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Article

# Nanoceria Ameliorates Fibrosis, Inflammation, and Cellular Stress in Experimental Chronic Pancreatitis

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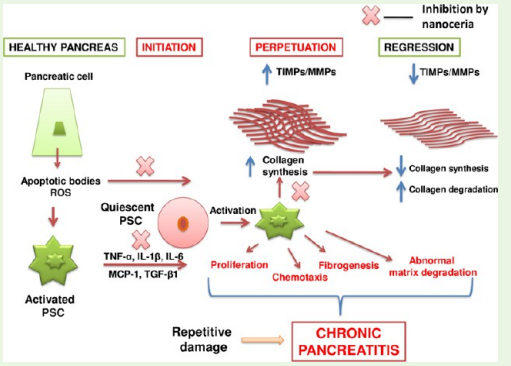
**ABSTRACT:** Chronic pancreatitis (CP) is an inflammatory, irreversible disorder of the pancreas which leads to organ atrophy and poses high risk for the development of pancreatic cancer. Given the lack of clinically approved therapy, we explored the pharmacological potential of the nanoparticles of cerium oxide (nanoceria, NC) against animal models of CP. Nanoceria ameliorated the features of CP as evident from biochemical parameters. It inhibited the inflammatory cytokines and chemokines by abrogation of macrophage signaling. Further, NC attenuated the fibrogenesis by inhibition of TGF-$\beta$ signaling, endoplasmic reticulum stress, and epithelial-to-mesenchymal transition. Our findings reveal the anti-CP potential of the novel redox regenerative nanoceria against two models of CP.




**KEYWORDS:** *nanoceria, chronic pancreatitis, pancreatic fibrosis, pancreatic stellate cells, inflammatory signaling*



## 1. BACKGROUND

Chronic pancreatitis (CP) is an inflammatory disorder of the pancreas characterized by persistent injury response leading to excessive extracellular matrix (ECM) deposition[1] with a significantly high risk of transition to pancreatic cancer.[2] The literature suggests high predisposition of chronic smokers and alcoholics (70%) to CP and higher incidence among men compared to women, although off late contrasting trends were reported.[3] CP is recognized by progressive fibrosis of the pancreas, pain, and exocrine and endocrine pancreatic dysfunction. The clinical manifestations of CP include recurring attacks of acute pancreatitis initially, pain, scarring of pancreas tissue, calcification, and impaired glucose homeostasis during the mature stage.[3,4]

The hallmark feature of CP is the persistent injury to the exocrine pancreas characterized by inflammation, resolution, and the regeneration which ultimately causes incremental damage to the pancreas. It is recognized by acinar cell atrophy, and activation of quiescent pancreatic stellate cells (PSCs) to activated PSCs leads to ECM deposition; however, the underlying pathological mechanisms of CP remain elusive.[5] The current consensus on the pathology of CP suggests the intertwined role of oxidative stress, endoplasmic reticulum (ER) stress, inflammation, epithelial-to-mesenchymal-transition (EMT), and fibrosis.[6]

Oxidative stress and ER stress play a central role in the pathology of CP and drive the chronic inflammatory response. The macrophages, neutrophils, and other inflammatory cells are responsible for the chronic inflammation of CP.[7] These cells secrete proinflammatory and profibrotic mediators which are responsible for the EMT of PSCs. Transforming growth factor-$\beta$ (TGF-$\beta$) acts as the master regulator of the fibrogenic response, mediates phosphorylation of the Smad proteins, and increases the synthesis of the matrix proteins like collagen type I, collagen type III, and fibronectin.[8] The pharmacological inhibition of TGF-$\beta$ has been shown to protect against pancreatic fibrosis upon chronic pancreatic injury.[9] Despite tremendous developments over past years, disease pathogenesis of CP is not completely elucidated; as a result, there is no clinically approved drug for the treatment of CP.

Cerium oxide nanoparticles or nanoceria (NC) are a well-known biomaterial with promising redox regenerative capabilities.[10] It has been reported to mimic superoxide dismutase (SOD) and catalase, two important physiological antioxidant enzymes owing to the differential ratio of $Ce^{+3}$ and $Ce^{+4}$ oxidation states.[11] Further, it has been shown to upregulate Nrf2 and downregulate the expression of NF$\kappa$B in various chronic inflammatory disorders including diabetes and metabolic disorders.[12] We have earlier reported that NC exhibits promising protective activity against cerulein induced AP by virtue of its SOD mimetic and anti-inflammatory activity.[13] These results formed the foundation for the current

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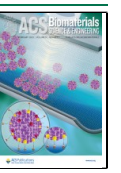




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work wherein we probed the detailed antifibrotic efficacy of NC against two animal models of CP with prominent pancreatic fibrosis.[14] The effects of NC on TGF-$\beta$ signaling pathway, fibrogenic signaling, and ER stress were evaluated with emphasis on its ability to halt fibrosis progression.

## 2. METHODS

**2.1. Chemicals and Reagents.** The following chemicals of molecular biology grade were purchased from Sigma-Aldrich, USA: bromophenol blue, direct red 80, glutathione, nanoceria, catalase, xylene, acetic acid, DPX mountant, Masson's trichrome staining kit, etc. L-arginine-HCl was purchased from SRL, Mumbai, India, and cerulein was procured from Ana Spec, France. All the other chemicals were of either molecular biology or analytic grade unless otherwise stated. The ELISA kits for cytokines (IL-1$\beta$, IL-6, IL-17, TNF-$\alpha$, and TGF-$\beta$1) were procured from Invitrogen, USA. The multiplex assay kit for an array of chemokines and cytokines was procured from Merck Millipore, USA. The primary antibodies for $\beta$-actin, collagen-3, $\alpha$-SMA, IL-1$\beta$, fibronectin (FN), *N*-cadherin, COX-2, and MMP2 were purchased from Santa Cruz Biotechnology, Santa Cruz, USA, whereas antibodies for CHOP, BiP, and calnexin were purchased from Cell Signaling Technology, USA. The primary antibody against NOX-4 and an ELISA kit for p65-NF$\kappa$B were purchased from Novus Biologicals, USA.

**2.2. Nanoparticle Characterization.** The nanoparticles were characterized using the important analytical techniques like FTIR, dynamic light scattering, powder XRD (pXRD), XPS, SEM, and TEM. The internalization of nanoparticles inside the Raw 264.7 macrophages was studied by TEM as per previous report.[13,15]

**2.3. Animals.** The L-arginine-induced CP was developed in male Wistar rats (200−250 g), and cerulein-induced CP was induced in male Swiss albino mice (20−25 g). The animals were purchased from Teena labs, Hyderabad, after approval from the Institutional Animal Ethics Committee (IAEC). The animals were humanely cared, maintained at optimal temperature and humidity, provided rodent food and purified water, and kept for 1 week acclimatization prior to starting the treatment with inducers and NC.

**2.4. Study Design.** The study was carried out in two animal models. The L-arginine-based CP study was carried out at two doses of NC (0.5 and 2 mg/kg) and consisted of four randomly divided animal groups ($n$ = 10): group 1: normal control, group 2: disease control, group 3: NC low dose (L-arginine-induced CP animals, 0.5 mg/kg NC, i.p., once daily for 9 weeks), and group 4: NC high dose (CP animals, 2 mg/kg NC, i.p., once daily for 9 weeks). The second study was carried out in cerulein-induced CP model with three groups ($n$ = 6): group 1: normal control, group 2: disease control, and group 3: NC (cerulein-induced CP animals, 2 mg/kg NC, i.p., once daily for 3 weeks). The L-arginine-induced CP was developed over a period of 9 weeks by giving weekly three times injections of L-arginine HCl (3000 mg/kg; i.p), which consisted of 7 weeks of inducer administration, followed by 2 weeks resolution period.[16] On the other side, the cerulein-induced CP was induced by five episodes of acute pancreatitis over 2 weeks, followed by 1 week resolution period. The blood glucose was measured using a digital glucometer, and the intraperitoneal glucose tolerance test was carried out by injecting 2 g/kg of glucose (i.p.) into animals after overnight fasting.[17] Blood glucose levels were measured at 0, 30, 60, and 90 min after glucose injection. Animals were euthanized and the pancreas were collected and properly stored for molecular biology and histological experiments.

**2.5. Estimation of Plasma and Tissue Biochemical Parameters.** The levels of important enzymatic markers of CP, that is, amylase and lipase, were measured as per the manufacturer's instructions by a colorimetric method using a commercial kit (Accurex Biomedical Private Limited, Mumbai, India), and the enzyme levels were expressed as international units per liter. The oxidative and nitrosative stress markers including malondialdehyde (MDA), glutathione (GSH), nitrite, catalase, and SOD in pancreas tissue were measured as per our earlier published protocols. The MDA content was estimated by carrying out TBARS method. 100 $\mu$L of tissue homogenate in ice-cold phosphate-buffered saline (PBS, pH 7.4) was mixed with 100 $\mu$L of SDS (8.1%), 750 $\mu$L of TBA (0.8%), and 750 $\mu$L of 20% glacial acetic acid (pH 3.5); the volume was made up to 2 mL with distilled water. Contents were boiled at 95 °C for 1 h and then cooled to room temperature and centrifuged to obtain supernatant, and the absorbance was measured at 532 nm. The amount of nitrite in the cell culture supernatant was estimated as described earlier.[18] The estimation of SOD was done by using a commercial kit by following the manufacturer's instructions. The catalase content was measured using PBS and $H_2O_2$ according to a previous method.[19] The pancreatic GSH content was measured using 0.05 mM Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) solution. Tissue supernatant was collected after thorough homogenization of pancreatic tissue in Tris buffer (pH 7.4), followed by addition of equal volumes of 10% trichloro acetic acid (TCA) for precipitation of proteins and centrifugation at 7,000 rpm at 4 °C for 5 min. GSH levels were calculated using a standard reference curve with reduced GSH. The absorbance was measured at 412 nm.

**2.6. Evaluation of Inflammatory Cytokine Levels and Multiplex-based Analysis of Proinflammatory Cytokines/Chemokines.** The proinflammatory cytokines and p65-NF$\kappa$B were measured in the pancreatic tissue lysates.[20] Briefly, the pancreas tissue was homogenized in RIPA buffer. The ELISA plates were coated overnight with the respective capture antibody, followed by washing (2x), blocking (1 h), and overnight incubation with samples and standards. Next, the plates were washed with buffer (3x), incubated with detection antibody (1 h), washed again (3x), incubated with HRP-linked antibody (30 min), washed (5x), and incubated with substrate solution (20 min), and finally, the reaction was stopped using 1M phosphoric acid. The results were expressed in pg/mg protein.[21] The simultaneous evaluation of levels of the panel of cytokines/chemokines was carried out by using a magnetic bead-based multiplex assay kit, and the analysis was carried out as per the manufacturer's instructions.

**2.7. Evaluation of Pancreatic Fibrosis by Hydroxyproline Assay.** The levels of hydroxyproline were measured as per the method reported by Woessner et al., with slight modifications. The pancreatic tissues were homogenized in PBS, and the protein content was assayed by Bradford reagent. The lysate was acid-hydrolyzed in 6M HCl for 90 min in an autoclave. Acid-digested tissue homogenate was oxidized by chloramine-T, followed by incubation for 20 min with Ehrlich's reagent. The sample absorbance was estimated at 550 nm, and the results were shown as $\mu$g hydroxyproline/mg protein.[22]

**2.8. Histological Evaluation and Immunohistochemical Analysis, Immunofluorescence.** The formalin-fixed pancreas tissues were used for the histological evaluation. Tissues were processed in the gradient of alcohol and embedded in paraffin wax. Then 5 $\mu$m sections were used for H&E staining to assess the pathological damage. Further, picrosirius red (PSR) staining and Masson's trichrome (MT) staining were performed as per established protocols to evaluate ECM deposition.[23] The pathological damage was observed using a light microscope, and the extent of fibrosis was quantified by Image J software.

The immunohistochemistry of the pancreatic tissue was carried out by activation of antigen sites using proteinase-K, followed by peroxidase deactivation by $H_2O_2$ and blocking by 3% bovine serum albumin. Then sections were incubated with primary antibody overnight, and the immunohistochemical positivity was developed using DAB chemistry.[24] On the other side, the immunofluorescence of the respective primary antibody against BiP was evaluated by using FITC-conjugated secondary antibody.[25]

**2.9. Immunoblotting.** The mechanistic effects of NC on CP were evaluated by immunoblotting performed as per previous reported methods.[13] The Image J software (NIH, USA) was used for the densitometric analysis of the blots. $\beta$-Actin was used as the house keeping protein for the normalization. The results were depicted as fold change with respect to normal control.

**2.10. Statistical Analysis.** Statistical analysis was performed on at least six samples per group, and the results were represented as mean


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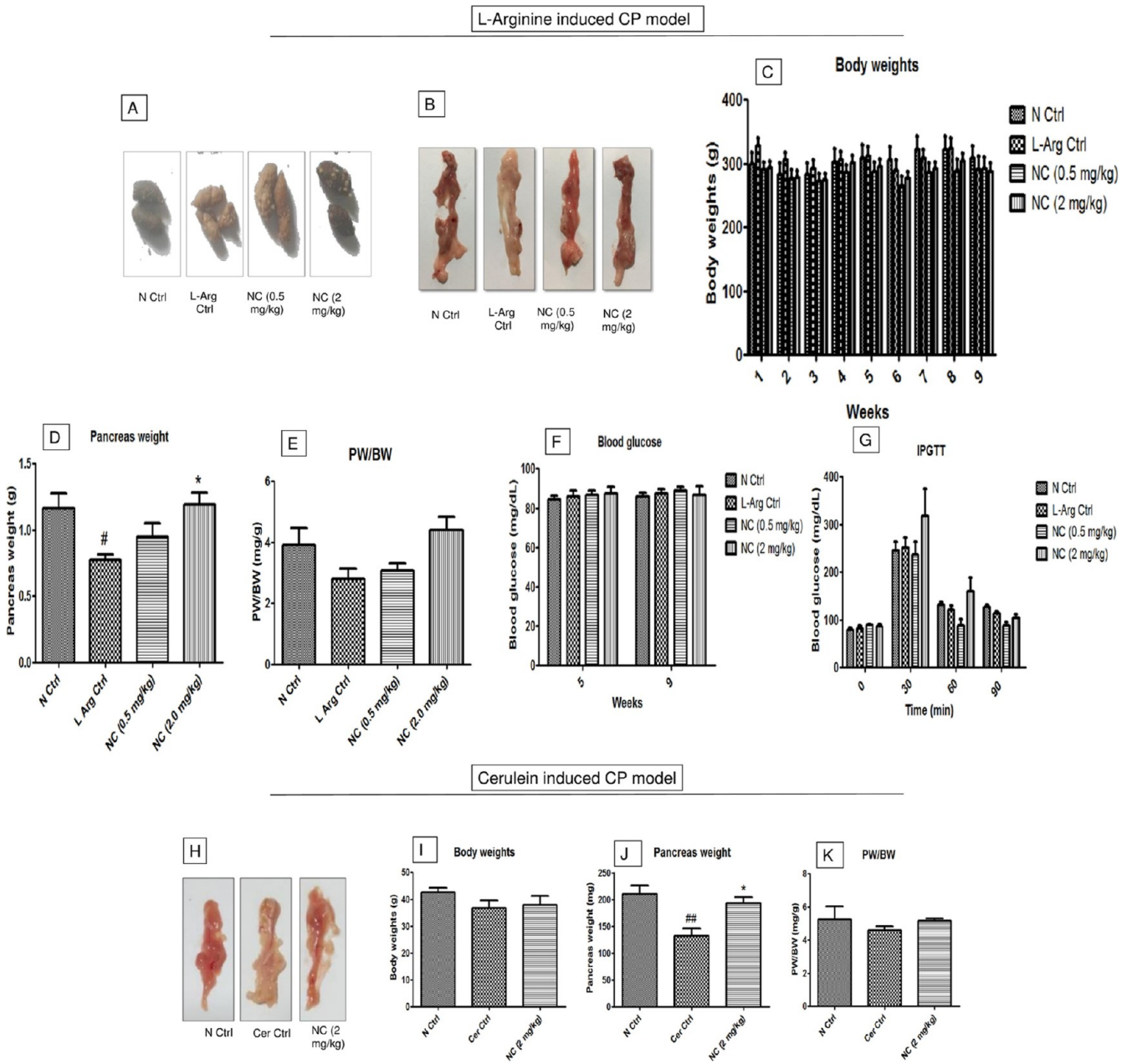


**Figure 1.** Effect of the pharmacological intervention of NC on various parameters of chronic pancreatitis. (A) Feces color, (B) pancreas morphology of L-arginine CP study, (C) body weights, (D) pancreas weight, (E) pancreas weight/body weight (PW/BW), (F) blood glucose, (G) IPGTT, (H) pancreas morphology of cerulein CP study, (I) body weights of cerulein CP study, (J) pancreas weight, and (K) pancreas weight/body weight (PW/BW). All the values are represented as mean ± SEM ($n = 6$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs N Ctrl; $^{*}p < 0.05$; $^{***}p < 0.001$, vs L-arg Ctrl/Cerulein Ctrl.

± standard error of mean (SEM). Tukey's multiple comparison test was used for post hoc analysis, and the variations among different groups were analyzed by one-way analysis of variance (ANOVA) using the Graph Pad Prism, version 5.0. The $P$ values < 0.05 were considered significant.

## 3. RESULTS

**3.1. NC Ameliorates the Biochemical Features and Oxidative Stress in Animal Models of Chronic Pancreatitis.** The characterization and internalization of NC are shown in the Supporting Information (Figure S1). The particle sizes were in the range of 100 ± 14 nm. We did not observe any pathological alterations in the hematological parameters in the studied animal groups in L-arginine-induced CP model as shown in Figure S2. NC intervention was found to ameliorate the biochemical features of CP. The fecal matter color was found to be slightly altered in the L-arginine-treated animals as shown in Figure 1A. The morphology of pancreas after study termination in both the models is shown in Figure 1B,H. Animal body weights in different animal groups of both the models are shown in Figure 1C,I. However, the pancreas weights were significantly reduced in the disease control animals, which might be due to gross pancreatic atrophy (Figure 1D,E,J–K). In contrast, NC intervention (2 mg/kg) led to significant improvement in pancreatic weights (Figure 1D,J). The results of pancreas weight/body weight index were non-significant (Figure 1E,K). Glucose levels and the glucose


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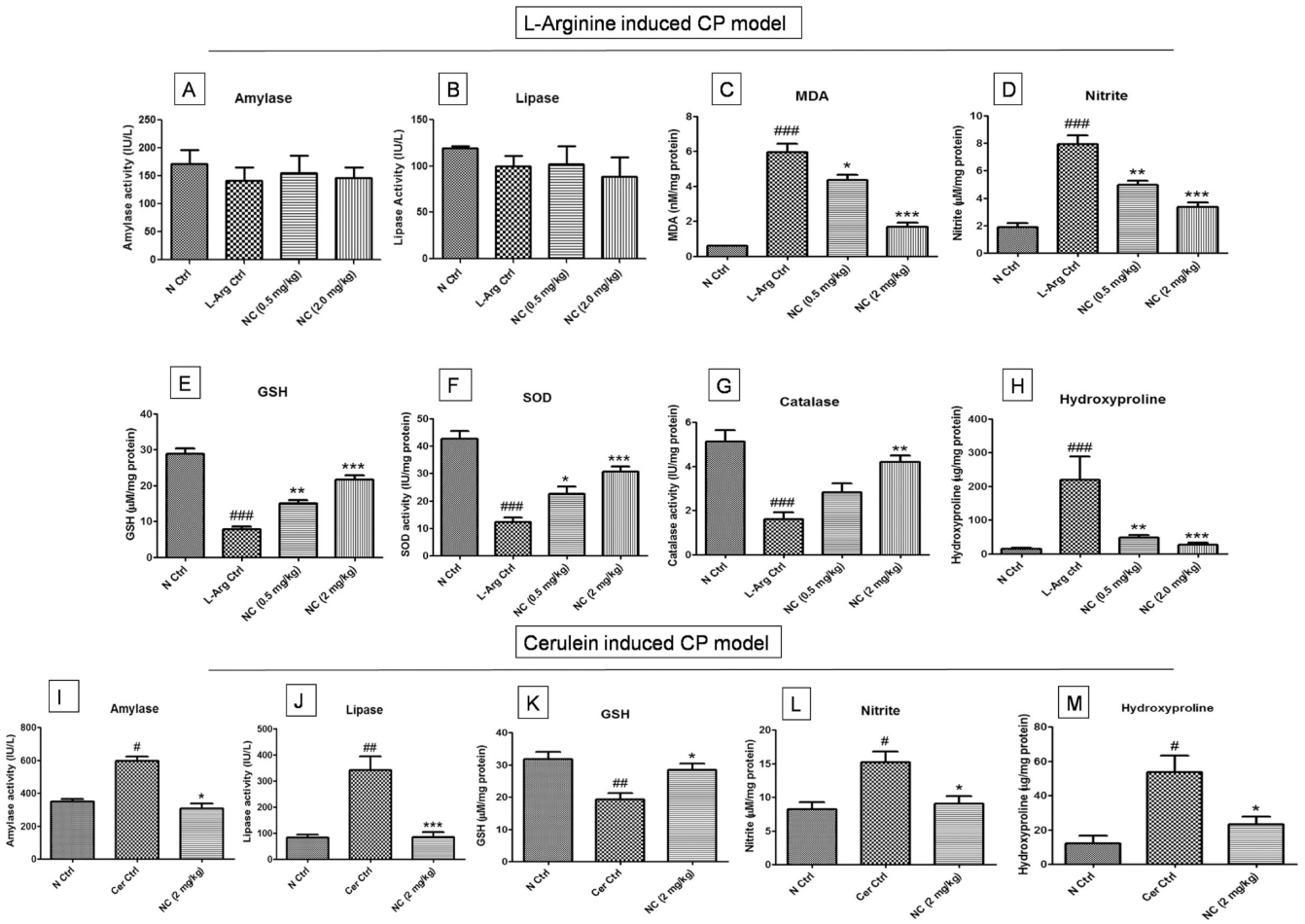


**Figure 2.** Effect of the pharmacological intervention of NC on biochemical markers of chronic pancreatitis. (A) Amylase, (B) lipase, (C) MDA, (D) nitrite, (E) GSH, (F) SOD, (G) catalase, (H) hydroxyproline, (I) amylase, (J) lipase, (K) GSH, (L) nitrite, and (M) hydroxyproline. All the values are represented as mean ± SEM ($n$ = 6). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs N Ctrl; $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, vs L-arg Ctrl/Cerulein Ctrl.

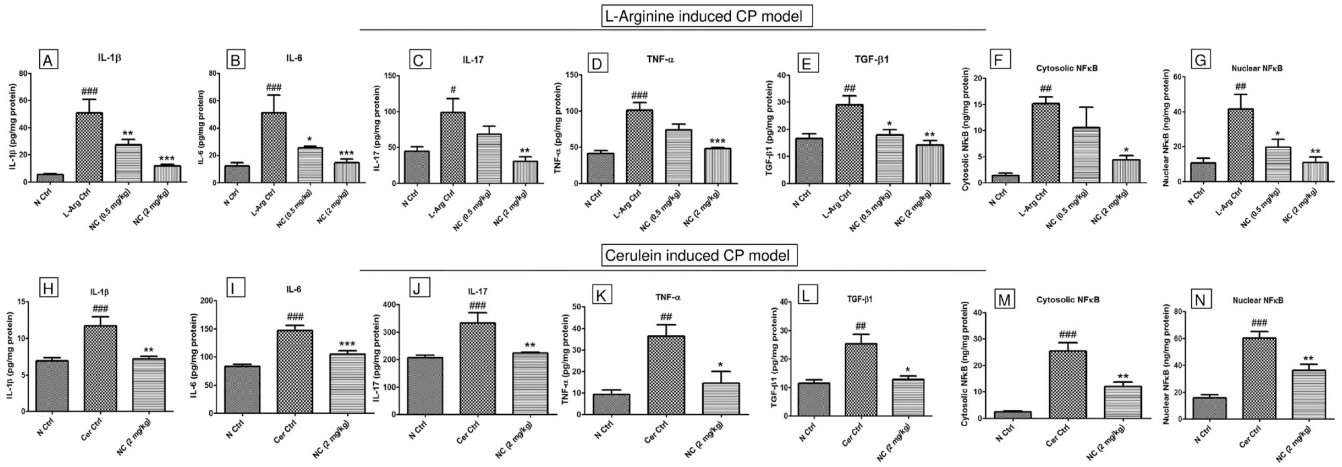


**Figure 3.** Effect of the pharmacological intervention of NC on inflammatory cytokines. (A) IL-1$\beta$; (B) IL-6; (C) IL-17, (D) TNF-$\alpha$, (E) TGF-$\beta$1, (F) cytosolic NF$\kappa$B, (G) nuclear NF$\kappa$B, (H) IL-1$\beta$, (I) IL-6; (J) IL-17, (K) TNF-$\alpha$, (L) TGF-$\beta$1, (M) cytosolic NF$\kappa$B, and (N) nuclear NF$\kappa$B. We observed that there was a significant elevation in the levels of the inflammatory cytokines in the disease control groups of both the animal models. However, NC intervention significantly reduced the levels of the studied inflammatory cytokines. Values are represented as mean ± SEM ($n$ = 5). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs N Ctrl; $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, vs L-arg Ctrl/Cerulein Ctrl.

clearance were found to be normal in all the treated groups, indicating insignificant damage to the endocrine gland (Figure 1F,G). We found insignificant changes in amylase and lipase levels in the L-arginine-induced CP model (Figure 2A,B), whereas the levels were significantly higher in cerulein-challenged animals (Figure 2I,J). The animals treated with



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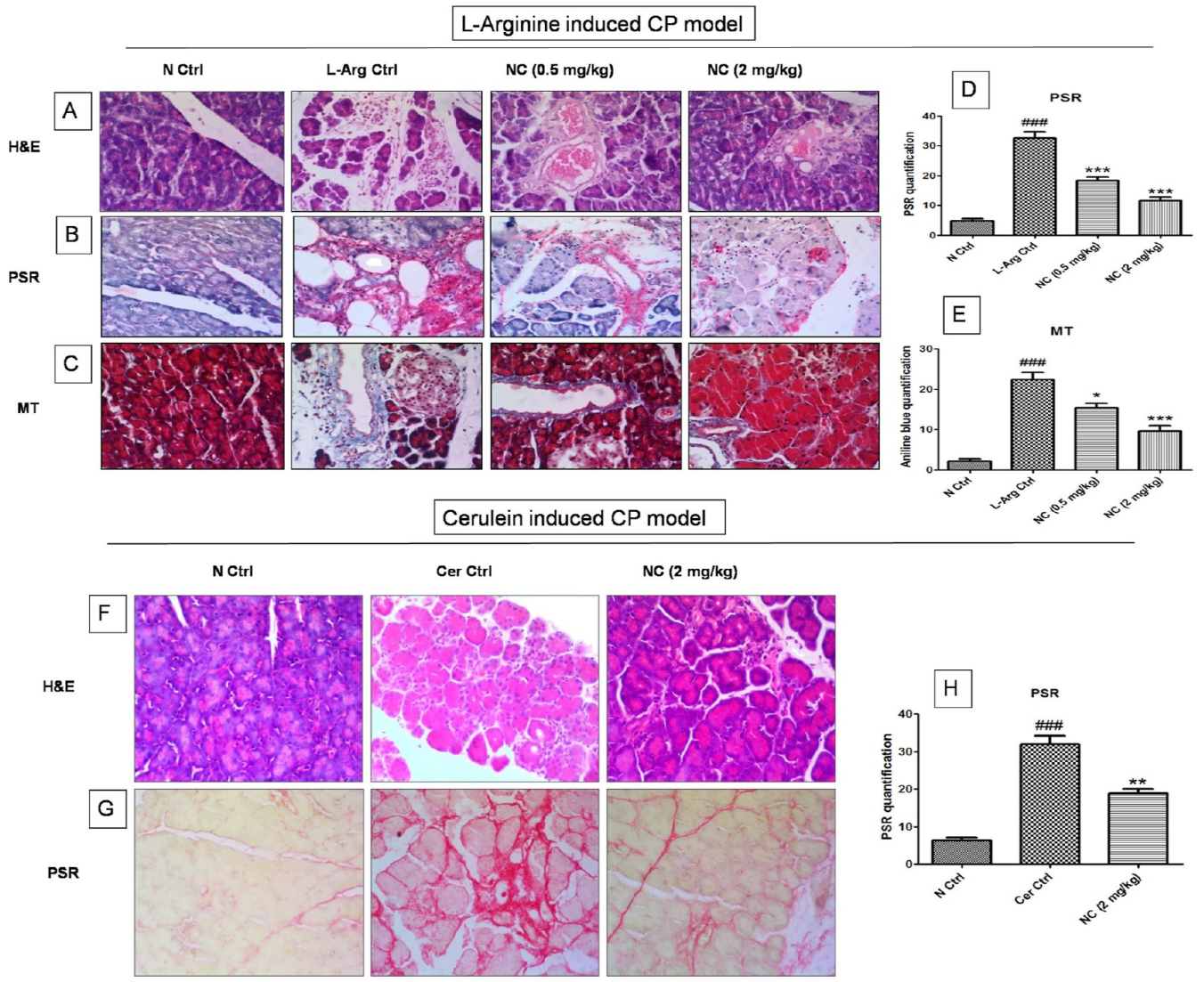


**Figure 4.** Effect of NC intervention on histological features in animal models of CP. (A) H&E staining; (B) PSR staining; (C) MT staining; (D,E) quantification of collagen by PSR and MT staining, respectively; (F) H&E staining of cerulein-induced CP study groups; (G) PSR staining and (H) quantification of collagen by PSR. All the values are represented as mean ± SEM ($n$ = 6). $^{###}p < 0.001$ vs N Ctrl; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs L-arg Ctrl/Cerulein Ctrl.

NC exhibited reduced levels of amylase and lipase in the cerulein-induced model, whereas the levels were similar in different treatment groups in L-arginine model (Figures 1A,B and 2I,J). Furthermore, oxidative and nitrosative stresses play a crucial role in the origin and progression of CP. In the L-arginine-induced CP model, we found a significant increase in the levels of MDA (Figure 2C) and nitrite (Figure 2D) in pancreas homogenates, whereas the levels of glutathione (Figure 2E), SOD (Figure 2F), and catalase (Figure 2G) were significantly reduced in the disease control group pancreatic homogenates compared to the normal control group. On the other side, the treatment with NC protected the animals from the oxidative and nitrosative stresses as evident from the reduced levels of MDA (Figure 2C) and nitrite (Figure 2D) and increased levels of GSH (Figure 2E), SOD (Figure 2F), and catalase (Figure 2G). Similarly, the levels of GSH were significantly increased (Figure 2K) and nitrite levels were significantly decreased (Figure 2L) in the NC-treated animals in cerulein model. Further, the levels of hydroxyproline were found to be significantly increased in both the models (Figure 2H,M), whereas NC intervention led to a significant reduction the levels of hydroxyproline, an important collagen marker (Figure 2H,M). These biochemical findings provide insights into the potential of NC to abrogate the pathological phenotype of CP and reduction of associated clinically relevant biomarkers.

**3.2. NC Abrogates the Inflammatory and TGF-$\beta$ Signaling in Animal Models of CP.** Chronic persistent inflammation is central to the pathology of CP. Hence, we measured the levels of inflammatory cytokines in the CP animals. We found significantly increased levels of IL-1$\beta$, IL-6, IL-17, TNF-$\alpha$, and TGF-$\beta$1 (studied by ELISA) in the disease control group of both the animal models (Figure 3). NC intervention led to promising anti-inflammatory activity as shown by reduction in the levels of IL-1$\beta$ (Figure 3A,H), IL-6 (Figure 3B,I), IL-17 (Figure 3C,J), TNF-$\alpha$ (Figure 3DK), and TGF-$\beta$1 (Figure 3E,L) in both the animal models. The levels of cytosolic (Figure 3F,M) and nuclear p65-NF$\kappa$B (Figure 3G,N) were found to be significantly increased in the disease control group of both the animal models. In contrast, the levels


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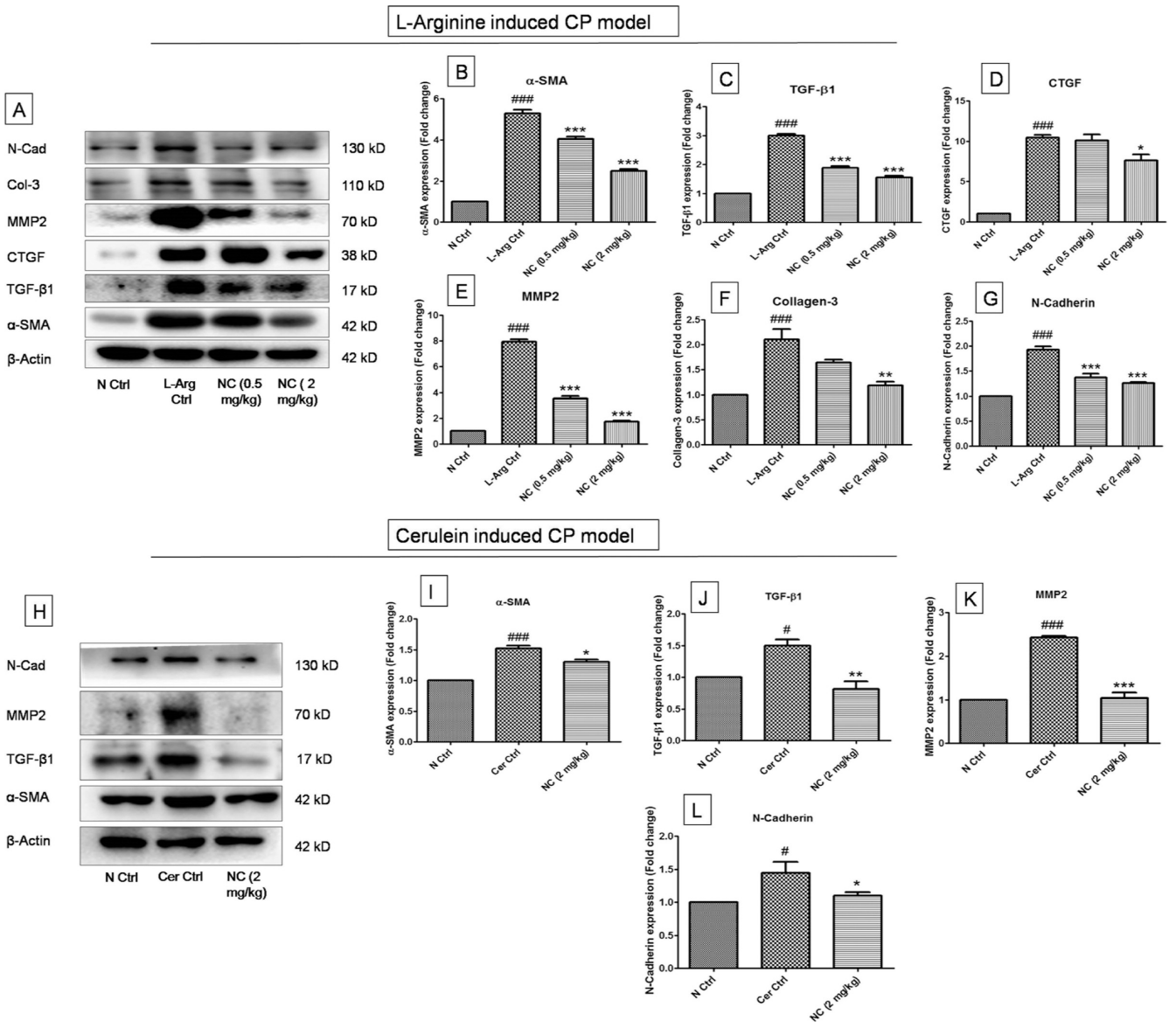


**Figure 5.** Effects of NC on fibrotic signaling in animal models of CP. (A) Blot panel of L-arginine-induced CP study groups, protein expression of (B) $\alpha$-SMA, (C) TGF-$\beta$1, (D) CTGF, (E) MMP2, (F) Collagen-3, (G) *N*-Cadherin, (H) blot panel of cerulein-induced CP study groups, (I) $\alpha$-SMA, (J) TGF-$\beta$1, (K) MMP2, and (L) *N*-Cadherin in whole-pancreas homogenates of different treatment groups, as determined by SDS-PAGE and western blotting. The results clearly show the upregulation of the expression of fibrotic proteins like *N*-Cadherin, Col-3, MMP2, CTGF, TGF-$\beta$1, and $\alpha$-SMA in the disease control groups as compared to the normal control group. In contrast, the expression of studied proteins was markedly reversed by NC treatment. Values are represented as mean ± SEM. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs N Ctrl; $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ vs L-arg Ctrl/Cerulein Ctrl.

of cytosolic (Figure 3F,M) as well as nuclear p65-NFκB (Figure 3G,N) were significantly decreased in the NC-treated animals in both the models, indicating the partial role of NFκB inhibitory activity of NC behind the observed protective effects.

Furthermore, we probed the detailed effect of NC on the macrophage-mediated chemokine and cytokine signaling using a magnetic bead-based cytokine array kit. The levels of the studied inflammatory cytokines and chemokines were markedly increased in the disease control groups as shown in supplementary Figure S3A−X. On the other side, NC proved beneficial as evident from the reduction in the levels of an array of chemokines and cytokines as shown in supplementary Figure S3A−X.

The TGF-$\beta$ signaling acts as the master regulator of fibrosis and EMT via both the canonical and non-canonical pathways. The phosphorylation of Smad proteins is regulated by TGF-$\beta$ and is involved in the canonical signaling, whereas the Smad-independent pathway is characterized by Akt and Erk phosphorylation. We studied the effects of NC on TGF- $\beta$ signaling by a magnetic bead-based cytokine array kit. The expression of TGF-$\beta$ type II receptor was markedly increased in the disease control group of both the models. The treatment with NC exhibited promising antifibrotic effects as demonstrated by reduction in the expression of TGF-$\beta$ type II receptor (Figure S4A,G). NC was found to attenuate both the Smad-dependant and Smad-independent pathways as observed by reduced levels of p-Smad-2, p-Smad-3, Smad-4, p-Erk 1/2, and p-Akt (Figure S4). These findings clearly point to the


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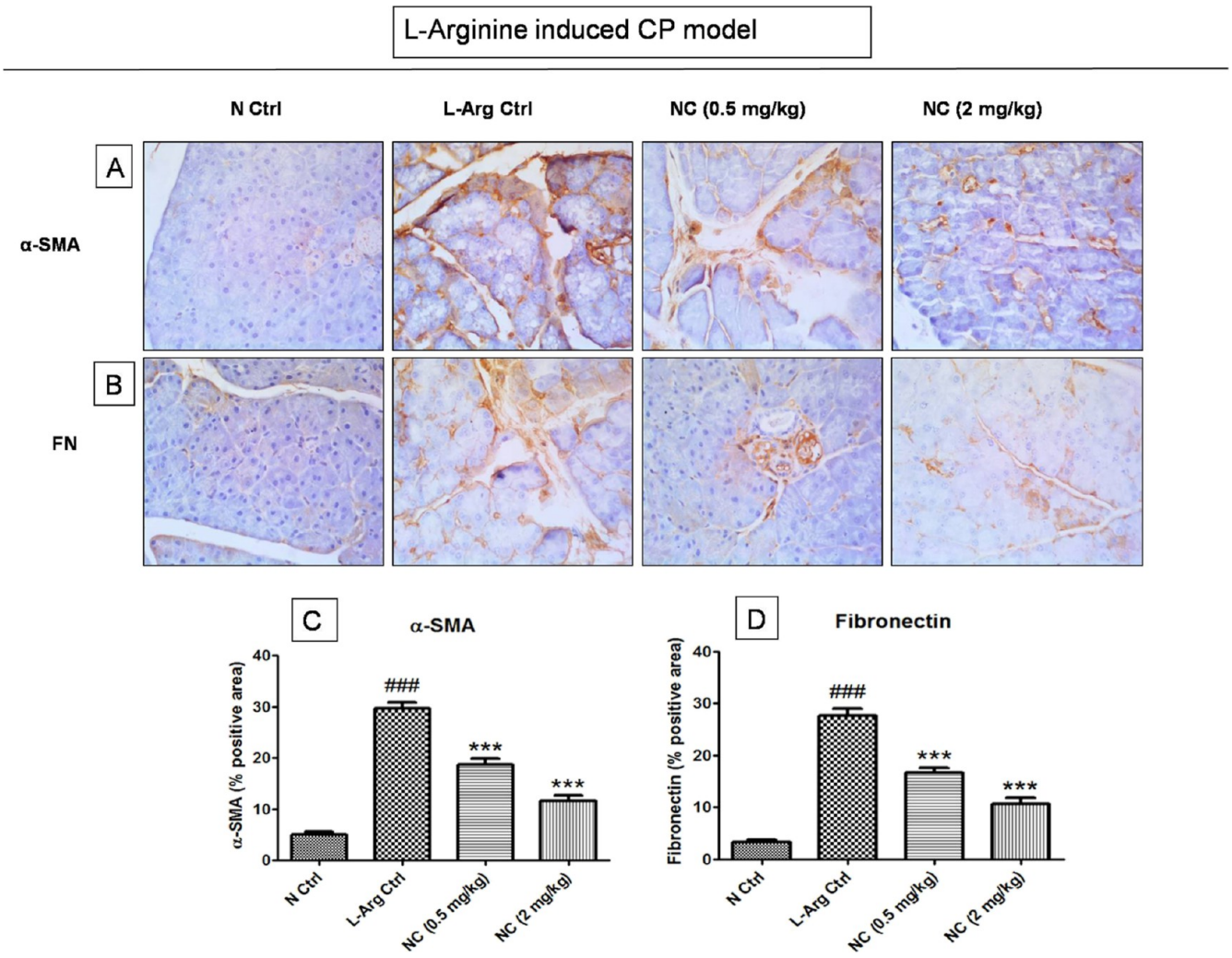


**Figure 6.** Effect of NC intervention on immunohistochemical markers of EMT in L-arginine-challenged rats. (A) $\alpha$-SMA, (B) fibronectin (FN), (C) quantification of $\alpha$-SMA, and (D) quantification of fibronectin. We found that the expressions of the fibrogenic proteins $\alpha$-SMA and fibronectin were significantly upregulated in the L-arginine-induced chronic pancreas disease control pancreas specimens as evident from the higher brown-colored stain. In contrast, pancreas specimens of animals treated with NC showed significantly lower expression of these fibrotic proteins, indicating potential antifibrotic activity of NC. Values are represented as mean ± SEM. $^{###}p < 0.001$, vs N Ctrl; $^{***}p < 0.001$, vs L-arg Ctrl.

potent anti-inflammatory and antifibrotic effects of NC in halting pathology of pancreatic fibrosis during CP via attenuation of TGF-$\beta$ signaling in CP.

**3.3. NC Reduces Extracellular Matrix Deposition, Suppresses Fibrotic Signaling, and Halts the Progression of Epithelial-To-Mesenchymal Transition during CP.** CP is recognized by activation of PSCs, the cells mainly responsible for excessive ECM deposition. Herein, we performed the histological analysis of pancreas by H&E staining and found a disrupted pancreatic architecture in the disease control pancreas in both the models, whereas NC treatment notably protected the pancreas from pathological L-arginine and cerulein-induced damage (Figure 4A,F). Furthermore, the ECM deposition was significantly increased in the disease control animals as observed by red-colored fibers in PSR (Figure 4B,D,G,H) and blue color in Masson's trichrome staining (Figure 4C,E). However, NC intervention attenuated the progression of ECM deposition and the accumulation of collagen fibers was significantly reduced in the pancreatic sections of NC-treated animals as evident from PSR (Figure 4B,D,G,H) and Masson's trichrome staining results (Figure 4C,E). These results indicate that NC potentially reduced the deposition of ECM and ameliorated the CP associated damage.

Next, we studied the expression of fibrotic proteins involved in EMT to probe the mechanism of action of NC. The expressions of $\alpha$-SMA, TGF-$\beta$1, CTGF, MMP2, collagen-3, and *N*-Cadherin were found to be increased in the CP model groups (Figure 5A−L). In contrast, NC intervention significantly reduced the expression of $\alpha$-SMA (Figure 5A,B,H−I), TGF-$\beta$1 (Figure 5A,C,H,J), CTGF (Figure 5A,D), MMP2 (Figure 5A,E,H,K), collagen-3 (Figure 5A,F), and *N*-Cadherin (Figure 5A,G,H,L). Further, the immunohistological analysis of $\alpha$-SMA and fibronectin supported these findings (Figure 6A−D). The expressions of $\alpha$-SMA (Figure 6A,C) and fibronectin (Figure 6B,D) were found to be significantly lower in the NC-treated animals. These results provide insights into the role of EMT and ECM synthesis inhibition as the mechanism of the observed antifibrotic effects of NC.

**3.4. NC Intervention Reduces the Expression of Oxidative and Endoplasmic Reticulum Stress in Animal**


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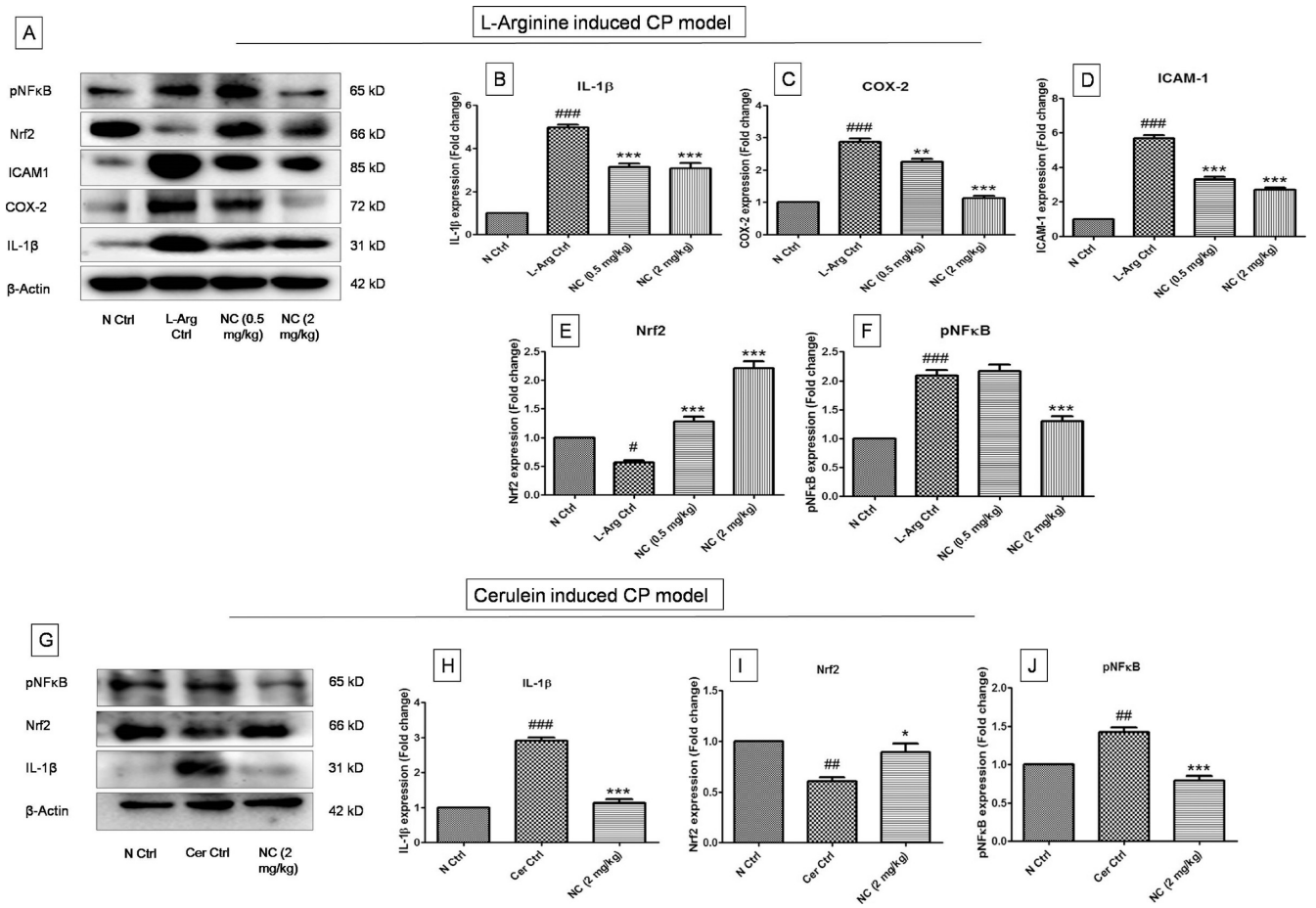


**Figure 7.** Effects of NC on inflammatory signaling in models of CP. (A) Blot panel of L-arginine-induced CP study groups, (B) IL-1$\beta$, (C) COX-2, (D) ICAM-1, (E) Nrf2, (F) pNFκB, (G) blot panel of cerulein-induced CP study groups, (H) IL-1$\beta$, (I) Nrf2, and (J) pNFκB. Protein expression of p-NFκB, Nrf2, ICAM1, COX-2, IL-1$\beta$, and $\beta$-Actin in whole-pancreas homogenates of different treatment groups, as determined by SDS-PAGE and western blotting. The results clearly show that the expressions of inflammatory mediators like pNFκB, ICAM1, COX-2, and IL-1$\beta$ were significantly increased in the disease control group, whereas the expression of Nrf2 significantly reduced in the disease control group. In contrast, the expression of studied proteins was markedly reversed by NC treatment. Values are represented as mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs N Ctrl; $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ vs L-arg Ctrl/Cerulein Ctrl. Abbreviations: COX-2, cyclooxygenase-2; ICAM1, intercellular adhesion molecule 1; p-NFκB, phospho-nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2 (NFE2)-related factor 2.

**Models of CP.** Oxidative stress and inflammation play a significant role in the development and progression of CP. We studied the expression of some of the important markers. The expression of IL-1$\beta$ correlated with the ELISA findings, with significant improvements in NC-treated animals (Figure 7A,B,G,H). The expressions of COX-2 and interstitial cell adhesion molecule 1 (ICAM1) were significantly increased (Figure 7A,C,D) in the disease control groups of both the models. Further, the expression of antioxidant protein nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) was significantly reduced (Figure 7A,E,G,I) and the expression of pro-oxidant transcription factor p65-nuclear factor kappa-light-chain-enhancer of activated B cells (p65-NFκB) was found to be significantly increased (Figure 7A,F,G,J) in the disease control groups. In contrast, NC treatment enhanced the expression of Nrf2 (Figure 7A,E,G,I) and reduced the expression of p65-NFκB (Figure 7A,F,G,J) in both the models. These results provide evidence that the pharmacological effects of NC are mediated by inhibition of NFκB pathway and upregulation of the Nrf2 pathway.

ER stress is associated with perturbed redox balance and leads to significant pathological damage during the progression of CP. We evaluated the expression of critical ER stress markers in the pancreatic lysates and tissue sections of NC-treated animals. Strikingly, we found a significant increase in ER stress marker proteins like BiP (Figure 8A,B,E,G), CHOP (Figure 8A,C), and calnexin (Figure 8E,H) in disease control pancreatic samples of both the models, signifying the vital role of ER stress in the pathology of CP. In contrast, NC intervention ameliorated the expression of these ER stress markers (Figure 8A−H). The immunofluorescence-based expression of BiP correlated well with the immunoblotting with a significant reduction in the NC-treated group pancreas (Figure 8I). Further, the expression of NOX-4 was found to be significantly increased in both the models, whereas NC treatment led to significant reduction (Figure 8A,D,E,F). These results reveal that NC inhibited the ER stress in a NOX-4/TGF-$\beta$-dependent manner in two animal models of CP and protected the pancreas from pancreatic fibrosis.


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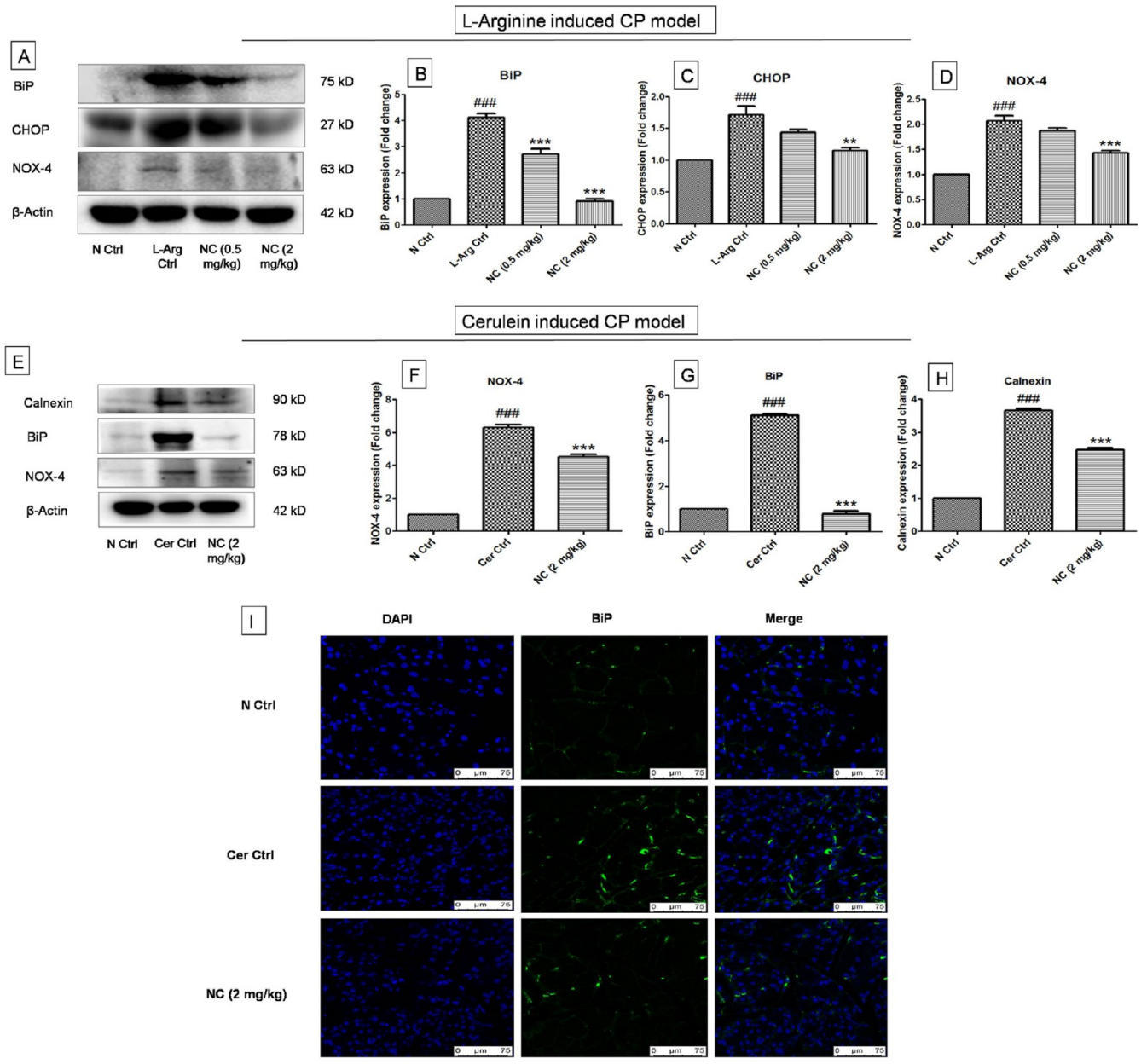


**Figure 8.** Effects of NC on ER stress markers in animal models of CP. (A) Blot panel of L-arginine-induced CP study groups, the protein expression of various markers of ER stress, (B) BiP, (C) CHOP and (D) NOX-4, (E) blot panel of cerulein-induced CP study groups, (F) NOX-4, (G) BiP, (H) calnexin, and (I) immunofluorescence-based expression of BiP. The expression of studied ER stress markers was found to be significantly increased in the disease control group, indicating that ER stress plays a major role in the pathology of chronic pancreatitis. The expressions of BiP, CHOP, and NOX-4 were significantly reduced in the NC-treated groups, showing that NC possesses promising activity to inhibit ER stress and this activity is partially responsible for the observed antifibrotic activity. Values are represented as mean ± SEM. $^{###}p < 0.001$, vs N Ctrl; $^{**}p < 0.01$, $^{***}p < 0.001$ vs L-arg Ctrl/Cerulein Ctrl.

## 4. DISCUSSION

CP is a complex inflammatory disease of unknown etiology, although addiction to alcohol and tobacco smoke are the prime reasons.[26] CP is driven by persistent inflammation and reaches the scarring stage which finally ends in the atrophy of the exocrine as well as the endocrine gland. The leukocytes are the primary inflammatory cells, particularly the macrophages, which trigger the differentiation of the inactive PSCs to the active myofibroblast form of PSCs by releasing of inflammatory cytokines and chemokines.[27] The TGF-$\beta$ signaling plays a pivotal role in the pathology of CP and is well known to be stimulated by various stimulants including cerulein and L-arginine.[28] Repeated bouts of AP are the key to the progression of chemically induced CP in animal models characterized by marked damage to the exocrine arm of the pancreas through fibrosis, calcification, and atrophy.[29] NC is a well-recognized SOD mimetic with promising anti-inflammatory, antidiabetic, antineurodegenerative, anticancer, and other activities.[30] We studied the pharmacological effect of NC against two animal models of CP, namely, L-arginine and cerulein-induced CP, with a focus on the impact on TGF-$\beta$-mediated EMT signaling and oxidative and ER stress.

As per our earlier reports, the physicochemical features of NC were similar in the current study.[13] The pancreas weight


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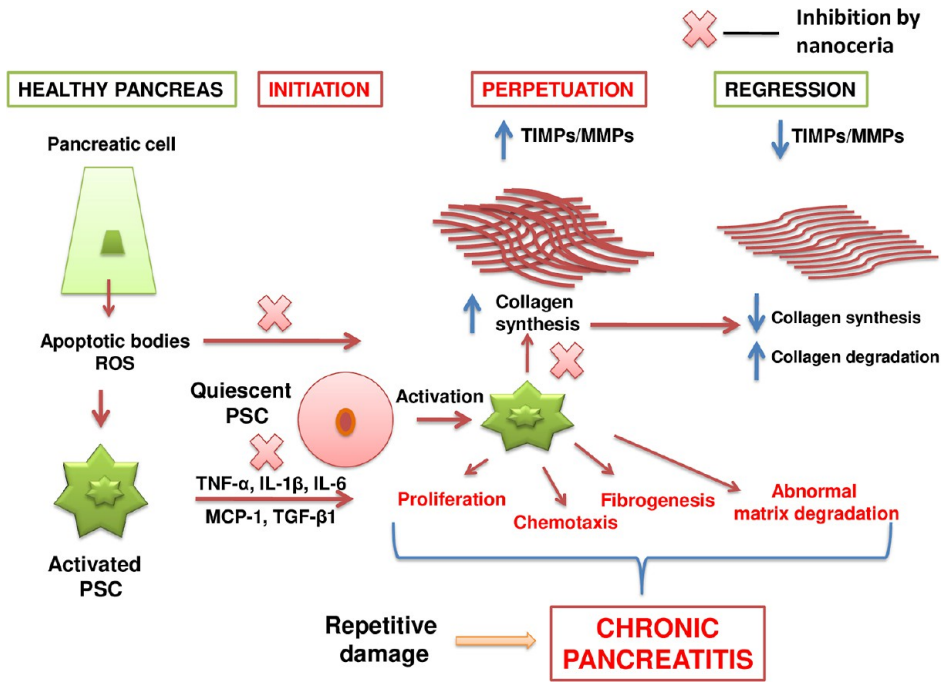


**Figure 9.** Proposed hypothesis for the therapeutic effectiveness of NC against chronic pancreatitis. CP is hallmarked by chronic inflammation which culminates after repeated bouts of acute inflammation. The acute inflammation phase stimulates the inflammatory cells to release inflammatory cytokines and chemokines at a rapid pace. However, if the inflammation persists, the pro-inflammatory cytokine levels are maintained for a prolonged duration. TGF-$\beta$ acts as the master regulatory fibrogenic cytokine and stimulates the activation of PSCs into a more secretory and motile phenotype that possesses the features of myofibroblasts. The process of activation of fibroblasts to myofibroblasts is called as epithelial-to-mesenchymal transition. The PSCs secrete the fibrogenic proteins like collagen, fibronectin, elastin, and others at a rapid pace owing to hypersecretory activity. Among various matrix proteins, collagen constitutes the major proportion and is largely responsible for the features of fibrosis during pancreatic fibrosis. NC, owing to its strong anti-inflammatory activity and redox regenerative capabilities, halts the progression of pancreatic chronic inflammation and in turn reduces the pancreatic fibrosis.

was significantly reduced in the disease control group in both the models, a feature showing pancreatic atrophy. The pancreatic zymogens are important for proper digestive activity.[31] However, in human CP conditions, abnormally high amylase and lipase may be present or not. We found insignificant alterations in these enzymes in the L-arginine-induced CP model, whereas the levels were strikingly increased in the cerulein-induced CP model. This might be due to the different origin points, molecular pathogenesis, and duration of the models. NC was found to ameliorate the disturbed enzyme levels in the cerulein-induced CP, whereas the levels were similar among all the groups in L-arginine model. Furthermore, type 3c diabetes is a disorder which often results from the progression of CP.[32] NC has been reported to ameliorate streptozotocin-induced type 1 diabetes by reduction of apoptosis and attenuation of Nrf2/NFκB pathway.[33] Further, cubical NC was reported to halt $H_2O_2$-induced cellular oxidative stress in INS1 $\beta$-cells.[34] However, in the current study, we did not observe any abnormal glucose levels in the two models and the glucose clearance was normal. These results indicate that in both the models, exocrine dysfunction was the primary feature without any significant damage to the endocrine gland.

Next, the involvement of ROS in the observed protective anti-CP activity of NC was probed. It is well known fact that ROS and nitrosative stress play the integral role of initiators of cellular stress during the pathogenesis of CP.[35] Dysregulated redox balance is the end result of this malfunctioned physiological response, which primarily propagates from the mitochondria and the ER. As NC has potent antioxidant and SOD mimetic activity, we observed a significant reduction in the highly reactive lipid peroxidation product MDA and improved levels of physiological antioxidant GSH in the NC-treated animals. NC intervention was found to abrogate the nitrosative stress as evident from reduction in the levels of nitrite radical, indicating its ability to scavenge peroxynitrite radicals also supported by earlier studies. Furthermore, owing to its potent SOD and catalase mimetic activity, NC significantly improved the levels of these antioxidant enzymes in the treated animals.

Chronic inflammation is the hallmark feature of CP and is responsible for the progression of the pathogenesis of this potentially fatal and irreversible pancreatic disorder. The macrophages release a plethora of inflammatory cytokines and chemokines which govern the cellular inflammatory response during acute as well as chronic disorders. Further, the PSCs are known to play a key role in the pancreatic fibrosis and are intricately regulated by macrophage signaling.[28] The alternatively activated macrophages have been proved to aggravate the pancreatic fibrosis.[36] Our previous study on AP revealed the macrophage inhibitory potential of NC via modulation of inflammatory response and reduction of mitochondrial stress. Herein, we evaluated the impact of NC on an array of inflammatory chemokines and cytokines. In line with earlier findings, NC exhibited strong anti-inflammatory effects on macrophage-mediated chemokine and cytokine signaling by inhibiting the NFκB pathway in the pancreatic tissue of CP animals. In addition, NC improved the levels of the anti-inflammatory cytokine IL-10 in the pancreatic tissue. This observation points out the importance of macrophage signaling


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inhibition behind the observed protective effect of NC against CP.

TGF-$\beta$ is a cytokine which acts as a growth factor and is involved in various cellular responses including the growth, proliferation, differentiation, and apoptosis. It has been reported to aggravate fibrosis and drives the progression of both L-arginine and cerulein-induced CP. Our results indicated that NC treatment was found to disrupt the TGF-$\beta$-mediated Smad signaling in the CP pancreas. Furthermore, NC intervention inhibited the Akt and Erk phosphorylation, indicating the inhibition of Smad-independent pathway behind the observed anti-CP activity. Further, the process of EMT leads to generation of highly motile and matrix-secreting myofibroblast phenotypes. In the case of CP, PSCs play the key role of ECM synthesis and deposition.[37] We observed a significant increase in the expression of $\alpha$-SMA, fibronectin, collagen-3, and MMP2 in the diseased animals. However, NC treatment led to attenuation of EMT culminating in the form of reduction of these markers of fibrosis. In addition, the levels of hydroxyproline correlated with these findings.

ER stress has been associated with the overall cellular stress and is often observed in oxidative stress-driven disorders like CP.[38] We found significantly high levels of the ER stress markers BiP, CHOP, and calnexin in the disease control animals. In contrast, owing to its strong antioxidant effect, NC was found to reduce the ER stress markers. Further, NOX-4 is associated with TGF-$\beta$ cross-talk and worsens the fibrosis prognosis. We found that the expression of NOX-4 was significantly increased in the disease control animals, whereas the NC intervention significantly reduced the expression of this critical mediator of cellular oxidative stress. These results indicate the potential of NC as a strong cell stress reducer and implicate promising anti-CP activity by inhibition of inflammation, fibrosis, and pancreatic ER stress. Figure 9 depicts the hypothetical scheme depicting the probable mechanism of action of NC.

NC has been widely explored in a plethora of preclinical studies for its potential pharmacological efficacy. The translation potential of NC remains high owing to its strong anti-inflammatory effects and promising redox regenerative capabilities. NC effectively bio-distributes in different organs of the body to provide the pharmacological effects. There are studies which suggest toxicological effects of NC in different in vitro and in vivo models.[39] However, it is considered one of the safest metal nanoparticles. Further, a detailed preclinical toxicity study of 29 metal oxide nanoparticles demonstrated NC to be among the safest metal oxide nanoparticles.[40] Our study provides the foundation for the future detailed investigations of NC in the context of chronic inflammation and fibrosis. Detailed mechanistic studies with isolated PSCs and acinar cells can provide insights into the sub-cellular interactions and shed light on the molecular effects. It will be essential to perform further detailed chronic studies for the potential long-term safety of NC on all the major organs to move to the next steps of its possible clinical translation.

## 5. CONCLUSIONS

The current study sheds light on the pharmacological potential of NC in amelioration of chronic inflammatory insult of the pancreas. NC was found to reduce the inflammatory signaling in two rodent models of CP. We report that NC significantly reduced the progression of fibrosis during CP by reduction of PSC activation, inhibition of TGF-$\beta$ signaling, and oxidative stress and ER stress in a TGF---NOX-4-dependent manner. Our study opens new avenues for the further exploration of this rare earth nanoparticles against other chronic inflammatory conditions.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

Data are available on request.

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.2c00933.

Results; nanoparticle characterization; physicochemical characterization and internalization of NC; effect of the pharmacological intervention of NC on various hematological parameters in L-arginine-induced CP model; effect of the pharmacological intervention of NC on an array of inflammatory cytokines as studied by Milliplex bead array-based assay; and effect of the pharmacological intervention of NC on an array of TGF-$\beta$ pathway as studied by bead-based Milliplex array-based assay in rodent models of CP (PDF)

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### Author Contributions

A.K. performed animal experimentation, data collection, and analysis and wrote the manuscript. M.A.S. helped in the animal experimentation. C.G. supervised the work, provided resources, and corrected the manuscript.

### Notes

Disclosures. Ethics conduct of research. All the animal studies were performed after the approval from Institutional Animal Ethics Committee (IAEC).

The authors declare no competing financial interest.

Consent for publication. All the authors have read the manuscript and agreed to its publication.

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