Original Article

In Vitro Inhibition of Human Sperm Creatine Kinase by Nicotine, Cotinine and Cadmium, as a Mechanism in Smoker Men Infertility

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Abstract

Background: Nicotine, cotinine and cadmium are harmful components of cigarettes that have an effect on human reproductive function. Although the effects of cigarette smoke on male reproductive function is characterized in several articles its mechanism of action is still unknown.
In the present study, we investigate the effect of nicotine, cotinine and cadmium on human sperm creatine kinase activity *in vitro*.
Materials and Methods: Total creatine kinase activity is measured in sperm homogenates after chromatography on a diethylaminoethyl cellulose (DEAE-32) column.
Results: We show that creatine kinase activity is significantly inhibited by nicotine (44%), cotinine (39%) and cadmium (65%) at a concentration of 60 µg/ml. Kinetic studies reveal that the inhibitory effect of nicotine, cotinine and cadmium are competitive in relation to creatine phosphate.
Conclusion: Considering the importance of creatine kinase activity for normal sperm energy metabolism, our results suggest that inhibition of this enzyme by nicotine, cotinine and cadmium may be an important mechanism in infertility amongst male smokers. However, further investigations are needed to elucidate the exact mechanism of cigarette effect on male reproductive function at the molecular level.

Keywords: Sperm, Creatine kinase, Nicotine, Cotinine, Cadmium

Introduction

Infertility is common in couples of childbearing age. Approximately one half of these cases are related to a male infertility factor (1). Cigarette smoking is a serious health problem of our society which, according to the World Health Organization (WHO), approximately one third of the world's population above 15 years of age smokes. It is known that cigarette smoke is a cell mutagen and carcinogen that may adversely affect male fertility (2). The effects of smoking on male reproductive function have been widely studied in various mammalian species (3-4). Tobacco combustion yields about 4000 compounds; the smoke can be divided into a gaseous phase and a particulate phase. The principal harmful components of the gaseous phase are carbon monoxide, nitrogen oxide, ammonia and volatile hydrocarbons. The main components of the particulate phase are nicotine and cadmium (2). Nicotine is quickly absorbed through the respiratory tract, mouth mucosa and skin. About 80% to 90% of nicotine is metabolized by body organs; mainly by the liver, but also by the kidneys and lungs (5). Its major metabolite is cotinine. Nicotine and its metabolites have been detected in serum, urine, saliva, milk and more recently they have been found at significant levels in smokers' seminal plasma and in subjects exposed to environmental tobacco smoke (6).
Creatine kinase (CK, EC 2.7.3.2) is a thiol enzyme which plays a central role in energy homeostasis in cells such as spermatozoa that display high and variable rates of ATP turnover. Creatine kinase catalyzes the following reversible reaction:

$$\text{Phosphocreatine} + \text{MgADP} + H^+ \longleftrightarrow \text{Creatine} + \text{MgATP}$$

In effect, the creatine kinase reaction functions as a temporal and spatial ATP buffering system (7). Sperm moves using ATP and a phosphoryl creatine shuttle as an energy source (8). Therefore, proper function of creatine kinase is the main factor of energy preparation for sperm movement. While much research has been done on the effects of cigarette nicotine on male reproductive function (3-6), nevertheless, data concerning the effect of cigarettes and/or its compounds on sperm creatine kinase activity is limited. Thus, the aim of this study is to evaluate the *in vitro* effects of nicotine, cotinine and cadmium on creatine kinase activity isolated from human sperm.

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Materials and Methods

Materials

ADP, AMP, NADP, glucose 6-phosphate dehydrogenase, hexokinase, Triton X-100 and diethylaminoethyl cellulose (DEAE-32) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), nicotine, cotinine, cadmium and bovine serum albumin (BSA) were obtained from Merk Chemical Co. (Darmstadt, Germany). All other reagents used were of the highest grade and purity available. All solutions were prepared in doubly distilled-deionized water.

Subjects

Ten healthy non-smokers, aged 25 to 35 years, not normally exposed to passive smoking and not treated medically in the 6 months prior to the study, were selected from men attending the Laboratory of Seminology (Razi Laboratory, Ahwaz, Iran). All procedures were approved by international guidelines, the institute Research ethics and use Committee of Ahwaz Joundishapour University Medical Sciences.

The samples were collected by masturbation following a 3 day period of sexual abstinence. Routine semen analysis was performed, according to the WHO guidelines (9). Only subjects with the following seminal characteristics were included in the study: volume ≥3.0ml; sperm concentration/ml $\geq 50\times10^6$; forward motility ≥60%; atypical forms ≤40%.

Purification of creatine kinase from human sperm

All procedures were carried out at 4°C. Creatine kinase was isolated from human sperm, as previously described (8). In short, fresh semen (after liquefaction) was washed in at least 10 volumes of ice cold 30 mM tris-HCl, 80 mM NaCl, 40 mM KCl, 0.1mM $CaCl_2$, pH 8.2 and centrifuged at 5000 g for 20 minutes. The resulting pellet was extracted in ice cold 50 mM sodium phosphate, 150 mM NaCl, 0.2 mM EDTA, 1 mM sodium azide, 1% Triton X-100, pH 7.2. The extract was centrifuged at 20,000 g for 30 minutes. The supernatant that was collected essentially contained all of the creatine kinase. The supernatant was applied to a diethylaminoethyl cellulose (DEAE-32) column (2×20 cm) equilibrated with 0.02% tris, pH 8.0. The column was eluted with tris buffer (0.02%, pH 8.0) and a gradient of NaCl ranging from 0 to 0.6 M (8). Subsequently, 1ml fractions were collected and monitored for protein content at 280 nm. In each step an aliquot of the samples was removed for total protein determination (10), creatine kinase activity (11) and electrophoresis on polyacrylamide gel (12).

Creatine kinase activity determination

Creatine kinase activity during different steps of purification was measured by the Rosalki method (11). This method was based on reduction of NADP. The increase in optical density (OD) at 340 nm, which depends upon NADP reduction, was followed by spectrophotometry and provided a measure of creatine kinase activity. According to this method, one international unit of creatine kinase is that amount of enzyme which will utilize 1μmol of creatine phosphate substrate per minute at 25°C and pH 6.8. For each micromole of creatine phosphate consumed, 1 μmol of NADP was reduced. Since the reduction of 1 μmol of NADP in 1 ml of solution produced an increase in OD of 6.22 in cells of 1 cm light path at 340 nm, the reduction of 1 μmol in 3 ml test volume would produce an increase in OD of 2.07. Therefore, the number of micromoles of NADP reduced per minute in the test cuvette was equal to $\frac{\text{observed } \Delta OD/min}{2.07}$. Under assay conditions the number of micromoles of NADP was reduced by 100μl of solution obtained from each step of human sperm creatine kinase purification. The equivalent number of micromoles of NADP reduced per minute from 1 L of solution was equal to. Therefore, creatine kinase activity of the obtained solutions was equal to $\frac{\text{observed } \Delta OD/min \times 10000}{2.07}$.

Therefore creatine kinase activity of the obtained solutions is equal to the observed ΔOD/min×4800.

Effect of nicotine, cotinine and cadmium on human sperm creatine kinase activity

The effects of nicotine, cotinine and/or cadmium on creatine kinase activity were examined by incubation of 1 unit creatine kinase (10 μg/ml) with different concentrations (0-100 μg/ml) of the above chemicals in tris buffer (0.1 M, pH 6.8) at 25°C for 5 minutes. Chemicals (nicotine, cotinine and cadmium) were dissolved in distilled deionized water, as all chemicals were water soluble. The degree of inhibition of creatine kinase activity by different concentrations of nicotine, cotinine and cadmium was determined at 340 nm as previously described (11). Inhibition kinetic was determined by incubation of 1 unit creatine kinase with 2 mM nicotine, 3 mM cotinine and/or 3 mM cadmium in the presence of concentrations of 0 to 10 mM creatine phosphate (as creatine kinase substrate).

Statistical analysis

All data sets were expressed as mean±standard

deviation (SD). Creatine kinase activity in the absence (as control) and/or presence of nicotine, cotinine and/or cadmium was compared using analysis of variance (ANOVA). Differences in means were judged significant at $p<0.05$.

Results

The results of creatine kinase purification from human sperm are summarized in Table 1. As shown in this table, 5 mg of creatine kinase displayed 20 units of activity with a specific activity of 4 units per mg. The creatine kinase activity was observed between fractions 28 to 62 with a double peak (Fig 1).

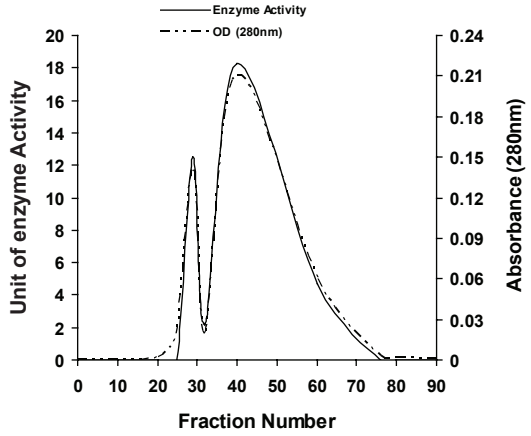

Fig 1: Variation of protein amounts in the elution fragments from DEAE-32 column creatine kinase activity. DEAE-32 column (2×20 cm) equilibrated with 0.02% tris, pH 8 and eluted with same buffer and a linear gradient of 0 to 0.6 M of NaCl. The flow rate was 1 ml/min.

Furthermore, the results of SDS-PAGE showed a single protein band (Fig 2) with molecular weight of 82 KD, which confirmed the purification stages (Fig 3).

A series of experiments was performed to examine the influence of nicotine, cotinine and cadmium on human sperm creatine kinase activity. The extent of inhibitory effect of the above chemicals is shown in Fig 4.

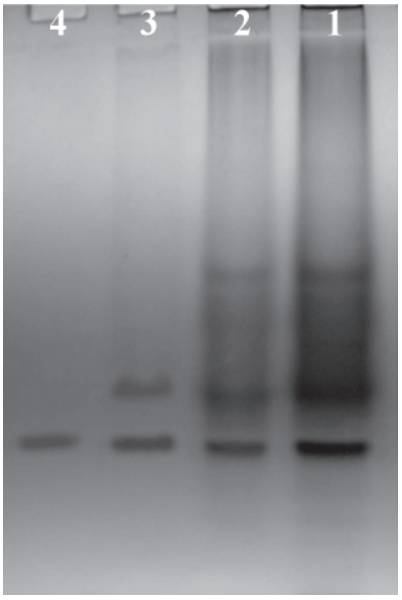

Fig 2: 7.5% SDS-PAGE analysis of the purified human sperm creatine kinase. Sample of sperm extracts step (lane 1), sample of supernatant phase (lane 2), sample of pre-chromatography phase (lane 3) and sample of ost-chromatography phase (lane 4).

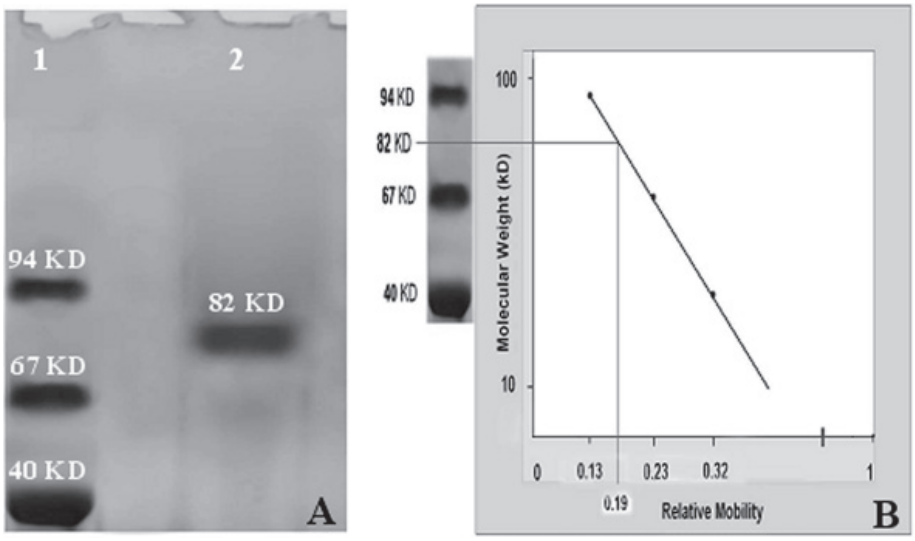

Fig 3: The comparison of electrophoretic mobility (A) and determination of molecular weight (B) of human sperm creatine kinase (lane 2, A) with markers (lane 1, A) of known molecular weight (Taq DNA polymerase, 94 KD, bovine albumin serum, 67 KD, and superoxide dismutase, 40 KD) on 7.5% polyacrylamide gel.

Table 1: Purification steps of creatine kinase from human sperm.

Purification steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Fold
Sperm extracts	120	117	44	0.38	100	1.00
Supernatant	85	59	30	0.51	68	1.34
Before chromatography	43	21	26	1.24	59	2.43
After chromatography	25	5	20	4.00	45	10.5

This figure shows that nicotine, cotinine and cadmium decrease human sperm creatine kinase activity in a dose dependent manner. In this study, 20 to 100 µg/ml of cadmium, in comparison to the control (without chemicals), showed a significant inhibition of creatine kinase activity by the ANOVA test, $p<0.002$. According to this study 60 µg/ml concentrations of cadmium, nicotine and cotinine are able to reduce creatine kinase activity by approximately 65%, 44% and 39%, respectively (Fig 4).

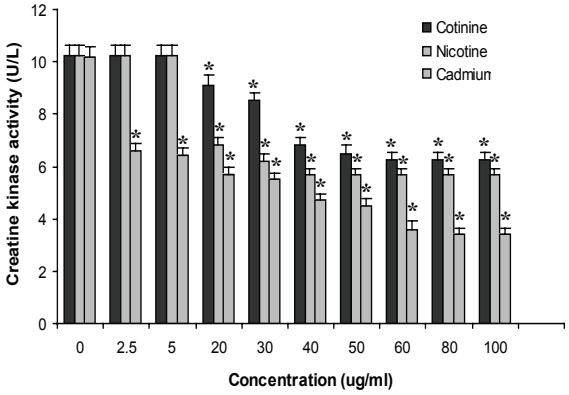

Fig 4: The effect of 2.5 to 100 µg/ml concentrations of cotinine, nicotine and cadmium on human sperm creatine kinase activity. Data points are Mean±SD (n=3). *p<0.002 compared with control (in absence of chemicals).

We also investigated the inhibitory effect of nicotine (2 mM), cotinine (3 mM) and cadmium (3 mM) on creatine kinase activity plots (activity vs. substrate concentration) using the Michaelis-Menten equation model (Fig 5A). Data from the resulting control curve (with no inhibitor) fit the Lineweaver-Burke model equation yielding an estimated K_m for creatine phosphate of 620 µM under these assay conditions (Fig 5B). Thus nicotine, cotinine and cadmium inhibited human sperm creatine kinase activity in a range of substrate (creatine phosphate) concentrations (0-10 mM) (Fig 5). From Lineweaver-Burke equation parameters, estimated K_i values for cotinine, nicotine and cadmium were 3.7 mM, 5.9 mM and 6.25 mM, respectively (Fig 5B). Figure 5B analysis also suggested a probable competitive mechanism for cotinine, nicotine and cadmium mediated inhibition.

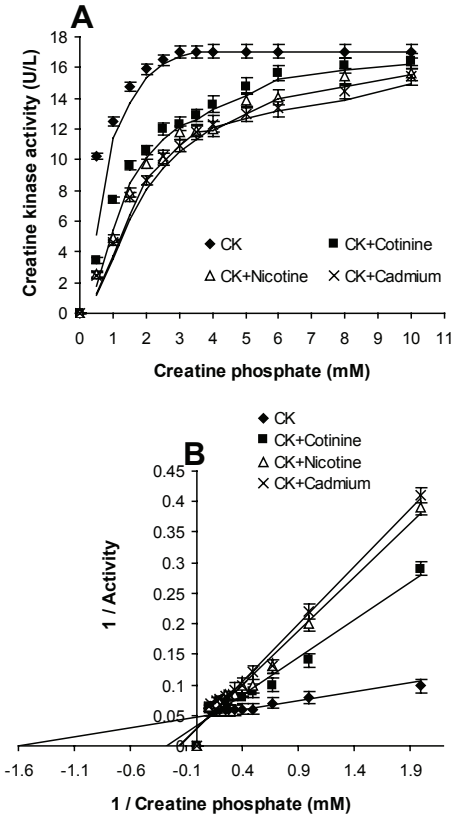

Fig 5: Cotinine, nicotine and cadmium-mediated inhibition of human sperm creatine kinase in a range of substrate concentrations (0–10 mM). (A) Concentration of 3 mM cotinine, 2 mM nicotin and 3 mM cadmium were used; (B) Lineweaver-Burke plots reflect a probable competitive type inhibition of human sperm creatine kinase by cotinine, nicotine and cadmium. Values on graphs are Mean±SD (n=3).

Discussion

Cigarette smoke contains a mixture of harmful components such as carbon monoxide, ammonia, volatile hydrocarbons, aldehyde, nicotine and cadmium. Nicotine and cadmium are the most abundant particulate organic substances found in cigarette smoke. Inhaled nicotine is swiftly oxidized to

its main metabolite, cotinine. In smokers, cadmium, nicotine and/or cotinine have been detected in seminal plasma in a dose related association with cigarette consumption (13). Furthermore, passage of the smoke metabolites through the blood testis barrier has been suggested (14). In the literature there are several studies that demonstrate cigarette smoking is associated with a reduction in fertilization rates (15), reduction in semen volume, semen concentration and sperm motility (16). It is known that cigarette smoke is associated with an overall reduction in fertility in men but the mechanism is not clear. Creatine kinase is an enzyme that plays a central role in energy homeostasis in sperm (1). Thus the main objective in the current study is to evaluate the effect of nicotine, cotinine and cadmium on human sperm creatine kinase activity in an *in vitro* model. Our experiments have been performed with various concentrations of nicotine, cotinine and cadmium (0-100 µg/ml) on human sperm creatine kinase activity, as a key enzyme necessary for normal sperm energy metabolism. We initially observed that nicotine, cotinine and cadmium significantly ($p<0.002$) inhibited creatine kinase activity in sperm homogenates by up to 44%, 39% and 65% in a dose dependent manner, respectively. Furthermore, the kinetic analysis of the effects of nicotine, cotinine and cadmium on creatine kinase activity also indicated a competitive inhibition. Nicotine is the main alkaloid constituent of tobacco that is based on the five member pyrrolidine and six member pyridine structures. This alkaloid is a subgroup of pyridine and piperidine alkaloids that is derived from vitamin B_3 (nicotinic acid) (17). In most of the people nicotine is 70% to 80% metabolized to cotinine by oxidation (18). Therefore, we suggest that nicotine and its metabolite (cotinine) may inhibit human sperm creatine kinase activity by competitive inhibition of NADP. Cadmium is a toxic heavy metal that displaces zinc and other divalent ions in metalloenzymes, thereby inhibiting enzymatic activity (19-20). From our data, it is apparent that human sperm creatine kinase is inhibited by cadmium to a maximum of 65%. The inhibition of this enzymatic activity may be due to displacing Mg atoms that are required for optimal creatine kinase activity. Several enzymes are known to be inhibited by nicotine, cotinine and/or cadmium, such as carbonic anhydrase (21), glucose 6-phosphate dehydrogenase (22), glutathione reductase (23), aromatase (24), delta-aminolevulinic dehydratase and superoxide dismutase (25-26). These reports show that nicotine, cotinine and cadmium inhibit enzymatic activity in tissues by different mechanisms. Thus, our study results are in agreement with other findings.

Conclusion

In conclusion, the results obtained in the present study show that nicotine, cotinine (as an oxide metabolite of nicotine) and cadmium decrease human sperm creatine kinase activity *in vitro*. These data indicate that cadmium has a more harmful effect. Thus creatine kinase may have the potential to be a sensitive biomarker of exposure to nicotine, cotinine and cadmium and its inhibition in sperm by these chemicals may be an important mechanism in infertility amongst male smokers. However, further investigations are still needed to confirm the role played by nicotine, cotinine and cadmium in male infertility.

Acknowledgments

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