

Chondroitin-4-Sulphate Reduced Oxidative Injury in Caerulein-Induced Pancreatitis in Mice: The Involvement of NF- κ B Translocation and Apoptosis Activation

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Activation of nuclear factor κ B (NF- κ B) and caspases may greatly amplify inflammation and cell damage in addition to that directly exerted by free radicals. Since reactive oxygen species (ROS) are involved in acute pancreatitis, we studied whether the administration of chondroitin-4-sulphate (C4S), in addition to its antioxidant activity, was able to modulate NF- κ B and caspase activation in an experimental model of caerulein-induced acute pancreatitis in mice. Hyperstimulating doses of caerulein (50 μ g/kg), five injections per mouse given at hourly intervals produced the following: high serum lipase and amylase activity; lipid peroxidation, evaluated by 8-isoprostane concentrations; loss of antioxidant defenses such as glutathione reductase (GR) activity; NF- κ B activation and loss of cytoplasmic I κ B α protein; increases in tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), caspase-3, and caspase-7 gene expression and their related protein; accumulation and activation of neutrophils in the damaged tissue, evaluated by elastase (ELA) determination; and pancreatic injury, evaluated by histologic analysis. Pretreatment of mice with different doses of C4S, given 1 hr before caerulein injections and 1 and 2 hrs after the last caerulein injection, reduced lipid peroxidation, inhibited NF- κ B translocation and cytoplasmic I κ B α protein loss, decreased TNF- α , IL-6, and caspase gene expression and their related protein levels, limited endogenous antioxidant depletion, and reduced tissue neutrophils accumulation and tissue damage. Since molecules with antioxidant activity can block NF- κ B and apoptosis activation, we suggest that C4S administration is able to block

NF- κ B and caspase activation by reducing the oxidative burst.
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Key words: acute pancreatitis; oxidative stress; glycosaminoglycans; caspases; antioxidants

Acute pancreatitis (AP) is an autodigestive and inflammatory disease that has been investigated for many years. In experimental pancreatitis induced by supramaximal doses of the cholecystokinin analogue caerulein, the secretory block is followed by lysosomal degradation of intercellular organelles within autophagic vacuoles in acinar cells. Activation of trypsin from its precursor trypsinogen is believed to be one of the principal events responsible for initiating damage (1). The initial stage of acute pancreatitis is characterized by interstitial edema coupled with infiltration of neutrophils and macrophages into the pancreatic tissue. These infiltrating inflammatory cells, particularly polymorphonuclear cells (PMNs), generate reactive oxygen species (ROS), which consequently destroy lipid membranes by peroxidation of fatty acids and trigger a variety of inflammatory processes (2). In addition, ROS induce phosphorylation of the I κ B- α inhibitor that in turn is responsible for NF- κ B activation that modulates the gene expression of inflammatory mediators including cytokines such as TNF- α and IL-6, chemokines, and nitric oxide (NO) (3). The greater production of inflammatory mediators results in complications that lead to local pancreatic inflammation progressing to a systemic inflammatory reaction, called multiple organ dysfunction syndrome (4). Thus, ROS-induced oxidative stress is considered to play an important pathophysiologic role in perpetuating pancreatic inflammation and in developing extrapancreatic complications. Nevertheless, several reports have shown that antioxidant therapies scavenging ROS ameliorate acute pancreatitis damage by reducing NF- κ B activation and by subsequently inhibiting the expression of inflammatory mediators (5, 6).

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Apoptosis activation in AP may contribute to acinar cell damage and impaired function. During this process, caspases are activated through a protease cascade, whereby the inactive pro-enzyme is cleaved to form subunits of the active heterotetrameric protease (7). Several distinctive changes take place during apoptosis; these include nuclear changes such as chromatin condensation, internucleosomal DNA degradation, and nuclear lobulation and fragmentation. There is a large body of evidence indicating that cell exposure to ROS, besides producing a direct chemical injury to cell components, also induces apoptosis that leads to cell disruption and tissue necrosis (8). Recent studies have reported that apoptosis stimulated by oxidative stress may be inhibited by a number of antioxidant molecules (9, 10).

Most biological molecules have more than one function. In particular, many molecules have the ability to directly or indirectly scavenge free radicals and thus act as antioxidants in living organisms. The increase of these molecular levels during oxidative stress seems to be a biological response that may protect cells from oxidation in synergy with other antioxidant defense systems. One of these structures is the glycosaminoglycan (GAG) chondroitin-4-sulphate (C4S), a biomolecule that has increasingly gained the interest of many research groups due to its antioxidant activity (11–15).

In light of these findings, the aim of this study was to investigate whether the antioxidant property of C4S, previously evaluated in caerulein-induced acute pancreatitis in rats (16), involves NF- κ B and caspase modulation using a model of caerulein-induced acute pancreatitis in mice.

Materials and Methods

Animals. CD-1 male mice 6–7 weeks old (Harlan Italy, Correzzana, Italy) with a mean weight of 25–30 g were used in our study, and maintained under climate-controlled conditions with a 12-hr light:dark cycle. The animals were fed standard rodent chow and given water *ad libitum*. The health status of the animal colony was monitored in accordance with Italian Veterinary Board guidelines. Mice were divided into the following groups: (i) control ($n = 20$); (ii) C4S (120 mg/kg) ($n = 20$); (iii) caerulein ($n = 24$); (iv) caerulein + C4S (30 mg/kg) ($n = 20$); (v) caerulein + C4S (60 mg/kg) ($n = 20$); (vi) caerulein + C4S (120 mg/kg) ($n = 20$).

Materials. C4S from bovine trachea and caerulein were obtained from Sigma-Aldrich S.r.l. (Milan, Italy). Mouse TNF- α and IL-6 polyclonal antibodies were obtained from Chemicon International Inc. (Temecula, CA), and caspase-3 and caspase-7 polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies were obtained from Imgenex Corporation (San Diego, CA). All other reagents used were purchased from Fluka, a division of Sigma-Aldrich S.r.l. (Milan, Italy).

Induction of Acute Pancreatitis. Mice were in-

jected intraperitoneally with caerulein at a dose of 50.0 mg/kg body weight five times at hourly intervals. Animals were maintained on a free diet and water *ad libitum* throughout the experiment. Mice were then sacrificed under ether anesthesia 5 hrs after the last caerulein injection, at which time blood was collected from the inferior vena cava and the pancreas was isolated. The blood collected was then separated into serum. The isolated pancreases were maintained at 4°C for histologic and biochemical evaluations.

C4S Treatment. On the day of the experiment, mice were randomized to receive treatment with C4S at doses of 30.0 mg/kg, 60.0 mg/kg, and 120.0 mg/kg. The first C4S administration was carried out 1 hr before the first caerulein injection, the second and the third doses of C4S were administered 1 hr and 2 hrs after the last caerulein injection. C4S was dissolved in saline solution (0.9% NaCl) and administered intraperitoneally in a volume of 1.0 ml/kg body weight.

Serum Lipase and Amylase Determination. Lipase and amylase activity were evaluated in order to estimate the degree of pancreatic injury. Serum samples (100 μ l) obtained at the end of the experiments were used for this assay. Activity levels were assayed spectrophotometrically using commercial clinical test kits (Roche Diagnostic, Milan, Italy).

NF- κ B p50/65 Transcription Factor Assay. NF- κ B p50/65 DNA binding activity in nuclear extracts of pancreatic tissue samples was evaluated in order to measure the degree of NF- κ B activation. The assay combines the principle of the electrophoretic mobility shift assay (EMSA) with the enzyme-linked immunosorbent assay (ELISA). Analysis was performed in line with the manufacturer's protocol for a commercial kit (NF- κ B p50/65 Transcription Factor Assay Colorimetric, Chemicon International). In brief, the pancreases of the animals were removed at the end of the experimental phase and maintained at 4°C, washed in ice-cold 10 mM Tris-HCl, pH 7.4, and blotted on absorbent paper. Samples were then trypsinized and plotted in order to isolate hepatic cells. Cytosolic and nuclear extraction was performed by lysing the cell membrane with an apposite hypotonic lysis buffer containing protease inhibitor cocktail and tributylphosphine (TBP) as reducing agent. The lysate was then incubated in the buffer on ice and centrifuged at 250 g . After adding two volumes of buffer, a series of drawing and ejecting actions were then performed using a syringe with a small gauge needle. This step was carried out 5 times. After centrifugation at 8000 g , the supernatant containing the cytosolic portion of cell lysate was recovered and stored at -70°C for subsequent analysis. The pellet containing the nuclear portion was then resuspended in the apposite extraction buffer and the nuclei were disrupted by a series of drawing and ejecting actions. After gentle stirring for 40 mins, the nuclei suspension was centrifuged at 16,000 g . The supernatant fraction was the nuclear extract. After the determination of protein concentration and adjustment to a final concentration of approximately 4.0 mg/ml, this extract

was stored in aliquots at -80°C for the successive NF- κB assay. The analysis comprised a series of control steps achieved by adding the following components to the nuclear extract in order to obtain the transcription factor assay as normal, positive control, specific competitor control and negative control: (i) HeLa (human epithelial carcinoma cell line) whole cell extract (TNF- α treated), (ii) transcription factor assay probe, (iii) NF- κB competitor oligonucleotide, and (iv) NF- κB capture probe and enhanced transcription factor assay buffer. After incubation with primary and secondary antibodies, color development was observed following the addition of the substrate TMB/E (Sigma-Aldrich). Finally, the absorbance of the samples was measured using a spectrophotometric microplate reader (DAS Instruments, Rome, Italy) set at λ 450 nm. Values are expressed as relative optical density (O.D.)/mg protein.

I κ B α Assay. I κ B α loss was quantified in pancreatic tissue samples in order to confirm NF- κB activation. The test is based on the solid phase sandwich ELISA assay. The cytosolic fraction, obtained during the nuclei extraction procedure for NF- κB assay, was used for I κ B- α evaluation. The assay was carried out using a commercial kit (Total Human BioAssay ELISA Kit, United States Biological Inc., Swampscott, MA) (I κ B α). Briefly, 100 μl of standards, samples, and controls were added to each well of the coated microplate. After 2 hrs incubation at room temperature, the microplate was decanted and the liquid discarded. Wells were washed four times. Subsequently, 100 μl of anti-I κ B- α antibodies were added to each well. After 1 hr incubation at room temperature, the liquid was again removed from the wells. Wells were washed four times and 100 μl of anti-rabbit IgG-HRP was added. After further incubation for 30 mins and having washed the wells four times, 100 μl of stabilized chromogen was added. The absorbance was measured using a spectrophotometric microplate reader (DAS Instruments) set at λ 450 nm. Values are expressed as relative O.D. per mg protein.

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative PCR Amplification. Total RNA was isolated from pancreatic tissue for reverse polymerase chain reaction (PCR) real-time analysis of TNF- α , IL-6, caspase-3, and caspase-7 (RealTime PCR System 7500, Applied Biosystems, Foster City, CA). Total RNA was isolated from pancreatic tissue using the Omnisol Reagent Kit (Euroclone, West York, UK). The first strand of cDNA was synthesized from 1.0 μg total RNA using a high capacity cDNA Archive kit (Applied Biosystems). β -actin mRNA was used as an endogenous control to allow the relative quantification of TNF- α , IL-6 and caspase mRNAs (17). Real-time PCR was performed on both targets and endogenous controls by means of ready-to-use assays (Assays-on-Demand, Applied Biosystems). The amplified PCR products were quantified by measuring the TNF- α , IL-6, caspase, and β -Actin mRNA-calculated cycle thresholds (C_T). The C_T values were plotted against log input RNA concentration in serially diluted total RNA of pancreatic tissue samples and used to

generate standard curves for all the mRNAs analyzed. The amounts of specific mRNA in samples were calculated from the standard curve and normalized with the β -actin mRNA. After normalization, the mean value of normal pancreatic cell target levels became the calibrator (one per sample) and the results are expressed as the n-fold difference relative to normal controls (relative expression levels).

Western Blot Assay of TNF- α , IL-6, Caspase-3, and Caspase-7 Proteins. For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, the pancreatic cells were washed twice in ice-cold phosphate-buffered saline (PBS) and subsequently dissolved in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). Aliquots of whole cell protein extract (10–25 μl /well) were separated on a mini-gel (10%). The proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NY) using a semidry apparatus (Bio-Rad Laboratories, Richmond, CA). The blots were flushed with double distilled H_2O , dipped into methanol, and dried for 20 mins before proceeding to the next step. Subsequently, the blots were transferred to a blocking buffer solution (1 $\times$ PBS, 0.1% Tween 20, 5% w/v nonfat dried milk) and incubated for 1 hr. The membranes were then incubated with the specific diluted primary antibody in 5% bovine serum albumin, 1 $\times$ PBS, and 0.1% Tween 20 in a roller bottle at 4°C overnight. After being washed in three stages in wash buffer (1 $\times$ PBS, 0.1% Tween 20), the blots were incubated with the secondary polyclonal antibody goat anti-rabbit conjugated with peroxidase, in TBS/Tween-20 buffer containing 5% nonfat dried milk. After 45 mins of gentle shaking, the blots were washed five times in wash buffer, and the proteins were made visible using a ultraviolet-visible transilluminator (EuroClone, Milan, Italy) and Kodak BioMax MR film (Kodak Imaging Network, Emeryville, CA). A densitometric analysis was also run in order to quantify each band (InterFocus Imaging, Ltd., Cambridge, UK).

Lipid Peroxidation Estimation. Evaluation of 8-isoprostane (8-IPE) in the pancreas was carried out to examine the extent of oxidation of hepatic cell phospholipids produced by oxygen radicals. The analysis was performed using an enzyme immunoassay (EIA) commercial kit (8-Isoprostane EIA kit, Cayman Chemical Company, Ann Arbor, MI). Briefly, the pancreases of the animals were removed at the end of the experimental phase and maintained at 4°C , washed in ice-cold 0.1 M phosphate buffer, pH 7.4, containing 1.0 mM EDTA and 10.0 μM indomethacin and blotted on absorbent paper. Each sample was then minced in ice-cold EIA buffer, containing 5.0 mg/ml butylated hydroxytoluene in ethanol, and homogenized in a 1:10 (w/v) ratio using an Ultra-Turrax homogenizer (IKA Works Inc., Wilmington, NC) maintained at 4°C . After centrifugation at 5000 g at 4°C for 5 mins, the supernatant was purified by affinity sorbent columns and the final eluent used for biochemical assay. Fifty microliters of

sample, 8-IPE AChE tracer, and 8-IPE antiserum were added to each well. The plate was then covered with plastic film and incubated at room temperature for 18 hrs. After rinsing the wells five times with wash buffer, 200 μ l of Ellman's reagent was added and the plate contents were gently mixed in the dark until coloration developed. Finally, the absorbance of each well was measured using a spectrophotometric microplate reader (DAS Instruments) set at λ 410 nm. A calibration curve of 8-IPE standard was also run for quantification. Values are expressed as relative O.D. per mg of protein.

Glutathione Reductase (GR) Determination. GR is a ubiquitous enzyme which catalyzes the reduction of oxidized glutathione (GSSG) into reduced glutathione (GSH). This enzyme is essential for the maintenance of the glutathione redox cycle. For GR evaluation, pancreatic tissue samples were first washed with cold isotonic saline solution containing 1.0 mM EDTA in order to remove erythrocytes. Each sample was then homogenized in a 1:10 (w/v) ratio in a solution containing 50 mM potassium phosphate, pH = 7.5, and 1.0 mM EDTA using an Ultra-Turrax homogenizer (IKA Works Inc.) maintained at 4°C. Subsequently, each sample was centrifuged at 8500 *g* at 4°C for 10 mins. The biochemical analysis was performed using a specific colorimetric assay (Bioxytech GR-340 assay kit, OxisResearch, Portland, OR). Briefly, an aliquot (0.2 ml) of each supernatant in a spectrophotometric cuvette was added to 0.4 ml of GSSG or potassium phosphate (sample blank). Having placed the cuvette in the spectrophotometer (DAS Instruments), 0.4 ml nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was added. The absorbance was then read at λ 340 nm for 5 mins and calculated as the rate of decrease in absorbance per minute. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of GR. The amount of pancreatic GR is expressed as mUnits/mg protein.

Elastase (ELA) Assay. ELA activity was evaluated as an indicator of neutrophil accumulation and activation in the pancreatic tissue. The analysis was performed using a fluorometric commercial kit (Elastase Assay Kit, EnzChek, Invitrogen, Eugene, OR). Briefly, the pancreases of the animals were removed at the end of the experimental phase, washed in ice-cold 10 mM Tris-HCl, pH 7.4, and blotted on absorbent paper. Samples were then homogenized, in the same washing buffer, in a 1:10 (w/v) ratio using an Ultra-Turrax homogenizer. After centrifugation at 5000 *g* at 4°C for 5 min, 100 μ l of each supernatant was added to a tube containing 450 μ l of diluted reaction buffer and 450 μ l of fluorescent-labeled elastin substrate. After 2 hrs incubation at room temperature, covered to protect from light, the fluorescence intensity was measured in a standard fluorometer at λ Ex 480 nm and λ Em 520 nm. A calibration curve of porcine pancreatic ELA standard was also run for quantification. Finally, a specific ELA inhibitor was used as control to eliminate interference exerted by other proteases.

Table 1. Effect of C4S^a Treatment on Serum Lipase and Amylase Activities in Mice After Caerulein Injection

Experimental group	Lipase (mean $\pm$ SD) ^{a,b}	Amylase (mean $\pm$ SD) ^b
Control	23.5 $\pm$ 4.7*	184.3 $\pm$ 41.2*
C4S (120 mg/kg)	25.2 $\pm$ 5.1*	178.4 $\pm$ 38.4*
Caerulein	169.2 $\pm$ 20.3*	1045.8 $\pm$ 121.1*
Caerulein + C4S (30 mg/kg)	121.6 $\pm$ 17.2**	770.2 $\pm$ 94.8**
Caerulein + C4S (60 mg/kg)	90.3 $\pm$ 15.1**	581.7 $\pm$ 71.5**
Caerulein + C4S (120 mg/kg)	66.5 $\pm$ 13.4**	394.7 $\pm$ 57.3**

^a C4S, chondroitin-4-sulphate; SD, standard deviation.

^b Values are the mean $\pm$ SD of seven experiments and are expressed as units/L.

* $P < 0.001$ vs. control; ** $P < 0.001$ vs. caerulein.

Values are expressed as fluorescent arbitrary units (FAU) at λ Ex 480 nm and λ Em 520 nm per mg protein.

Histologic Analysis. Histological examination was conducted in order to evaluate the degree of pancreatic damage. Pieces of tissue obtained from the pancreas of each mouse were removed and fixed by immersion in 10% neutral buffered formalin. The fixed tissues were embedded in paraffin and cut into 6- μ m sections. The sections were stained with hematoxylin-eosin and examined under a light microscope (Optech Instrument, Munich, Germany) connected to a digital camera (Coolpix 4500, Nikon, Tokyo, Japan).

Protein Analysis. The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Laboratories) with bovine serum albumin (BSA) as a standard in accordance with the published method (18).

Statistical Analysis. Data are expressed as mean $\pm$ SD of no less than seven experiments for each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was performed by one-way ANOVA followed by the Student-Newman-Keuls test. The statistical significance of differences was set at $P < 0.05$.

Statement of Animal Care. The studies reported in this manuscript were carried out in accordance with the Declaration of Helsinki and with the Guidelines for the Care and Use of Laboratory Animals.

Results

Lipase and Amylase Activity. Table 1 reports the changes in activity of both these pancreatic enzymes in serum of mice taken at the end of the experiment. Low lipase and amylase activity levels were seen in the groups not treated with caerulein. Instead, a marked increase in activity of both enzymes was found in the serum of caerulein-treated mice. The treatment with C4S significantly reduced lipase and amylase levels (Table 1). The reduction

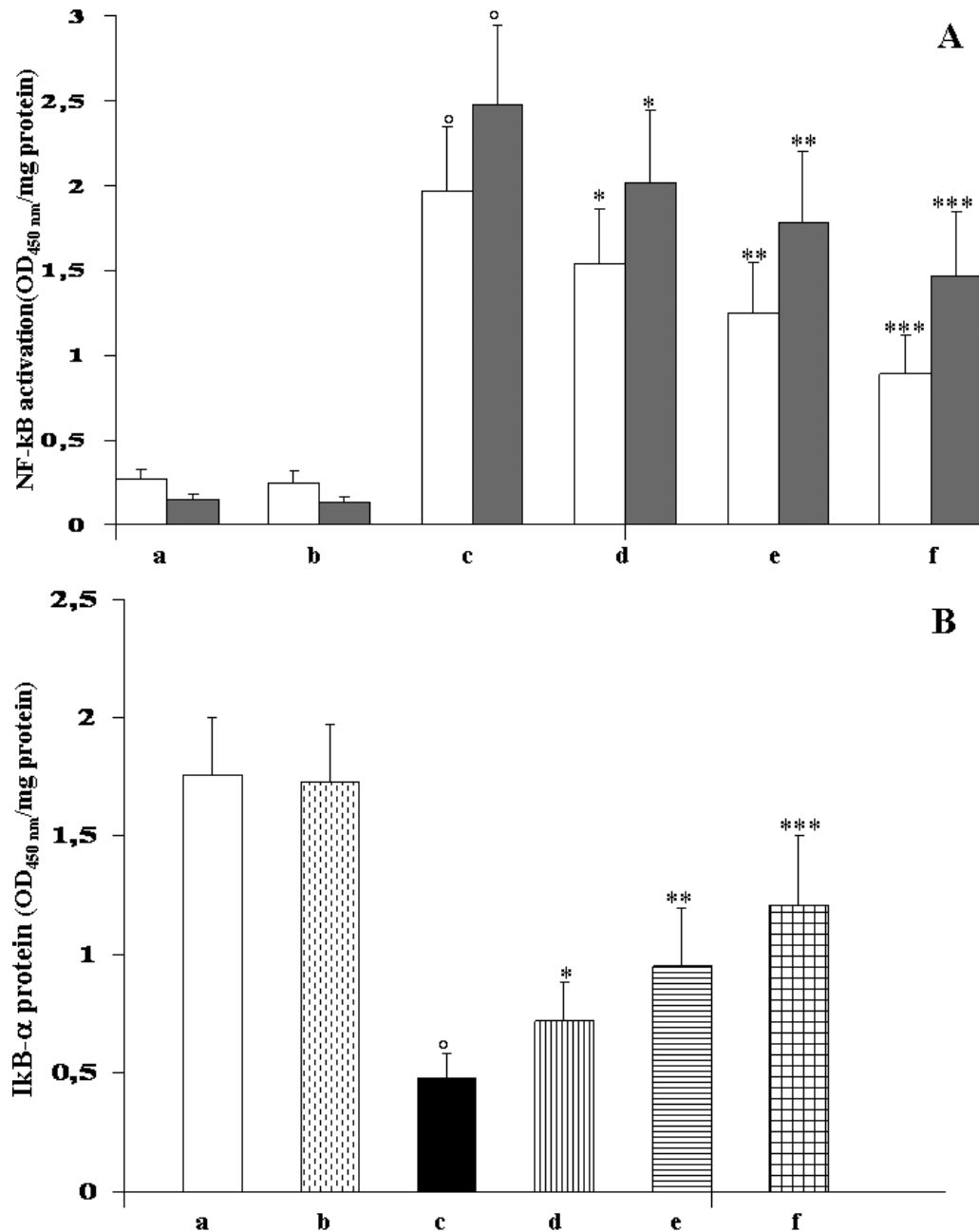


Figure 1. Effect of C4S treatment on pancreatic NF- κ B p50/65 transcription factor DNA binding activity (A) and I κ B- α protein degradation (B) in pancreatic tissue of mice injected with caerulein. In Panel A, white bars represent the p/50 subunit; black bars represent the p/65 subunit. (a) control; (b) C4S (120 mg/kg); (c) caerulein; (d) caerulein + C4S (30 mg/kg); (e) caerulein + C4S (60 mg/kg); (f) caerulein + C4S (120 mg/kg). Values are the mean $\pm$ SD of seven experiments and are expressed as O.D. at λ 450 nm/mg protein of nuclear extract (A) and as O.D. measured at λ 450 nm/mg protein (B). [°] $P < 0.001$ vs. control; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ vs. caerulein.

in lipase and amylase release by pancreatic cells was significant and dose-dependent.

NF- κ B DNA Binding Activity. Figure 1A shows the changes in NF- κ B p50/65 heterodimer translocation in the nuclear extract of pancreatic cells. NF- κ B DNA binding activity was present at very low levels in the pancreas of mice untreated with caerulein. In contrast, NF- κ B activation was markedly higher in caerulein-treated mice. The acute treatment of animals with C4S resulted in a significant

inhibition of NF- κ B DNA binding. The positive effect was seen for all the doses used (Fig. 1A).

Loss of I κ B α Protein in Hepatic Cell Cytoplasm. NF- κ B activation was also indirectly investigated by studying its inhibitory protein I κ B α in the acinar cell cytoplasm (Fig. 1B). Normal amounts of I κ B α protein were assayed in caerulein-untreated mice, while pancreatitis produced a significant reduction in I κ B α protein in the cytoplasm of pancreatic cells. The treatment of mice with

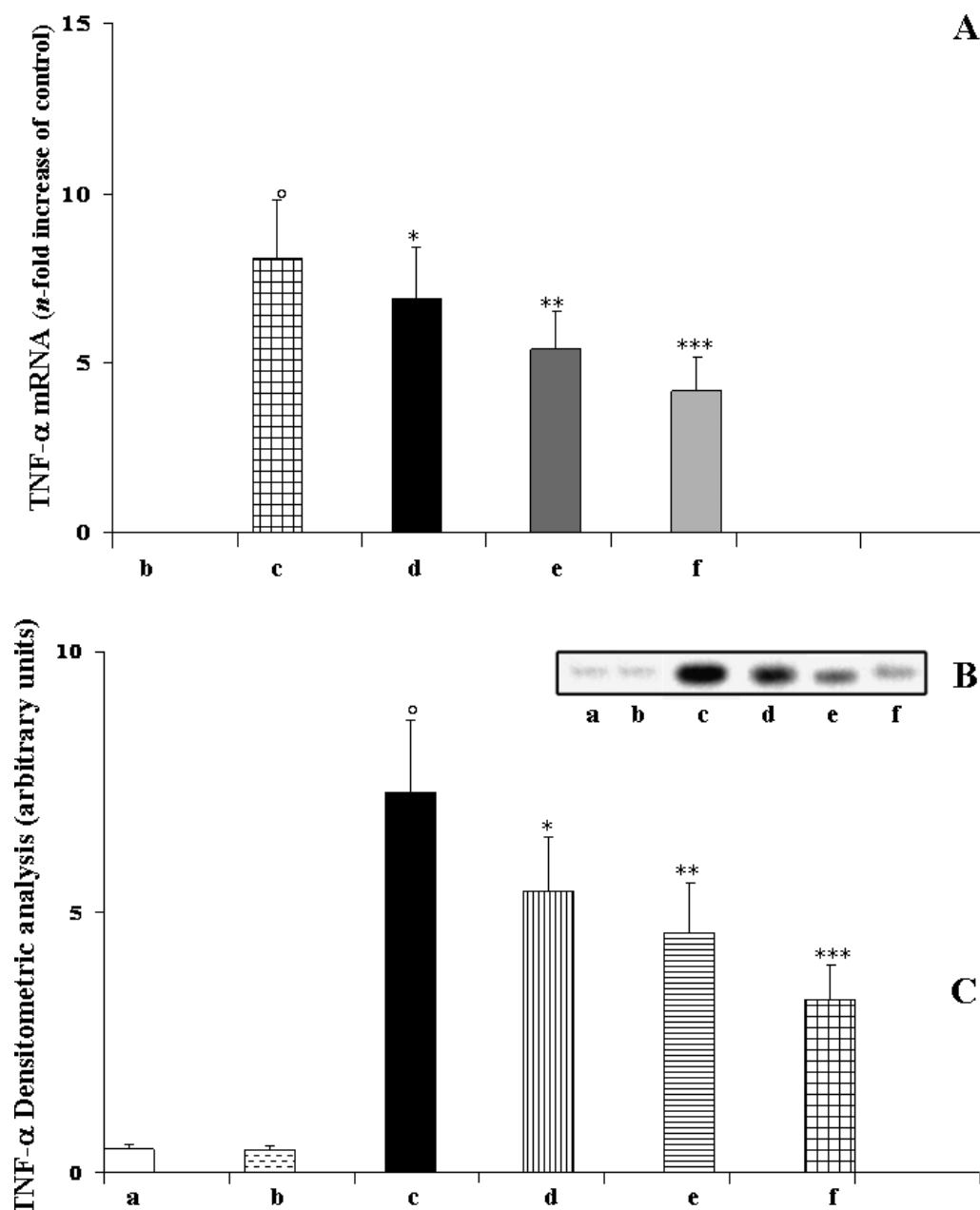


Figure 2. Effect of C4S treatment on pancreatic TNF- α mRNA expression (A) and related protein production (B, C) in pancreatic tissue of mice injected with Caerulein. (a) control; (b) C4S (120 mg/kg); (c) caerulein; (d) caerulein + C4S (30 mg/kg); (e) caerulein + C4S (60 mg/kg); (f) caerulein + C4S (120 mg/kg). Values are the mean $\pm$ SD of seven experiments and are expressed as the n-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for TNF- α protein levels. ° $P < 0.001$ vs. control; * $P < 0.05$, ** $P < 0.005$; *** $P < 0.001$ vs. caerulein.

C4S significantly blunted the phosphorylation of I κ B α protein in the cytoplasm of pancreatic cells.

TNF- α and IL-6 mRNA Expression and Protein Activity. TNF- α and IL-6 were evaluated because of their direct involvement in the prime and amplification of inflammation mechanisms (Figs. 2A, B, C and 3A, B, C). Quantification of gene expression (Figs. 2A, 3A) showed that the mRNA of these pro-inflammatory cytokines was not stimulated in caerulein-untreated mice. Otherwise, TNF- α and IL-6 expression were significantly upregulated

in the animals given only caerulein. The acute administration of C4S was able to reduce this mRNA increment. The rise in TNF- α and IL-6 expression, in mice with pancreatitis, correlated well with the increment in protein synthesis. This correlation also held for mice treated with C4S. In fact, the reduction in TNF- α and IL-6 expression was matched by a similar diminution in protein formation (Figs. 2B, C and 3B, C).

Caspase-3 and Caspase-7 mRNA Expression and Protein Activity. The apoptotic activators caspase-3

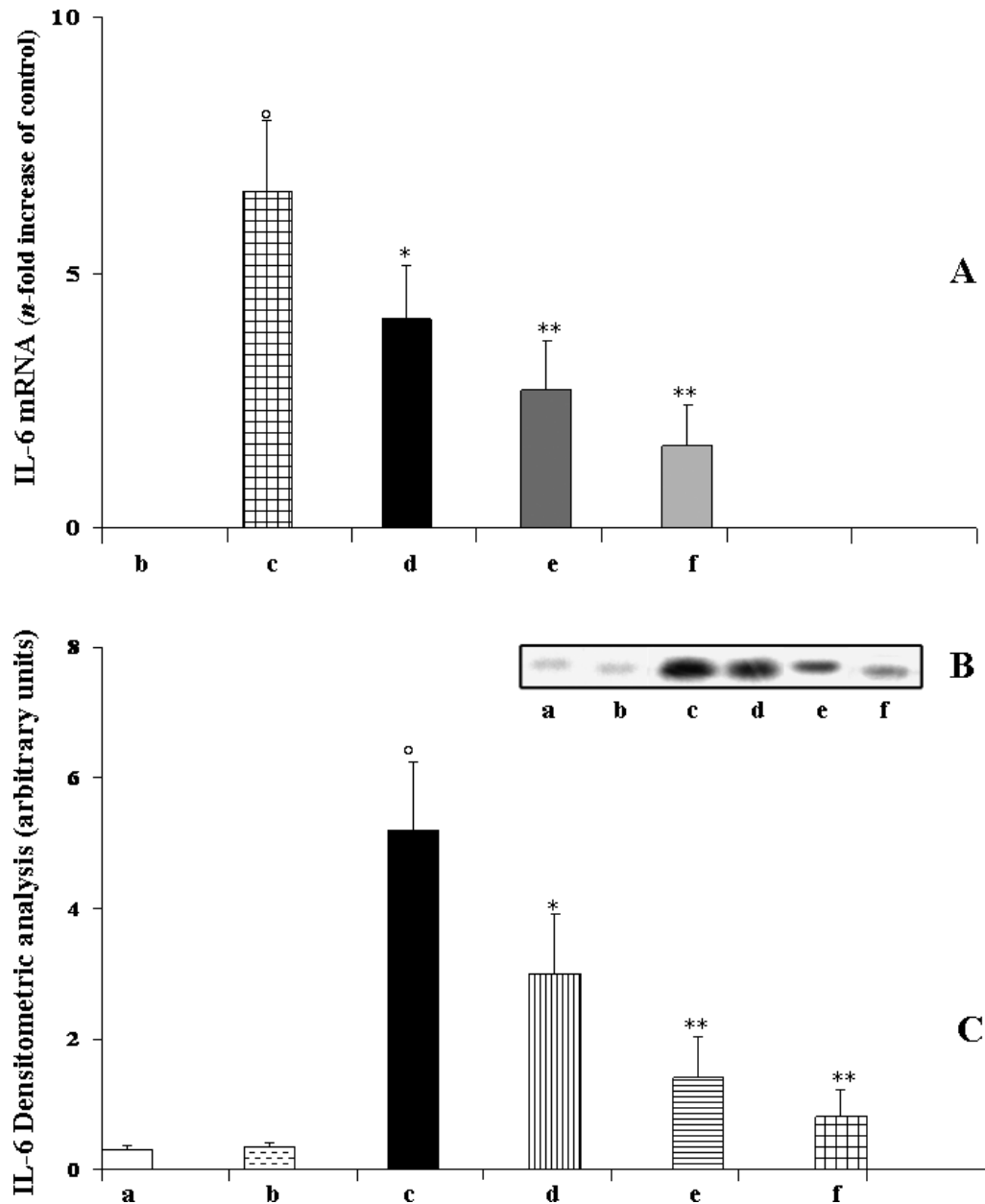


Figure 3. Effect of C4S treatment on pancreatic IL-6 mRNA expression (A) and related protein production (B, C) in pancreatic tissue of mice injected with Caerulein. (a) control; (b) C4S (120 mg/kg); (c) caerulein; (d) caerulein + C4S (30 mg/kg); (e) caerulein + C4S (60 mg/kg); (f) caerulein + C4S (120 mg/kg). Values are the mean $\pm$ SD of seven experiments and are expressed as the n-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for IL-6 protein levels. ^o $P < 0.001$ vs. control; * $P < 0.01$; ** $P < 0.001$ vs. caerulein.

and caspase-7 were evaluated by measuring mRNA expression and protein activity in order to estimate apoptosis in pancreatic tissue. Caspase-3 (Fig. 4A) and caspase-7 (Fig. 5A) mRNA evaluation and protein activity (Figs. 4B, C and 5B, C) in mice with pancreatitis showed a marked increase in the expression of the two apoptotic proteases. The treatment of animals with C4S was able to prevent caspase-3 and caspase-7 mRNA expression and new protein generation. Once again, caspase-3 and caspase-7 mRNA levels correlated well with the protein concentrations obtained by Western blot analysis.

8-IPE Evaluation. Determination of 8-IPE in the pancreatic tissue was carried out to estimate the degree of lipid peroxidation in pancreatic cell membranes (Table 2). Low levels of 8-IPE were measured in caerulein-untreated mice, whereas a significant increment in this marker was found in the pancreas of mice treated with caerulein alone. Acute treatment with C4S reduced 8-IPE levels by inhibiting membrane lipid peroxidation.

ELA Activity. Neutrophil infiltration to the damaged tissue contributes to the progression and the spread of inflammation (Table 2). Very low ELA activity was

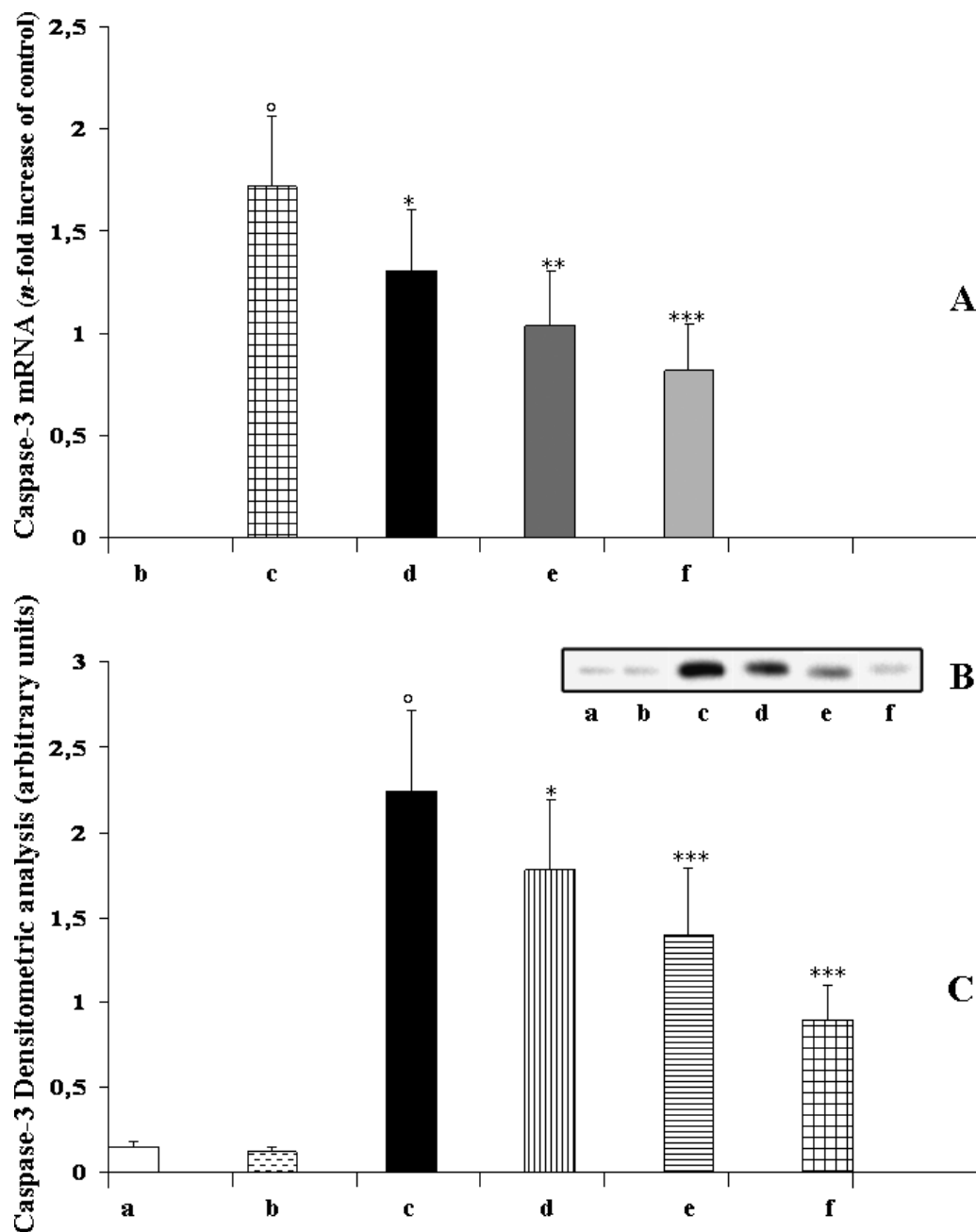


Figure 4. Effect of C4S treatment on pancreatic caspase-3 mRNA expression (A) and related protein production (B, C) in pancreatic tissue of mice injected with caerulein. (a) control; (b) C4S (120 mg/kg); (c) caerulein; (d) caerulein + C4S (30 mg/kg); (e) caerulein + C4S (60 mg/kg); (f) caerulein + C4S (120 mg/kg). Values are the mean $\pm$ SD of seven experiments and are expressed as the n-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for caspase-3 protein levels. ° $P < 0.001$ vs. control; * $P < 0.05$, ** $P < 0.005$; *** $P < 0.001$ vs. caerulein.

measured in caerulein-untreated mice. In contrast, elevated ELA activity levels were assayed in the group only given caerulein. The administration of C4S had a beneficial effect by reducing PMN accumulation in the pancreas as demonstrated by the drop in ELA activity levels.

Glutathione Reductase Activity. The concentrations of GR (Table 3) were assayed in order to evaluate the maintenance of the glutathione redox cycle downregulated by pancreatitis. A significant reduction in GR was observed in

the pancreatic tissue obtained from mice given only caerulein. Once again, the treatment with C4S restored activity levels of this important enzyme to a significant extent.

Histology. Figure 6 reports the histologic changes evaluated at the end of the experiment. Panels A and B show a representative pancreas section from a caerulein-untreated mouse. The cells appear integral, without edema and hemorrhagic alterations. Panel C presents a section of pancreas from a caerulein-treated mouse. Severe interstitial

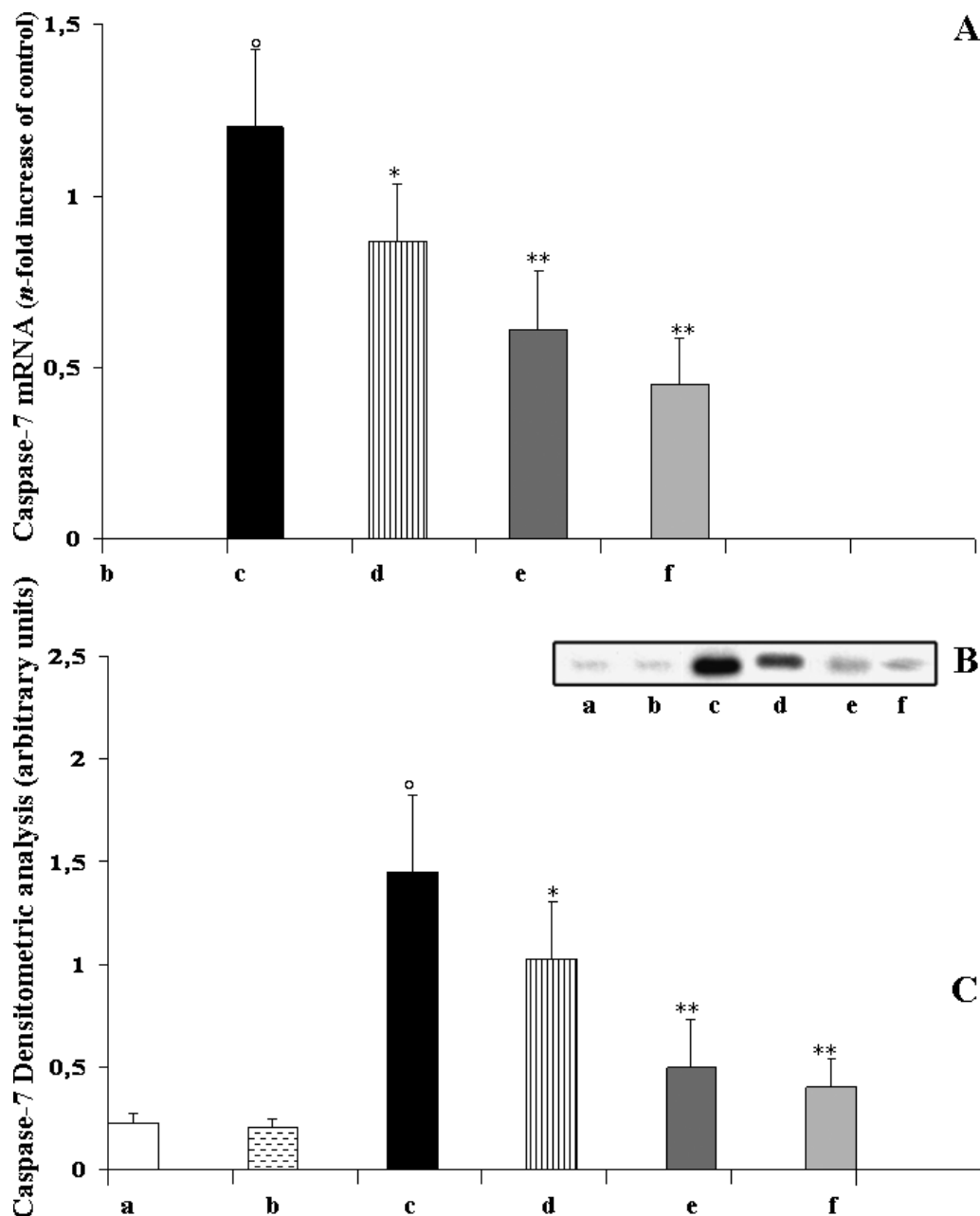


Figure 5. Effect of C4S treatment on pancreatic caspase-7 mRNA expression (A) and related protein production (B, C) in pancreatic tissue of mice injected with caerulein. (a) Control; (b) C4S (120 mg/kg); (c) caerulein; (d) caerulein + C4S (30 mg/kg); (e) caerulein + C4S (60 mg/kg); (f) caerulein + C4S (120 mg/kg). Values are the mean $\pm$ SD of seven experiments and are expressed as the n-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for caspase-7 protein levels. $^{\circ} P < 0.001$ vs. control; $^{*} P < 0.01$; $^{**} P < 0.001$ vs. caerulein.

edema and hemorrhagic signs with diffuse infiltrate of leukocytes, in particular a massive influx of inflammatory cells, pancreatic hyperplasia, and accumulation of abundant monocyte and PMN cells in the interstitial space are evident (indicated by arrow). In panels D, E, and F, representative sections of pancreases from mice receiving caerulein and C4S treatment show that a gradual reduction in pancreatic lesions occurred, with minimal evidence of inflammation or interstitial edema (indicated by arrows) (Fig. 6).

Discussion

The present study clearly demonstrates that exogenous C4S treatment has a therapeutic effect in the protection of the development of severe acute experimental pancreatitis in mice. The main findings of this study are the following: first, the antioxidant role of C4S in acute experimental pancreatitis, as previously reported (16, 19), is confirmed, although in the previous experiment a rat model was used. Secondly, C4S, at a range of doses, showed a marked inhibitory effect

Table 2. Effect of C4S^a Treatment on 8-Isoprostane Content and Elastase Levels in Pancreatic Tissue of Mice After Caerulein Injection

Experimental group	8-Isoprostane (mean $\pm$ SD) ^{a,b}	Elastase (mean $\pm$ SD) ^b
Control	1.5 $\pm$ 0.2*	0.25 $\pm$ 0.03*
C4S (120 mg/kg)	1.6 $\pm$ 0.3*	0.23 $\pm$ 0.03*
Caerulein	44.7 $\pm$ 7.9*	1.98 $\pm$ 0.43*
Caerulein + C4S (30 mg/kg)	29.2 $\pm$ 7.0**	1.20 $\pm$ 0.34**
Caerulein + C4S (60 mg/kg)	24.1 $\pm$ 6.1***	0.88 $\pm$ 0.27***
Caerulein + C4S (120 mg/kg)	18.3 $\pm$ 5.7***	0.65 $\pm$ 0.22***

^a C4S, chondroitin-4-sulphate; SD, standard deviation.

^b Values are the mean $\pm$ SD of seven experiments and are expressed as pg/mg protein for 8-isoprostane and as fluorescence arbitrary units at Ex 480 nm and Em 520 nm/mg protein for elastase.

* $P < 0.001$ vs. control; ** $P < 0.005$; *** $P < 0.001$ vs. caerulein.

on NF- κ B and caspase activation. The reduction/inhibition of these mechanisms led to improved biochemical and histologic parameters in pancreatic mice.

Pancreatic cells produce large amount of ROS at an early stage of acute pancreatitis (20). ROS directly attacks lipids, proteins in the biological membranes, and other biological structures very close to the site of their production. Under normal conditions, a natural system of scavengers and antioxidants counteracts the cytotoxicity of ROS generated by molecular oxygen in the mitochondria. During acute pancreatitis, the production of ROS is greatly increased by activated leukocytes, and intrinsic defense mechanisms are able to bring about changes in the cytoskeleton and membrane of acinar cells. ROS and activated pancreatic enzymes that are leaked from the disrupted cells also damage capillary endothelium leading to increased capillary permeability and edema (2). It is also known that pro-inflammatory and cytotoxic cytokines play

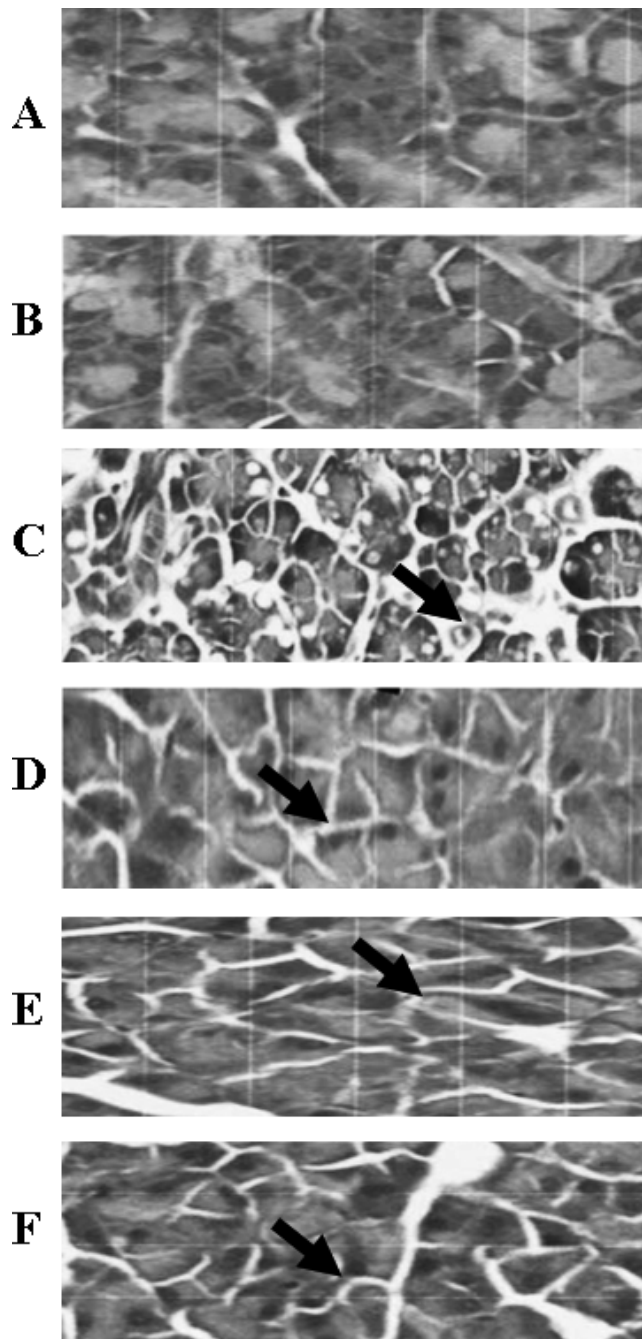
Table 3. Effect of C4S^a Treatment on Glutathione Reductase Activity in Pancreatic Tissue of Mice After Caerulein Injection

Experimental group	Glutathione reductase (mean $\pm$ SD) ^{a,b}
Control	1.89 $\pm$ 0.34*
C4S (120 mg/kg)	1.86 $\pm$ 0.31*
Caerulein	0.57 $\pm$ 0.11*
Caerulein + C4S (30 mg/kg)	0.91 $\pm$ 0.16**
Caerulein + C4S (60 mg/kg)	1.23 $\pm$ 0.18**
Caerulein + C4S (120 mg/kg)	1.48 $\pm$ 0.21**

^a C4S, chondroitin-4-sulphate; SD, standard deviation.

^b Values are the mean $\pm$ SD of seven experiments and are expressed as mUnits/mg protein.

* $P < 0.001$ vs. control; ** $P < 0.001$ vs. caerulein.

**Figure 6.** Effect of C4S on histologic analysis of pancreatic tissue from an untreated mouse. (A) control; (B) C4S (120 mg/kg); (C) caerulein; (D) caerulein + C4S (30 mg/kg); (E) caerulein + C4S (60 mg/kg); (F) caerulein + C4S (120 mg/kg). H&E $\times 100$.

an important role in the cell injury during acute pancreatitis (21, 22). Even though the relationship between oxidative stress, cytotoxic cytokines, and acinar cell injury has not been fully clarified, NF- κ B is considered to play a pivotal role at this stage (23). The characteristic oxidative burst induced by caerulein in pancreatic cells markedly stimulates NF- κ B translocation into the nucleus (23). The synthesis of ROS, in fact, subsequently activates NF- κ B, which has been known to contribute to vicious cycles of inflammatory

reactions (24). Overactivation of the fine-tuned apoptotic process can lead to significant acinar cell damage. ROS can also play significant roles in promoting apoptosis (7). It has been reported that molecules capable of scavenging ROS may reduce NF- κ B and apoptosis activation in acute pancreatitis (5, 6, 9, 10).

Chondroitin sulphate (CS), a complex GAG extracted and purified from various tissues, is a ubiquitous component of all connective tissue extracellular matrix (ECM), where it serves a number of functions mainly covalently attached to proteins in the form of proteoglycans (PGs). CS consists of alternating disaccharide units of glucuronic acid and galactosamine and is attached to serine residues of the protein cores *via* a tetrasaccharide linkage (25). Despite the simplicity of the backbone structure, the CS molecule is complex enough to carry biological information and thus to affect many biological functions. In recent years, CS has become a focus of attention by virtue of its important roles in wound healing: promoting neurite outgrowth, axonal regeneration, cell adhesion, cell division, and the regulatory roles of growth factors (25).

In our study, the treatment of acute pancreatitis in mice with various doses of C4S was able to prevent cell damage induced by cerulein treatment. In addition to the antioxidant effect of this GAG, the focus of this study was the C4S activity on NF- κ B and apoptosis modulation. We hypothesize that the inhibition of NF- κ B DNA binding to the nucleus is probably the consequence of C4S-reduced ROS production in pancreatic tissue. NF- κ B activation requires sequential phosphorylation and degradation of I κ B α , which disappears from the cytoplasm in the end. Since ROS are able to activate this pathway, C4S also prevents the loss of the inhibitory protein from the cytoplasm by preventing ROS generation. Although several papers have reported that CS may also directly inhibit NF- κ B activation and apoptosis (26, 27), we suggest that the same concept hypothesized for NF- κ B DNA binding exerted by ROS may be extended to apoptosis activation. In fact, restricted ROS production was able to reduce the caspase activation pathway. C4S thereby reduced damage by not only reducing ROS generation in acinar cells, but also by inhibiting NF- κ B and apoptosis activation that contribute greatly to exacerbate pathological conditions in acute pancreatitis. In addition, it is widely known that oxidative stress produces a series of reactive intermediates such as H₂O₂, O₂⁻, OH $\cdot$, lipid peroxides, and NO, whose chemical action may directly damage the cell or may produce injury indirectly by activating a multitude of other different mechanisms, including NF- κ B and caspase activation. Both these mechanisms disrupt the cell by activating inflammatory processes or *via* apoptosis. Thus, the oxidative damage overall is due to both direct and indirect mechanisms. Previously published data suggest that ROS are involved in the NF- κ B and apoptosis activation during inflammation and particularly in acute pancreatitis (23). Therefore, antioxidants that, for instance, prevent lipid peroxidation by reducing the detrimental OH $\cdot$ action, have

no effect on H₂O₂ or O₂⁻ production. Direct damage to the cellular membrane is thereby prevented. However, NF- κ B and caspase activation may be induced by H₂O₂ or O₂⁻. In such cases, these antioxidants have no effect on NF- κ B and apoptosis induction during the oxidative process. The same concept may be extended to antioxidants that, for instance, scavenge only H₂O₂ or other specific ROS. As regards C4S, it has been hypothesized that its antioxidant effect is exerted by chelating transition metal ions. Since HaberWeiss and Fenton reactions are primed by transition metal ions such as Fe⁺⁺, and the effect of the chelation of this metal ion reduces oxidative stress by eliminating the product of this reaction, it is reasonable to hypothesize that it is more efficient to act by blocking this reaction rather than scavenging the reactive activity of the products formed. C4S activity would therefore be more efficient than a conventional antioxidant because the inhibition of the oxidative burst does not prime NF- κ B and apoptosis. However, this evidence has yet to be fully confirmed since other studies have reported that the antioxidant activity of C4S derives from the direct neutralization of ROS due to its chemical interaction, mainly with the OH $\cdot$ radical (28, 29). If this were so, the antioxidant effect of C4S would be independent from NF- κ B and caspase activation. However, the treatment of mice with C4S was able to protect acinar cells from oxidative injury and also to inhibit NF- κ B DNA binding and apoptosis *via* two separate mechanisms. This study is a further confirmation that the antioxidant effect of C4S is due to the blocking of Haber-Weiss and Fenton's reactions by metal ions chelation. Nevertheless it does not exclude the possibility that C4S may exert a direct effect on NF- κ B and apoptosis inhibition.

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