology or in Western blotting experiments for comparison. Three groups of rats entered the study (n = 9). We decided not to interfere with immediate repair processes after pancreatitis. Therefore, rats in the tocotrienol group were daily supplemented with TRF (Tocomin 50; Carotech, Perak, Malaysia) to deliver 100 mg/kg body wt of tocotrienols. TRF was administered by gavage at dusk only during 1 wk before arginine administration and from day 5 after the last arginine injection. This dose stands below the level of toxicity (120 mg/kg) reported by Nakamura et al. (26). Arginine pancreatitis was induced in the control group as above, but in place of TRF rats received coconut oil supplemented with 0.25 mg of α-tocopherol (both from Sigma-Aldrich, St. Louis, MO) to comply with the recommended vitamin E daily allowances issued by the National Research Council (18 mg/kg of rat diet) (45). Rats in the basal group...
received daily sham saline injections and 0.5 ml of saline by gavage. For a more precise control on vitamin E intake, rats were fed a vitamin E-deficient diet (TD.88163, Harlan Laboratories) during the two treatment periods. To discriminate between prophylactic and therapeutic effects of TRF, independent groups of rats underwent the same experimental procedures except that TRF (and coconut oil or saline) was only given either before or after pancreatitis induction. Animals were euthanized 15 days after the last arginine injection. Blood was collected from cardiac puncture using heparinized needles and syringes. Plasma was obtained after low-speed centrifugation and was freed from platelets by centrifugation at 5,800 rpm at 4°C to avoid interference with the TGF-β1 assay. The pancreas was removed, weighed, and sliced. One piece was placed in fresh PBS-buffered 2% paraformaldehyde, pH 7.4, for 4 h and embedded in paraffin. The rest of the pancreas was snap frozen in liquid nitrogen and stored at −80°C until use.

TGF-β1, amylase, and hydroxyproline assays. Samples and standards were acidified for 1 h and then neutralized to pH 7.4 immediately before loading for TGF-β1 assay (Rat TGF-β1 ELISA; Bender MedSystems, Vienna, Austria).

Pancreatic homogenates (100 mg/ml) were prepared by serial use of a motor-driven shearer (Tissue Tearer 985–370; Biospec Products, Drewel, WI) and glass-Teflon potter in cold buffer (50 mM sodium phosphate pH 7.3, 120 mM NaCl, 0.01% soyaehematin proteinin). Homogenates were treated with 0.2% Triton X-100 at 37°C for 20 min before measurement of amylase activity in supernatants. Results are provided as U/mg dry weight. To calculate dry weight, 200 μl of the initial homogenate were desiccated to dryness at 120°C for 16 h and then weighed. Amylase activity was measured by an enzymatic colorimetric assay (Sentinel, Milan, Italy).

Hydroxyproline was determined from crude homogenates as described (14) as a measure of ECM deposition. Protein concentration was assessed with the bichinchonic acid protein assay reagent (Pierce Biotechnology, Rockford, IL).

Oxidative stress and matrix metalloproteinase activity. To assess the levels of oxidative stress in tissue samples, lipid peroxidation was determined by measuring the formation of thiobarbituric-reactive substances (TBARS) as described (14). Because increased matrix metalloproteinase (MMP) activity (particularly MMP-9, but also MMP-2) seems to promote ECM remodeling and fibrosis in injured tissues (22, 47), MMP-2/9 activity was measured in pancreatic homogenates using the EnzCheck Gelatinase/Collagenase Assay Kit from Invitrogen Life Technologies (Carlsbad, CA) according to manufacturer’s instructions.

Morphology and morphometrical analysis of ECM. Several sections from each sample were stained with hematoxylin-eosin using standard protocols. To assess ECM, five nonconsecutive sections were stained with picro-Mallory trichrom fight (BioOptica, Milano, Italy). RGB images were captured from 10 consecutive nonoverlapping fields (×200) on an Olympus BX-61 microscope fitted with UPLAN FLN objectives and an Olympus DP70 camera (Olympus, Tokyo, Japan). Images were saved in tagged image file format (TIFF) at 2,040 × 1,536 pixels. The blue component of each image was extracted and the area fraction determined using Image J software (NIH, Bethesda) (37) with a threshold color plug-in as described (31). Hue, saturation, and brightness adjustments were maintained constant throughout the entire series of images. For each animal the area fraction occupied by ECM in each picture was averaged to obtain the fibrosis area fraction. All calculations were performed on an iMac 7.1 computer with a 20-inch monitor display.

Antibodies. The following antibodies and dilutions were used: rabbit anti-collagen type I (1:10,000) from Rockland Immunochemicals for Research (Gilbertsville, PA) incubated at room temperature for 4 h. Mouse monoclonal anti-fibronectin ab6328 (1:400), rabbit monoclonal anti-Sma3 ab40854 (1:5,000), and rabbit monoclonal anti-phosphorylated Smad3 (pSmad3) ab52903 (1:2,000) were purchased from Abcam (Cambridge, MA) and incubated overnight at 4°C. Monoclonal anti-α-SMA A2547 (1:2,000) and polyclonal rabbit anti-glia fibrillary acidic protein (GFAP) Z0334 (1:400) were from Sigma-Aldrich. Biotin-conjugated AffiniPure secondary antibodies were from Chemicon International (Temecula, CA). Goat anti-type III collagen (1330–01) was from Southern Biotech (Birmingham, AL). Alexa Fluor 647 chicken anti-goat IgG (A21469) was from Invitrogen.

Western blotting. Total pancreatic homogenates were prepared in ice-cold radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mg/0.4 ml) with protease inhibitors (Complete Mini; Roche Applied Science, Sant Cugat, Spain). Homogenates were rotated on a Labrotor rotator (Labnet International, Woodbridge, NJ) at 4°C for 45 min and centrifuged at 20,000 g for 10 min. Homogenates were then mixed with NuPAGE LDS sample buffer with NuPAGE reducing agent (Invitrogen), vortexed, heated at 70°C for 10 min, and centrifuged at 13,000 g for 5 min at 4°C. Proteins (50–100 μg) were fractionated in 4%–12% Bis-Tris NuPAGE gels (Invitrogen) and transferred to nitrocellulose filters by electroblotting. After being blocked with 5% skim milk in 4 mM Tris base, 100 mM NaCl, pH 7.5, membranes were washed in the same buffer containing 0.05% Tween 20, incubated with primary antibodies, and for 1 h with horseradish peroxidase-labeled secondary antibody. Reactions were revealed with the Supersignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology), and bands were visualized using a Fujifilm Image Reader LAS-3000 phosphoimager (Fujifilm Photo Film, Tokyo, Japan).

Collagen III immunofluorescence. Paraffin sections were deparaffinized and heated in an autoclave at 120°C for 1 min in 0.01 M citrate buffer, pH 6.0, and blocked with 1% BSA in PBS containing 0.2% Triton for 1 h. Anti-collagen III antibody (1:20) was incubated at room temperature for 1 h. After 3 PBS washes, Alexa Fluor 647 anti-goat IgG (1:500) was incubated for 30 min. Sections were washed and mounted in Vectashield mounting medium with Dapi (Vector Laboratories, Burlington, CA) before being examined in a confocal microscope (FV1000, Olympus) fitted with UPLAN objectives.

Assessment of collagen network complexity and inhomogeneity by fractal analysis. For quantitative assessment of collagen network architecture, fractal analysis was performed on confocal images immunostained with collagen III. Five confocal TIFF images for each section were acquired from consecutive nonoverlapping fields at ×400 magnification. Images were processed using Image J software and the FracLac plug-in as described to characterize microvascular morphology (20, 23). Briefly, images were converted into binary and skeletonized before calculating fractal dimension and lacunarity by the box-counting method (8). Mean values were obtained for each animal.

Isolation, culture, treatment, and immunocytofluorescence of PSCs. PSCs were isolated and cultured as described (38). Briefly, the pancreas was excised, and cells were dispersed by a combination of enzymatic and mechanical methods. PSCs were separated using Optiprep density gradient centrifugation and grown in DMEM/F12 medium on glass coverslips at 37°C. Starting from day 0 after isolation, PSCs were treated either with vehicle (0.1% alcohol) or TRF at the indicated concentrations for 5 days. Activation of PSCs was confirmed by immunocytofluorescence evidence of α-SMA and GFAP expression. Paraformaldehyde-fixed cells were permeabilized with 0.25% Triton X-100 for 10 min and incubated either with anti-α-SMA (1:500, Ref. F3777 from Sigma-Aldrich) or anti-GFAP (1:250, Ref. Z0334 from Dako, Glostrup, Denmark) at 4°C overnight. Bound antibodies were revealed by incubating cells with secondary antibodies from Invitrogen (Alexa Fluor 594 rabbit anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG, both at 1:1,000) at room temperature for 1 h. Coverslips were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen), and images were acquired on the Olympus BX-61 fluorescence microscope mentioned above.
Statistical analysis. Data are expressed as means ± SE. Differences of means were analyzed by ANOVA followed by the Newman-Keuls Multiple-Comparison Test. Statistical significance was defined as a \( P \) value <0.05. Correlations between quantitative variables were examined by the Pearson’s coefficient, except for hydroxyproline when Spearman’s coefficient was used because data failed to pass the normality test. The area under the curve was used to compare weight gain rates. All data analyses were performed using GraphPad Prism statistical (GraphPad Software, San Diego, CA).

RESULTS

Repeated arginine pancreatitis induces morphological features of chronic pancreatitis. Light microscopy of pancreatic samples disclosed typical features of chronic pancreatitis (Fig. 1B). The severity of pathological findings revealed some variability among animals. Seriously affected animals exhibited acinar cell atrophy with altered ductal morphology, tubular complexes, inflammatory cell infiltration, increased ECM, acinar replacement by adipocytes, and neural hypertrophy, all of which are typical features of human chronic pancreatitis (6, 21, 42, 51). Acinar cell remnants embedded in severely affected areas were found close to islets (Figs. 1B and 2, A–D).

Rats lost weight during pancreatitis inductions (Fig. 1C) but improved gain rates thereafter. No statistical differences were found between TRF-treated and untreated animals at individual time points or after computing the area under the curve. One animal in each treatment group died (11.1%), with deaths occurring during pancreatitis inductions or at the early recovery period. Neither TRF nor coconut oil had any impact in the blood levels of cholesterol, triglycerides, glucose, or amylase (data not shown).

TRF counteracts pancreatic atrophy and reduces fibrogenic activity in chronic pancreatitis. TRF largely improved overall pancreatic morphology, including features of atrophy and fibrosis (Fig. 2, E and F) compared with controls (Fig. 2, C and D). Accordingly, parameters that quantitate pancreatic acinar atrophy, such as pancreatic weight and amylase, also improved upon TRF treatment (Fig. 3).

Fibrosis was assessed using three approaches: hydroxyproline was determined in pancreatic homogenates, connective tissue was quantitated by morphometry (fibrosis area fraction), and collagen and fibronectin were estimated by Western blotting. As expected from morphological inspection, TRF reduced
hydroxyproline compared with controls (Fig. 3). This finding was supported by quantitative morphometry, which showed a marked reduction in fibrosis area fraction in TRF-treated animals (Fig. 3). In addition, TRF also reduced the increased activity of MMP-2/-9 that are frequently found enhanced in tissues with substantial fibrogenic responses (22, 47) (Fig. 3). Moreover, TRF reduced collagen and fibronectin in pancreatic homogenates (Fig. 4C) compared with both coconut oil-treated rats (controls) and rats on a normal diet undergoing arginine pancreatitis.

Fifteen days after the last pancreatitis induction, oxidative stress was still clearly patent in arginine chronic pancreatitis, as shown by increased lipoperoxides (TBARS) in pancreatic samples, probably reflecting persistent fibrogenic activity and tissue remodeling (Fig. 4A). TRF largely reduced oxidative stress.

The expression of α-SMA is a reliable marker for activated PSCs (25). GFAP is also expressed in stellate cells, particularly in the acute response to injury. GFAP expression declines in the chronic response, often as α-SMA further increases (1, 28). Interestingly, chronic arginine pancreatitis was associated with an increased expression in α-SMA and with a concomitant reduction in GFAP (Fig. 4C), as it happens in chronic fibrogenic diseases. TRF returned both α-SMA and GFAP expression to basal levels.

TGF-β1 is a key cytokine-governing fibrogenesis that is persistently elevated in chronic pancreatitis and in impaired pancreatic regeneration (14). Indeed, TGF-β1 was found to be increased in arginine chronic pancreatitis (Fig. 4B). Furthermore, TGF-β1 pathways appeared to be activated in chronic pancreatitis because pSmad3 expression raised in pancreatic homogenates (Fig. 4B), findings that coincided with increased α-SMA expression and fibrosis. Both TGF-β1 and pSmad3 were largely reduced by TRF.

Disordered collagen network architecture is restored by TRF. Fibrosis denotes increased ECM, but it also stands for erosion of its organized architecture. Fibrilar proteins such as collagen conform webs surrounding groups of parenchymal cells. To quantitate the degree of impairment of collagen network architecture in chronic pancreatitis, we performed fractal analysis on images acquired from collagen III-immunostained sections. Fractal analysis can return measures of fractal dimension and lacunarity. Fractal dimension measures changing patterns with amplification and, therefore, assesses image complexity. For collagen III-outlined images, fractal dimension gives an estimate for fiber-layering structure with values running from 1 to 2, with the higher value representing greater complexity. Lacunarity measures image inhomogeneity from 0 to 1, with higher values denoting less homogeneity. It quantitates how different one part of an image is from another part.

Collagen III immunostaining illustrated the changing pattern of collagen network structure in chronic pancreatitis (Fig. 5, A and B).

![Fig. 3. TRF mitigates pancreatic atrophy and fibrogenic activity. Arginine-induced chronic pancreatitis resulted in pancreatic atrophy as assessed by reduction in both pancreatic weight and amylase activity. TRF treatment ameliorated every parameter of pancreatic atrophy measured. TRF also reduced the increased fibrogenic activity induced by repeated arginine pancreatitis as assessed by measuring pancreatic hydroxyproline, matrix metalloproteinase (MMP)-2/-9 activity, and fibrosis area fraction (n = 8; *P < 0.05 vs. all other groups).](image-url)

![Fig. 4. TRF attenuates the rise in pancreatic oxidative stress (thiobarbituric-reactive substances, TBARS) and in serum transforming growth factor (TGF)-β1 induced by arginine (A and B; n = 8; *P < 0.05 vs. all other groups). C: Western blotting of collagen I, fibronectin, α-smooth muscle actin (α-SMA), giall fibrillary acidic protein (GFAP), Smad3, and phosphorylated Smad3 (pSmad3) in pancreatic specimens from arginine chronic pancreatitis (arg), normal pancreas (basal), arginine chronic pancreatitis treated with coconut oil (arg+co), and arginine chronic pancreatitis treated with TRF (arg+TRF).](image-url)
TRF treatment tended to restore the distorted collagen mesh and to recover basal architecture (Fig. 5C). Fractal dimension and lacunarity data supported these findings by quantitating the degree of collagen network disorganization. Both parameters were impaired in chronic pancreatitis and improved after TRF (Fig. 5, D and E).

**Correlations of quantitative variables.** Individual data were used to calculate correlation coefficients of quantitative parameters estimating pancreatic chronic damage. Pancreatic weight, amylase content, hydroxyproline, TGF-β1, fibrosis area fraction, fractal dimension, and lacunarity values were compared with each other. The strongest positive correlation was obtained between fibrosis area fraction and lacunarity (Fig. 6A), and the strongest negative correlation was between fibrosis area fraction and pancreatic weight (Fig. 6B). In addition, fibrosis area fraction also showed positive correlations with TGF-β1 \((r = 0.66; P = 0.002)\) and fractal dimension \((r = 0.59; P = 0.01)\) and a strong negative correlation with pancreatic weight \((r = -0.79; P = 0.002)\).

**Evaluation of TRF ability to block PSCs activation.** We have shown that TRF induces active PSC cell death (38), but the effects of TRF on PCS activation from quiescence have not been addressed so far. This question was raised because the beneficial effects of TRF on arginine pancreatitis could be explained, at least in part, by a hypothetical ability of TRF in blocking PSCs activation. To this end, we followed an in vitro elementary approach: freshly isolated PSCs were treated with TRF, and expression of both α-SMA and GFAP was examined 5 days later. As shown in Fig. 7, TRF did not seem to block PSC activation because PSCs treated with both 10 and 20 μM TRF clearly expressed α-SMA and GFAP to a similar extent as control cells.

**Relative efficacy of prophylactic (before only) or therapeutic (after only) TRF administration.** TRF was given to independent groups of rats either only before or only after pancreatitis induction. Prophylactic, but not therapeutic, TRF partly recapitulated TRF effects obtained with the combined treatment,
but to a lesser extent (Fig. 8). Although both groups of treatment were not fully comparable because the period of rat manipulation differed with respect to onset of pancreatitis inductions, the degree of damage seemed to be less severe when rats were manipulated only after pancreatitis inductions. We can only speculate that the daily stress caused by forced oral feeding before pancreatitis could have had an adverse impact in the severity of pancreatic lesions. In this regard, it has been shown that experimental acute pancreatitis gets worse in the setting of previous chronic stress (3).

**DISCUSSION**

In this work we provide evidence to conclude that oral tocotrienols promote beneficial effects in the management of chronic pancreatitis because chronic damage induced by repeated arginine pancreatitis was greatly improved by oral supplementation with TRF. Tocotrienols reduced the degree of pancreatic atrophy, ECM accumulation, fibrogenic MMP activity, TGF-β signaling, and α-SMA expression and returned GFAP expression to basal reference levels. Additionally, we assessed the effects of tocotrienols on collagen network architecture using image fractal analysis of immunolabeled collagen fibers. This approach has allowed us to demonstrate tocotrienol protection of the collagen network architecture from the distorting effects induced by arginine pancreatitis.

TRF is highly enriched in tocotrienols. Tocomin 50% typically contains 395 mg/g of mixed tocotrienols. It also contains 100–140 mg/g d-α-tocopherol and small amounts of squalene, mixed carotenoids, and coenzyme Q10. We previously demonstrated the cytotoxic effects of TRF on activated PSCs are mainly attributable to β-, γ- and δ-tocotrienol (38). We found that tocotrienols were able to elicit autophagy and apoptosis in these cells but did not cause cytotoxicity in quiescent PSCs or in acinar cells. Interestingly, activated PSCs proliferate in an oxidative stress environment (as cancer cells do), but the potent antioxidant α-tocopherol did not induce significant PSC death. Indeed, tocotrienols exert cytotoxicity over a number of cancer cells in vitro, including pancreatic cancer cells (16), but not in normal parenchymal cells. Despite these cytotoxic actions, humans have been consuming tocotrienols from rice, barley, and palm oil for ages without signs of adverse effects. On the contrary, tocotrienols lower cholesterol blood levels, prevent
cardiovascular disease and brain cell damage, inhibit carcinogenesis, and exhibit anti-inflammatory properties (27, 32, 34, 40, 52). The beneficial actions of tocotrienols do not seem to be simply attributable to their antioxidant capacity because α-tocopherol (which displays equivalent antioxidant potency) may counteract tocotrienol-induced effects (40, 41).

To test the potential benefits of tocotrienols on chronic pancreatitis, we discarded the model we had previously described because we found that cyclosporin A inhibits tocotrienol-induced cell death in vitro (38, 48). Some other experimental models induce the development of chronic-like pancreatitis after surgical manipulations (35), after administration of toxic compounds that also affect other organs (43), or under specific genetic backgrounds (29). We chose to test tocotrienols on the rat model of pancreatic damage induced by repeated arginine pancreatitis on the basis of previously published work and on our own observations (Figs. 1B and 2, A–D). Administration of high doses of L-arginine is known to cause acute pancreatitis in rats and mice (9, 46). The pancreas seems to be the only organ developing inflammatory damage, but the mechanism by which arginine induces acinar cell toxicity is not clear: cytoskeletal changes, oxidative stress, inflammatory mediators, NO, and arginine have been implicated in its etiopathogenesis (44). The fact that arginine pancreatitis induces long-term pancreatic lesions has been long noticed, but it has not been studied in detail. Dawra et al. (9) demonstrated increased collagen after acute arginine pancreatitis, and some authors have previously claimed arginine pancreatitis could be used as a model for chronic pancreatitis (46). Here we show excessive ECM accumulation and collagen network distortion in addition to acinar atrophy (all three typical features of human chronic pancreatitis) after repeated arginine pancreatitis in rats. Some other findings support the chronic nature of the model, such as persistent MMP-2/-9 activity, enhanced TGF-β signaling (14), neural hypertrophy (6), and reduction of GFAP expression coinciding with increased α-SMA (1, 28). All these findings were largely prevented by TRF.

The therapeutic strategy we designed merits some remarks. If we consider chronic pancreatitis as the result of repeated intermittent injuries, administration of TRF before pancreatitis could provide some protection by allowing TRF to be incorporated into cell membranes. Additionally, because activated PSCs are needed to fulfill physiological repair mechanisms at the injured pancreas, either large doses of tocotrienols or tocotrienol administration during the acute phase of pancreatitis may interfere with appropriated repair by eliminating active PSCs. We have no data to support these assumptions, and a new series of experiments is needed to solve this particular issue.

To assess the relative efficacy of the two treatment periods (before and after pancreatitis induction) on the overall beneficial effects of TRF administration, we conducted a new series of experiments. As shown in Fig. 8, prophylactic TRF administration appeared to best recapitulate the beneficial effects of the combined treatment. However, the degree of improvement over nontreated animals was not as good as with the combined treatment approach, suggesting that both treatment periods are required for full TRF efficacy. This treatment schedule fits well with the most accepted theory on the etiopathology of human disease, which considers chronic pancreatitis as the result of repeated events of tissue injury and poor healing. Continuous TRF treatment could help to reduce the severity of tissue damage and to increase pancreatic recovery.

Combined TRF treatment to L-arginine pancreatitis resulted in better preservation in acinar cell mass, as shown in Figs. 2 and 3. Because peri-insular acini are most protected from L-arginine damage (Figs. 1–2 and Ref. 15), and tocotrienols were previously found to elevate insulin sensitivity (7, 13), a possible mechanism for TRF acinar cell protection could be a TRF-induced increase in insulin sensitivity in these cells that could result in improved acinar cell survival.

TRF-induced prevention of activation of quiescent PSCs could also explain TRF protection. The results of our experiments on the activation of PSCs in vitro indicate that TRF does not seem to block PSC activation. A new series of experiments will be needed to further explore this issue.

Besides increased ECM, fibrosis also stands for the loss of the well-structured collagen arrangement. Therefore, we also quantitated the beneficial effects of tocotrienols on pancreatic damage at the level of collagen network organization. In the normal pancreas, collagen is layered as thin interconnected bundles that surround parenchymal structures, composing geometrical shapes of repeating patterns (25). Patterns of nonlinear scaling rules define fracts, which can be measured in terms of complexity and homogeneity. Fractal geometry has been used to quantify biological processes, such as tumor boundaries, vascular architecture, angiogenesis (8, 10, 23, 24), bone tissue organization during regeneration (39), and liver fibrosis in rats and humans (5, 12, 25). Fractal analysis of collagen images provided quantitative measurements for the morphological observations. As a result, we can now conclude that arginine pancreatitis disturbs collagen architecture by increasing its complexity and inhomogeneity, and tocotrienol treatment tends to restore collagen network organization.

From all the parameters measuring pancreatic damage and the effects of tocotrienol treatment, fibrosis area fraction and fractal lacunarity gave the best positive correlation coefficient (Fig. 6A). Both parameters also positively correlated with TGF-β1 and fractal dimension, and they both negatively correlated with pancreatic weight and pancreatic amylase content. The fact that morphometrical data, which give estimates of the amount of collagen deposition, and fractal analysis data, which give numerical values to collagen network disposition, correlated so well adds relevance to the selection of parameters we measured because these two quantitative variables measure different, yet complementary, dimensions of fibrosis. Moreover, both variables also showed good correlation with both pancreatic weight and amylase content, two accurate measures of pancreatic atrophy, which is also a hallmark of chronic pancreatitis.

On the contrary, hydroxyproline values correlated poorly with all the variables examined, except for TGF-β1 and amylase content. Of notice, hydroxyproline levels did not correlate with fibrosis area fraction, pancreatic weight, lacunarity, or fractal dimension, parameters that give good estimates of chronic pancreatic damage. This apparent contradiction can be explained by the fact that hydroxyproline measurements are high when fibrosis is present but also when collagenase activity is increased, as in tissue remodeling evolving in normal wound healing (35, 53). Therefore, hydroxyproline may not always accurately reflect the fibrotic burden of the tissue.
In summary, we have shown that a diet enriched in tocotrienols has the potential to ameliorate biochemical and morphological quantitative parameters of experimental chronic pancreatitis, in conjunction with reduced TGF-β signaling and PSC activation. On the basis of the above statements, we can suggest that it would be worth conducting clinical trials on tocotrienols for the long-term treatment of chronic pancreatitis in humans.

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DISCLOSURES
No conflict of interest, financial or otherwise, is declared by the authors.

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