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Dimethyl Fumarate Ameliorates Acute Pancreatitis in Rodent

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Objectives: Pancreatitis is a complex inflammatory disorder, ranging from a mild attack, to severe and potentially fatal condition. Dimethyl fumarate (DMF), a potent antioxidant and anti-inflammatory, has been used medicinally for decades. The purpose of this study was to test the hypothesis that treatment with DMF may ameliorate acute pancreatitis (AP) in a rodent model.

Methods: Rats were treated with DMF (25 mg/kg) 24 hours prior to AP induction with L-arginine (3 g/kg). At 72 hours, the pancreas was processed for histology. Serum amylase, lactate dehydrogenase, pancreatic trypsin, and lipid peroxidation product (malondialdehyde) were evaluated. Key cytokines and chemokines in the supernatant of lipopolysaccharide-stimulated splenocytes were also determined.

Results: Pancreata from DMF-treated rats showed reductions in the severity of inflammatory cell infiltration, acinar damage, perilobar edema, and cell necrosis. This was associated with significantly lower amylase and malondialdehyde but not lactate dehydrogenase or trypsin levels. The apoptotic pancreatic cells (cleaved caspase 3 positive) were significantly lower in the DMF-treated rats. Lipopolysaccharide-stimulated splenocytes treated with DMF produced a significantly lower amount of key inflammatory mediators.

Conclusion: Administration of DMF attenuates AP in rats.

Key Words: dimethyl fumarate, reactive oxygen species, pancreatitis, oxidative stress, cytokine, inflammation

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Acute pancreatitis (AP) is characterized by the sudden onset of local pancreatic inflammation from intrapancreatic activation of digestive enzymes. The disease carries a spectrum of severity from mild self-limiting to a highly morbid and even fatal illness. It is believed that the initial event occurs from the disruption of acinar cells, leading to the leakage of enzymes, resulting in auto-digestion of the pancreas. The activated enzymes cause a local inflammatory response, which if severe can result in further recruitment of inflammatory mediators and the development of a systemic response leading to multiorgan system failure and death.^{1,2}

Fortunately, because of advances in critical care, the mortality rate has decreased over the last decade; however, the incidence of AP remains high worldwide.³ Recent reports have revealed an increase in the incidence of pancreatitis in developed countries including the United States.^{4,5} Injury to the pancreatic acinar cells causes a cascade of events that includes production of reactive oxygen species (ROS), resulting in the oxidation of lipids and proteins and disruption of the pancreatic membranes.⁶ Increased

production of ROS and the resulting oxidative stress play a major role in the pathogenesis of tissue damage in AP. Under normal condition, cells respond to ROS by up-regulating expression of cytoprotective and antioxidant enzymes and related molecules such as superoxide dismutase, catalase, glutamate-cysteine ligase catalytic (GCLC), and glutathione (GSH) peroxidase, which work in concert to neutralize ROS. However, when production of ROS exceeds the antioxidant capacity, it results in oxidative stress, inflammation, and tissue damage.⁷

Currently, the treatment of AP in the United States is limited to supportive care. Although there have been numerous trials exploring the efficacy of antioxidants or anti-inflammatory drugs in the treatment paradigm of AP, the results remain inconclusive. Dimethyl fumarate (BG-12) is the methyl ester of fumaric acid and was initially recognized for its anticancer effects.⁸ Later, it became widely used in Europe for the treatment of psoriasis under the trade name Fumaderm.⁹ Most recently, BG-12 received Food and Drug Administration approval in the United States for the treatment of patients with multiple sclerosis (MS). Dimethyl fumarate is a unique and potent antioxidant and anti-inflammatory drug whose mechanism of action has yet to be elucidated. The drug has been effective for decades for the treatment of other acute and chronic inflammatory conditions; however, it has never been examined specifically in the setting of pancreatitis.

The aim of the present study was to test the hypothesis that treatment with dimethyl fumarate may attenuate severity of AP in experimental animals by enhancing cellular antioxidant and anti-inflammatory machinery.

MATERIALS AND METHODS

In Vivo AP

Rodent studies were performed in accordance with the Institutional Animal Care and Use Committee of University of California, Irvine (Irvine, Calif). Male Sprague-Dawley rats (control-normal rats, n = 3; L-arginine, n = 12; L-arginine + dimethyl fumarate [DMF], n = 12; n = 27) were purchased from Charles River (Wilmington, Mass). Experimental agents used to produce AP were purchased from Sigma (St Louis, Mo) unless otherwise specified. Rats (250–300 g) were fed ad libitum on a standard diet with free access to water. They were maintained on a 12-hour light-dark cycle. Experimental animals were given oral DMF (25 mg/kg) dissolved in methylcellulose and fed via oral gavage 24 hours prior to initiating AP and daily thereafter until the animals were killed.

L-Arginine

Twenty percent L-arginine was dissolved in normal saline and filtered through a syringe filter with pH adjusted to 7.0. The solution was administered to nonfasted rats in 2 intraperitoneal injections at a dose of 3 g/kg body weight, each injection separated by 1 hour. Animals, control and experimental, received buprenorphine (Reckitt Benckiser, Richmond, Va) pain medication (0.01 mg/kg) intramuscularly twice a day and regular food and water. Because of the experimental end points, anti-inflammatory medications were

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not administered to the animals. Experimental animals continued to receive daily DMF via gavage along with a standard diet. Control animals (L-arginine, $n = 12$) received daily methylcellulose. The animals were killed after L-arginine induction at 24 hours for biochemical assessments ($n = 15$; control, $n = 3$; L-arginine, $n = 6$; L-arginine + DMF, $n = 6$) or 72 hours for histology ($n = 12$; L-arginine, $n = 6$; L-arginine + DMF, $n = 6$).¹⁰

Histology

Rat pancreata were fixed in 10% buffered formalin, embedded in paraffin blocks, and sectioned. The pancreas tissue was processed for hematoxylin-eosin staining using standard techniques. Using Schmidt criteria, interstitial edema, leukocyte infiltration, acinar cell destruction, and total scores were evaluated individually by 2 pathologists blinded to the source of the histology sections they evaluated. Acute pancreatitis was scored using a quantitative grading system as described by Schmidt et al.¹¹ Classification (Table 1) is based on the presence of edema, leukocyte infiltration, acinar cell necrosis, and hemorrhage.

Immunohistochemistry for Cleaved Caspase 3

The detection system was DAKO LSAB2 system—horseradish peroxidase kit using the manufactures protocol (K0675; DAKO, Carpinteria, Calif). Briefly, following deparaffinization and rehydration in clearing solution and graded alcohol series, samples were used for heat-induced antigen retrieval procedure in citrate buffer (pH 6) for 15 minutes on a hot plate. We blocked nonspecific binding sites with protein block solution (#HK112-9 K; BioGenex, Fremont, Calif) for 1 hour and endogenous peroxidase activity by 3% H₂O₂ for 10 minutes in room temperature. Sections were incubated overnight at 4°C with primary antibody (cleaved caspase 3, rabbit mAb, 1/800; Cell Signaling, Danvers, Mass). After washing in Tris-buffered solution, slides were incubated with ready-to-use secondary antibody for 15 minutes and then incubated with peroxidase substrate-chromogen solution (DAB) for 5 to 7 minutes. Sections were counterstained with Meyer hematoxylin for 60 seconds, rinsed with tap water, dehydrated in graded alcohol series, cleared with clearing solution, and then covered with permount cover slips. Apoptotic cells were identified as those with a cytoplasmic brown stain. Cleaved caspase 3-positive pancreatic acinar cells (%) were quantified on 400 \times magnification by 2 pathologists. Caspase 3 index was defined as the proportion of caspase-positive cells per high-power field in a minimum of 3 high-power fields. The results corresponded to the average number of positive acinar cells per field.¹²

Serum Amylase

Whole blood was obtained from anesthetized rats via cardiac puncture just before the animals were killed. Blood was then centrifuged (3000 revolutions/min for 20 minutes), and the supernatant collected. Amylase level in serum was determined to indicate the severity of pancreatitis using the Phadebas Amylase test (Magle AB, Lund, Sweden). Supernatant was incubated

with a 45-mg blue starch tablet, which became hydrolyzed by α -amylase to form water-soluble blue fragments. The absorbance (620 nm) was measured as a function of the α -amylase activity and expressed as units per liter.

Pancreatic Malondialdehyde Content

Pancreatic malondialdehyde (MDA) was measured by thiobarbituric acid colorimetric method using MDA assay kit (Cayman Chemical Company, Ann Arbor, Mich). Harvested pancreata (100 mg) were placed in lysis buffer (RIPA; Thermo Scientific, Piscataway, NJ) and homogenized (Power Gen; Fischer Scientific, Pittsburgh, PA). Samples were then centrifuged at 1200 revolutions/min at 4°C. The absorbance of the supernatant was measured by spectrophotometry at 535 nm for MDA content, as MDA reacted with thiobarbituric acid and turned pink after a 1-hour boil. The MDA concentration was calculated from the standard curve and expressed as micrometers.

Serum Lactate Dehydrogenase

Lactate dehydrogenase (LDH) was measured using a kit, In Vitro Toxicology Assay Kit, Lactate Dehydrogenase (TOX7; Sigma). Lactate dehydrogenase assay mixture was added (1:2) to serum samples on a 96-well plate and covered with aluminum foil for 30 minutes at room temperature. The reaction was terminated with addition of 1 N HCl (1:10) to each well. The absorbance was then measured at 490 nm and then again at 690 nm and subtracted. The concentrations were compared with a standard curve, and LDH was expressed as milliunits per milliliter.

Pancreatic Trypsin

Trypsin level was measured using a Trypsin Activity Assay Kit (ab102531; Abcam, Cambridge, Mass) according to manufacture specifications. Pancreatic tissue (100 mg) was placed in RIPA buffer (RIPA; Thermo Scientific) and homogenized (Power Gen, Fischer Scientific). Samples were combined with trypsin assay buffer and incubated for 10 minutes at 25°C and measured at time zero at absorbance 405 nm; this was followed by 1-hour incubation at room temperature and a second measurement at 405 nm (time 1). These measurements were compared with a standard curve to measure the trypsin activity in milliunits per milliliter.

Inflammatory Mediators From Lipopolysaccharide-Stimulated Splenocytes

Spleens were removed from normal adult rats under general anesthesia just before the animals were killed. Spleens were cut into tiny morsels with a standard 15-blade scalpel. The spleens were then placed in an erythrocyte lyses buffer (1.5 M NH₄CL, 100 mM KHCO₃, 100 mM EDTA-2Na adjusted to a pH of 7.2).¹³ Whole splenocytes were stimulated with endotoxin lipopolysaccharide 1 μ g/mL (LPS; Sigma) and incubated for 24 hours with or without DMF 20 μ M. The supernatant was then placed on a standard rat cytokine kit (Ray Biotech, Norcross, Ga). The

TABLE 1. Quantitative Grading Score for Pancreatitis

Score	0	1	2	3
Interstitial edema	None	Interlobular	Lobule involved	Isolated island-like acinar cells
Leukocyte infiltration	None	<20%	20–50%	>50%
Acinar cell necrosis	None	<5%	5–20%	>20%
Hemorrhage	None	1–2 Points	3–5 Points	>20%

cytokines/chemokines: activin A, agrin, CINC-1, CNTF, Fas-ligand, granulocyte-macrophage colony-stimulating factor, intercellular adhesion molecule 1, interferon γ , interleukin 1 α (IL-1 α), IL-4, IL-6, IL-13, leptin, macrophage inflammatory protein 3 α , receptor for advanced glycation end products, tissue inhibitor of metalloproteinases, tumor necrosis factor α (TNF- α), and vascular endothelial growth factor were analyzed. Supernatant was placed on special membranes and blocked and washed several times followed by the addition of a biotin conjugate anticytokine mixture incubated at room temperature for 2 hours. This is followed by the addition of conjugated horseradish peroxidase-labeled streptavidin (horseradish peroxidase-streptavidin) and incubation at room temperature for 2 hours. Membranes were washed and processed with detection buffer and exposed with x-ray Film (Kodak, Rochester, NY). Analysis was carried out using Image Quant TL 7.0 (GE Healthcare Life Sciences, Pittsburgh, Pa).¹⁴

Statistical Analysis

Student *t* test and 1-way analysis of variance were used in statistical analysis of the data using Excel for Windows software (Microsoft, Redmond, Wash). $P \leq 0.05$ was considered significant. Data are expressed as mean \pm SD.

RESULTS

DMF Ameliorated AP in Rodents

Treatment with oral DMF in experimental pancreatitis induced by L-arginine resulted in significant reductions in infiltrating inflammatory cells, acinar architectural damage, edema, and necrosis (Figs. 1A, B). The total severity score was statistically significant for L-arginine versus L-arginine + DMF (Table 2; $P < 0.001$).

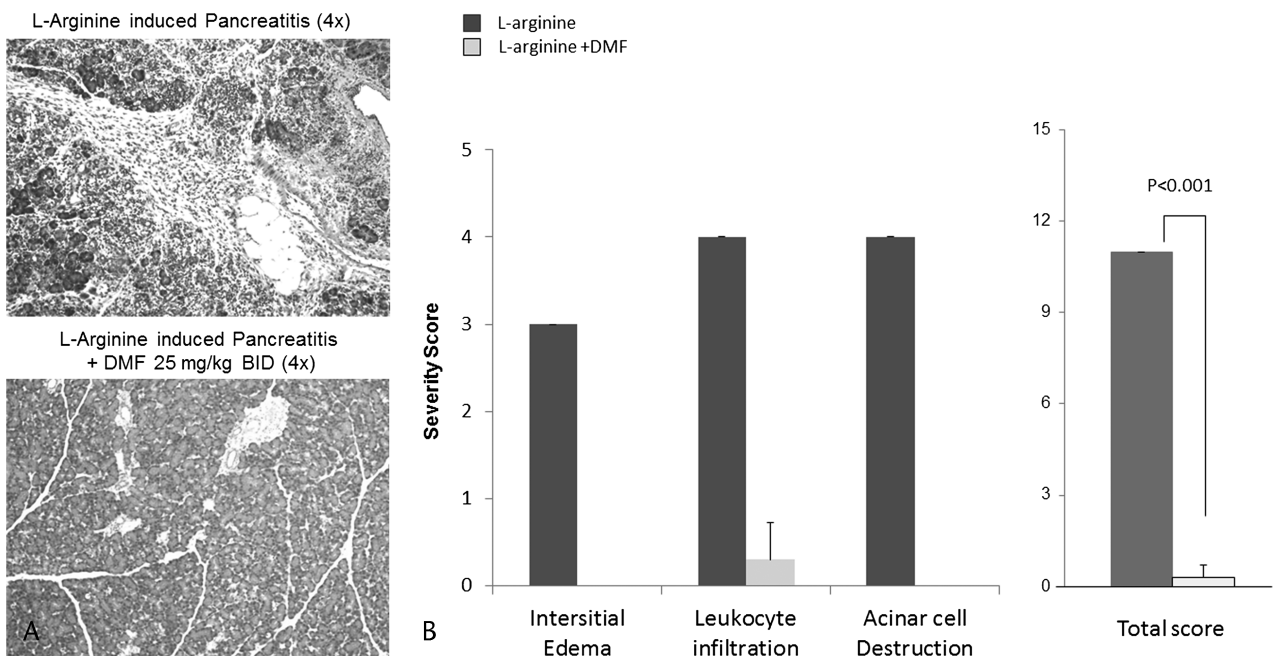


FIGURE 1. A, Histology of L-arginine-induced pancreatitis. Representative photomicrograph of rat pancreatic hematoxylin-eosin stained sections. Dimethyl fumarate supplementation in rats significantly reduced pancreatic infiltration of inflammatory mediators, destruction of normal acinar architecture, peribulbar edema, necrosis of cells, and surrounding fat in a rodent model of pancreatitis induced by L-arginine. B, Quantitative pancreatitis score: interstitial edema, leukocyte infiltration, and acinar cell destruction. Pancreas histology slides were evaluated by 2 blinded pathologists and given a score based on severity from 0 to 3 based on 3 criteria: edema, infiltration, and acinar cell destruction. Based on quantitative scores for all 3 criteria, there was a significantly higher score for L-arginine compared with L-arginine + DMF ($P < 0.001$).

Treatment With Oral DMF Significantly Reduced the Proportion of Caspase 3-Positive Cells

Cleaved caspase 3 antibody was used for histological identification of pancreatic acinar cell apoptosis.¹⁵ Cleaved caspase 3 was used to evaluate apoptotic cells in the pancreas of rats induced with L-arginine. Compared with the untreated group, DMF-treated rats showed significantly lower caspase-positive cells in pancreatic sections (Fig. 2, Table 3; $P < 0.001$).

Treatment With Oral DMF Significantly Decreased Serum Amylase But Not LDH

The serum amylase but not LDH in the DMF-treated rats was significantly lower after 24 hours of induction with L-arginine when compared with the untreated group (Fig. 3, Table 3). Lower amylase level in the DMF-treated rats is consistent with the observed histological improvement in rats with L-arginine-induced pancreatitis.

Treatment With Oral DMF Significantly Decreased Pancreatic MDA But Not Trypsin

Malondialdehyde is an indicator of lipid peroxidation and cellular damage under oxidative stress. The pancreatic MDA levels in the DMF-treated rats were significantly lower after 24 hours of AP induction with L-arginine when compared with the untreated group (Fig. 3, Table 3; $P < 0.05$). However, there was no significant difference in trypsin levels; however, there was a propensity for lowered trypsin levels in the experimental groups.

TABLE 2. Histological Grading Score for L-Arginine–Induced Pancreatitis

Groups	Interstitial Edema	Leukocyte Infiltration	Acinar Cell Destruction	Hemorrhage	Total	<i>P</i>
L-Arginine n = 6	3	4	4	0	11	<0.001
L-Arginine + DMF n = 6	0	0.3 ± 0.42	0	0	0.3 ± 0.42	

DMF Significantly Lowered Production of Inflammatory Mediators in LPS-Treated Rodent Splenocytes

To confirm DMF's ability to reduce inflammatory mediators, whole splenocytes from normal rats were treated with LPS alone or with DMF+ LPS 1 µg/mL in vitro for 24 hours. Lipopolysaccharide-stimulated splenocytes were used to mimic an infection that can occur in the setting of clinical pancreatitis. Dimethyl fumarate significantly decreased the production of inflammatory mediators (activin A, agrin, CINC-1, CNTF, Fas-ligand, granulocyte-macrophage colony-stimulating factor, intercellular adhesion molecule 1, interferon γ , IL-1 α , IL-4, IL-6, IL-13, leptin, macrophage inflammatory protein 3 α , receptor for advanced glycation end products, tissue inhibitor of metalloproteinases, TNF- α , vascular endothelial growth factor) in the supernatant of rodent splenocytes treated with LPS (Fig. 4).

DISCUSSION

In this study, we evaluated the effects of DMF on a rodent model of AP induced by L-arginine. Our data showed that DMF attenuated the severity of tissue injury and inflammation, reduced many inflammatory mediators, and lowered specific biochemical

markers of pancreatitis. Careful histological examination of the pancreas and pancreatitis scoring criteria revealed that DMF significantly reduced acinar cell destruction. The MDA and cleaved caspase 3 levels in the pancreatic tissue were significantly lower in the DMF-treated compared with the untreated rats pointing to the efficacy of DMF in attenuating oxidative stress and cell injury in the rats with AP. Lastly, the serum amylase level, which was significantly elevated in the untreated rats, was reduced to the normal control rat values in the DMF-treated rats.

Dimethyl fumarate is a potent anti-inflammatory drug; as such, it can be assumed that it would exert protected effects against a number of inflammatory conditions such as pancreatitis. To our knowledge, DMF has not been previously investigated for the treatment of either clinical or experimental pancreatitis. We aimed to assess DMF's protection against experimental AP induced by L-Arginine.

Dimethyl fumarate has been widely used in Europe for the treatment of psoriasis vulgaris and psoriatic arthritis, which are chronic inflammatory disorders, for over 20 years. Although repurposed for human use for decades, the exact mechanism of action of DMF is not clearly elucidated. The beneficial action of DMF is linked to its ability to stimulate production of anti-inflammatory cytokines in leukocytes and inhibit keratinocyte proliferation. In initial investigations, monomethylfumarate, the

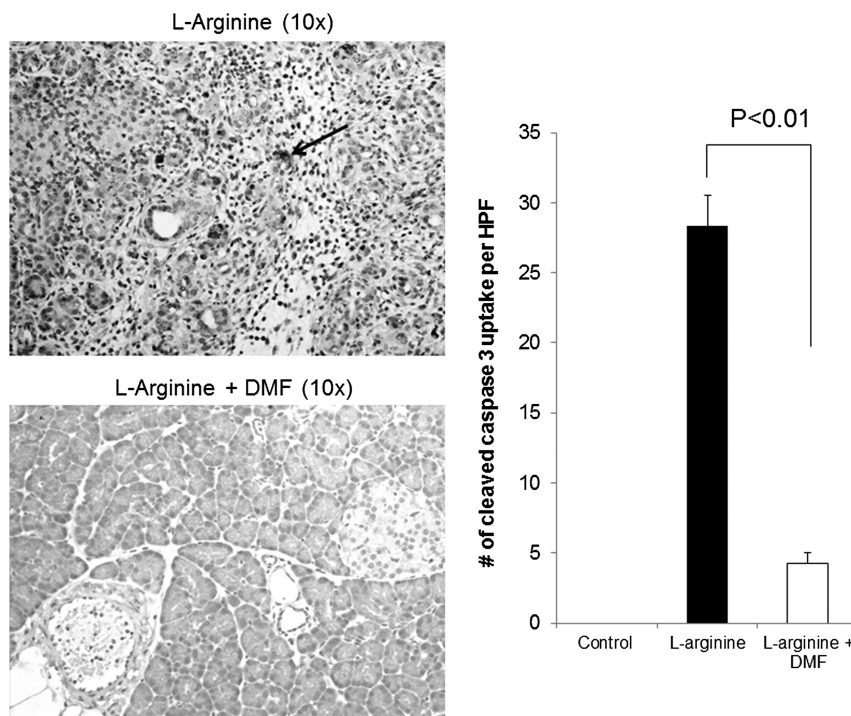


FIGURE 2. Cleaved caspase 3 immunohistochemistry in L-arginine–induced pancreatitis. Representative photomicrograph of pancreas histology stained with cleaved caspase 3 antibody. In rats treated with DMF, the caspase 3 staining of the pancreas revealed significantly lower cleaved caspase 3–positive cells when compared with the L-arginine group ($P < 0.001$). The arrow reveals an area of brown cytoplasmic uptake indicating cleaved caspase 3. The slides were reviewed by 2 blinded pathologists who scored the slides based on standardized criteria. Data are representative of 3 independent experiments.

TABLE 3. Biochemical Parameters for L-Arginine–Induced Pancreatitis

Biochemical Parameter	Control (n = 3), Mean	L-Arginine (n = 6), Mean ± SD	L-Arginine + DMF (n = 6), Mean ± SD	P (L-Arginine vs L-Arginine + DMF)
Caspase 3 (n per high-power field)	0	28.33 ± 2.22	4.25 ± 0.75	0.001
Serum amylase, U/L	700	4015 ± 440	1970 ± 260	0.014
Pancreatic MDA, μ M	1.9	5.9 ± 1.26	3.75 ± 0.38	0.04
Serum LDH, mU/mL	60.12	75.65 ± 9.58	68.13 ± 24.64	0.17
Pancreatic trypsin, mU/mL	3.67	70.18 ± 72	19.05 ± 11	0.18

most active metabolite of DMF, was found to selectively up-regulate expression of T_H2 cytokines and suppress a T_H1 response.¹⁶ Dimethyl fumarate also down-regulates expression of several chemokines that are involved in the pathogenesis of psoriasis.^{17,18} Another study, by Ghoreschi et al, found that in mice the supplementation of fumarate caused type II dendritic cells to produce IL-4, resulting in T_H2 expansion. Multiple sclerosis and psoriasis are 2 diseases in which IL-12 and IL-23 promote pathogenic T helper cell differentiation.^{19,20} Furthermore, previous studies have found that DMF specifically inhibits transcription of NF- κ B's target gene products.²¹ Nuclear factor κ B plays a major role in transcriptional regulation of inflammatory cytokines as well as in cell differentiation and apoptosis. In a study by Meilbutz et al,²² isolated rat endothelial cells were stimulated with TNF- α , which resulted in translocation of cytoplasmic NF- κ B

into the nucleus. Tumor necrosis factor α –induced nuclear translocation of NF- κ B was inhibited by pretreatment with DMF. In another study, DMF was shown to inhibit phosphorylation of I κ B, which led to a significant reduction in cardiac damage in an experimental mouse model.²³

In a study by Lehmann et al,²⁴ DMF was found to induce immunosuppression via GSH depletion and induction of a potent endogenous antioxidant, heme oxygenase 1. Others have also postulated that DMF exerts its antioxidant effects as a synthetic Nrf2 activator. The Nrf2 pathway is the principal regulator of the antioxidant and anti-inflammatory responses by mediating the transcription of genes encoding hundreds of antioxidant and phase II detoxifying enzymes.^{25,26} In summation, the effects of DMF on experimental models have been attributed to multiple factors, one being activation of Nrf2, leading to up-regulation of

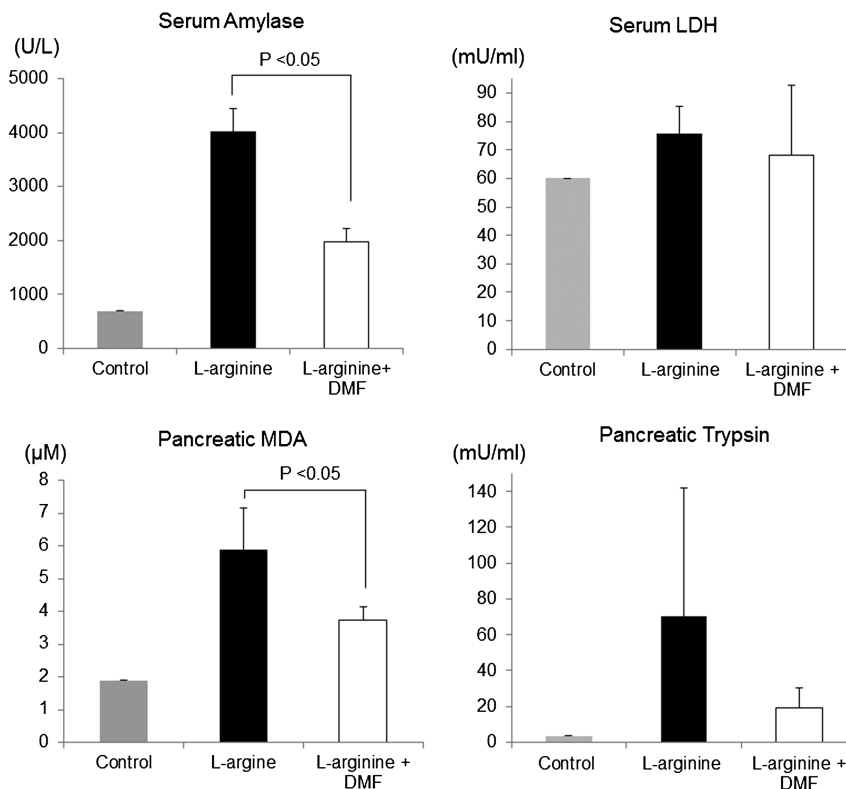


FIGURE 3. Serum amylase and pancreatic MDA level. Serum amylase and LDH and pancreatic MDA and trypsin were determined at 24 hours after induction of L-arginine. Compared with the control L-arginine rats, DMF-treated rats resulted in a significant decrease in serum amylase levels and pancreatic MDA concentration ($P < 0.05$). Although there was a trend toward lower trypsin and LDH in DMF-treated rats, the results were not statistically significant. Data are representative of at least 3 independent experiments.

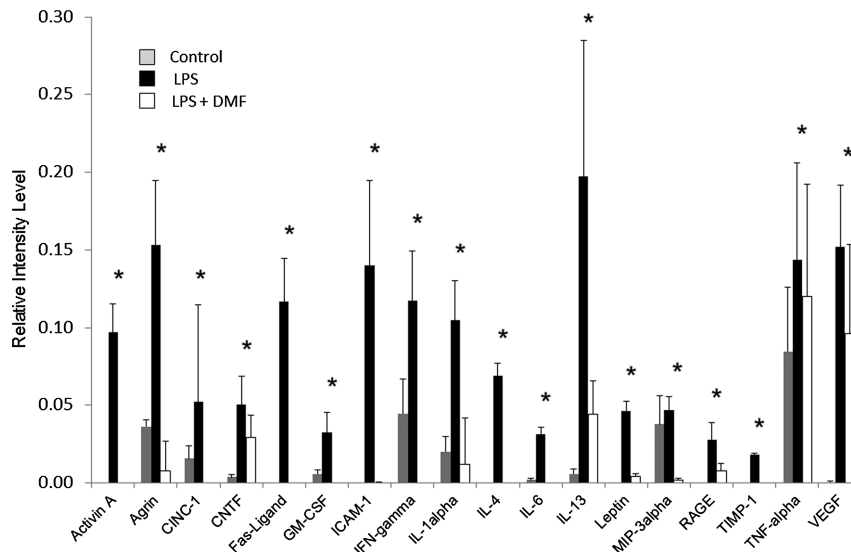


FIGURE 4. Inflammatory mediator levels from LPS-stimulated rat splenocytes treated with DMF. Rat splenocytes were isolated from normal rats and stimulated with LPS. They were cultured for 24 hours with or without DMF. Dimethyl fumarate significantly decreased the production of key inflammatory mediators related to AP. Data are representative of 6 independent experiments.

antioxidant and anti-inflammatory mediators, and down-regulation of proinflammatory cytokines and ROS-producing pathways.^{27–29}

Dimethyl fumarate has recently been investigated clinically in the United States for its neuroprotective effects. In a phase 3 trial for relapsing-remitting MS, oral DMF significantly reduced the proportion of patients who had a relapse, the rate of disease progression, the annual relapse rate, and the number of gadolinium-enhancing lesions and new or enlarging T2-weighted hyperintense lesions on magnetic resonance imaging.⁷ In previous investigation, neuroprotection was attributed predominately to the activation of the Nrf2-keap1 pathway.^{16,28,29} Studies have revealed increased cellular levels of nicotinamide adenine dinucleotide phosphate dehydrogenase quinone 1 (NQO1), heme oxygenase 1, and GCLC subunit. Glutathione is the main cellular antioxidant against ROS; thus, up-regulation of GCLC, the rate-limiting enzyme in GSH biosynthesis, would increase the cells' ability to survive and combat oxidative stress.^{16,29} Given the positive outcomes of the recent clinical trial of DMF, the Food and Drug Administration recently approved its use for the treatment patients with MS.

Taken all together, studies in experimental animals and humans have demonstrated strong antioxidant, anti-inflammatory, and cytoprotective effects of DMF, which are mediated by activation of Nrf2, inhibition of NF- κ B, and suppression of T helper 1 response. As noted above, oxidative stress and inflammation play a major role in the pathogenesis and progression of AP and pancreatic tissue damage. Intense oxidative stress and inflammation in our animals with AP were accompanied by impairment of the endogenous anti-inflammatory and antioxidant responses. Given the proven efficacy of DMF in enhancing the antioxidant and anti-inflammatory defense systems in various other inflammatory disorders in humans and experimental animals, it is not surprising that it proved effective in attenuating oxidative stress, inflammation, and tissue injury in our rats with AP.

CONCLUSIONS

In conclusion, DMF administration, a widely used antioxidant and anti-inflammatory medication, resulted in significant amelioration of pancreatic tissue damage, inflammation, and oxidative stress in experimental AP.

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