

Research Article

Effect of CK2 Inhibitor in Caerulein Induced Acute Pancreatitis in a Mouse Model

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ABSTRACT

Background: The pathogenesis of caerulein induced acute pancreatitis involves reactive oxygen species, inflammation, and necrosis of the exocrine pancreas. Protein kinase CK2 is formally known as casein kinase2. It is a phosphorylating enzyme of serine/threonine containing proteins. This enzyme plays an important role in the regulation of oxidative stress, inflammation, and apoptosis. While the importance of CK2 in the cancer biology, cell cycle regulation is undisputed, in pancreatic inflammation particularly acute pancreatitis remains elusive. Here, we anticipated the hypothesis to clarify the role of CK2 in acute pancreatitis by using its inhibitor (TBBt, Tetra bromo benzotriazole).

Results: In this study, acute pancreatitis in male swiss albino mice was induced by eight hourly intraperitoneal (i.p.) injection of caerulein (50µg/kg/hr). TBBt administered after 60min of last caerulein injection at three different doses (10, 20, and 30 mg/kg/hr, i.p.) for 3 hrs. Dose dependent effect of TBBt was observed against acute pancreatitis. At the dose of 20mg/kg/hr, it has shown protective effect by altering oxidative stress, physical, and biochemical parameters. Furthermore, it altered inflammatory (p-p65 NF-κB, p-Iκβ, COX-2, IL-6, and TNF-α), apoptotic and antiapoptotic mediators (p53, caspase3, PARP, Bcl2, and Sir2), and p47phox subunit of NADPH oxidase. However, when dose shifted to 30mg/kg/hr, it upholds acute pancreatitis by altering above parameters and mediators.

Conclusions: These results indicate that at the dose of 20mg/kg/hr, TBBt gives protection against pancreatitis by altering oxidative stress, inflammation, and apoptosis. In contrast, at the dose of 30mg/kg/hr, it aggravates acute pancreatitis by increasing oxidative stress.

Key words: Acute pancreatitis, casein kinase2, inflammation, oxidative stress and apoptosis.



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INTRODUCTION:

Acute pancreatitis (AP) is a disease of the exocrine pancreas with an inflammation and varying severity [1]. Although, the pathogenesis of AP is still a matter of debate, most hypotheses were based on the concept of premature activation of digestive zymogens in the pancreatic acinar cells [2]. It is the initial and an initiative pathological event in AP, leads to pancreatic cell death by autodigestion [1, 3].

Regardless of an early changes in AP, the pathogenesis a three-phase continuum: local inflammation in pancreas, leads to generalized inflammatory response, and a final stage of multiple organ failure (systemic inflammatory response syndrome, SIRS) [4,5]. Numerous aetiological factors implicated in the pathology of AP, with alcohol abuse and gallstone formation accounts more than 80% of cases [6].

The extent of pancreatic damage in AP is associated with an increased reactive oxygen species (ROS), which are released by the activity of NADPH oxidase. This enzyme activates nuclear factor-kappaB (NF- κ B)/Rel family transcription factors [7], which are key regulatory proteins of genes involved in the diseases of inflammation and cell survival [8-11]. Tumour necrosis factor- α (TNF- α) is a member of cytokine family inflammatory mediator, which is involved in local as well as systemic inflammation either directly or indirectly by the activation of NF- κ B[12]. In addition, the type of cell death in pancreatic acinar cells is a hallmark of an experimental AP, which decides the severity of disease [13]. In caerulein induced AP in mice, cell death is mainly through the necrosis rather than apoptosis. Alterations in apoptotic (caspase3 and p53) and anti-apoptotic (Bcl2) proteins may modify the severity of AP. It has previously been reported that shifting of acinar cell death from necrosis to apoptosis reduces the disease severity in AP [14, 15].

Protein kinase CK2 (EC 2.7.11.1) is formally known as casein kinase2; ubiquitously expressed serine/threonine selective protein kinase. It is a tetramer, consists of two catalytic α/α' and two regulatory β subunits and ATP/GTP acts as a co-factor [16]. CK2 is constitutively active protein kinase but the regulatory mechanism of this enzyme is still a subject of discussion [17]. CK2 has been implicated in cell differentiation, cell proliferation and DNA repair, regulation of circadian rhythm, and in other cellular processes. It has also previously been reported in the regulation of oxidative stress, inflammation, and apoptosis [18]. It is negatively regulates NADPH oxidase and apoptosis and positively associated with inflammation [10, 19].

TBBt (4, 5, 6, 7-Tetra bromo benzotriazole) is a selective and potent inhibitor of CK2. It acts by reversible and competitive inhibition of ATP/GTP binding to CK2 [20, 21]. Inhibition of CK2 by TBBt is known to induce programmed cell death, activates NADPH oxidase, and inhibits inflammation [22].

Based on above point out evidences, we hypothesized to clarify the CK2 inhibitor role in caerulein induced acute pancreatitis in a mouse model.

METHODS

Materials and Animals

Caerulein was purchased from Bachem AG (Bubendorf, Switzerland). Plasma α -amylase assay kit was purchased from Accurex

Biomedical Pvt. Ltd (India). All other reagents utilized were purchased from Sigma unless otherwise specified. All animals for experiment were approved by the Institutional Animal Ethics Committee (IAEC/11/28) and abide by guidelines of NIH on handling of experimental animals. Male swiss albino mice weighing 22-30g were provided by central animal facility of the institute (NIPER-Mohali). Animals were kept in controlled environmental conditions of temperature ($22\pm 2^\circ\text{C}$) and relative humidity ($50\pm 5\%$), and 12hr light/dark cycle under supervision of trained veterinarian.

Experimental design

Animals were acclimatized for one week prior to experiment. Mice were assigned randomly to control or experimental groups. A total of 42 animals were used; saline group (n=6), caerulein group (n=6), caerulein group treated with TBBt (n=6) (10mg/kg/hr, for 3hrs, i.p.), caerulein group treated with TBBt (n=6) (20mg/kg/hr, for 3hrs, i.p.), saline group treated with TBBt (20mg/kg/hr, for 3hrs, i.p.; for immunoblotting), caerulein group treated with TBBt (30mg/kg/hr, 3hrs, i.p.), and saline group treated with TBBt (30mg/kg/hr, 3hrs, i.p.; for immunoblotting). Animals were given eight hourly i.p. injection of normal saline (0.9% wt/vol NaCl) or saline containing caerulein (50 μ g/kg/hr). TBBt was injected after 60min of last saline/caerulein injection in experimental groups for 3hrs. One hour after last injection of saline/TBBt, animals were killed by an i.p. injection of pentobarbital sodium (150mg/kg). Blood samples were drawn from the right ventricle using heparinized syringes and centrifuged (2,000g for 10min, 0-4 $^\circ\text{C}$). Thereafter, plasma and pancreatic samples were stored at -80 $^\circ\text{C}$.

Intrapancreatic edema assessment

Pancreatic tissue was clipped of fat and weighed (wet weight). Thereafter, the samples were dried at 80 $^\circ\text{C}$ for 48hrs and reweighed (dry weight). The pancreatic water content was calculated as the difference between wet and dry weight of pancreas. Increased water content in pancreas is considered as an indicative of development of the pancreatic edema, expressed in mg.

Estimation of plasma α -amylase and lipase activity

Plasma α -amylase and lipase activity (expressed as IU/L and U/L respectively) were determined by α -amylase and lipase assay kits (supplied by Accurex Biomedical Pvt. Ltd, India). Briefly, blood samples were drawn from the right ventricle using heparinized syringes and centrifuged (2000g for 10min, 0-4 $^\circ\text{C}$).

Thereafter, the assay was performed by protocol described in assay kits.

Measurement of superoxide dismutase levels in pancreas

Superoxide dismutase (SOD) levels were measured according to the method described by Kono *et al.* [23] where the reduction of nitrazoblue tetrazolium (NBT) is inhibited by SOD. This was measured by using spectrophotometer at 560nm. Briefly, the reaction was initiated by the addition of hydroxylamine HCl to the reaction mixture containing NBT and nuclear fraction of pancreatic homogenate. The superoxide dismutase activity expressed as units/mg of protein.

Measurement of reduced glutathione and thiobarbituric acid reactive substrate levels

For measurement of reduced glutathione (GSH) level, pancreatic samples were homogenized in an ice-cold homogenizing buffer (pH 7.2) combined with sulphosalicylic acid. This homogenate was used for measurement of GSH content. GSH level was measured by the method described by Ellman *et al.* [24]. The thiobarbituric acid reactive substrate (TBARS) assay used for the measurement of lipid peroxidation. The extent of lipid peroxidation was measured according to the method described by Oshawa *et al.* [25]. Both GSH and TBARS levels were expressed as $\mu\text{M}/\text{mg}$ of protein.

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured by using lucigenin-enhanced chemiluminescence method [26]. Briefly, pancreatic homogenate was prepared in a 1ml of lysis buffer (consist of 20mM KH_2PO_4 , pH 7.0, 1mM EDTA, 1mM phenyl methyl sulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ aprotinin, and 0.5g/ml leupeptin). Homogenates were subjected to low speed centrifugation (at 800g for 10min, 0-4°C) to remove unbroken debris and cells, and aliquots were used immediately. Shortly, 100 μl of homogenate was added to 900 μl of phosphate buffer (50mM), EDTA (1mM, pH 7.0), sucrose (150mM), lucigenin (5 μM), and NADPH (100 μM). Photon emission was measured every 60sec interval for 10min. There was no calculable activity in the absence of NADPH. A buffer blank of <5% cell signal was subtracted from each reading. NADPH activity was expressed in radio frequency units as RFU/min.

Measurement of peroxide production by 2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) assay

Production of peroxides including hydrogen peroxide (H_2O_2) and peroxyxynitrite were measured in pancreatic homogenates by using 2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) assay method as described previously [1]. Briefly, 100 μl of pancreatic homogenate was incubated with DCFH-DA (16 $\mu\text{g}/\text{ml}$, final concentration) for 20min at 37°C. DCFH-DA was oxidized by peroxides present in the pancreatic homogenate to highly fluorescent compound, 2, 7-dichlorofluorescein (DCF), which was quantified by using spectrofluorometer at 485nm excitation and 535nm emission wavelengths for 30min with the interval of 30sec. The results were expressed as CL (counts/30sec).

Measurement of protein kinase CK2 activity

Protein kinase CK2 activity in cytosolic fractions of pancreas was measured by using CK2-specific synthetic peptide substrate [Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu] as described previously [27]. Briefly, the reaction mixture consists of Tris-HCl (30mM, pH 7.4) MgCl₂ (5mM), dithiothreitol (1mM), NaCl (150mM), [γ -³²P]ATP (specific radioactivity 3x10⁶ dpm/nmol of ATP, 0.05mM) and decapeptide substrate (1mM). The reaction was initiated by the addition of pancreatic homogenate containing 5 μg of protein to reaction mixture and was carried out over a time course of 0 to 30min at 37°C. At appropriate times, aliquots were placed on whatman P-81 paper, and the reaction was stopped by placing papers in a 10mM phosphoric acid. Unreacted [γ -³²P]-ATP was removed from papers by washing twice with phosphoric acid followed by two washes with 15% acetic acid, then acetone. The amount of radioactive ³²p incorporated into CK2 substrate peptide was determined by using liquid scintillation spectrometry. CK2 activity was expressed as nmol of ³²p transferred/mg of protein/hr.

Protein isolation and immunoblotting

Isolation of proteins and immunoblotting was performed by the method described previously [28]. For immunoblotting, the proteins were transferred onto the nitrocellulose membrane (Pall Corporation, Pensacola, FL). The analysis was performed by using different primary antibodies namely: anti-TNF- α (goat, 26/17kD, 1:500, Santa Cruz, CA), anti-NF- κB (rabbit, 65kD, 1:1000, Santa Cruz, CA), anti p-(p65)NF- κB (rabbit, 65kDa, 1:500, Santa Cruz, CA) anti-caspase3 (goat, 17/20kD, 1:500, Santa Cruz, CA), anti-I $\kappa\text{B}\alpha$ (rabbit, 39kD, 1:500, Santa Cruz, CA), anti-actin (rabbit, 42kD, 1:1000, Santa

Cruz, CA), PARP (rabbit, 85/116 kDa, 1:200, cell signalling), anti-Sir2 (rabbit, 110kDa, 1:1000), anti-COX-2 (rabbit, 70-72kDa, 1:2000, Santa Cruz, CA), anti-IL-6 (goat, 21.5/28kDa, 1:2000, Santa Cruz, CA) and HRP-conjugated secondary antibodies (Santa Cruz, CA), and detected with enhanced chemiluminescence (ECL) method (Amersham Pharmacia Biotech UK Ltd, Little Chalfont). Immunoblots were quantified by using densitometry analysis (Image J, NIH, USA).

Morphological examination to determine acinar cell necrosis

Pancreatic samples isolated were subjected to fix in 4% wt/vol neutral phosphate-buffered formalin for overnight and subsequently dehydrated through a series of graded ethanol. After impregnation in paraffin wax, tissue samples were set into blocks. Pancreatic sections (3 μ m) were stained with hematoxylin/eosin (H&E) and examined by light microscopy using a leica microscope (objective lens magnification of x100). Changes in histology of pancreas were assessed in at least 18 randomly selected tissue sections from each group. The degree of acinar cell necrosis was scored by using schmidt scoring system ranging from 0 to 4, with 4 being most severe [29]. It was determined by both number of necrotic cells per high-powered field and its distribution (focal or diffuse).

Measurement of apoptotic cell death by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay in pancreatic acinar cells was performed by the method described previously [30]. Briefly, mice were euthanized by using pentobarbital sodium (150mg/kg). Isolated pancreas were fixed in a 10% formalin solution overnight, then embedded in paraffin and cut into thin (3 μ m) sections. DNA fragmentation was assessed by using TUNEL assay. Total cell population and TUNEL positive cells were counted using image J analysis (Carl Zeiss, AxioImager M1, Germany), and images were acquired by using a charged coupled device (CCD) camera. TUNEL assay was performed with 6 mice per group. 4 sections were prepared from the pancreas of each mouse. Finally, the average numbers of TUNEL positive cells per 100 pancreatic acinar cells were calculated and expressed as percent of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive.

Statistical analysis

Data were shown as the Mean \pm S.E.M with statistical analysis performed by one-way ANOVA using Tukey's post hoc test (GraphPad Prism 5.00; GraphPad, San Diego, CA). Histological damage was analyzed by one-way ANOVA followed by Kruskal-Wallis test. An independent two-way student's t-test was used when there were two groups to be compared. The null hypothesis was rejected if P value was <0.05 and the difference was regarded as significant.

RESULTS

Mice treated with caerulein showed morphological changes in pancreas and altered in physical, and biochemical parameters.

Mice treated with caerulein showed a significant (P<0.01) increase in pancreatic wet weight, and pancreas/body weight ratio compared with saline control. Morphological evidence of pancreatic inflammation was assessed by change in colour and increased water content of pancreas. The wet-to-dry weight ratio of pancreas is an indication of intrapancreatic edema, which was also increased significantly (P<0.01) following caerulein treatment. Increased secretion of digestive enzymes is the sign of early AP. Mice treated with caerulein showed higher (P<0.01) plasma α -amylase and lipase activity compared with saline control (Table 1).

TBBt showed dose dependent effect on physical and biochemical parameters in caerulein treated mice.

Mice showed a significant (P<0.01) increase in pancreatic weight [Fig. 1A], pancreas/body weight ratio [Fig. 1B], intrapancreatic edema [Fig. 1C], plasma α -amylase [Fig. 1D], and lipase [Fig. 1E] activity following caerulein treatment. TBBt was decreased all these parameters significantly (P<0.01) at 20mg/kg/hr dose [Fig. 1A–Fig. 1E]; however there was no significant effect at 10mg/kg/hr [Fig. 1A–Fig. 1E]. In contrast, TBBt at the dose of 30mg/kg/hr, increased above parameters but it was not significant compared with saline control [Fig. 1A–Fig. 1E].

TBBt prevents oxidative stress at 20mg/kg/hr, but upholds at 30mg/kg/hr in caerulein treated mice.

Mice treated with caerulein showed a significant decrease in pancreatic SOD and GSH levels and increase in TBARS compared with saline control. TBBt significantly (P<0.01) increased pancreatic SOD [Fig. 1G] and GSH [Fig. 1H] and decreased TBARS levels [Fig. 1F] at 20mg/kg/hr, but at 30mg/kg/hr, it decreased

SOD [Fig. 1G] and GSH [Fig. 1H] and increased TBARS levels [Fig. 1F].

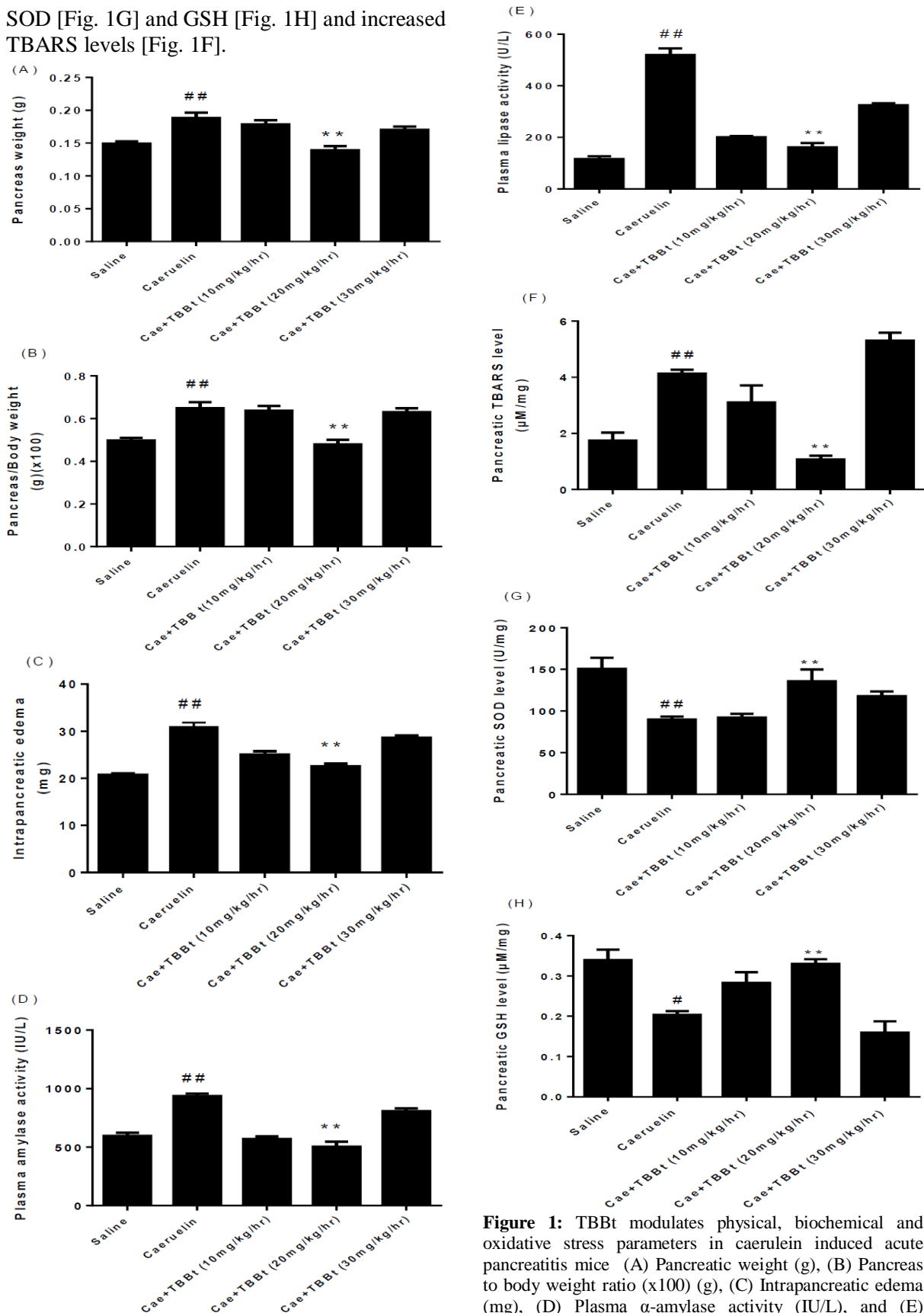


Figure 1: TBBt modulates physical, biochemical and oxidative stress parameters in caerulein induced acute pancreatitis mice (A) Pancreatic weight (g), (B) Pancreas to body weight ratio (x100) (g), (C) Intrapancreatic edema (mg), (D) Plasma α -amylase activity (IU/L), and (E) Plasma lipase activity (U/L), (F) Pancreatic thiobarbituric acid reactive substances (TBARS) (μ M/mg), (G) Pancreatic superoxide dismutase (SOD) (U/mg), (H) Pancreatic reduced glutathione (GSH) (μ M/mg). All values are represented as Mean \pm S.E.M. (n=6) ##P<0.01, #P<0.05 versus saline control. **P<0.01, *P<0.05 versus caerulein control.

We further confirmed an increased production of ROS through lucigenin (NADPH oxidase) and DCFDA assays. Lucigenin assay is used for an estimation of ROS produced by NADPH oxidase whereas DCFDA assay is used for measurement of ROS generated by inflammatory mediators. In both the assays we observed decreased levels of ROS production in pancreas at 20mg/kg/hr [Fig. 3A & B] but not at 30mg/kg/hr [Fig. 3A & B] dose of TBBt.

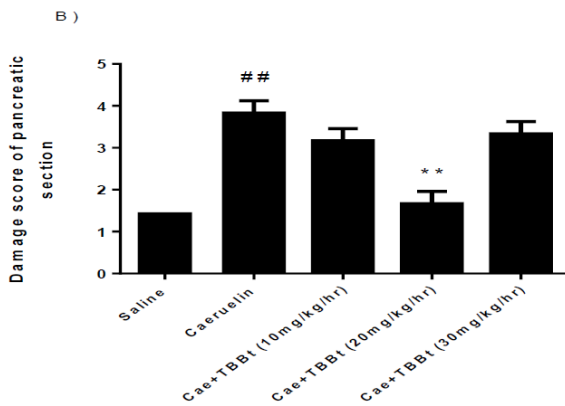
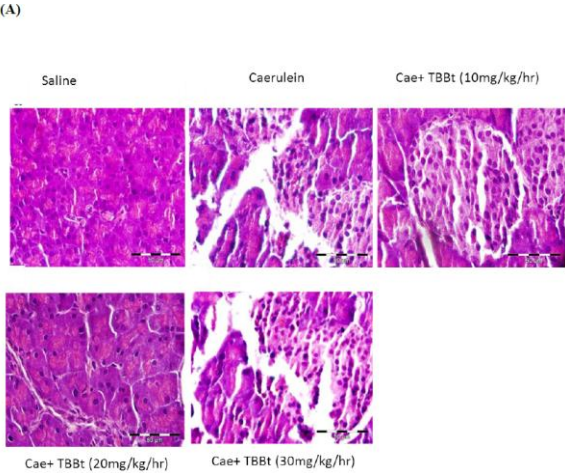


Figure 2: TBBt alters pancreatic damage in caerulein induced acute pancreatitis mice (A) Representative images of pancreatic sections stained with hematoxylin and eosin (original magnification of 100x), (B) Damage score of pancreatic sections as described in methods. All values are represented as Mean ± S.E.M. (n=6) ^{##}P<0.01, [#]P<0.05 versus saline control. ^{**}P<0.01, ^{*}P<0.05 versus caerulein control.

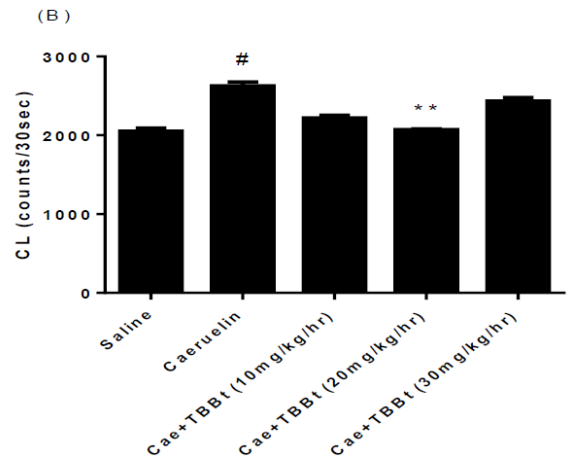
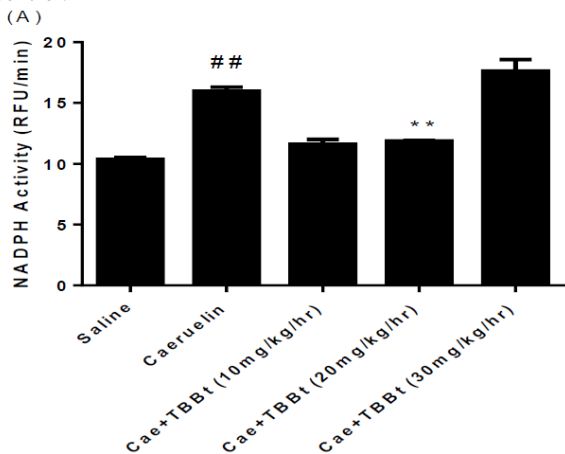


Figure 3: Measurement of the oxidative stress by lucigenin and DCFDA staining assay in acute pancreatitis mice (A) DCFDA staining and (B) Lucigenin assay. The results shown were representative of five different groups. All values are represented as Mean ± S.E.M. (n=6) ^{##}P<0.01, [#]P<0.05 versus saline control. ^{**}P<0.01, ^{*}P<0.05 versus caerulein control.

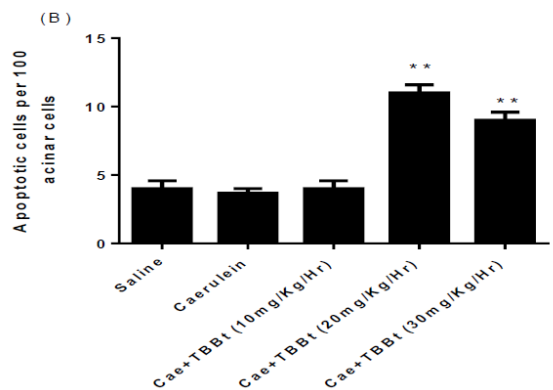
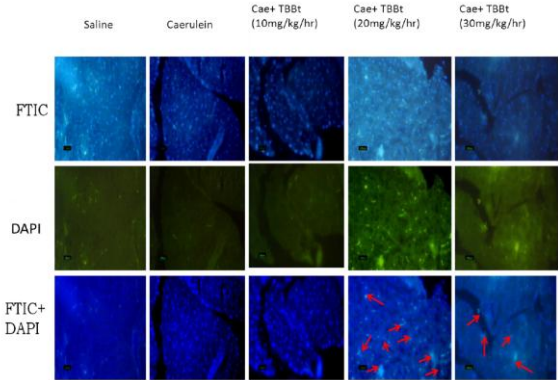


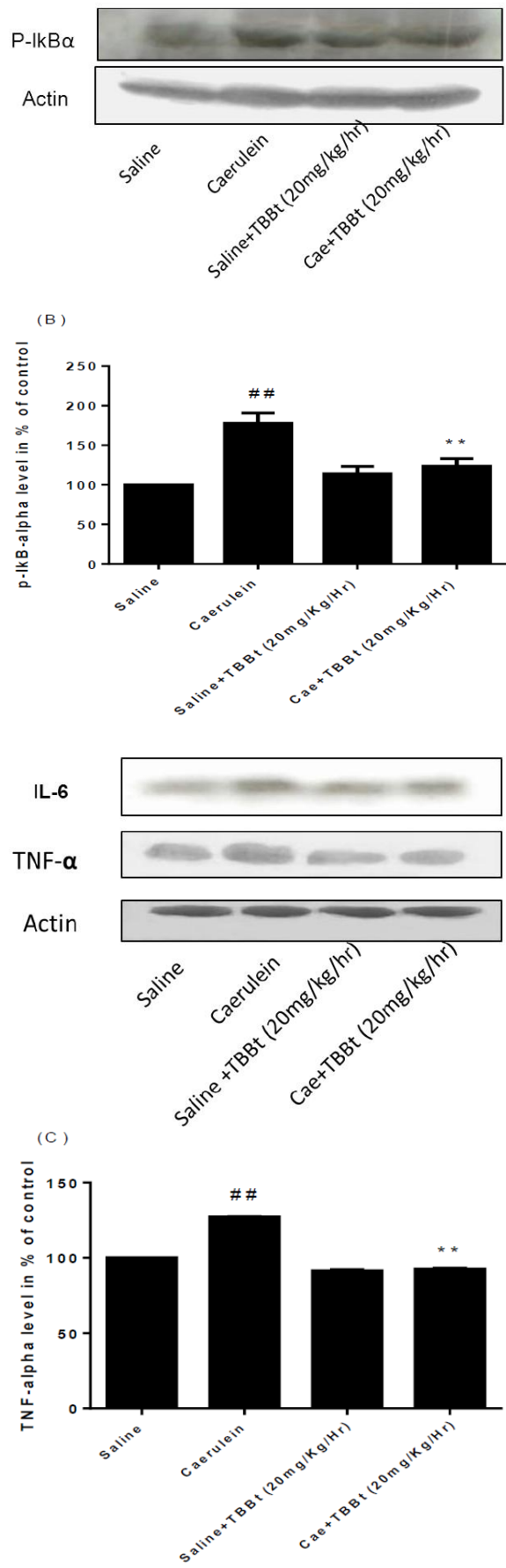
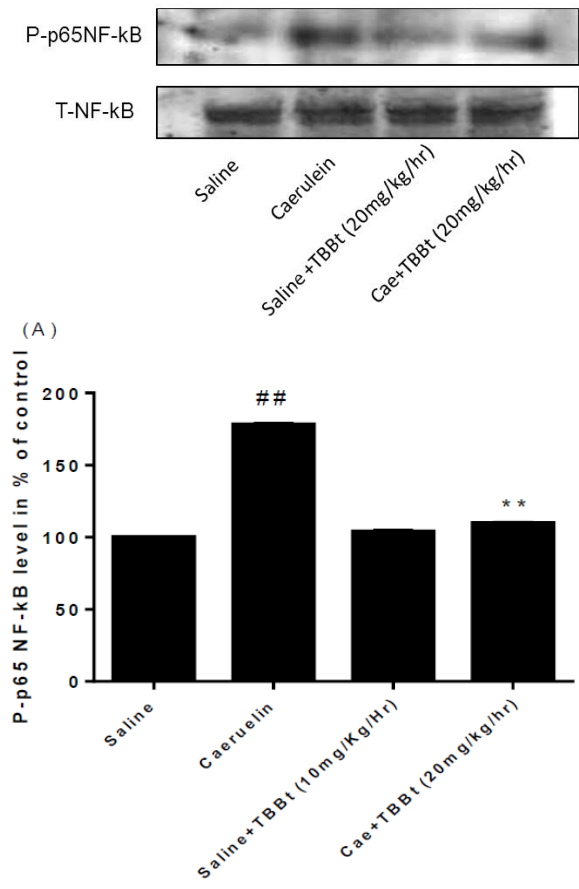
Figure 4: Effect of TBBt treatment on the frequency of TUNEL positive cells (A) Representative photomicrographs showing apoptotic acinar cells having fragmented DNA as revealed from TUNEL assay (B) The graph illustrates the percent of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells per 100 acinar cells. All values are represented as Mean ± S.E.M. (n=6) ^{**}P<0.01, ^{*}P<0.05 versus caerulein group. ^{##}P<0.01, [#]P<0.05 versus saline group

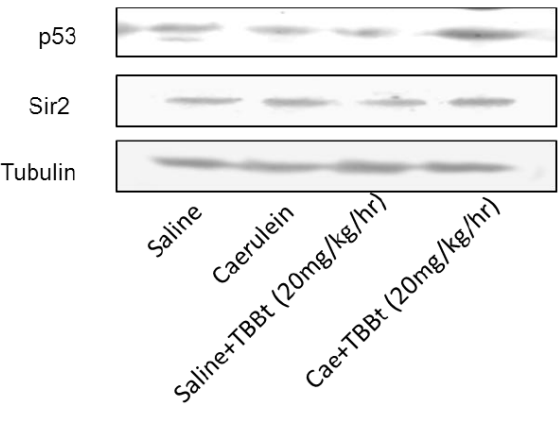
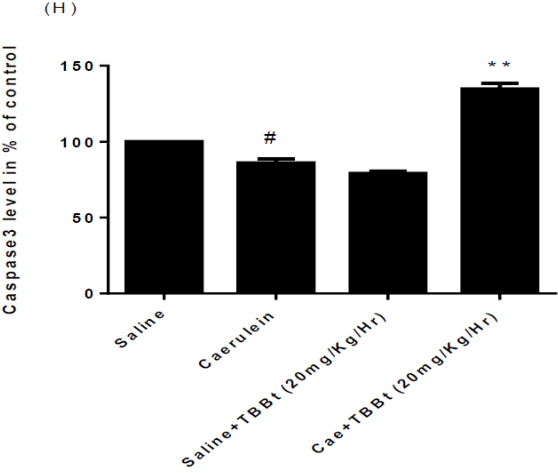
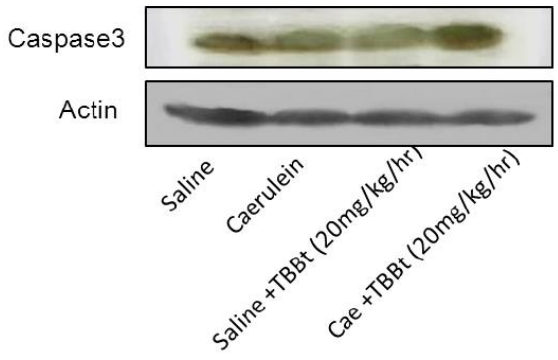
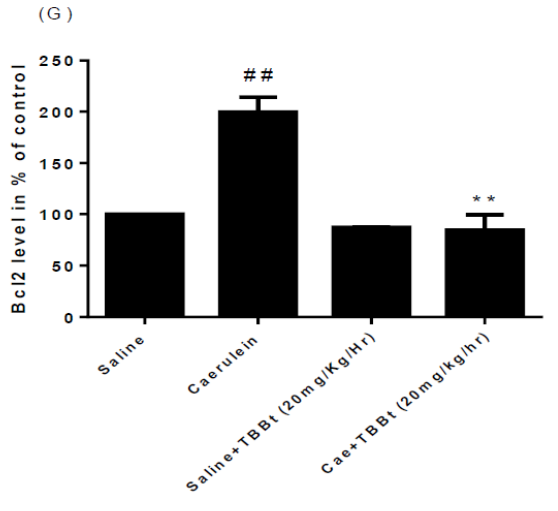
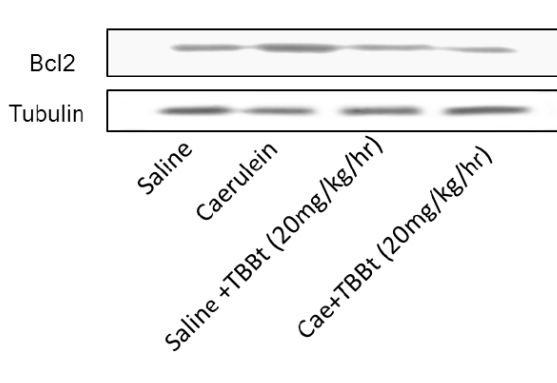
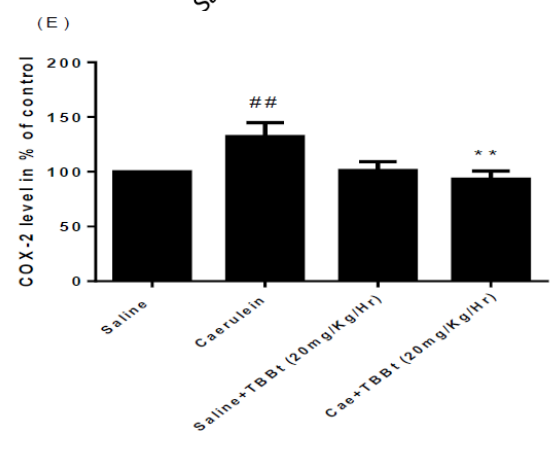
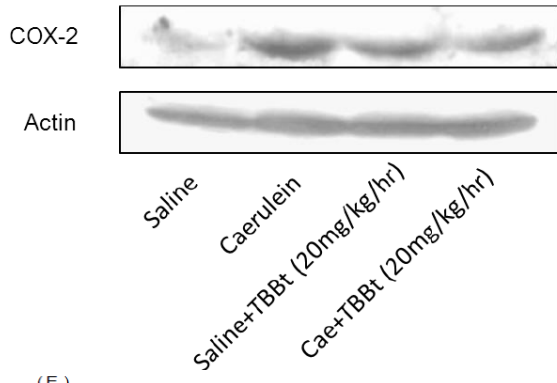
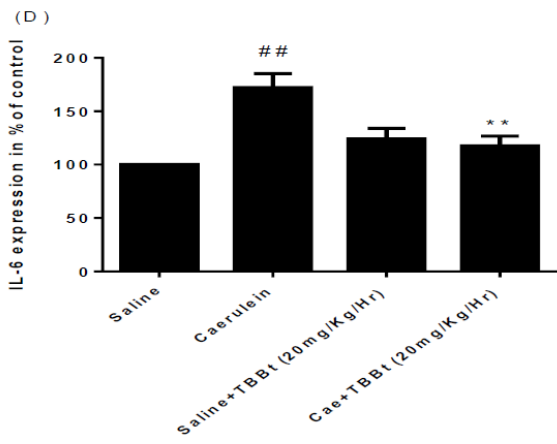
Pancreatic injury in acute pancreatitis

H&E staining of the pancreas showed tissue damage as expected, with acute pancreatitis resulting in edema formation, acinar cell necrosis, and leukocyte infiltration as compared to saline control. TBBt treated mice scored significantly less at 20mg/kg/hr but no change at 10mg/kg/hr as compared to caerulein control. These results were confirmed our biochemical observations and suggest that TBBt prevents progression of caerulein induced AP at 20 mg/kg/hr. In contrast, mice treated with TBBt at 30mg/kg/hr further increased pancreatic damage as compared to caerulein control [Fig. 2A].

TBBt alters inflammation and necrosis associated with acute pancreatitis following caerulein treatment

Mice treated with caerulein showed significantly higher pancreatic levels of p-NF-κB (p65 subunit), p-IκBα, cytokines such as TNF-α, IL-6 and COX-2 compared with saline control. Treatment with TBBt at 20mg/kg/hr results in significant ($P < 0.01$) decrease in p-(p65) NF-κB [Fig. 5A], p-IκBα [Fig. 5B], TNF-α [Fig. 5C], IL-6 [Fig. 5D], and COX-2 [Fig. 5E] but no change in total NF-κB level [Fig. 5A].





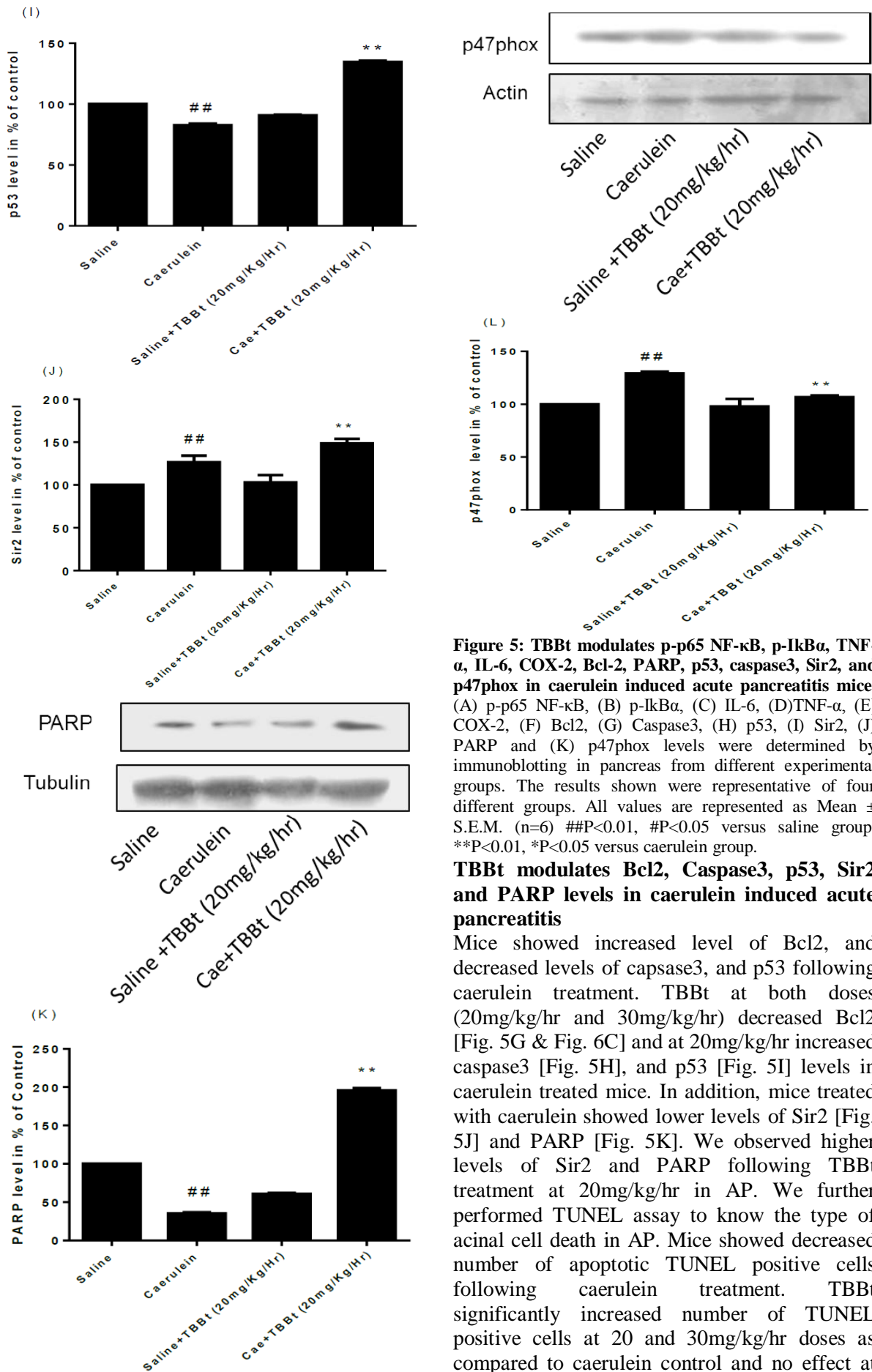


Figure 5: TBBt modulates p-p65 NF-κB, p-IκBα, TNF-α, IL-6, COX-2, Bcl-2, PARP, p53, caspase3, Sir2, and p47phox in caerulein induced acute pancreatitis mice. (A) p-p65 NF-κB, (B) p-IκBα, (C) IL-6, (D)TNF-α, (E) COX-2, (F) Bcl2, (G) Caspase3, (H) p53, (I) Sir2, (J) PARP and (K) p47phox levels were determined by immunoblotting in pancreas from different experimental groups. The results shown were representative of four different groups. All values are represented as Mean ± S.E.M. (n=6) ##P<0.01, #P<0.05 versus saline group. **P<0.01, *P<0.05 versus caerulein group.

TBBt modulates Bcl2, Caspase3, p53, Sir2 and PARP levels in caerulein induced acute pancreatitis

Mice showed increased level of Bcl2, and decreased levels of capsase3, and p53 following caerulein treatment. TBBt at both doses (20mg/kg/hr and 30mg/kg/hr) decreased Bcl2 [Fig. 5G & Fig. 6C] and at 20mg/kg/hr increased caspase3 [Fig. 5H], and p53 [Fig. 5I] levels in caerulein treated mice. In addition, mice treated with caerulein showed lower levels of Sir2 [Fig. 5J] and PARP [Fig. 5K]. We observed higher levels of Sir2 and PARP following TBBt treatment at 20mg/kg/hr in AP. We further performed TUNEL assay to know the type of acinal cell death in AP. Mice showed decreased number of apoptotic TUNEL positive cells following caerulein treatment. TBBt significantly increased number of TUNEL positive cells at 20 and 30mg/kg/hr doses as compared to caerulein control and no effect at 10mg/kg/hr of TBBt [Fig. 4A&B].

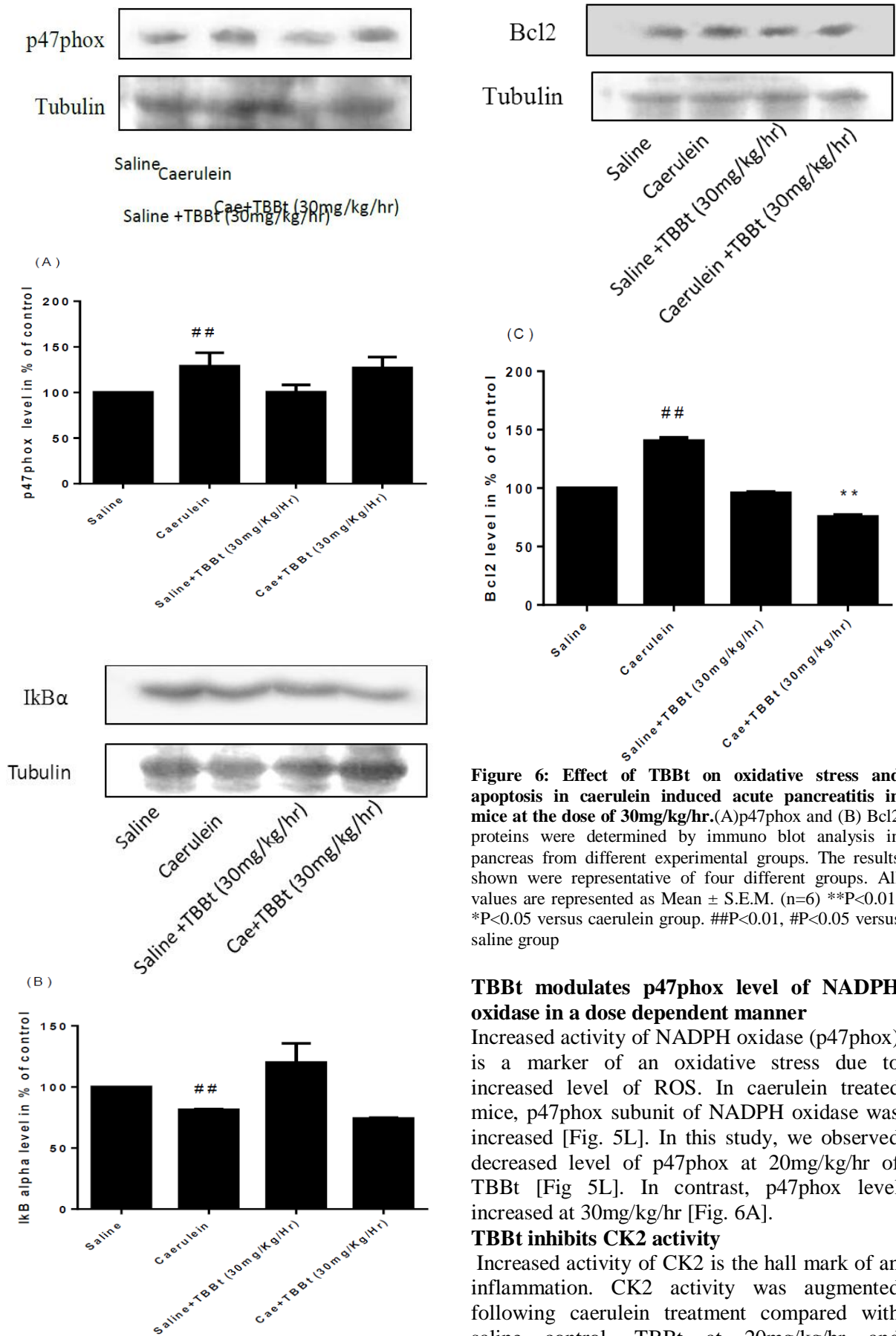


Figure 6: Effect of TBBt on oxidative stress and apoptosis in caerulein induced acute pancreatitis in mice at the dose of 30mg/kg/hr.(A)p47phox and (B) Bcl2 proteins were determined by immuno blot analysis in pancreas from different experimental groups. The results shown were representative of four different groups. All values are represented as Mean ± S.E.M. (n=6) ^{**}P<0.01, ^{*}P<0.05 versus caerulein group. ^{##}P<0.01, [#]P<0.05 versus saline group

TBBt modulates p47phox level of NADPH oxidase in a dose dependent manner

Increased activity of NADPH oxidase (p47phox) is a marker of an oxidative stress due to increased level of ROS. In caerulein treated mice, p47phox subunit of NADPH oxidase was increased [Fig. 5L]. In this study, we observed decreased level of p47phox at 20mg/kg/hr of TBBt [Fig 5L]. In contrast, p47phox level increased at 30mg/kg/hr [Fig. 6A].

TBBt inhibits CK2 activity

Increased activity of CK2 is the hall mark of an inflammation. CK2 activity was augmented following caerulein treatment compared with saline control. TBBt at 20mg/kg/hr and 30mg/kg/hr diminished CK2 activity

significantly ($p < 0.01$) while at 10mg/kg/hr it has no effect [Fig. 7].

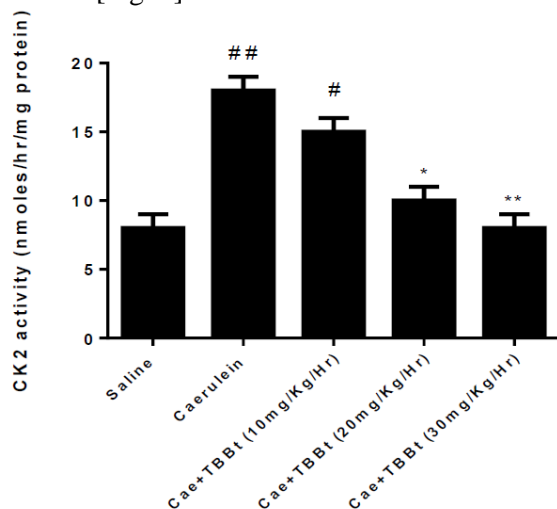


Figure 7: Effect of TBBt on CK2 activity in caerulein induced acute pancreatitis mice. All values are represented as Mean \pm S.E.M. (n=6) ## $P < 0.01$, # $P < 0.05$ versus saline control. ** $P < 0.01$, * $P < 0.05$ versus caerulein group.

DISCUSSION

This report is the first study that uses CK2 inhibitor (TBBt) in a model of caerulein induced AP in mice to elucidate its role in the pathogenesis of acute pancreatitis. Little is known about CK2 role in inflammatory diseases. Increased expression and activity of the CK2 was observed in animal model of chronic colitis and *in vitro* studies of intestinal epithelial cells [31]. CK2 inflammatory role was also reported in glomerulonephritis, atherosclerosis, autoimmune encephalomyelitis, and thermal and inflammatory pain [32, 33]. Based on existing literature on CK2 inflammatory role, we hypothesized to clarify its role in acute pancreatitis by using CK2 Inhibitor (TBBt).

Experimental AP is characterized by an increased pancreatic weight, pancreatic edema, acinar cell necrosis, and leukocytes infiltration into pancreas, and the elevation of an α -amylase and lipase activity in plasma [34]. CK2 is an ATP/GTP dependent protein kinase plays an important role in the phosphorylation of NF- κ B, I κ B α and NADPH oxidase. CK2 negatively regulates NADPH oxidase subunits by phosphorylation. CK2 also controls an apoptosis by stabilising p53, decreasing Bcl2, and increasing caspase3 levels in acinar cells [35]. Here we provide the evidence of dose dependent effect of TBBt against caerulein induced AP in mice.

We targeted CK2 enzyme by using TBBt, an experimental drug and performed our study using three different doses (10mg/kg/hr,

20mg/kg/hr, and 30mg/kg/hr for 3hrs post treatment). Among these, TBBt at the dose of 20mg/kg/hr showed beneficial effect but not at the dose of 10mg/kg/hr against caerulein treated mice, so we omit this dose from experiment for immunoblotting studies. In contrast, at 30mg/kg/hr of TBBt, it worsened AP condition by increasing oxidative stress, inflammation and necrosis, and decreasing apoptosis.

Caerulein treated mice showed an increased pancreatic weight indicating an edematous condition and an elevation of plasma α -amylase and lipase activity. In this study, treatment with TBBt at the dose of 20 mg/kg/hr resulted in a significant decrease in pancreatic weight, pancreas/body weight ratio, intrapancreatic edema, plasma α -amylase, and lipase activity as compared to caerulein control. In contrast, at 30mg/kg/hr treatment there was no difference as compare with caerulein control or further worsened the disease severity in mice.

Superoxide dismutases (SODs) and reduced glutathione (GSH) are endogenous antioxidants protect cells from damage by scavenging ROS and peroxides. SODs and GSH reduce the severity of AP by decreasing oxidative stress [12, 36]. In addition, the extent of lipid peroxidation is an oxidative stress marker in AP [37]. Thiobarbituric acid reactive substances (TBARS) are by products of lipid peroxidation. Decreased levels of SODs and GSH, and increased TBARS level was implicated following caerulein treatment. TBBt significantly improved the GSH and SODs content and decreased TBARS level at 20mg/kg/hr but it upholds an oxidative stress at 30mg/kg/hr by altering GSH, SODs, and lipid peroxidation.

NF- κ B is a transcription factor can play key role in an inflammation by increasing inflammatory mediators [38]. It regulates the synthesis of TNF- α , IL-6, and COX-2, which play an important role in the pathogenesis of AP [39, 40]. CK2 increases the proteosomal degradation of I κ B α by phosphorylation, makes the availability of free NF- κ B in the cytoplasm (NF- κ B is associated with I κ B α in cytoplasm) [10, 41]. The p65 subunit of NF- κ B is also undergoes phosphorylation by CK2 that allows p-p65 (NF- κ B) into the nucleus, which in turn activates inflammatory mediators such as TNF- α , and IL-6 and COX-2 [42, 43]. Our data showed that TBBt attenuated increased levels of p-p65 NF- κ B, p-I κ B α , IL-6, COX-2 at the dose of 20mg/kg/hr. NF- κ B is also indirectly responsible for cell death through necrosis by increasing

inflammation. We observed reduced acinar cell necrosis by histology studies at 20 mg/kg/hr dose of TBBt, but it upholds or further worsened necrosis at 30 mg/kg/hr.

NADPH oxidase is the major enzyme responsible for production of ROS. Increased activity of the p47phox of NADPH oxidase subunit is a marker for an oxidative stress and has previously been reported in pathology of AP [26], which indirectly activates NF- κ B through generation of ROS [44, 45]. CK2 negatively regulates NADPH oxidase, particularly p47phox and finally oxidative stress. Our data showed that TBBt inhibits p47phox at 20 mg/kg/hr but activates at 30mg/kg/hr. These results suggest that the extent of CK2 inhibition by TBBt at 20mg/kg/hr is not sufficient to inhibit p47phox; leads to lack of control on p47phox and produces beneficial effect in AP. On the other hand, TBBt at the dose of 30mg/kg/hr was substantially high resulting in activation of p47phox, leads to increased oxidative stress in AP.

In experimental AP, pancreatic cell death occurs through both necrosis and apoptosis [46]. It is mainly through necrosis rather than apoptosis and severity of AP depends on the extent of necrosis [47]. In caerulein induced AP, decreased level of p53 takes place, which is negatively associated with Bcl2 and positively with cytochrome c [48]. The cytochrome c indirectly regulates caspase3 resulting pancreatic necrosis [49, 50]. p53 is an apoptotic protein which is stabilized by phosphorylation at serine 386/392 position by CK2[51]. Caspase3 and Bcl2 proteins are downstream mediators of the p53 also regulated by CK2. Sir2 (Silent information regulator 2 protein) and PARP (Poly ADP Ribose Polymerase) also provides protection against acinar cell death caused by necrosis and promotes programmed cell death (apoptosis) [52]. Our data showed that TBBt increases p53 and caspase3 and decreases Bcl2 in caerulein induced AP mice. TBBt also augments Sir2 and PARP, which indirectly gives protection towards pancreatic acinar cells by promoting apoptotic cell death.

CONCLUSION

By observing these results, we conclude that TBBt acts by three mechanisms. At 20mg/kg/hr dose, it inhibits an inflammation by targeting NF- κ B and its downstream mediators. It also gives protection against acinar cell necrosis by increasing Sir2 and PARP. At the dose of 30mg/kg/hr, it activates NADPH oxidase, which in turn increases oxidative stress. In contrast, at

both doses (20mg/kg/hr and 30mg/kg/hr) it activates apoptotic pathway that directly or indirectly upsurges caspase3 and p53, and decreases Bcl2 levels; this shifts acinar cell death from necrosis to apoptosis. These observations can help to further insight into the role of CK2 in acute pancreatitis.

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