Background

Chronic pancreatitis (CP) is a progressive inflammatory disease characteristically accompanied by irreversible functional (exocrine and endocrine pancreatic insufficiency) and structural damages (ductal irregulation and dilatation, calcification)[1] of the pancreas. It is well documented that smoking is an independent risk factor of CP and the risk of CP increases in a dose-dependent manner[2]. However, the exact mechanism of pathological cellular events generating by cigarette smoke, which can lead to development of pancreatitis, is unresolved. Human **pancreatic ductal epithelial** cells (PDEC) produce an alkaline fluid which is essential for normal digestion and crucially important for the maintaining the integrity of the pancreas. One of the key proteins in pancreatic ductal secretion is cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. Our group has recently showed that the other risk factor of CP, alcohol disrupts expression and localization of the CFTR, which appears to contribute to the development of pancreatitis[3]. Notably it has been shown that the **HCO_3^- concentration in pancreatic juice is reduced in CP**[4], however, the exact mechanism is unclear. Therefore, comprehensive analysis of the effects of smoking on pancreatic ductal system and function of CFTR was crucially important since it may offer potential **therapeutic targets in CP.**

Results and discussion

1. Smoking decreases CFTR expression and activity in human subject

The elevated sweat Cl^- concentration (Cl^-_{sw}) is correlate with the dimished CFTR absorptive activity (hiv 9) which is typical in CF patients. In our study, Cl^-_{sw} was 40.45±1.7 mmol/L in non-smoker patients without CP. In a smoker, without CP group, the Cl^-_{sw} was significantly elevated (50.05±2.74 mmol/L), suggesting inhibition of CFTR by smoking (Fig 1A). It is documented, that trafficking of CFTR is largely compromised in CP (Ko et al OTKA). In accord with this finding, further elevation of the Cl^-_{sw} was observed in non-smoker patients with CP (60.46±4.1 mmol/L). Additional inhibiton of CFTR absorptive activity was detected in a smoker, CP goup, which was indicated the highest Cl^-_{sw} (67.42±4.2 mmol/L).

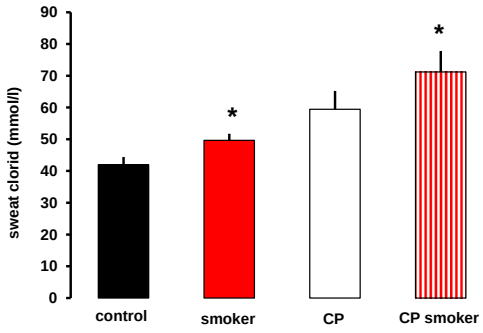


Figure 1. *Smoking inhibits CFTR function*

Next, we determined the effects of smoking on mRNA expression of CFTR. 3D human pancreas organoid culture (Fig 2.A) and CAPAN-1 cells (Fig 2.B) were incubated with CSE for 24 h. In both cell type, CFTR mRNA expression was significantly lower after CSE treatment compaerd to control (Fig 2).

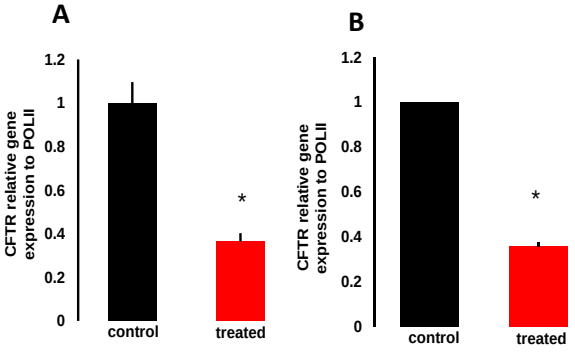


Figure 2. Smoking inhibits CFTR mRNA expression

After that, we detected the effect of smoking on CFTR localization in the pancreas using tissue samples from non-smoker (Fig 3.A) and smoker (Fig 3.B) cadaver donors, and from non smoker (Fig 3.C) and smoker (Fig 3.D) CP patients. CFTR expression on the apical membrane of pancreatic ducts was significantly reduced in smoker groups compared to non smoker patients in both patients with

and without CP. (Fig 3) However, cytoplasmic density of CFTR did not change between the non smoker and smoker patients with CP.

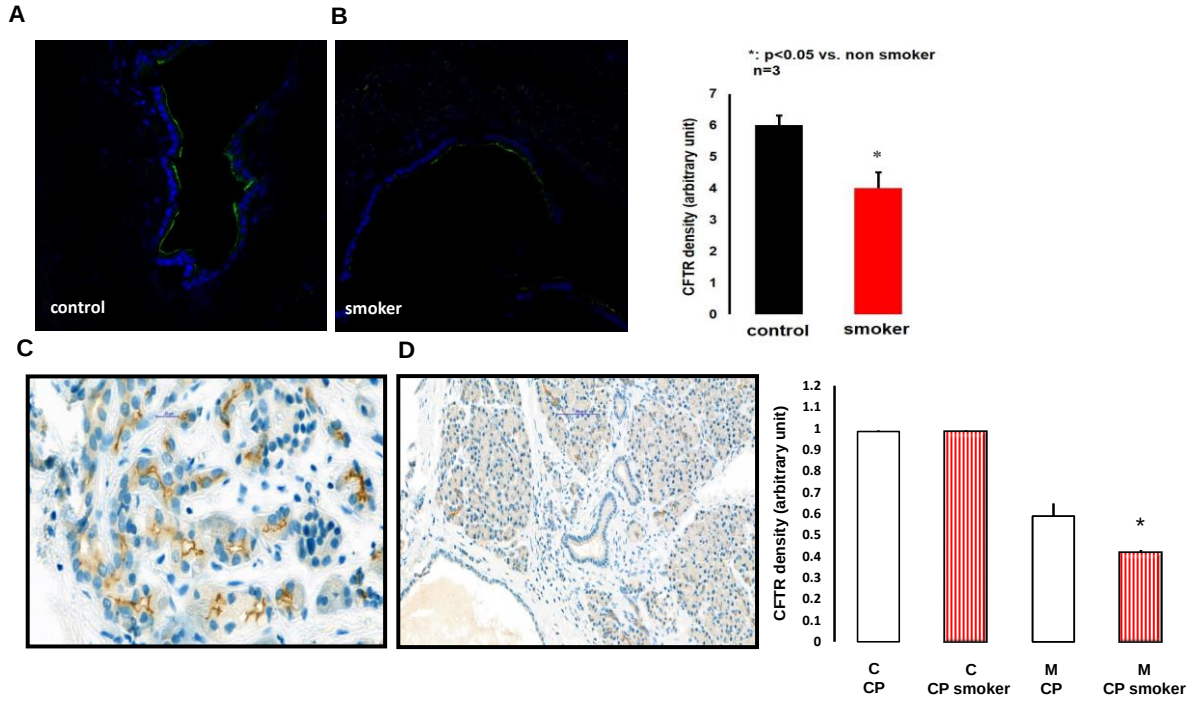


Figure 3. Smoking reduced the expression of CFTR on the apical membrane of pacreatic ductal cells.

Inhalation of many different toxins, such as heavy metals can be responsible for smoking-related diseases. Therefore, we analized 20 different heavy metal content of human serum samples. In case of mercury (Fig 4.A) and cadmium (Fig 4.B) the difference were significant between the non-smoker (Cd: 0,098±0,0058 ng/ml; Hg: 14.83±0.98 ng/ml) and smoker groups (Cd: 0,159±0,0,01 ng/ml; Hg: 23.05±2.22 ng/ml). Therefore, Cd concentration was analyzed in human tissue samples from non smoker and smoker cadaver donors. One order of magnitude higher amount of Cd was measured than in serum samples, which indicate accumulation of Cd in pancreas. Significantly higher level of Cd was detected in a tissue from smoker patients (3.38±0.38 µg/ml) compared to non smoker (1.06±0.61 µg/ml) (Fig 4.C.)

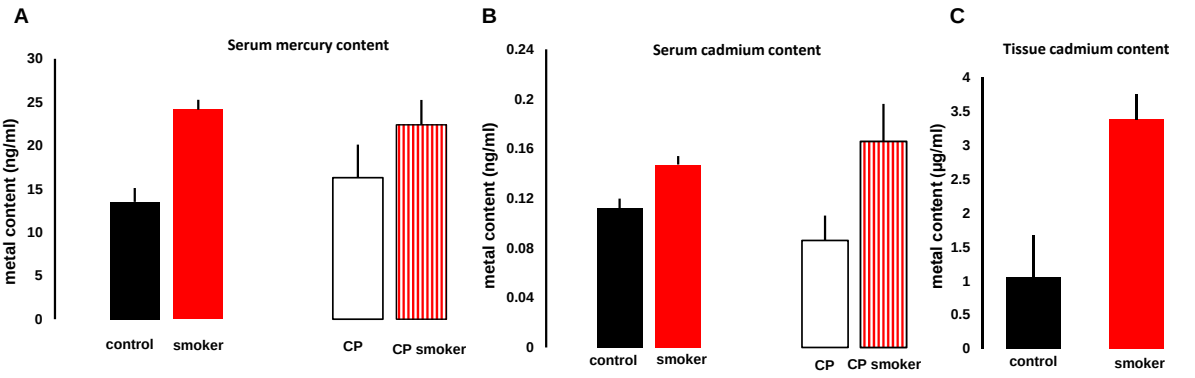


Figure 4. Cadmmium and mercury concentration is significantly higher in serum and tissue samples from smoker patients.

2. Whole-body smoking reduce pancreatic fluid and bicarbonate secretion and inhibit CFTR Cl^- channel expression and function

In the next step, animal model was used to clarify the details of our human results. To mimic the effect of smoking guinea pig was exposed to whole-body smoke exposure for 6 weeks.

At the beginning of the *in vitro* fluid secretion experiments, the ducts were perfused with standard HEPES solution and then the perfusion was changed to standard HCO_3^-/CO_2 solution. An increase in the luminal volume in HCO_3^- solution was detected. Then the ducts were stimulated with 5 µM forskolin, which resulted in a further increase in luminal volume in non-smokers. In contrast, this rapid elevation was not observed in ducts isolated from guinea pigs smoked for 6 weeks. Therefore, the fluid secretion is significantly reduced in smoked animals (Fig 5).

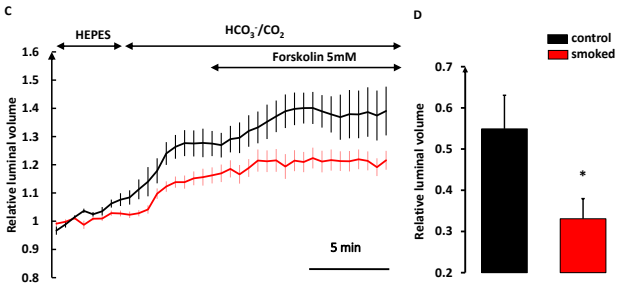


Figure 5. Whole body smoke reduces pancreatic fluid secretion.

After characterization of fluid secretion, we also examined the effect of smoking on HCO_3^- secretion. Pancreatic ductal HCO_3^- secretion was measured by using luminal Cl^- removal technic, where the initial rate of intracellular pH elevetion reflects the activity of luminal Cl^-/HCO_3^- exchangers an CFTR. Similarly to ductal fluid secretion, the duct isolated from smoked guinea pigs had significantly lower levels of alkalosis, so HCO_3^- secretion was reduced by smoking compared to control animals (Fig 6).

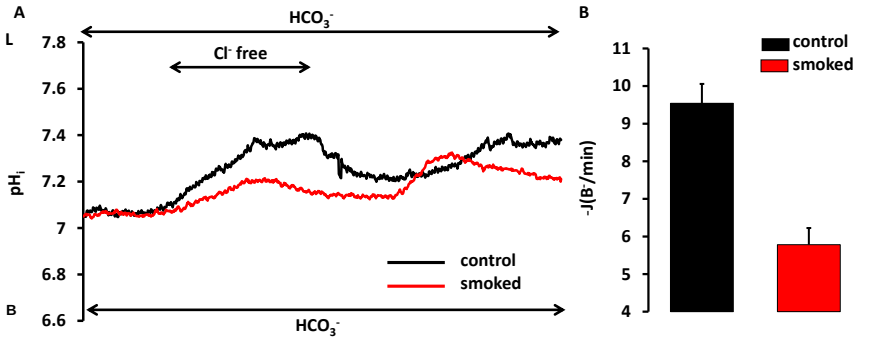


Figure 6. *Whole body smoke reduces pancreatic bicarbonate secretion.*

Subsequently, we examined the activity of CFTR, another major channel involved in HCO_3^- secretion. Using the whole cell configuration of the patch clamp technique, we demonstrated that 5 µM forskolin stimulates CFTR Cl^- current in non-smoked, control guinea pig pancreatic ductal cells. However, this stimulatory effect was significantly lower in smoked guinea pig cells (Fig 7).

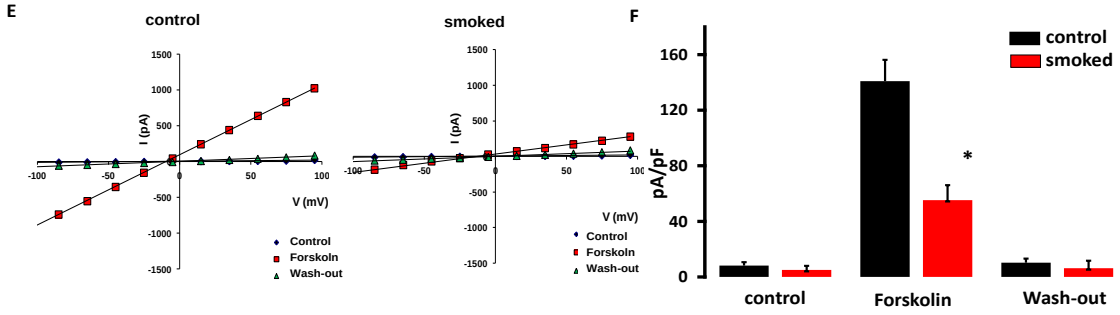
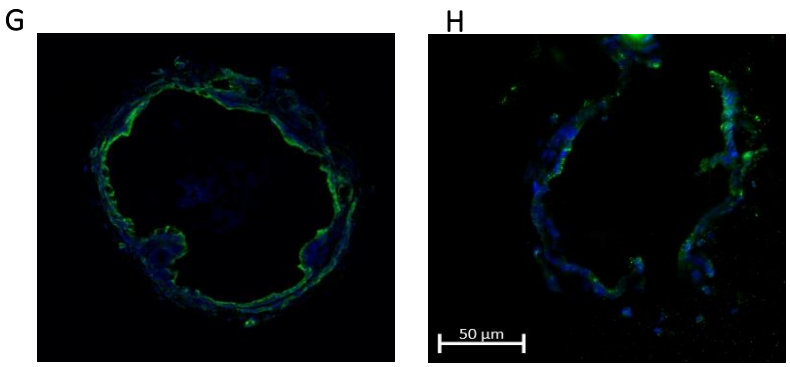


Figure 7. Smoking decreases CFTR Cl^- current.



The localization of CFTR on the apical membrane of the pancreatic ductal epithelial cells was significantly reduced in smoked guinea pigs (Fig. 8H) compared to control animals (Fig. 8G)

Figure 8. Smoking significantly reduced the expression of CFTR.

3. Whole-body smoking reduce pancreatic fluid and bicarbonate secretion and inhibit CFTR Cl^- channel expression and function

To elucidate the mechanism underlying the observed effect, we examined how the Ca^{2+} homeostasis, ATP levels, and mitochondrial membrane potential of ductal epithelial cells changed after smoking. In our experiments, we found that the maximum magnitude of the Ca^{2+} signal induced by 500 µM CDC was significantly smaller in pancreatic ductal epithelial cells isolated from a smoked animal.(Fig 9A, B).

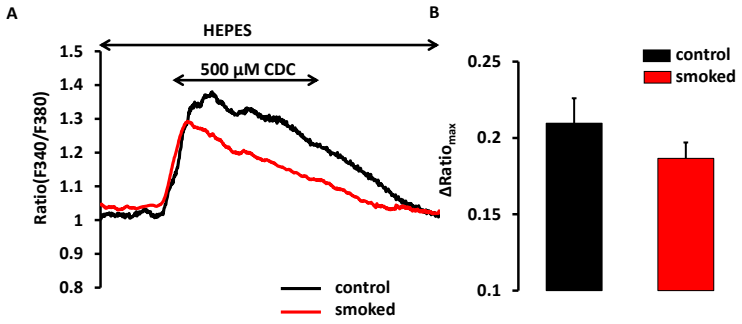


Figure 9. Smoking decreases Ca^{2+} signal in pancreatic ductal cells.

A similar result was obtained for ATP levels. Administration of a mixture causing total ATP depletion in the cell (DOG + CCCP + IAA) resulted in significantly lower ATP levels in pancreatic ductal fragments isolated from a smoked animal compared to control animals (Fig 10). We further examined how smoking affects mitochondrial function and found that smoking significantly reduced mitochondrial membrane potential compared to control animals (Fig 10).

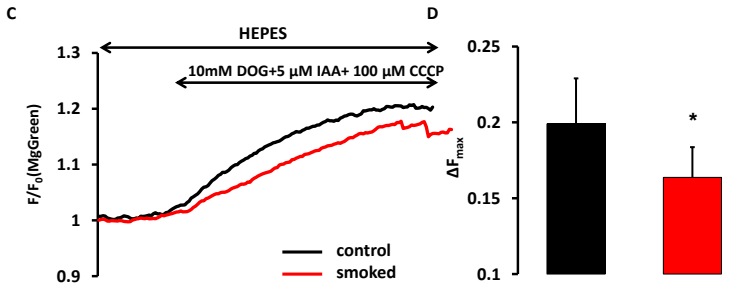


Figure 9. Smoking decreases ATP level in pancreatic ductal cells.

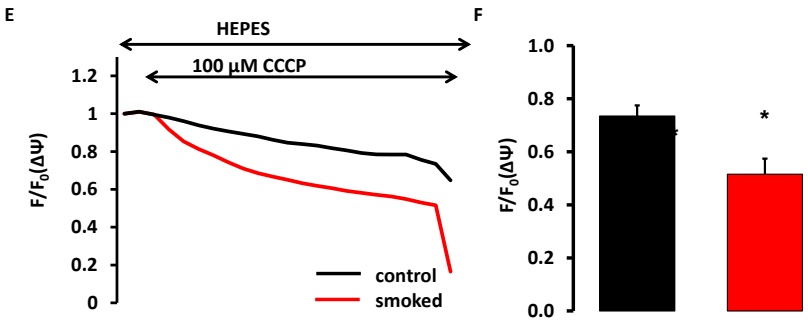
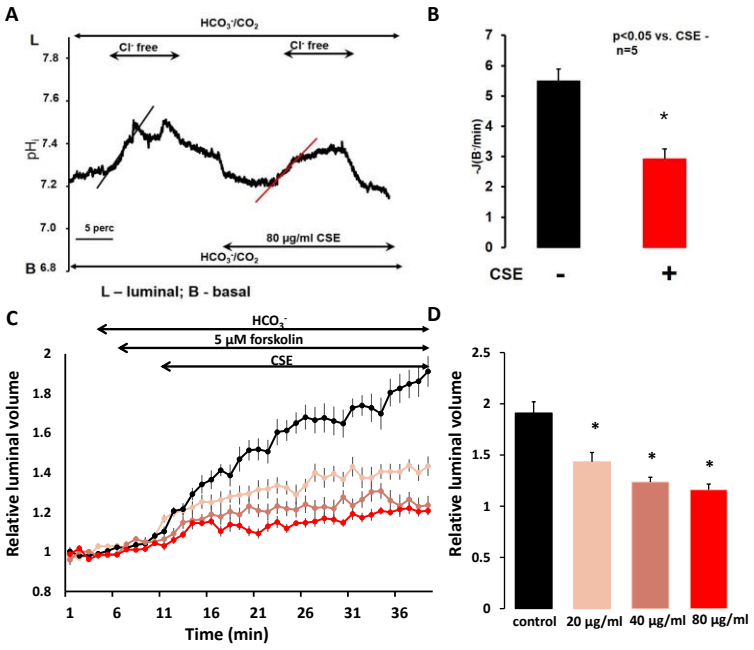


Figure 10. Smoking decreases mitochondrial membrane potential in pancreatic ductal cells.

To further characterize the effect of smoking, we developed an *in vitro* model. Cigarette smoke extract (CSE) was prepared by leading the smoke of 15 research cigarettes into distilled water and then measuring the dry matter content of the extract. Based on literature data, CSE was used at concentrations of 20, 40, and 80 µM in the experiments. In the first step, we examined whether CSE incubation affects the viability of ductal epithelial cells. We demonstrated that CSE treatment did not cause either apoptosis or necrosis in ductal epithelial cells, so it will be suitable for our experiments.

In this experimental set-up, ductal fragments were isolated from the pancreas of control animals, and pancreatic ductal fluid and HCO_3^- secretion, CFTR expression and function, and Ca^{2+} and cAMP levels, mitochondrial morphology and function were measured after CSE treatment.



To clarify our questions, we used the methods mentioned above. Our results showed that, similarly to smoking, CSE dose-dependently inhibited fluid (Fig 11A, B) and HCO_3^- secretion (Fig 11 C,D) by pancreatic ductal epithelial cells and CFTR protein expression in the apical membrane and function of ductal epithelial cells (Fig 12). Furthermore, we showed that lower intracellular Ca^{2+} (Fig 13A) and cAMP levels (Fig 13B) were observed in cells as a result of CSE treatment, and CSE significantly reduced the mitochondrial membrane potential (Fig 13C) of pancreatic ductal epithelial cells.

Figure 11. Cigaretta smoke extract decreases fluid and bicarbonate secretion in pancreatic ductal cells.

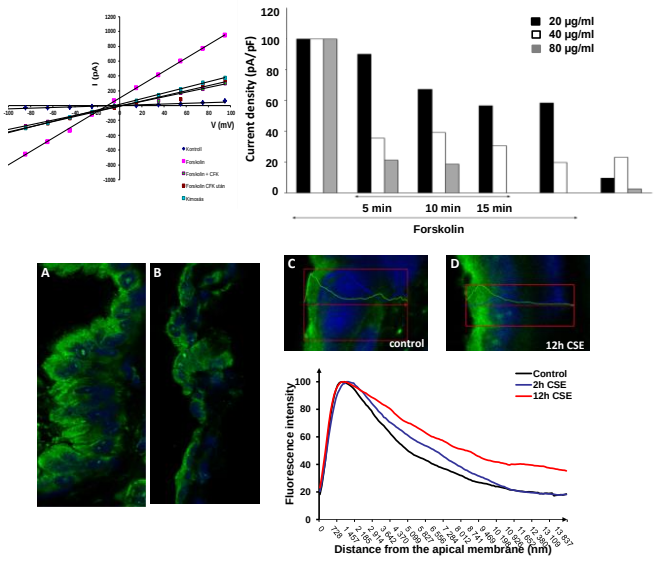


Figure 12. Cigaretta smoke extract decreases CFTR function and expression in pancreatic ductal cells.

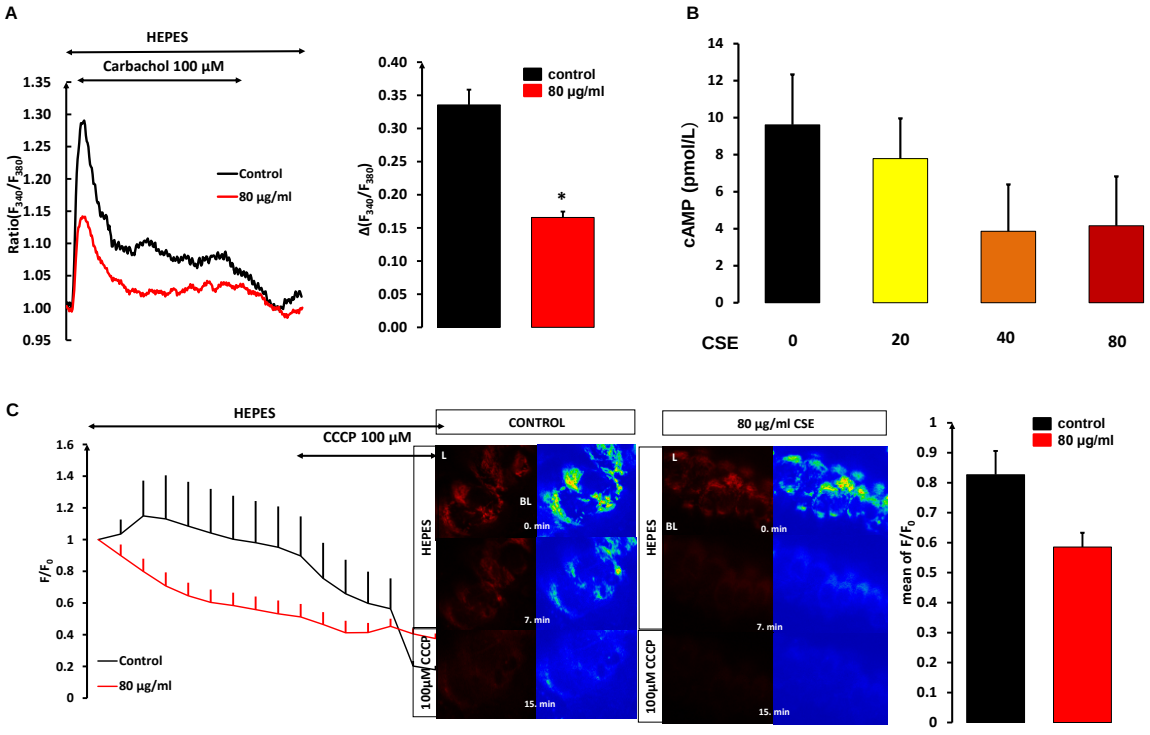


Figure 13. Smoking decreases Ca^{2+} signal (A), cAMP level (B) and mitochondrial membrane potential (C) in pancreatic ductal cells.

Cigarette smoke contains more than 4,000 ingredients. One of the most abundant component is nicotine, so we examined the effect of nicotine on bicarbonate secretion in ductal cells. We showed that the anion exchange function in the apical membrane of pancreatic ductal epithelial cells was not affected by nicotine treatment, however, it inhibited the function of transporters in the basement membrane involved in bicarbonate uptake (Fig 13).

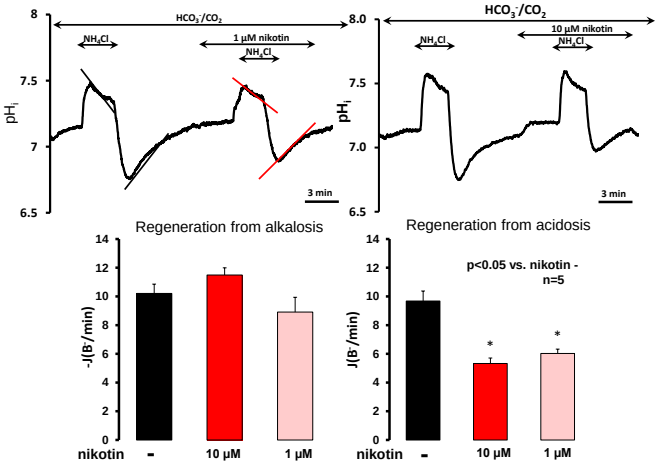


Figure 13. Nicotine inhibits bicarbonate uptake, but not secretion in pancreatic ductal cells.

Then we examined, how Cd and Hg influence CFTR protein expression, bicarbonate secretion, and cAMP levels in pancreatic ductal epithelial cells. We used the concentrations which were measured earlier from serum and tissue samples. For both heavy metals, we obtained a dose-dependent inhibition of bicarbonate secretion (Fig 14 A, B), cAMP levels (Fig 14C) and CFTR expression (Fig 14D) in pancreatic ductal cells.

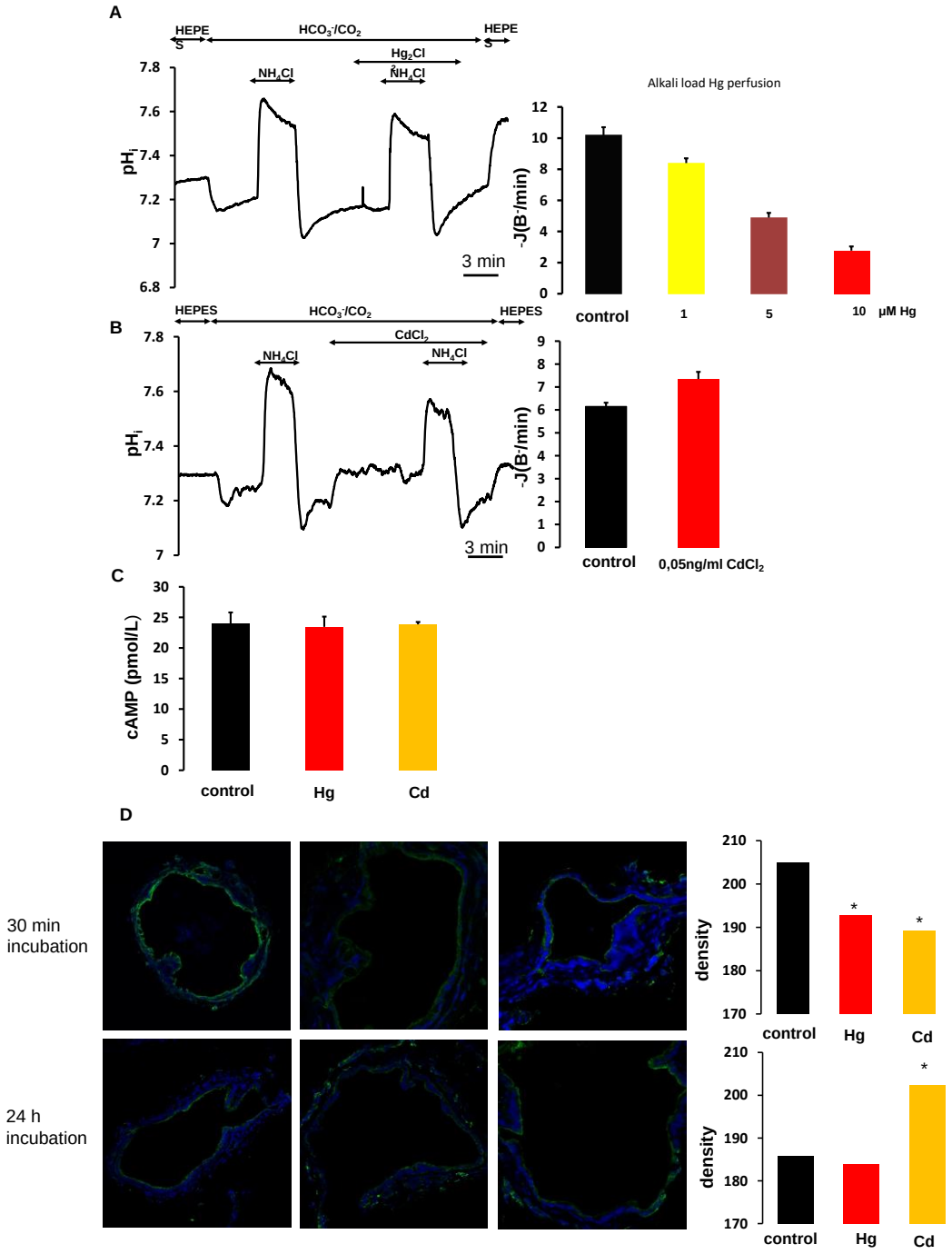


Figure 14. Cadmium and mercury inhibits bicarbonate secretion (A, B), cAMP level (C) and CFTR expression (D) in pancreatic ductal cells.

To sum up our results, we were able to characterize the effect of smoking on the function of pancreatic ductal epithelial cells. We have shown that smoking inhibits the physiological function of pancreatic ductal epithelial cells, reduces the expression and function of transporters involved in fluid and bicarbonate secretion, especially the CFTR chloride channel, and reduces Ca^{2+} and cAMP levels in the cells. In addition, it inhibits mitochondrial function, thereby damage the energy balance of cells. We were also able to identify two heavy metal components of cigarette smoke (cadmium, mercury) that may be responsible for the effects of smoking. Our results can contribute to understand the role

of smoking in the development of CP. Our results may open up new therapeutic possibilities in the treatment of CP.