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Lead Inhibits Human Sperm Functions by Reducing the Levels of Intracellular Calcium, cAMP, and Tyrosine Phosphorylation

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It is well known that there has been a worldwide decrease in human male fertility in recent years. One of the main factors affecting this is environmental pollution. Lead is one of the major heavy metal contaminants that threaten the health of animals and human beings in China. It preferentially accumulates in male reproductive organs and can be up to 10 μM in human seminal plasma. Lead impairs mammalian spermatogenesis and sperm quality *in vivo*. It also inhibits sperm functions *in vitro* but the underlying mechanisms remain unclear. Therefore, we aimed to investigate the *in vitro* toxicity of lead on human sperm functions and to elucidate the underlying mechanisms. Semen samples were collected from 20 healthy volunteers with different careers and backgrounds living in Nanchang, Jiangxi. Human sperm suspensions were treated with different concentrations of lead acetate (0, 0.5, 2.5, 10, 50, and 100 μM) and the viability, motility, capacitation and progesterone-induced acrosome reaction were examined. Treatment with 10-100 μM lead acetate dose-dependently inhibited total and progressive motility measures, capacitation and progesterone-induced acrosome reaction. It also dose-dependently decreased the intracellular concentrations of cyclic adenosine monophosphate (cAMP) and calcium ($[Ca^{2+}]_i$), and reduced the tyrosine phosphorylation of sperm proteins, all of which are thought to be key factors in the regulation of sperm function. Our findings suggest that lead inhibits human sperm functions by reducing the levels of sperm intracellular cAMP, $[Ca^{2+}]_i$ and tyrosine phosphorylation of sperm proteins *in vitro*.

Keywords: $[Ca^{2+}]_i$; cyclic adenosine monophosphate; human sperm function; lead acetate; protein-tyrosine phosphorylation
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Introduction

It is well known that there has been a worldwide decrease in human male fertility in recent years. One of the main factors affecting this is environmental pollution (Auger et al. 2001). Heavy metals are the most persistent pollutants because they are barely biodegraded once introduced into the environment. They are toxic to all forms of life because they form complex compounds within the cell (Tchounwou et al. 2012). Lead is a typical heavy metal and stored richly in mineral resources around China. For socio-economic development, the lead mineral resources were exploited and utilized. Nonetheless, mineral extraction causes the influx of lead into all environmental and biological systems in China (Li et al. 2014). Lead can accumulate in the human body over a lifetime (Awadalla et al. 2011). It has highly toxic effects on all mammals. Many reports have shown the toxic effects of lead on bone, hematopoiesis, and renal function, and on the digestive, immune and nervous systems (Gurer-Orhan et al. 2004; Ekong et al. 2006; Ravibabu et al. 2015). Additionally, lead preferentially accumulates in male reproductive organs and leads to adverse effects on the male reproductive system (Benoff et al. 2000).

Many previous studies on the reproductive toxicity of lead on male reproduction have focused on passive changes in spermatogenesis *in vivo* induced by exposure to lead. In animal models, lead exposure can decrease mouse sperm counts and increase the proportions of spermatozoa with abnormal morphology after a single intraperitoneal injection of 100 mg lead acetate per kg of body weight (Acharya et al. 2003). Lead can also affect mouse sperm motility and

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acrosome integrity after subcutaneous injections of 100 mg lead chloride per kg body weight for 4 consecutive days (Oliveira et al. 2009). Oral administration of 6 mg lead acetate per kg body weight per day to rats for 8 weeks significantly reduced epididymal sperm counts and motility, and caused abnormal sperm morphology (Jegede et al. 2015). In addition, a study performed in red deer indicated an association between an increase in testicular lead levels and damage to sperm chromatin and sperm membrane integrity (Castellanos et al. 2015). As in animal models, lead exposure also disturbs human spermatogenesis and impairs sperm quality. Lead might also act as a biomarker in studies evaluating human semen quality and reproductive endocrine function (Telisman et al. 2000). Thus, Gennart et al. (1992) suggested that long-term exposure to lead could cause asthenozoospermia and oligospermia once the blood concentration reached 61 μg/dl. Kasperczyk et al. (2008) described decreases in human sperm motility and increased sperm lipid peroxidation in lead-exposed men with blood level values above 40 μg/dl. Furthermore, men with idiopathic infertility had high levels of lead in their semen, which were correlated with impairments in sperm motility, higher sperm DNA fragmentation and elevated reactive oxygen species levels in semen (Taha et al. 2013). All these findings indicate that lead disturbs spermatogenesis and affects mammalian sperm function *in vivo*. Nevertheless, semen needs to be ejaculated into the female reproductive tract to effect fertilization, and active sperm motility, capacitation and the acrosome reaction are essential for this (Yanagimachi 2011). Therefore, studying the effect of lead on these functions of ejaculated spermatozoa will provide new insights for understanding the toxicity of lead against male reproductive functions. Although a few studies have shown that lead could affect the motility and acrosome reaction of human spermatozoa separated from frozen-thawed semen *in vitro* (Benoff et al. 2003), the underlying mechanism is still unknown.

Here, we evaluated the *in vitro* effects of lead on the viability, motility, capacitation, and progesterone-induced acrosome reaction of human spermatozoa. To explore the possible mechanisms involved, we measured the intracellular levels of cyclic adenosine monophosphate (cAMP), calcium ($[Ca^{2+}]_i$) and protein tyrosine phosphorylation, which are known to play vital roles in the regulation of sperm function.

Materials and Methods

Sperm sample collection and treatments

Semen samples were collected by masturbation from 20 volunteers with different careers and backgrounds living in Nanchang, Jiangxi, P.R. China. These men had known reproductive histories during the previous 2 years and good semen quality according to World Health Organization (WHO) laboratory manual for the examination and processing of human semen (http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/). The collection of semen samples and experiments in this study were approved by the Institutional Ethics Committee for human experimentation of Jiangxi Provincial Maternal and Child Health Hospital.

In this study, synthetic human tubal fluid (HTF) medium (4.7 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 106 mM NaCl, 5.6 mM D-glucose, 1.5 mM NaH_2PO_4, 1 mM sodium pyruvate, 41.8 mM sodium lactate, 25 mM $NaHCO_3$, 1.33 mM glycine, 0.68 mM glutamine, 0.07 mM taurine, non-essential amino acids (1:100 dilution) and 3% (w/v) HSA, and adjusted pH 7.4 with NaOH) is used as a capacitating medium because it contains the key factors for capacitation, $NaHCO_3$ and HSA. Here, we used HS medium (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, and 1 mM Na-pyruvate at pH 7.4 with NaOH) as the non-capacitating medium. These mediums can also mimic the physiological environments in the female reproductive tract. Spermatozoa were purified by direct swim-up as described previously (Luo et al. 2015).

Lead acetate (Sigma-Aldrich, St. Louis, MO, USA), frequently used in previous studies (Acharya et al. 2003; Benoff et al. 2003; Jegede et al. 2015), was used to investigate the *in vitro* toxicity of lead on human spermatozoa. Based on the reported lead levels in human seminal plasma (range 0.05-10 μM, normal < 1 μM) (Benoff et al. 2000, 2003), we used a range of concentrations to examine the effect on ejaculated human spermatozoa. Thus, 50-μl aliquots of purified sperm suspensions from single donors were mixed with equal volumes of HTF and/or HS mediums containing different concentrations of lead acetate to final doses of 0.5, 2.5, 10, 50, and 100 μM and incubated at 37°C in 5% CO_2 in humidified air for different times according to the experimental protocol used.

Determination of sperm viability and motility

Sperm suspensions were treated with different concentrations of lead acetate for 1 h in HS medium (a non-capacitating medium), and for 4 and 8 h in HTF medium (capacitating medium), which represent non-capacitating, capacitating and post-capacitating stages, respectively. Sperm viability was determined using eosin-nigrosin staining kits (Nanchang Royo Biotech Co. Ltd., Nanchang, P.R. China) according to the manual. Heads of dead spermatozoa stain red with eosin, while viable cells are not stained. The percentage of viable spermatozoa in each suspension was calculated. Sperm motility was measured with a computer-assisted semen analysis (CASA) system (WLJY-9000, WeiLi Co., Ltd., Beijing, P.R. China). An aliquot of 10 μl of each sperm sample was transferred to a sperm-counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel) and total motility and progressive motility were analyzed. At least 200 spermatozoa were counted for each assay.

Evaluation of capacitation and the acrosome reaction

For determining the effects of lead acetate on sperm capacitation and the spontaneous acrosome reaction, sperm suspensions were capacitated in HTF medium containing different concentrations of lead acetate for 4 h. To evaluate effects on the progesterone-induced acrosome reaction, suspensions were first capacitated in HTF medium for 4 h and then 20 μM progesterone plus different concentrations of lead acetate were added for a further 1 h. Capacitation and acrosome reaction were evaluated using chlortetracycline (CTC) staining as described (DasGupta et al. 1993). Briefly, the CTC solution was prepared freshly and contained 750 μM CTC (Sigma-Aldrich), 130 mM NaCl, 5 mM cysteine and 20 mM Tris-HCl (pH 7.8). Fifty microliter treated spermatozoa were stained with equal volume of CTC solution

for 5 min. The stained spermatozoa were fixed by paraformaldehyde and examined with a Leica DM2500 Upright Microscope using an Hg excitation beam passed through a 340-380 nm filter and fluorescence emission via a DM 400 dichromatic mirror (Leica "A" filter, Germany). A total of 200 spermatozoa were counted to assess the different CTC staining patterns as follows: "F" represents the characteristics of non-capacitating spermatozoa; "B" represents the capacitating but acrosome-intact spermatozoa; and "AR" corresponds to spermatozoa that had undergone acrosomal exocytosis. The capacitating spermatozoa were quantified as the sum of "AR" and "B".

Determination of intracellular cAMP content

The intracellular cAMP content was determined using a competitive immunoassay as described previously (Jansen et al. 2015). Sperm suspensions were adjusted to a concentration of 1×10^7 cells/ml with HTF medium containing different concentrations of lead acetate. After incubation for 1 h at 37°C in 5% CO_2 in humidified air, the suspension was quenched with 0.5 M $HClO_4$. Samples were frozen in liquid N_2, thawed, and neutralized by the addition of 0.24 M K_3PO_4. The salt precipitate and cell debris were pelleted by centrifugation at 12,000 *g* for 10 min at 4°C. The cAMP content in the supernatant was examined using a cAMP-Screen cAMP immunoassay system (Thermo Fisher Scientific, Waltham, MA, USA) in 96-well plates in an EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA) according to the user's manual.

Measurement of human sperm $[Ca^{2+}]_i$

Changes in sperm $[Ca^{2+}]_i$ were measured in 96-well plates in an EnSpire® Multimode Plate Reader (Perkin Elmer) as described previously (Strunker et al. 2011). Briefly, sperm samples were adjusted to 1×10^7 cells/ml and loaded with 5 μM of the fluorescent Ca^{2+} indicator Fluo-4, AM (Molecular Probes, Eugene, OR, USA) in the presence of 0.05% Pluronic F-127 (Molecular Probes) in non-capacitating HS medium at 37°C for 30 min in the dark. After incubation, excess dye was removed by centrifugation at 300 *g* for 5 min and the pellet was resuspended in HS medium to 1×10^7 cells/ml. Each well was filled with 50 μl of the sperm suspension; fluorescence was excited at 503 nm and emission was recorded at 525 nm. Fluorescence was recorded before and after the injection of 50 μl (1:1 dilution) aliquots of solutions containing different concentrations of lead acetate. The sperm $[Ca^{2+}]_i$ values were calculated by the formula $\Delta F/F_0$ (F_0, the mean fluorescent intensity before adding the chemicals; F, the fluorescent intensity recorded at each time; $\Delta F = F–F_0$).

Protein-tyrosine phosphorylation analysis

Samples were incubated in HTF medium containing different concentrations of lead acetate at 37°C in 5% CO_2 in humidified air for 4 h. After incubation, spermatozoa were precipitated by centrifugation at 12,000 *g* for 5 min at 4°C. Sperm proteins were isolated according to ref (Luo et al. 2015). A phosphatase inhibitor was added to the lysis buffer. The protein concentrations were determined using the bicinchoninic acid (BCA) method (Thermo Fisher Scientific). Aliquots of 50 μg protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Little Chalfont, UK). Following the transfer, the membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature, then incubated with the anti-phosphotyrosine 4G10 (1:1,000 dilution; Merck, Darmstadt, Germany) or anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH, 1:20,000 dilution; Proteintech, Wuhan, P.R. China) primary antibodies overnight at 4°C and incubated with an HRP-conjugated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific) for 1 h at room temperature. After incubation, the membranes were visualized using enhanced chemiluminescent (ECL) detection kits (Thermo Fisher Scientific) according to the manufacturer's instructions. Western blot intensities were quantified using Image J software (http://rsb.info.nih.gov/ij/download.html).

Statistical analysis

Data are expressed as the mean ± standard error (SEM). All data are normally distributed by Shapiro-Wilk test ($P > 0.05$). Differences between the means of controls and samples were assessed using unpaired One-way ANOVA analysis. Statistically significant differences were determined at $P < 0.05$ using GraphPad Prism statistical software (version 5.01; http://www.graphpad.com/prism).

Results

Lead treatment decreased sperm motility

Previous studies have showed that a significant correlation was found between the concentration of lead in blood and an impaired of human sperm total motility and progressive motility (Gennart et al. 1992; Telisman et al. 2000; Kasperczyk et al. 2008; Taha et al. 2013). Therefore, we examined the effect on human sperm motility in this study. The results showed that 10-100 μM lead acetate dose-dependently inhibited both total motility and progressive motility (Fig. 1A, B), consistent with a previous study on frozen-thawed semen *in vitro* (Benoff et al. 2003). In addition, one hour of lead exposure to human spermatozoa in non-capacitating stage caused decreases in sperm motility but extension of the exposure time did not result in any further inhibition of motility of capacitating human spermatozoa (Fig. 1A, B). These results indicate that lead inhibits human sperm motility via a short-term (< 1 h) regulatory mechanism, and that the inhibitory effect is not dependent on the state of capacitation. In addition, we also examined whether lead was spermicidal *in vitro*. However, sperm viability was not significantly affected by lead acetate exposure (Fig. 1C).

Lead inhibited capacitation and progesterone-induced acrosome reaction

Capacitation and the acrosome reaction are essential for fertilization in the female reproductive tract. They are also used as functional indicators of sperm quality (De Jonge 2005; Yanagimachi 2011). We tested whether lead acetate affected capacitation and the acrosome reaction of human spermatozoa *in vitro*. Sperm capacitation was dose-dependently reduced by lead acetate (10-100 μM) (Fig. 2A). As with the effects of lead on sperm capacitation, the acrosome reaction induced by progesterone, an important physiological hormone regulating sperm function (Baldi et al. 2009), was also dose-dependently inhibited by 10-100 μM lead acetate (Fig. 2B). In contrast, lead treatment did not affect the spontaneous acrosome reaction rate (Fig. 2C),

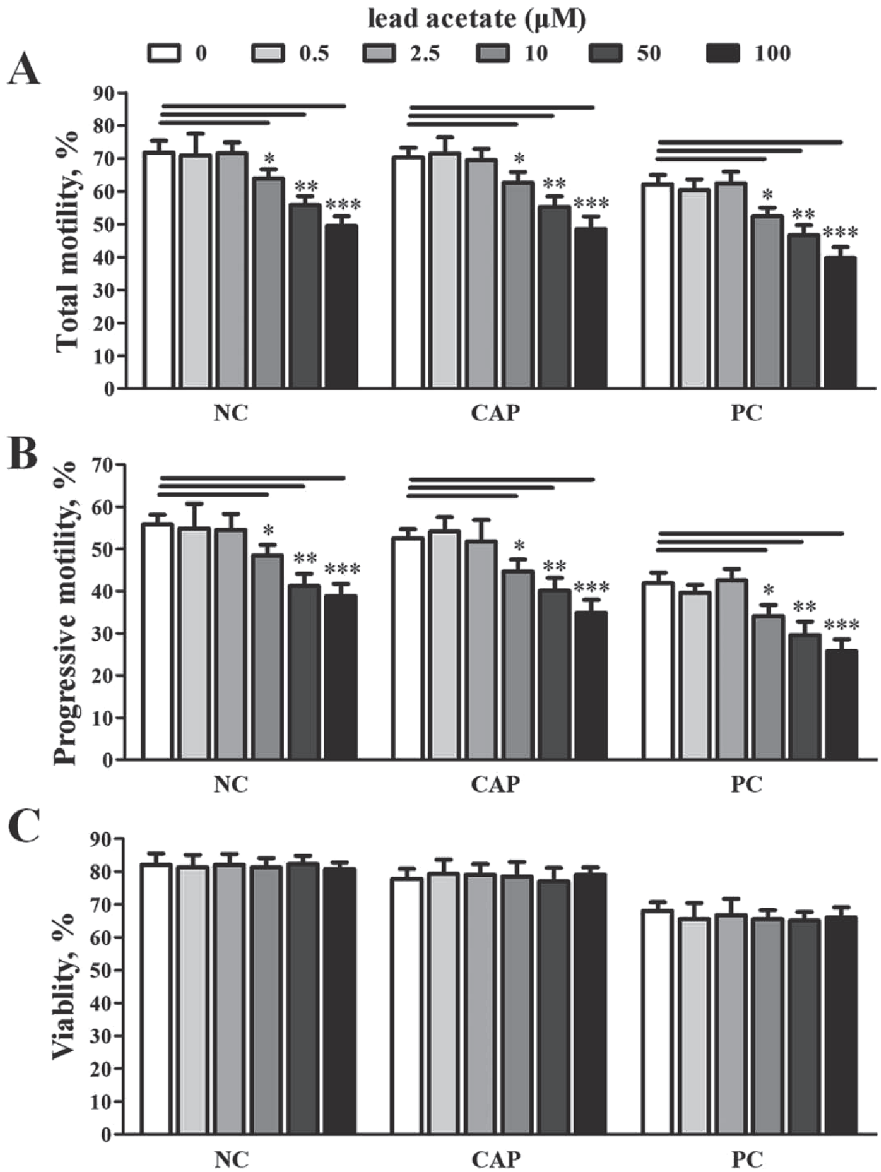

Fig. 1. The effect of lead acetate on human sperm motility and viability *in vitro*.
Sperm suspensions were treated with lead acetate for 1 h in HS medium (a non-capacitating medium), and for 4 and 8 h in HTF medium (capacitating medium), which represent non-capacitating (NC), capacitating (CAP) and post-capacitating (PC) stages, respectively. After incubation, the total motility (A), progressive motility (B) viability (C) were analyzed by Computer-Assisted Sperm Analysis (CASA) and eosin staining, respectively. A minimum of 200 sperm were counted for each assay. Bar: mean ± SEM. $n = 9$. Statistically significant differences between controls (0 μM) and samples (0.5-100 μM) were determined at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

although a previous study showed that this increased the spontaneous acrosome reaction rate of human spermatozoa prepared from frozen-thawed semen. (Benoff et al. 2003).

Lead reduced intracellular cAMP levels

Lead inhibited human sperm motility in this study by short-term (< 1 h) treatment. It is known that cAMP, an important second messenger, can stimulate a series of intracellular signaling pathways to regulate cell function within a few hours (Formosa and Vassallo 2014). It is also essential for sperm motility (Buffone et al. 2014; Jansen et al. 2015). Thus, it was reasonable to test whether lead treatment impaired human sperm motility by affecting the intracellular cAMP level. A competitive immunoassay was performed to measure the cAMP levels in human sperm samples exposed to different concentrations of lead acetate in HTF medium for 1 h. As shown in Fig. 3, 10, 50 and 100 μM lead acetate dose-dependently reduced sperm intracellular cAMP by 20%, 33% and 45%, respectively, compared with the control. These results were consistent with the effects of lead on sperm motility, suggesting that it inhibits human sperm motility by an intracellular cAMP-related mechanism.

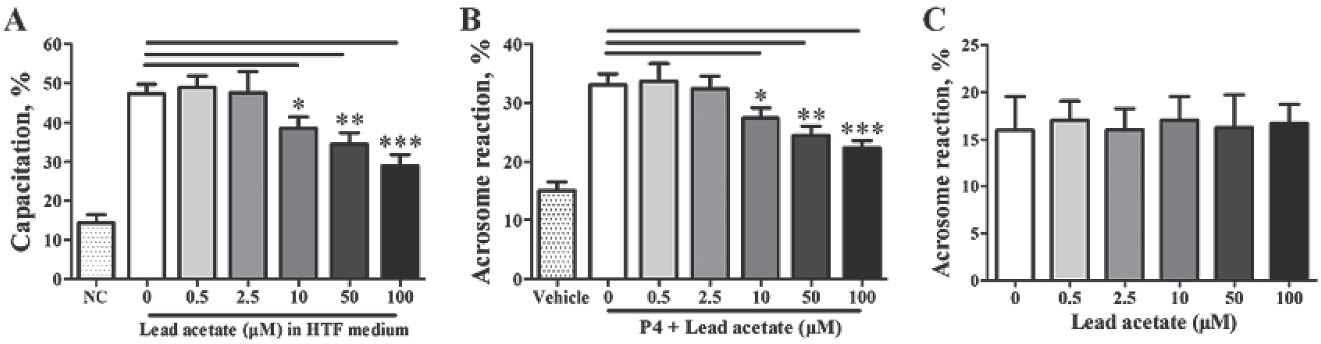

Fig. 2. The effect of different concentrations of lead acetate on capacitation and acrosome reaction of human spermatozoa *in vitro*.
The ejaculated human spermatozoa were purified and capacitated in HTF medium containing the 0-100 μM lead acetate at 37°C, 5% CO_2 incubator for 4 h. As the non-capacitating control, human spermatozoa were incubated in non-capacitating HS solution for 1 h (NC). For examining the progesterone (P4)-induced acrosome reaction, human spermatozoa were first capacitated in HTF medium at 37°C in a 5% CO_2 incubator for 4 h, then vehicle (0.1% DMSO, a negative control) and 20 μM P4 plus 0-100 μM lead acetate were added and incubated for 1 h. The ratios of capacitation (A), progesterone-induced acrosome reaction (B) and spontaneous acrosome reaction (C) of human spermatozoa were determined by CTC staining. A minimum of 200 spermatozoa were counted for each assay. Bar: mean ± SEM. $n = 9$. Statistically significant differences between controls (0 μM) and samples (0.5-100 μM) were determined at $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

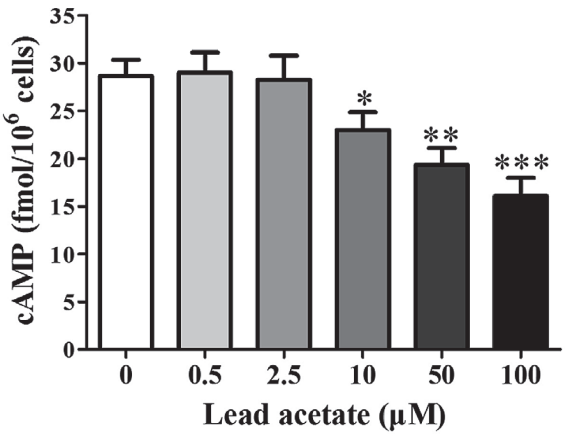

Fig. 3. The effects of lead acetate on intracellular cAMP of human spermatozoa *in vitro*.
Human ejaculated spermatozoa were incubated with different doses of lead acetate (0-100 μM) in HTF medium at 37°C in a 5% CO_2 incubator for 1 h. The intracellular cAMP was determined using cAMP-Screen cAMP immunoassay system described in Materials and Methods. Bar: mean ± SEM. $n = 9$. Statistically significant differences between controls (0 μM) and samples (0.5-100 μM) were determined at $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Lead lowered human sperm $[Ca^{2+}]_i$

It is believed that mature mammalian spermatozoa are transcriptionally inactive (Braun 1998). Thus, regulation of nuclear gene expression cannot modulate sperm function, while signaling pathways stimulated by signaling molecules such as cAMP and Ca^{2+} serve as the main regulators (Abou-haila and Tulsiani 2009). Our present data had showed that lead dose-dependently reduced the sperm intracellular cAMP *in vitro* (Fig. 3). Therefore, we examined the effect of lead on intracellular Ca^{2+} concentrations. The sperm $[Ca^{2+}]_i$ level was reduced by lead acetate (2.5-100 μM) within 1 min (Fig. 4A), and inhibition was intensified with increased lead concentrations up to 50 μM, at around 50% of control values (Fig. 4B). Additionally, the progesterone-induced sperm $[Ca^{2+}]_i$ increase was inhibited by lead acetate (2.5-100 μM) dose-dependently (Fig. 4C, D). Thus the toxic effect of lead acetate on human sperm functions might partially result from this reduction in sperm $[Ca^{2+}]_i$.

Lead suppressed the tyrosine phosphorylation in human sperm proteins

Phosphorylation of mammalian sperm tyrosine is a critical event during capacitation, and is involved in modulating sperm function (Visconti 2009). Moreover, a previous study reported that cAMP and Ca^{2+} are correlated with tyrosine phosphorylation levels in spermatozoa (Leclerc et al. 1998). Here we found that 10-100 μM lead acetate significantly reduced human sperm intracellular cAMP and $[Ca^{2+}]_i$ levels (Figs. 3 and 4) and inhibited human sperm functions (Figs. 1 and 2). Therefore, we speculated that the tyrosine phosphorylation level of human sperm proteins might be affected by lead *in vitro*. To test this, we analyzed the tyrosine phosphorylation levels in human sperm proteins after being treated with lead acetate in HTF capacitating medium at 37°C in 5% CO_2 in humidified air for 4 h. The tyrosine phosphorylation levels were detected by western blotting. The level of tyrosine phosphorylation in capacitating spermatozoa suspensions was higher than in the non-capacitating spermatozoa (Fig. 5A). Compared with the control, treatment with 10 μM of lead acetate slightly decreased the level of tyrosine phosphorylation and higher doses (50 and 100 μM) decreased it substantially (Fig. 5A). The maximum inhibition was approximately 50% of control values when quantifying the western blots

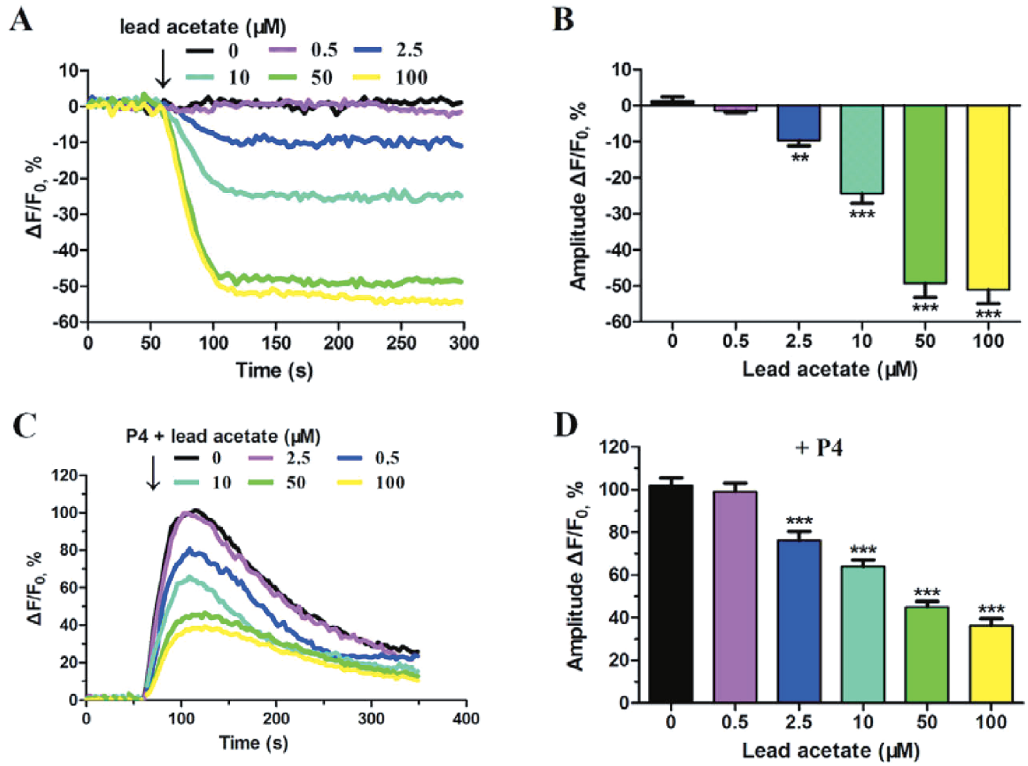

Fig. 4. The effect of different concentrations of lead acetate on human sperm $[Ca^{2+}]_i$.
Human sperm $[Ca^{2+}]_i$ was monitored after loading cells with 5 μM Fluo-4, AM and the fluorescence intensity of the spermatozoa was detected by the EnSpire® Multimode Plate Reader described in Materials and Methods. A, A time-course curve showed the real-time changes of sperm $[Ca^{2+}]_i$ treated by 0-100 μM lead acetate. Arrows indicated the time of adding lead acetate. B, The statistical analysis of the effects of lead on sperm $[Ca^{2+}]_i$ was calculated as the $\Delta F/F_0$ at the time frame of 120 s indicated in Fig. 4A. C, A time-course curve showed that 0.5 μM progesterone (P4)-induced sperm $[Ca^{2+}]_i$ increases were inhibited by lead. D, The statistical analysis of the effects of lead on P4-induced sperm $[Ca^{2+}]_i$ increases was calculated as the $\Delta F/F_0$ at the time frame of 120 s indicated in Fig. 4C. Bar: mean ± SEM. $n = 9$. Statistically significant differences between controls (0 μM) and samples (0.5-100 μM) were determined at $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

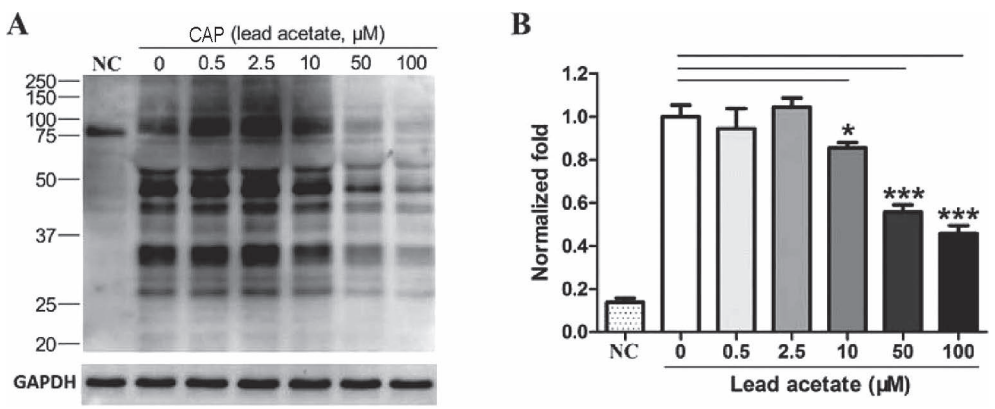

Fig. 5. The effect of different concentrations of lead acetate on tyrosine phosphorylation of human sperm proteins.
Human spermatozoa were capacitated in HTF medium containing different concentrations (capacitating, 0-100 μM) of lead acetate at 37°C in a 5% CO_2 incubator for 4 h, and then sperm proteins were isolated. NC (non-capacitating) represents human sperm incubated in HS non-capacitating solution as a negative control. The tyrosine phosphorylation was measured by western blot using anti-phosphotyrosine monoclonal antibody 4G10 (A) and quantified with the total intensities of the bands normalized to the loading control, GAPDH, using Image J software (B). Bar: mean ± SEM. $n = 6$. Statistically significant differences between controls (0 μM) and samples (0.5-100 μM) were determined at $^*P < 0.05$ and $^{***}P < 0.001$.

using Image J (Fig. 5B). Additionally, we did not found any tyrosine phosphorylated proteins specific to lead exposure. These results imply that lead affects the tyrosine phosphorylation of most function-related proteins by inactivating tyrosine kinase in human sperm. These results were consistent with our functional analysis and imply that impaired tyrosine phosphorylation might also be involved in lead-mediated inhibition of human sperm function.

Discussion

Many studies have reported that lead disturbs spermatogenesis and impairs mammalian sperm quality *in vivo*. Few studies have reported the *in-vitro* effects of lead on mature mammalian spermatozoa, although normal function is essential for fertilization. As far as we know, only one study has examined the toxicity of lead on human spermatozoa *in vitro* (Benoff et al. 2003). However, those results might not reflect the entire impact of lead on human spermatozoa because the authors used cells recovered from frozen-thawed semen samples. Therefore, to study the effect of lead on human spermatozoa, we used freshly ejaculated semen samples. We found that treatment with 10-100 μM lead acetate decreased sperm motility (Fig. 1A, B), consistent with the results above. More importantly, we found that treatment with lead acetate at 10 μM was sufficient to inhibit capacitation and the progesterone-induced acrosome reaction (Fig. 2A, B). These findings have not been reported before. As reported previously, lead levels in human seminal plasma can reach up to 10 μM (Benoff et al. 2000, 2003). Thus, our findings imply that lead can inhibit human sperm functions even after ejaculation and reduce male fertility once it accumulates in the environment of maturing spermatozoa, such as the seminal plasma and female reproductive tract fluids.

Mature spermatozoa have highly condensed nuclei that lack active transcriptional machinery. Signaling molecules such as cAMP and Ca^{2+} play important roles in the modulation of mammalian sperm function (Braun 1998). Changes in the levels of these second messengers can trigger a series of intracellular signals, such as tyrosine phosphorylation, and thereby regulate sperm function (Leclerc et al. 1998). In this study, we tested whether the inhibitory effect of lead on human spermatozoa was correlated with changes in the levels of these second messengers. Interestingly, we found that lead dose-dependently reduced the intracellular cAMP and $[Ca^{2+}]_i$ levels and progesterone-induced $[Ca^{2+}]_i$ increases (Figs. 3 and 4). The tyrosine phosphorylation levels of human sperm proteins were also decreased by lead exposure *in vitro* (Fig. 5). These results might explain the decreased sperm motility and inhibition of capacitation and the progesterone-induced acrosome reaction. However, the spontaneous acrosome reaction was not affected by lead exposure (Fig. 2C), although the sperm $[Ca^{2+}]_i$ was reduced by lead (Fig. 4). In fact, the mechanism underlying how and why spontaneous acrosome reaction happens is complicated. The spontaneous acrosome reaction is more likely affected by the sperm membrane integrity. In this study, the result of eosin staining showed that lead did not affect the sperm membrane integrity (Fig. 1C), which may explain why lead had no effect on spontaneous acrosome reaction of human spermatozoa. These observation are consistent with our previous studies that reduction of sperm $[Ca^{2+}]_i$ but not alteration of sperm membrane integrity did not affect spontaneous acrosome reaction (Luo et al. 2015, 2016).

Previous reports indicated that intracellular cAMP and $[Ca^{2+}]_i$ are critically involved in lead-mediated signaling pathways and are significantly decreased by lead treatments in rat brain (Ferguson et al. 2000; Chang et al. 2005). Herein, we found that lead-induced inhibition of human sperm functions are also mediated by intracellular cAMP and $[Ca^{2+}]_i$. Taken together, these results suggest that the lead-induced decreases in cAMP and $[Ca^{2+}]_i$ are common ways of regulating cell functions. It is reported that lead decreased intracellular cAMP by inhibiting the activity of adenylate cyclase (AC), which is the key enzyme involved in synthesizing cAMP (Rodrigues et al. 1999; Chang et al. 2005). Despite AC is also found in human spermatozoa and involved in regulation of human sperm function (De Jonge et al. 1991), Although AC is also found in human spermatozoa and is involved in regulating sperm function, whether it participates in the lead-caused cAMP decreasing requires further study. Furthermore, a previous study revealed that lead could inhibit voltage-dependent calcium channel currents in nerve cells (Busselberg et al. 1991; Gawrisch et al. 1997). In human spermatozoa, the voltage-dependent calcium channel, CatSper, is regarded as the main channel to mediate Ca^{2+} influx and regulate progesterone-induced acrosome reaction (Strunker et al. 2011; Tamburrino et al. 2014). Hence, lead treatment probably decreased $[Ca^{2+}]_i$ and inhibited the progesterone-induced acrosome reaction via a CatSper-related mechanism.

In conclusion, we examined the effects of lead acetate on human sperm function and conclude that *in-vitro* exposure of human spermatozoa to lead inhibits sperm motility, capacitation and progesterone-induced acrosome reaction by reducing sperm intracellular cAMP, $[Ca^{2+}]_i$ and protein-tyrosine phosphorylation. Our study provides new understanding of the toxicity of lead to the male reproductive system and sheds light on the underlying mechanisms.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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