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Calcium Administration Augments Pancreatic Injury and Ectopic Trypsinogen Activation after Temporary Systemic Hypotension in Rats

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Background: **Calcium infusion and hypotension have been described as the most important risk factors for pancreatic injury after cardiopulmonary bypass.**

Methods: **Rats were randomly allocated to three experimental groups undergoing either sham operation and saline infusion (Control, n = 30), hemorrhagic reduction of mean arterial pressure to 30 mmHg for 30 min alone (hypotension, n = 51), or hypovolemic hypotension followed by bolus infusion of $CaCl_2$ (200 $mg \cdot kg^{-1}$; hypercalcemia, n = 85). Serum ionized calcium, amylase activity, trypsinogen activation peptide in pancreatic tissue homogenates, pancreatic wet/dry weight ratio, histologic changes, and mortality were assessed for 24 h.**

Results: **Control rats showed no significant changes of any parameter throughout the experiments. In contrast, hypotension significantly increased serum amylase ($P < 0.001$), tissue trypsinogen activation peptide ($P < 0.01$), wet/dry weight ratio ($P < 0.001$), and histologic scores for edema ($P < 0.001$) and pancreatic necrosis ($P < 0.05$). Subsequent $CaCl_2$ administration transiently increased $[Ca^{2+}]$ ($P < 0.001$) with the concentration rapidly returning to baseline within 3 h. That infusion of $CaCl_2$ further increased amylase ($P < 0.05$), tissue trypsinogen activation peptide ($P < 0.05$), wet/dry weight ratio ($P < 0.001$), and histologic evidence of pancreatic edema ($P < 0.05$) and acinar necrosis ($P < 0.05$) when compared with hypotension alone. Whereas all Control animals survived the experiments, 22% ($P < 0.05$) and 47% ($P < 0.05$ *vs.* hypotension) of animals died in the hypotension and hypercalcemia groups, respectively.**

Conclusions: **Temporary hypotension alone causes ectopic trypsinogen activation and lethal acute pancreatitis. Superimposed hypercalcemia significantly aggravates hypotension-induced pancreatic injury and mortality in rats. (Key words: Ions: calcium; hypercalcemia. Cardiopulmonary bypass. Hypovolemic hypotension. Pancreas: acute pancreatitis; trypsinogen activation.)**

ACUTE pancreatitis is a well-recognized complication after cardiopulmonary bypass (CPB).[1–7] Although diagnosed infrequently, it assumes clinical importance because of the frequency of CPB procedures and its association with an unfavorable clinical course and mortality as great as 86%.[2,4] Evidence of acute pancreatitis in approximately 25% of autopsies of patients dying after cardiac surgery further underlines the relevance of this complication.[3,6] Several causative factors have been described, including hypothermia,[8] nonpulsatile blood flow,[9] complement activation,[10] and pancreatic ischemia.[1–7] Considering the well-described pathogenetic role of ischemia in acute pancreatitis[11,12] many authors have suggested pancreatic ischemia, which results from temporary hypotension during CPB,[13,14] as a primary factor for pancreatic complications after CPB.[1–6] A recent prospective clinical study suggested administration of a large dose of calcium during separation from CPB is an equally significant risk factor.[7] Large calcium doses are routinely administered in many centers during emergence from CPB because serum $[Ca^{2+}]$ can decrease during CPB[15] and postoperative cardiovascular function can benefit from the inotropic and vasopressor effects of calcium.[16,17] However, other studies reported that the serum ionized calcium concentration does not change[17,18] or even increases[19] during CPB and that calcium infusion is not beneficial[20,21] and may in fact adversely affect postop-

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erative cardiac recovery.[22,23] Based on these conflicting data, the routine administration of calcium salts after CPB is still openly discussed.[24,25] Because the risk for postoperative pancreatic complications would principally influence the indication for routine calcium administration, we investigated the effect of calcium infusion on the ischemic pancreas with emphasis on ectopic protease activation, which is thought to be a critical event in the pathophysiology of pancreatitis.[26,27] We hypothesized that the administration of calcium chloride to rats subjected to hemorrhagic hypotension would worsen pancreatic injury and exacerbate mortality.

Materials and Methods

Experimental Protocol

Experiments were carried out according to National Institutes Health guidelines for the care and use of laboratory animals and approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital. Male Sprague-Dawley rats (weighing 260–360 g) were obtained from Charles River Laboratories (Wilmington, MA). Anesthesia was initiated with vaporized ether and maintained by intraperitoneal injections of 20 $mg \cdot kg^{-1}$ pentobarbital (Anpro Pharmaceuticals, Arcadia, CA) and 40 $mg \cdot kg^{-1}$ intramuscular ketamine (Ketalar, Parke-Davis, Morris Plains, NJ). On the day before the experiment, a polyethylene catheter (Intramedic, ID 58 mm, Clay Adams, Parsippany, NJ) was introduced into the left internal carotid artery and advanced into the aorta for blood sampling, infusions, and monitoring of arterial pressure. The catheter was tunneled subcutaneously to the suprascapular region and brought out through a steel tether, allowing unrestrained activity of the animal. Animals were then allowed to stabilize and fasted overnight with water *ad libitum*. The next day, animals were randomly allocated to a Control group receiving no further treatment (n = 30), hypotension group undergoing reduction of mean arterial pressure to 30 mmHg for 30 min by controlled hemorrhage *via* the arterial catheter, and subsequent reinfusion of the heparinized blood (Elkins-Sinn, Cherry Hill, NJ, 75 U/ml; n = 51) and a hypercalcemia group in which the hypotensive period and reinfusion was followed by a bolus injection of 200 $mg \cdot kg^{-1}$ $CaCl_2$ over 2 min (n = 85). Mean arterial pressure and heart rate were monitored using an Electrodyne ST-219 transducer (Becton-Dickinson, Parsippany, NJ).

Assays

Serum ionized calcium concentration was measured with an ion-selective electrode (Nova 2, Nova Biomedical, Waltham, MA) after reinfusion of the withdrawn blood (baseline), 5 min, 20 min, 1 h, 3 h, 6 h, and 24 h. Concentrations of serum amylase were determined at baseline, 20 min, 6 h, and 24 h according to the method of Ceska *et al.*[28] Electrophoretic separation of serum amylase isoenzymes was performed in 7% polyacrylamide gel (pH 8.3). Serum samples were compared to secretions obtained by direct aspiration from the pancreatic and salivary ducts of control animals. Control serum was included in every experiment and all specimens were run in duplicate. After completion of electrophoresis, amylase activity was assayed in sequential segments of the gel using a previously described calorimetric technique.[29]

For measurement of trypsinogen activation peptide (TAP) concentrations in pancreatic tissue animals were killed at baseline, 1, 6, and 24 h, the pancreas resected, and two tissue portions (0.18–0.35 g) excised. Specimens were immersed in 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.3) containing 20 mM ethylenediaminetetraacetic acid and remaining protease activity immediately denatured by heat (100°C for 15 min). Each sample was then homogenized in a Brinkman Polytron (Brinkman Instruments, Westbury, NY) for 30 s with subsequent centrifugation (1,500 rpm, 10 min, 4°C). The resulting supernatants were coded, and stored at −20°C until assayed. Samples were simultaneously assayed for free TAP using a competitive enzyme-linked immunosorbent assay.[30,31]

Wet/Dry Weight Ratio

At the designated time points (baseline, 1, 6, and 24 h) the entire gland was resected in a standardized fashion by the same investigator, trimmed of fat, blotted dry, and weighed. Pancreatic water content was determined by calculating the ratio of the initial weight of the pancreatic specimen (wet weight) to its weight after incubation at 210°C for 12 h (dry weight).

Histopathologic Analysis

Specimens of pancreatic tissue from each time point were fixed in 10% phosphate-buffered formalin (pH 7.5), embedded in paraffin, and stained with hematoxylin and eosin for evaluation by light microscopy. The extent of interstitial edema formation, acinar cell necrosis, parenchymal hemorrhage, and inflammatory in-

filtration was quantitated by a pathologist unaware of the identity of the specimens using a previously described scoring system (range 0–4).[32]

Statistical Analysis

The results are presented as mean ± SEM of at least five different experiments in each group at each time point. Differences between experimental groups were tested with one-way analysis of variance directly or after calculating the area under the curve (when multiple time points were obtained), using Student's *t* test with Bonferroni correction for individual differences. Statistical significance of changes from baseline values within one experimental group was investigated by paired Student's *t* test. Proportional differences between the individual groups were tested by χ^2 analysis. Differences were considered significant if *P* values of <0.05 were obtained.

Results

Circulatory Parameters, Serum Ionized Calcium

Mean arterial pressure of all animals before hemorrhage averaged 121 ± 7 mmHg without significant differences between experimental groups. The average blood volume withdrawn to achieve 30 mmHg was 9.4 ± 0.5 ml (hypotension) and 8.8 ± 0.4 ml (hypercalcemia; 49 ± 3% and 46 ± 2% of estimated circulating blood volume, respectively). After reinfusion of the withdrawn blood mean arterial pressure returned to 110 ± 10 mmHg in hypotensive and 107 ± 13 mmHg in hypercalcemic animals after 30 min. Subsequent injection of CaCl2 in the hypercalcemia group transiently increased mean arterial pressure to 142 ± 15 mmHg at 5 min ($P < 0.01$), and mean arterial pressure remained increased after 30 min (133 ± 12 mmHg, $P < 0.05$). Serum ionized calcium concentration after reinfusion of the withdrawn blood was 1.1 ± 0.1 $mmol \cdot l^{-1}$. The $CaCl_2$ bolus induced an immediate increase of $[Ca^{2+}]$ to 3.1 ± 0.2 $mmol \cdot l^{-1}$ after 5 min ($P < 0.001$), which decreased to 2.0 ± 0.1 $mmol \cdot l^{-1}$ after 20 min ($P < 0.001$ *vs.* baseline and 5 min) and to baseline concentrations (1.2 ± 0.1 $mmol \cdot l^{-1}$) within 3 h.

Serum Amylase Concentration and Isoenzyme Pattern

The serum amylase concentration in control animals remained constant during the experimental period (52 ± 5 $U \cdot l^{-1}$). In contrast, temporary hypotension immediately increased serum amylase at 20 min (103 ± 5 $U \cdot l^{-1}$, $P < 0.05$), and further to 156 ± 21 $U \cdot l^{-1}$ ($P < 0.001$) at 24 h. Addition of temporary hypercalcemia significantly increased serum amylase activity at 20 min above that brought about by hypotension alone (134 ± 11 $U \cdot l^{-1}$, $P < 0.05$). Concentrations at 24 h were still increased but not significantly different between the hypotension and hypercalcemia groups (fig. 1). Electrophoretic analysis of the serum amylase isoenzyme pattern demonstrated that the observed increase of serum amylase activity was caused by isoamylases originating from the pancreas (data not shown).

Ectopic Trypsinogen Activation

Concentration of TAP in pancreatic tissue homogenates of controls remained unchanged (83 ± 5 $nmol \cdot^{-1} \cdot g^{-1}$) throughout the experimental period (fig. 2). Temporary hypotension caused an increase of TAP concentrations at 6 h (299 ± 45 $nmol \cdot l^{-1} \cdot g^{-1}$, $P < 0.01$) that was maintained until 24 h (355 ± 77 $nmol \cdot l^{-1} \cdot g^{-1}$, $P < 0.001$). Superimposing calcium infusion resulted in increased intrapancreatic trypsinogen activation at 1 h (243 ± 35 $nmol \cdot l^{-1} \cdot g^{-1}$, $P < 0.05$ *vs.* hypotension at 1 h) while later changes were not significantly different from the hypotension group (fig. 2).

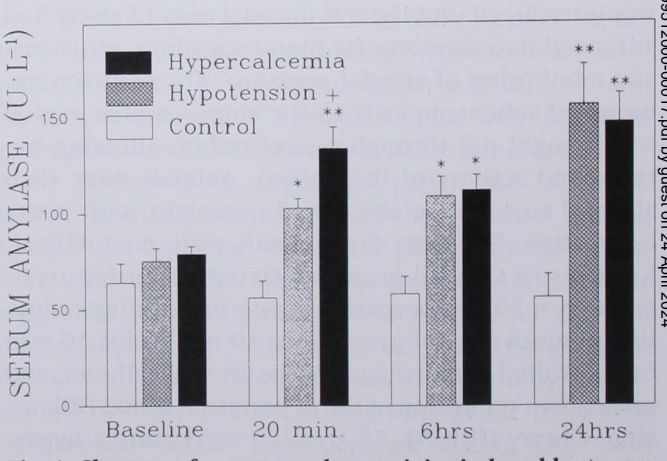

Fig. 1. Changes of serum amylase activity induced by temporary hypotension alone and combined with subsequent $CaCl_2$ infusion. Serum amylase increases immediately after temporary hypotension and further at 24 h. Superimposed $CaCl_2$ significantly increases the initial amylase response while later values are not significantly different. Mean ± SEM ($P < 0.001$, *$P < 0.05$ timepoint *vs.* baseline; †$P < 0.05$ hypercalcemia *vs.* hypotension).**

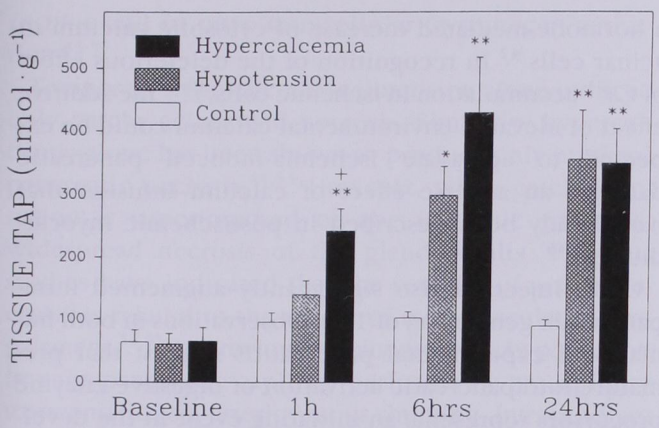

Fig. 2. Concentration of trypsinogen activation peptide in pancreatic tissue homogenates during the experimental period. Hypotension continuously increases intrapancreatic trypsinogen activation. Subsequent hypercalcemia accelerates TAP generation during the first 6 h ($P < 0.001$, *$P < 0.01$, †$P < 0.05$, *group vs control, †hypercalcemia *vs.* hypotension).**

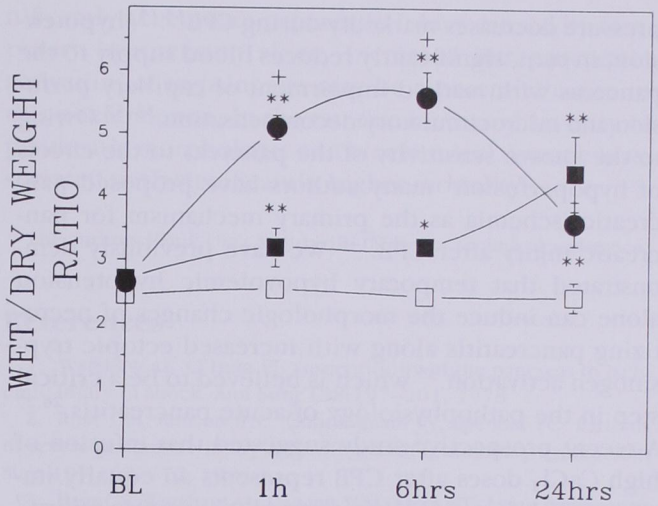

Fig. 3. Hypotension-induced increase of pancreatic wet/dry weight ratio after hypotension (closed squares) when compared to controls (open squares). The addition of hypercalcemia (closed circles) further increases this index of edema ($P < 0.001$, *$P < 0.01$, †$P < 0.001$ hypercalcemia *vs.* hypotension).**

Morphology and Histopathology

Macroscopic edema formation was absent in control preparations throughout the experiments (wet/dry weight ratio 2.3 ± 0.2). Temporary hypotension induced a significant increase of wet/dry weight ratio to 3.2 ± 0.3 at 1 h ($P < 0.001$) and 4.4 ± 0.6 at 24 h ($P < 0.001$). When calcium was administered, edema formation was significantly increased as reflected by a wet/dry weight ratio of 5.1 ± 0.2 after 1 h ($P < 0.001$ vs hypotension). The wet/dry weight ratio remained increased until 24 h (fig. 3). Light microscopy demonstrated normal pancreatic histologic appearance in the sham-operated animals. Histologic changes induced by hemorrhagic hypotension included interstitial edema formation at 1 h ($P < 0.001$), patchy acinar necrosis at 24 h ($P < 0.05$) (fig. 4), hemorrhage (11% of specimens), inflammatory infiltration (14%) and scattered vacuolar degeneration of acinar cells. Superimposed hypercalcemia significantly increased scores for edema at 1 h ($P < 0.001$) and necrosis at 24 h ($P < 0.001$) (fig. 4), whereas inflammatory infiltration (33%), hemorrhage (42%), and vacuole formation increased only nonsignificantly compared to hypotension.

Mortality

The observed morphologic and biochemical changes were associated with an increased mortality. While all control animals survived the experiments, 24-h mortality was 22% ($P < 0.05$) in hypotensive animals. When temporary hypotension was combined with calcium infusion mortality was significantly further increased to 47% ($P < 0.05$ hypercalcemia *vs.* hypotension).

Discussion

Despite their importance for postoperative morbidity and mortality, the mechanisms responsible for development of pancreatic injury after CPB are still poorly understood. It is well established that systemic arterial

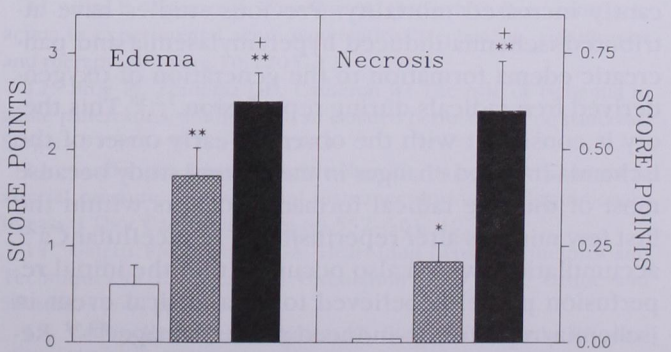

Fig. 4. Comparison of histologic scores for edema at 1 h and acinar necrosis at 24 h. While hypotension alone causes moderate changes of both parameters, superimposed calcium administration significantly aggravates the hypotension-induced histopathologic changes ($P < 0.001$, *$P < 0.05$, group *vs.* control, †$P < 0.001$ hypercalcemia *vs.* hypotension).**

pressure decreases markedly during CPB[13,14]; hypotension, in turn, significantly reduces blood supply to the pancreas with marked impairment of capillary perfusion and microcirculatory decompensation.[33–37] Owing to the known sensitivity of the pancreas to the effects of hypoperfusion[1] many authors have proposed pancreatic ischemia as the primary mechanism for pancreatic injury after CPB.[1–6] We have previously demonstrated that temporary hypovolemic hypotension alone can induce the morphologic changes of necrotizing pancreatitis along with increased ectopic trypsinogen activation,[35] which is believed to be a critical step in the pathophysiology of acute pancreatitis.[26,27] A recent prospective study suggested that infusion of high $CaCl_2$ doses after CPB represents an equally important risk factor for "postpump" pancreatic injury,[7] but was unable to establish causality. In the current study, we address this question. Because the conditions of CPB cannot be replicated in the rat, we chose as an experimental paradigm that of hemorrhagic shock, which has been shown to cause decreased pancreatic perfusion and pancreatitis.[11,35] We compared the effect on the pancreas of temporary hypovolemic hypotension alone or in conjunction with bolus infusion of calcium to simulate the clinical sequence of events during CPB.

The observed effects of systemic hypotension on serum amylase activity, ectopic trypsinogen activation, and pancreatic morphology in this study are similar to those we described previously as a consequence of pancreatic ischemia.[35] However, in the current study, subsequent administration of $CaCl_2$ significantly accelerated and accentuated the development of the hallmarks of pancreatic injury, resulting in a significantly increased mortality. Previous studies have attributed ischemia-induced hyperamylasemia and pancreatic edema formation to the generation of oxygen-derived free radicals during reperfusion.[12,38] This theory is consistent with the observed early onset of the ischemia-induced changes in the current study because most of the free radical formation occurs within the first few minutes after reperfusion.[39] Intracellular Ca^{2+} accumulation, which also occurs within the initial reperfusion phase, is believed to be a critical event in ischemia/reperfusion-induced tissue damage.[40,41] Reperfusion-induced elevation of intracellular [Ca^{2+}] is likely further increased by the acute elevation of blood ionized calcium because hypercalcemic environments increase acinar [Ca^{2+}].[42,43] In addition, calcium infusion induces release of cholecystokinin,[44] which also causes a hormone-mediated increase of cytosolic calcium in acinar cells.[42] In recognition of the deleterious effect of Ca^{2+} accumulation in ischemic cells,[40,41] the additive effect of elevated environmental calcium could be expected to aggravate ischemia-induced pancreatic damage, an adverse effect of calcium infusion that has already been described in postischemic myocardium.[45,46]

$CaCl_2$ injection also significantly augmented intrapancreatic generation of TAP. Observations in both human and experimental pancreatitis suggest that premature intrapancreatic activation of digestive enzyme precursors represents an initiating event in the development of acute pancreatitis.[26,27,35,43] Generation of trypsin plays a pivotal role in the activation process of pancreatic proteases due to trypsin's autocatalytic activity and ability to initiate the rest of the pancreatic enzyme cascade.[27] Under physiologic conditions, TAP is cleaved from trypsinogen by enterokinase in the intestinal lumen and subsequently is degraded by mucosal peptidases. However, when ectopic intrapancreatic activation occurs, TAP provides a quantitative index of active trypsin generation that correlates with the severity of pancreatic damage.[47,48] Under the conditions of this study, a small amount of TAP is found in pancreatic tissue at baseline, but these levels remained unchanged in controls throughout the experiments and are believed to represent minor physiologic autoactivation that occurs in the normal pancreas.[27,49] In contrast, pancreatic TAP concentration increased continuously after hypotension, and, as with amylase release and edema formation, $CaCl_2$ further accelerated the ischemia-induced generation of TAP in pancreatic tissue.

Because several studies have demonstrated that increased calcium concentrations increase trypsinogen autoactivation,[50,51] increased cytosolic [Ca^{2+}] in the ischemic acinar cells has been suggested as the mechanism of the ischemia-induced increase of trypsinogen activation.[35] Calcium is an essential cofactor in trypsinogen autoactivation.[27,52] It enhances the stability and activity of trypsin[52] by binding to the N-terminal aspartyl residues of trypsinogen activation peptides, thereby abolishing their inhibitory effect on autocatalytic activation.[50] Increased Ca^{2+} accumulation in acinar cells as a consequence of ischemia and subsequent hypercalcemia could therefore contribute to the observed acceleration of TAP generation. Preliminary data from our laboratory demonstrates that hypercalcemia alone increases acinar [Ca^{2+}], which in turn can increase

in vivo and *in vitro* intracellular trypsinogen activation.[43,53]

Temporary hypovolemic hypotension alone induced only patchy acinar cell necrosis. Similarly, hypercalcemia alone has been shown to produce only minimal pancreatic necrosis.[44,53] However, when calcium infusion is superimposed on the ischemic pancreas, widespread necrosis of the gland results. Previous studies have indicated that the severity of acinar necrosis is related to the extent of microcirculatory impairment.[54,55] As previously demonstrated, hypotension impairs pancreatic capillary perfusion by precapillary vasoconstriction, arteriovenous shunting, intracapillary clotting, and venous stasis.[1,11,34,35] Because hypercalcemia can promote platelet aggregation and blood coagulation[56] and decreases erythrocyte deformability through the Gardos effect,[57] it could foster further microcirculatory deterioration. Calcium-induced stimulation of precapillary resistance effectors could also contribute to the compromise of pancreatic capillary flow, an effect whose importance for progression to pancreatic necrosis has been shown for other therapeutic vasoconstrictors.[58]

Temporary hypotension itself caused death in 22% of animals. The prevalence of fatal outcome was more than doubled when calcium infusion was added to the initial ischemic insult. Inasmuch as preliminary experiments demonstrated that comparable infusions of $CaCl_2$ alone are not lethal,[53] these data illustrate the potentially serious consequences of calcium administration if the pancreas is already primed and susceptible because of an ischemic lesion.

The rats in this experimental study were normocalcemic after the period of hypovolemic hypotension and before the calcium infusion. While it could be argued that many patients after CPB are hypocalcemic (and thus presumably less susceptible to the development of hypercalcemia with calcium administration), an inevitable transient hypercalcemia will occur whenever calcium is given as a bolus as is commonly done.[25] Furthermore, many patients continue to receive $CaCl_2$ during the initial postoperative period without regard for measured concentrations of ionized calcium, even though normocalcemia may have been restored. Under these conditions of clinical practice, the experimental model used in this study would be both comparable and relevant to the previously demonstrated association between $CaCl_2$ administration and pancreatic injury.[7]

In summary, we were able to demonstrate that infusion of calcium after temporary hypotension can significantly accentuate pancreatic injury caused by temporary hypotension alone. Increased intrapancreatic activation of trypsinogen may be a potential cause of this phenomenon. In the rat, this increased severity of pancreatic morphologic and biochemical damage is associated with significantly higher mortality.

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