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† 八十六年度及以前的一般國科會專題計畫(不含產學合作研究計畫)亦可選擇適用，惟較特殊的計畫如國科會規劃案等，請先洽得國科會各學術處同意。

一、中文摘要

精子對卵子的穿透力，是評估精子功能的重要指標之一。當精子進入雌性生殖道後，精子會進行一連串反應，以便能穿透卵子，達到受精目的。這種反應於起始稱為精子復活化，當完全反應後稱為頂極反應。精子外膜反應的發生時間與位置，對精子是否能穿透卵子，達到受精目的，具有決定性的影響。研究結果發現，鉛暴露大鼠副睪中的精子，其復活化比率顯著增高。此外，副睪精子發生復活化與頂級反應率愈高，則對具有透明帶之卵子的穿透力卻愈差。此研究說明鉛暴露引起精子產生反應性氧化物質，影響精子功能，也包括可能導致副睪精子發生頂極反應，這種早發性的頂極反應可能影響精卵穿透力。近年來，不孕症的發生率不斷增加，相較於數十年前，人類精液品質似有惡化趨勢，究其原因與人類暴露於環境中的化學污染有關。本研究相關發現：(一)、鉛暴露可能透過精子產生反應性氧化物質的機轉，影響精子功能，(二)、鉛暴露可能與副睪精子發生早發性頂極反應有關，這種早發性的頂極反應可能影響精卵穿透力。此研究成果，將可提供工業衛生上，鉛暴露導致雄性生殖危害，另一個新的省思方向。

關鍵詞：鉛、精子、反應性氧化物質、復活化、頂級反應

Abstract

The relationships between sperm reactive oxygen species (ROS) generation, capacitation and acrosome reaction, and sperm-oocyte penetration rate (SOPR) were investigated to understand the effect of lead toxicity on sperm functions and the mechanisms of these effects. Male Sprague-Dawley rats received weekly intraperitoneal injection of 20 mg or 50 mg lead acetate/kg or sodium acetate/kg (control) for 6 weeks. In cauda epididymal sperm, the chmiluminescence was measured to evaluate the sperm ROS generation. Chlortetracycline fluorescence assay was used to study the status of capacitation and acrosome reaction on fresh cauda epididymal sperm after 24 hours of incubation with 5 mg/ml bovine serum albumin. In lead-exposed rats, the percentage of capacitation and the ROS generation were significantly increased in fresh cauda epididymal sperm. Sperm ROS was positively correlated with the percentage of both capacitated and acrosome reacted sperm. In summary, this study showed that male rats exposed to lead produced early onset of capacitation by one of the pathways of ROS generation. These effects might consequently result in premature acrosome reaction and reduced zona-intact oocyte-penetrating capability.

Keywords: Lead, Spermatoxoa, Reactive Oxygen Speceies, Capacitation, Acrosome Reaction

二、緣由與目的

Lead-induced infertility has been reported in rats (Puhac et al., 1963) and mice (Varma et al.,1974). Workers with elevated blood lead levels were known to have altered spermatogenesis and a substantial decrease in fertile capability compared to unexposed individuals (Lancranjan et al., 1975). Sokol et al. (1985) suggested that lead-induced suppression of spermatogenesis involved disruption of the hypothalamo-pituitary axis. However, it is

conceivable that lead might exert a direct toxic action on testes. Histologic evidence of testicular damage and inhibited spermatogenesis was shown in lead-exposed rats with blood lead concentrations of 50 μg/dl (Hilderbrand et al.,1973). Recently, reactive oxygen species (ROS) such as superoxide anion ($O_2^{-}\cdot$), hydroxyl radical ($OH^{-}\cdot$), and hydrogen peroxide (H_2O_2), known to cause oxidative damages to liver, brain, kidney, lung, and other organs (Weiss,1989;Halliwell,1994), were found induced in rat spermatozoa (Hsu et al., 1997).It was suggested that the induced ROS in rat spermatozoa subsequentIy reduced the sperm-oocyte penetration rate (SOPR)(Hsu et al.,1997).Excessive ROS formation could probably explain the reduced capability of spermatozoa of lead-exposed rats to fertilize eggs in vitro. However, the mechanisms of ROS-related SOPR reduction are not clear.

One possible explanation might be associated with the early changes in spermatozoa, which reduced their ability to penetrate oocytes. Lead exposure was shown to induce a premature acrosome reaction in the spermatozoa of mice and a delay in the fertilization of the oocyte (Johansson, 1989). During spermatozoa transport in the female genital tract, mammalian spermatozoa must undergo a complex process of development, called capacitation, to become capable of fertilizing the oocyte (Zaneveld et al.,1991). Under physiological conditions, when spermatozoa reach the zona pellucida, they will bind to the zona peIlucida and the acrosome reaction is initiated (Bieil & Wassarman, 1983). Spermatozoa are Presumed to carry on their acrosome-reacted head a hydrolytic enzyme that causes digestion of the zona pellucida. The enzyme could be a protease, an unfoldase, or any other enzyme that excludes intermoIecular bonding between components of the zona pellucida (Myles,1993).Although how sperm and egg actually fuse remains a mystery, there has been strong evidence that the acrosome reaction plays an important role in the fusion of sperm and oocyte.

The capacitation process is a prerequisite step for acrosome reaction and is accompanied by alterations in (1) membrane lipid, (2)changes in protein organization and localization (wolf, 1987), and (3)changes in ion fluxes, including calcium (Singh et al.,1978), sodium, potassium (Mrsny & Meizel,1981), and protons(Working & Meizel,1983). Capacitation isbelieved to be physiologically triggered by oviductal fluid, follicular fluid, and a cumulus matrix, which contain serum albumin (Yanagimachi, 1982), hydrolytic enzymes (Meizel,1984), glycosaminoglycans (Ball et al., 1982) or steroids (Meizel,1985). In capacitated spermatozoa incubated with bovine serum albumin, a spontaneous acrosome reaction was also shown to occur Yanagimachi,1982). There also have been studies suggesting that only acrosome-intact mouse spermatozoa can bind to the zona pellucida, and then timely acrosome reactions enable the spermatozoa to penetrate the zona pellucida (Sating & Storey,1979) . Since appropriate timing of the acrosome reaction was a major requirement for the fertilization of oocyte (Tesarik,1989), earlier exposure to chemicals capable of inducing the acrosome reaction might be deleterious to the fertilizing capability.

There is also evidence that ROS are involved in capacitation in vitro. Superoxide anion triggered capacitation in human spermatozoa (Delamirande & Gagnon, 1993). Low concentration of H_2O_2 induced capacitation. However, higher levels of H_2O_2 reduced spermatozoa penetrating and fertilizing oocytes (Oehninger et al.,1995). ROS were also shown negatively associated with penetration and fertilization in humans (Aitken & Fisher, l994) and rats (Hsu et al., 1997).Therefore, ROS might also be detrimental to these processes if given as an excessive amount or generated at nonphysiological time points.

It was not clear whether the effects of lead on sperm function capaciation, and acrosome reaction were related to ROS

generation. Hence this study examined the roles of capacitation, acrosome reaction, and ROS generation in lead-exposed rats. The capability of spermatozoa to penetrate the zona-intact oocyte was undertaken to determine the role of ROS lead-induced sperm toxicity.

三、材料與方法

Chemicals

Chlortetracycline, Tris buffer, L-cysteine, paraforamaldehyde, l,4-diazabieyelo [2.2.2] octane, glycerol, nitric acid (HNO_3),5-amino-2,3-dihydro-1,4-phthalhydrazide (luminol),ferrous sulfate ($FeSO_4$), dimethyl sulfoxide(DMSO), bovine albumin, postmenopausal serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), hyaluronidase, testosterone, ether, and mineral oil were obtained from Sigma Chemical Co. (St. Louis, MO).Whole-blood certified materials were obtained from Nycomed Pharma CO.(Oslo,Norway). Phosphate-buffered saline (PBS)and human tubule fluid (HTF)medium consisting of 105.6 mM NaCl, 5.06 mM KCl, 0 .73 mM $CaCI_2$, 1.l7 mM KH_2PO_4 , 1.01 mM $MgS0_4 \cdot 7H_2O$, 25.3 mM $NaHCO_3$,0.27 mM sodium Pyruvate, 21.6 mM sodium lactate, 5.56mM glucose,10001U/ml Penicillin, and 50 μg/ml streptomycin were from Gibco Life Technologies Ltd.(New York, USA).The medium had a pH 7.4, an osmolarity of 330 mOsm/L when gassed with 5% CO_2 and air, and was Prewarmed to a temperature of 34 ℃ or37℃.Lead acetate, Triton X-l00, and ammonium dihydrogen phosphate were obtained from Merck Co. (Darmstada, Germany) .

Animals and Treatment Regimens

Sprague-Dawley rats weighing 100-120 g were purchased from the Animal Center of National Cheng Kung University Medical Center (Tainan, Taiwan, Republic of Chin) and were housed in a rodent vivarium under a l2-h light,l2-h dark cycle and controlled temperature. Animals were housed in plastic cages and allowed to. acclimatize to their new environment for l4 d prior to initiation of treatment. Control or lead-exposed rats were administered weekly 20 or 50 mg/kg sodium acetate or lead acetate, respectively, intraperitoneally for 6 wk. The ip route was chosen as it is less stressful to rats and blood concentrations reached simulate human levels (Mobarak & P'an, 1984). There were six rats per group.

Blood Lead Analysis

The determination of blood lead was carried out by atomic absorption spectrophotometry (Perkin Elmer zeeman 5100；Perkin Elmer, Norwalk, CT) using an HGA-600 graphite furnace with Zeeman background correction. Blood was drawn by cardiac puncture in rats anesthetized with ether into lead-free heparinized blood collecting tubes. Blood samples were diluted l : 5 with a diluent containing 0.1% Triton X-l00 and 0.1% HNO_3 in distilled deionized water(Del-Rosario et al.,1982).The bsorption wave length was 228.3 nm. Blood lead standards included bovine whole-blood certified materials at concentrations from 5 μg/dl to 98 μg/dl. The r^2 of the calibration curve was at least. 995.Our analytical lab is certified and regularly passes proficiency testing by the Centers for Disease Control and Prevention (CDC) Blood Lead Laboratory System (BLLRS) program at Madison, Wisconsin.

Sperm Suspension Preparation

The rat sperm suspension preparation was performed using a modification of previous studies (Holloway et al., 1990； Sokol et al., 1994). The right cauda epididymis at termination was dissected from each male and transported to the laboratory in l ml 34 ℃ HTF buffer supplemented with 5mg/ml bovine albumin. The cauda epididymis was removed from the transport buffer, slashed, and placed in l ml HTF-albumin buffer, overlaid with mineral oil. A l :10 dilution of spermatozoa was prepared and an epididymal sperm count done with a hemocytometer. The motile epididymal sperm count was obtained form multiplying the epididymal sperm

count by percent motile spermatozoa. On the other hand, the concentration of spermatozoa was adjusted to $1*10^6$ and $10*10^6$ cells/ml for the assay of CTC fluorescence and sperm-oocyte penetration , respectively.

Sperm Lead Analysis

Sperm lead levels were analyzed by graphite furnace atomic absorption Spectrophotometer (Perkin Elmer Zeeman 5l00, HGA-600; Perkin Elmer, Norwalk, CT) and a L'vov platform was used for digested sperm cells (Stachel et al.,1989).In brief, epididymal spermatozoa were suspended in HTF buffer, washed three times with PBS, recentrifuged, and resuspended, The washed spermatozoa were adjusted to final concentration of $47*10^6$ sperm/ml with 60% HNO_3 and then put aside for 12h until the materials were acid-digested completely. The matrix modifier was 0.1% Triton X-l00 and 25% ammonium dihydrogen phosphate in 2% HNO_3. The polyethylene tubes used in the analyses were washed with 5%HNO_3 and rinsed with deionized water as a Precaution against contamination. Each sample was analyzed for lead content in 2 replicates, and the value was accepted only if the ROS was lower the 3%. In the absence of suitable reference materials, the accuracy of the measurements was monitored by use of spike recovery. The percentage of recovered lead was 102 ±4.3%.Sperm lead level was calculated in micrograms lead per 10^9 sperm.

Chlortetracycline Fluorescence Assay

At different time points (0, 2, 4, and 24 h) of incubation, aliquots of cauda epididymal spermatozoa were taken for the chlortetracycline (CTC) assay. The CTC fluorescence assay was used to study the status of capacitation and the extent of acrosome reaction in rats spermatozoa. The modification of Fraser and Herod (l990) and the Oberländer et al. (1996) Methods were used. CTC was prepared freshly at a concentration of 1.5 mM in 2O mM Tris buffer containing 130 mM NaCl and 5 mM L-cysteine. The pH was a adjusted to 7.8 and the solution was shielded from light at room temperature. Fifty microliters of the sperm suspension was mixed with 50μl of CTC solution. After 30 s,8μl of l2.5% (w/v) paraforamaldehyde in 0.5 mM Tris buffer (pH 7.4) was added. After mixing, 50μl of 0.22mM l,4-diazabicyclo[2.2 .2]octane in glycerol was introduced and mixed with the sperm suspension to retard fading of fluorescence. Fluorescence patterns of spermatozoa were analyzed with a Olympus BX-60 microscope (Tokyo) equipped with phase-contrast and epifluorescence optics. The excitation beam was passed through a bandpass filter of 400-410 nm and CTC fluorescence emission was observed through a DM 455 dichroic mirror.

A volume of 5μl sperm suspension was placed on a slide and covered with a 22 *22 mm coverslip. In each sample, a total of 100 cells was assessed and classified classified as having 1 of 3 staining patterns (Figure 1): (1) 〃 uncapacitated spermatozoa,〃 with fluorescence over the entire head;(2) 〃 intermediate spermatozoa, 〃 with a fluorescence-free, dark band in the postacrosomal region of the sperm head and considered as the general tozoa ；or (3) 〃 **acrosome-reacted spermatozoa,〃 with a dark head except for the tip, with retained some fluorescence. Each sample was calculated** by counting 100 spermatozoa in duplicate, and the intra-assay mean coefficients of variation values were 4.5% for assessment of uncapacitation, 9.7% for the intermediate stage, and 18.3% for acrosome-reacted spermatozoa. Acrosome-reacted spermatozoa were presented as percentage of capacitated spermatozoa, calculated by；

Percentage of capacitated spermatozoa(%)= 【1-portion of uncapacitated spermatozoa】 *100

Sperm Reactive Oxygen Species Assay

The sperm ROS assay was modified from our previous study (Hsu et al., 1997). in brief, the generation of ROS by rat

epididymal spermatozoa can be determined using luminol as the chemiluminescence pro be, which interacts with a variety of ROS with the emission of light. A volume of 300 μl of PBS-washed rat epididymal sperm suspension at a concentration of $47*10^6$ cells/ml were mixed with 100μl of 30 mM $FeSO_4$ and then treated with 50 μl of luminol stored as a 1 mM stock solution in DMSO. The Chemiluminescent signal was measured immediately with a computer-driven luminometer (Autolumat-LB 953; EG&G Berthold Co., Bad Wildbad, Germany), with the counts being integrated over a 60-speriod. For each experiment the background chemiluminescence was assessed prior
to luminol addition and was found to be less than l.7 counts/s, which was considered negligible. At the end of this period, the sperm ROS levels were measured into chemiluminescence counts per 10^6 sperm per second .

Sperm-Oocyte Penetration Assay

The rat sperm oocyte penetration assay was performed using a modification of previously published methodologies (Sokol et al., 1994). Control female Sprague-awley rats were superovulated at age 63-70 d weighing 330-380g. In the morning of d 1, the rats were injected with 25 IU of PMSG. On d 3, 52 h later, the animals were injected with 25 IU of hCG. Twenty hours later, the female rats were terminated and the oviducts dissected and placed into HIF-albumin buffer. The cumuli were dissected from the oviducts, collected in HIF-albumin buffer, and dissolved with 10mg/ml hyaluronidase. The zona-intact ova were rinsed twice in HIF-albumin buffer prior to incubation spermatozoa.

After adjusting $10*10^6$ sperm/ml with HIF-albumin buffer,10 μl of epididymal sperm suspension was added to 100μl of HIF-albumin buffer containing 10-15 zona-intact rat ova. Each culture well of the sperm-ova preparation was overlaid with mineral oil and incubated at37℃in 95% air/5% CO_2. After 48 h of insemination, the number of oocytes penetrated by spermatozoa was determined by Phase-contrast microscopy at 400X magnification. SPOR was used to evaluate the sperm-oocyte penetration capacity as described in the following equation:

SOPR(%)=【1-(number of not penetrated oocytes/ Number of total oocytes)】* l00

Statistical Analysis

All the value are presented as means ±SD. Student's t-test was used to compare body and cauda epididymis weights, sperm profile, sperm chemiluminescence counts, SOPR,blood and sperm lead levels, serum testosterone levels, and the percentage of uncapacitated, intermediate, and acrosome-reacted status in different time point between lead-exposed and control groups. The relationship among sperm chemiluminescence counts, the percentage of capacitated, acrosome-reacted spermatozoa, and sperm-oocyte penetration were determined by using linear regression of the JMP statistical package (SAS lnstitute, lnc., Cary, NC) on an Apple Macintosh computer. The criterion for significance was $p< .05$.

四、結果

Body and Tissue Weight

The food and water intake was monitored and recorded weekly during the experiment. There were no significant differences in food and drinking water intake between the lead-exposed and control groups. A significant decrease in body weight was found in rats receiving 20mg or 50 mg lead for 6 wk (Table 1), but cauda epididymis weights were not markedly altered.

Motility and Motile Epididymal Sperm Concentrations

Epididymal sperm counts were significantly decreased in rats treated with 50 mg/kg lead per week, but not in rats treated with 20 mg/kg lead (Table 1). Sperm motility was significantly reduced in rats treated with 50mg lead but not in rats

receiving 20mg/kg lead. The motile epididymal sperm counts from rats treated with 20 mg/kg and 50 mg/kg metal were significantly lower than those in their respective controls.

Blood and Sperm Levels

The blood lead levels averaged 63.7 μg/dl (range:52.5-66.2) and 105.5 μg/dl (range:90.0-112.0) in rats treated with 20 and 50 mg/kg weekly, respectively. The sperm lead levels averaged 1.23 μg/10^9 sperm (range:0.84-1.83) and 2.69 μg/10^9 sperm (range:2.34-3.53) in rats treated with 20 and 50mg/kg weekly, respectively. For the control animals, all blood lead levels were less than 3 μg/dl and sperm lead levels less than 0.08 μg/10^9 sperm (Table 1).

Status of Capacitation and Acrosome Reaction

The percentage of capacitation was significantly increased in fresh cauda epididymal spermatozoa in rats treated with 20 or 50 mg/kg lead (Table 2). The percentage of acrosome reaction was increased only in rats treated with 50 mg/kg metal.

The development of capacitation and acrosome reaction were compared between lead-exposed and control groups at different time point of incubation. In 20 mg/kg lead-exposed rats, the percentage of uncapacitated spermatozoa was significantly lower than those in their controls at 0, 2, 4, and 24 h of incubation (Figure 2A). There were no significant changes in the percentage of intermediate spermatozoa between 20 mg/kg lead exposed rats and their controls at different time point of incubation. The percentage of acrosome-reacted spermatozoa was significantly higher than those in their controls after the spermatozoa were incubated for 2, 4, and 24 h (Figure 2C).

TABLE1. Effects of Lead Exposure on Cartain Parameters in Male Sprague-Dawley Rats

	Treatment			
	20mg/kg		50mg/kg	
Parameter	Coutrol	Lead	Coutrol	Lead
Body weight(g)	436.7±27.5	347.2±44.2[a]	444.2±8.3	342.3±17.0[a]
Cauda epididymis weight(mg)	241.5±12.1	216.0±28.6	231.8±27.2	218.2±20.6
Epididymal sperm count (10^6 sperm/mg)	0.59±0.05	0.58±0.08	0.60±0.03	0.50±0.10[a]
Motility(%)	68.8±9.7	56.5±10.3	69.8±7.4	49.8±7.1[a]
Motile epididymal sperm count (10^6 sperm/mg)	0.41±0.06	0.32±0.04[a]	0.42±0.06	0.25±0.06[a]
Blood Pb(μg/dl)	1.5±0.4	63.7±9.3[a]	1.7±0.7	105.5±12.5[a]
Sperm Pb(μg/10^9 sperm)	0.03±0.01	1.23±0.43[a]	0.04±0.02	2.69±0.98[a]

Note.All values are expressed as mean±SD, n=6 rats for each group.
[a]Significant at $p<.05$ compared with the respective control group.

In50mg/kg lead-exposed rats, the percentage of uncapacitated spermatozoa was significantly lower than those in their controls at 0, 2, 4, and 24 h of incubation (Figure 3A). There were significant changes in the percentage of intermediate spermatozoa between 50 mg/kg lead-exposed rats and their controls at 0, 2, and 4 h of incubation (Figure 3B).The percentage of acrosome-reaced spermatozoa was significantly higher than those in theur controls after the spermatozoa were incubated for 0, 2, 4, and 24 h(Figure 3C)

TABLE2. Effects of Lead Exposure on Cartain Parameters in Male Sprague-Dawley Rats

	Treatment			
	20mg/kg		50mg/kg	
Parameter	Coutrol	Lead	Coutrol	Lead
Capacitated spermatozoa	16.3±2.8	24.7±43.5[a]	18.8±2.1	29.7±3.6[a]
Acrosome-reaced spermatozoa(%)	2.0±12.3	5.2±4.4	1.7±1.0	7.5±3.9[a]
Sperm chemiluminescence (counts/10^6 sperm/s)	7.6±3.7	18.9±7.6[a]	6.3±2.1	33.9±13.9[a]
Sperm-oocytee penetration rats(%)	88.3±9.8	64.3±16.6[a]	88.0±10.4	57.3±10.7[a]

Note.Capacitated spermatozoa(%)=【1-uncapacitated spermatozoa (proportion)】*100. All valuse are expressed as mean±SD, n=6 rats for each group.

[a] Significant at p<.05 compared with their respective control group.

Sperm Reactive Oxygen Species Generation

Sperm ROS generation measured with chemiluminescence counts integrated over 60 s in the 20 mg/kg lead-exposed rats were significantly higher than those in their respective controls (Table 2). In fresh cauda epididymal spermatozoa, the sperm chemiluminescence counts were positively associated with both the percentage of capacitage of capacitated spermatozoa (r^2= .529) (Figure 4A) and the percentage of acrosomereacted spermatozoa (r^2=.608) (Figure 4B).

Sperm-Oocyte Penetration Rate

Spermatozoa harvested from animals treated with 20 mg or 50 mg lead penetrated significantly fewer eggs after 48 h of insemination than did spermatozoa collected from their respective controls (Table 2). SOPR were negatively associated with both the percentage of capacitated spermatozoa (r^2 =..581) (Figure 5A) and the percentage of acrosome-reacted spermatozoa (r^2 =.196) (Figure 5B).

五、討論

In this investigation, the correlation between ROS generation and capacitation and acrosome reaction provided evidence that ROS mediated the toxicity of lead on spermatozoa by accelerating capacition and acrosome reaction. Preacitation need to be taken in concluding a direct effect of ROS on capacitation and acrosome reaction. However, the findings in this study were compatible with previous studies showing that lead-exposed mice were found to have a significantly increased frequency of acrosome-reacted spermatozoa (Johansson, 1989). Spermatozoa from lead-exposed rats had also significantly lower rates of penetrating or fertilizing eggs harvested from unexposed female rats in vitro (Sokol et al.,1994).Mammalian spermatozoa must undergo a period of preparation, including the capacitation and acrosome reaction, which normally occurs in the female reproductive tract, to be capable of fertilizing oocytes (Zaneveld et al., 1991). When and where the acrosome reaction occurs might be important factors for fertilizing the oocyte. In the hamster and mouse, there is evidence that the physiologically relevant acrosome eaction occurred after spermatozoa bound to the zona pellucida (Philips & Shalgi, 1980). Premature acrosome reaction therefore might reduce the capability of fertilization (Tesarik, 1989). If capacitation of spermatozoa occurs as early as in the cauda epididymis and before ejaculation, it may induce the premature acrosome reaction before the spermatozoa meet the egg and probably affects the fertilization. In this investigation, the observed decrease in the penetration of the zona-intact oocyte might be explained by lead-induced ROS-related early onset of capacitation and premature acrosome reaction. These might affect the capability of spermatozoa to become incorporated into the plasma of oocytes.

Data suggested that sperm ROS generation in the lead-exposed rats was significantly higher than those in the controls. A negative association between human sperm ROS generation and the

capacity for sperm-hamster oocyte fusion has been reported (Aitken et al., 1989). These results were consistent with our previous suggestion that lead exposure causes ROS generation in rat spermatozoa (Hsu et al., 1997). A species of ROS, superoxide anion, has been shown to trigger capacitation of human spermatozoa (DeLamirande & Gagnon, 1993). Studies in hamster suggested that H_2O_2 produced by spermatozoa played a significant role during the process of capacitation (Bize et al., 1991). Decrease of membrane thiols by dithiothreitol has been shown to inhibit the acrosome reaction of guinea pig spermatozoa (Fleming et al., 1982). It is conceivable that oxidation of membrane thiols by H_2O_2 generated by spermatozoa may play a role in sperm capacitation. However, micromolar concentrations of H_2O_2 are required stimulate capacitation, including enzymatic activation of guanylate cyclase (White et al., 1976), carrier-mediated glucose transport, glucose oxidation (Mukherjee, 1980), cyclooxygenase (Lands et al., 1984), and protein kinase C (Gopalarkrishna & Anderson, 1989). ROS facilitate the acrosome reaction through a promoting effect on the phospholipase A_2 (PLA_2) activity that was stimulated both by calcium and by the formation of lipid peroxidation within the plasma membrane (Goldman et al., 1992). Lipid peroxidation and ROS generation were significantly increased in defective or normal spermatozoa treated with the calcium ionophore A23187 (Aitken et al., 1989). Moreover, lead might have a calcium-related effect on lipid peroxidation and ROS generation. Lead had effects on plasmalemma or intracellular membranes (Simon, 1993) and impaired mitochondrial functions (Chavez et al., 1987) to enhance the permeability of the calcium. In vivo exposure to lead in rats significantly reduced the Ca^{2+}ATPase activity, resulting in an increase in intrasynaptosomal calcium and high levels of lipid peroxidation in nerve terminals (Sandhir et al., 1994).

In contrast, inhibitory protein might play an important role in prevent- ing premature capacitation. On mice spermatozoa, a protein bound to the acrosomal region of the sperm head membrane had been recognized (Okabe et al., 1986). The protein vanished from the membrane of the spermatozoa at capacitation. Fraser (1984) found that loss of a surface associated inhibitory component, which stabilized the membranes and prevented the acrosome reaction, was involved in the process of capacitation. Moreover, one of the porcine seminal proteins synthesized by the epithelium of the seminal vesicles, PSP-1, might also be involved in preventing premature acrosome reaction (Kwok et al., 1993) and seemed to have properties similar to the inhibitory protein. Lead was shown to affect protein synthesis at different concentrations (Vallee & Ulmer, 1972). It is possible that lead caused enhanced capacitation and premature acrosome reaction through disturbing the synthesis or the functions of such components.

A decrease in body weight was associated with lead exposure in this Study. Lead was found to lower the set point for food consumption and to reduce body weight in rats (Hammond et al.,1990). Although reduced nutrition was implicated as a cause of reduced sperm counts (Oldham et al., 1978), it is not clear whether motility or penetration capability is equally affected. However, there is no known report that reduced bodyweight accelerates capacitation or acrosome reaction in sperm. Further studies concerning effects of nutrition on sperm ROS and acrosome reaction are needed to clarify this issue. Epididymal and motile epididymal sperm counts were significantly decreased in rats treated with 50 mg/kg of lead for 6 wk where average blood lead levels were l05.5 ± 12.5 μg/dl and sperm lead levels2.69 ± 0.98 μg/10^9 sperm. This is comparable with our previous study showing that epididymal sperm counts and motile epididymal sperm counts were

decreased in rats treated with l0 mg/kg of lead for 9 wk where average blood lead levels were 48 ±4.3 μg/dl and sperm lead levels 0.88 ±0.16 $\mu g/10^9$ sperm, but not in rats treated with 10 mg/kg of lead for 6 weeks with average blood lead levels of 33.6 ± 6.1 μg/dl and sperm lead levels of 0.67±0.11 $\mu g/10^9$ sperm (Hsu et al., 1997). Different doses, as well as the duration of exposure, might have accounted for the observed differences. In our study, animals treated with 20 mg/kg and 50 mg/kg of lead for 6 wk had average blood lead levels of 63.7 ±9.3 μg/dl and l05.5 ± 12.5 μg/dl. Lancranjan et al. (1975) used clinical and toxicological criteria for the classification of workmen into four groups with blood lead levels as follows:(l) lead-poisoned workmen (74.5±26 μg/dl；(2) moderate (52.8 ±21 μg/dl) ; (3) slight (41±12 μg/dl)；or (4) nonexposed (23±14 μg/dl). They used the classification to evaluate lead toxicity in occupational exposure. Hirata et al. (1995) found that workers exposed to lead had blood lead levels from 38 μg/dl to l02 μg/dl in a Japanese factory manufacturing lead glass-based paints. A recent report on lead-exposed workers from two factories in Taiwan showed an average blood lead of 67± 26 μg/dl (Lai et al.,1997), indicating that probably l0% had a blood lead level of l00 μg/dl or higher. Therefore, in this study, the two levels of lead doses chosen could reflect the situation of moderate to high exposure of workers in Asian countries. On the other hand, serum testosterone level from 50 mg/kg lead-exposed rats were significantly lower than controls. Decreased testosterone levels could be caused by direct effects of lead on the hypothalamic-pituitary-testicular axis (Sokol et al., 1985). However, indirect effects of reduced body weight on the hypothalamus and secretion of luteinizing hormone (Walkden-Brown et al., 1994) could also have contributed to the reduced testosterone levels. A dose-dependent suppression of serum testosterone levels and spermatogenesis was shown in rats with blood lead levels of 34 ± 3 and 60 ± 4 μg/dl (Sokol et al., 1985). Testosterone and 5α-dihydrotestosterone present in human seminal plasma might also prevent premature capacitation before the spermatozoa reach the site of fertilization in vitro (Chan et al., 1983). It was noted that the serum testosterone levels were negatively associated with the percentage of acrosome-reacted spermatozoa in cauda epididymis. A possible explanation for this finding was that lead exposure reduced estosterone levels in blood and seminal plasma and therefore inactivated the inhibitory effects on premature acrosome reaction.

In conclusion, lead exposure might cause early onset of capacition, premature acrosome reaction, reduced serum testosterone levels, and ROS generation. The ROS-related early onset of capacitation in rat cauda epididymal spermatozoa resulted in premature acrosome reaction and reduced zona-intact oocyte penetration capability . Further studies are needed to determine which types of ROS, as well as whether testosterone is involved in the onset of the capacitation or/and acrosome μg/dl reaction.

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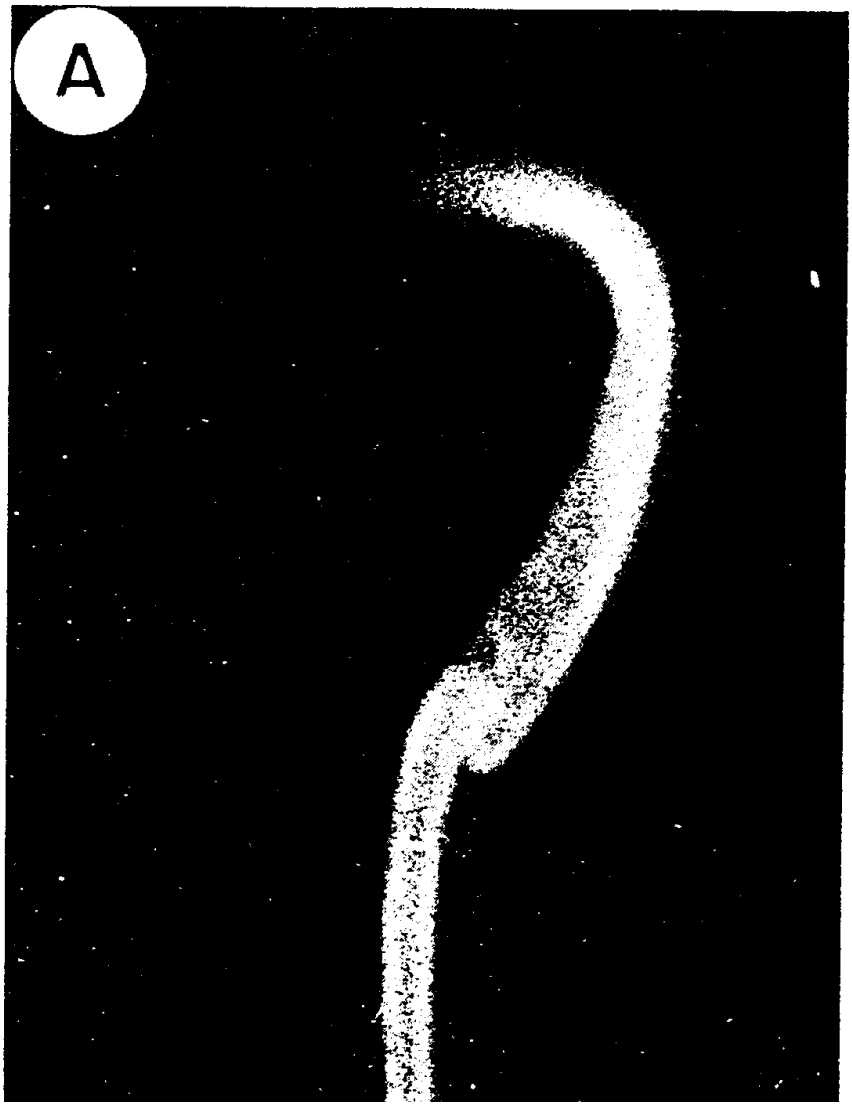


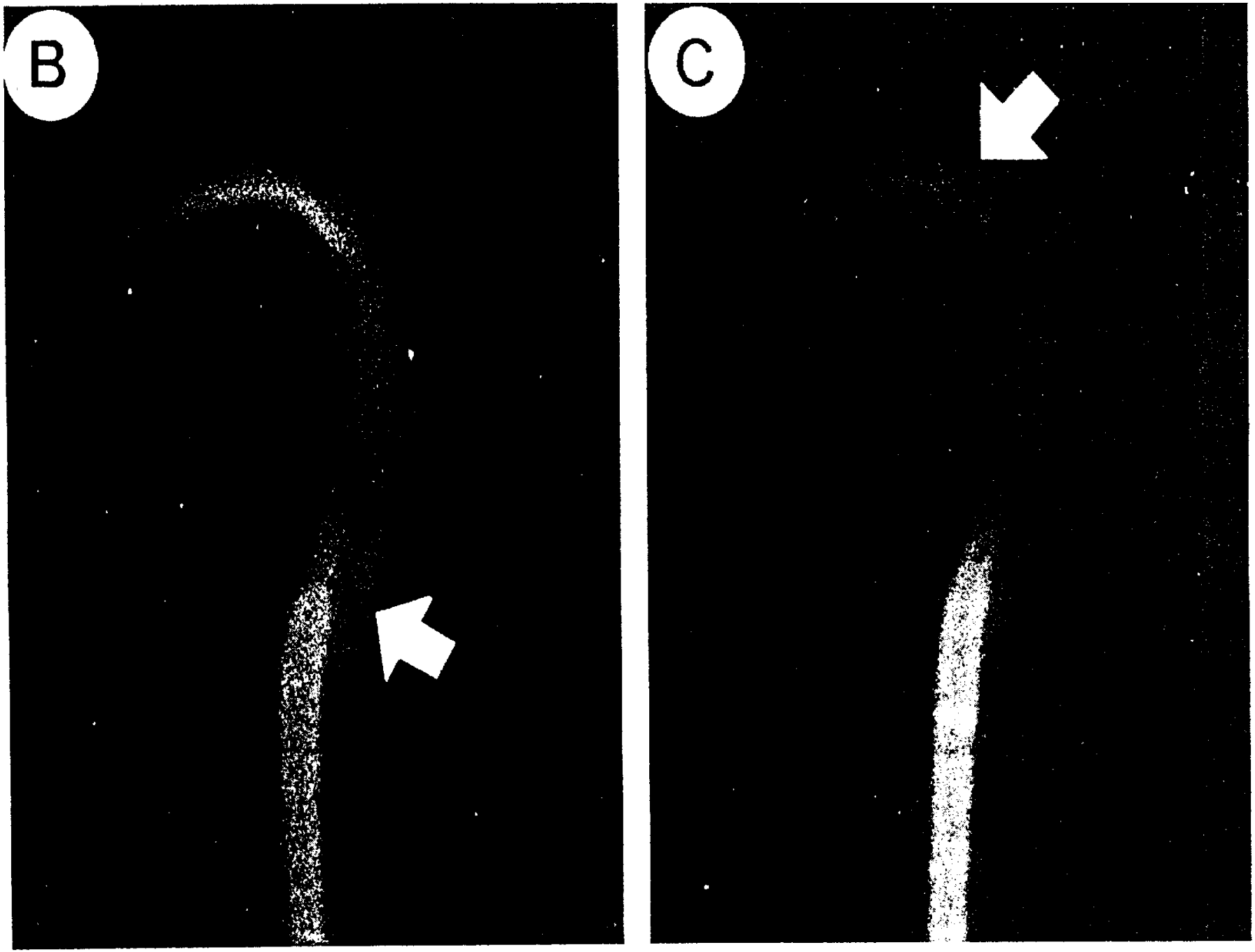
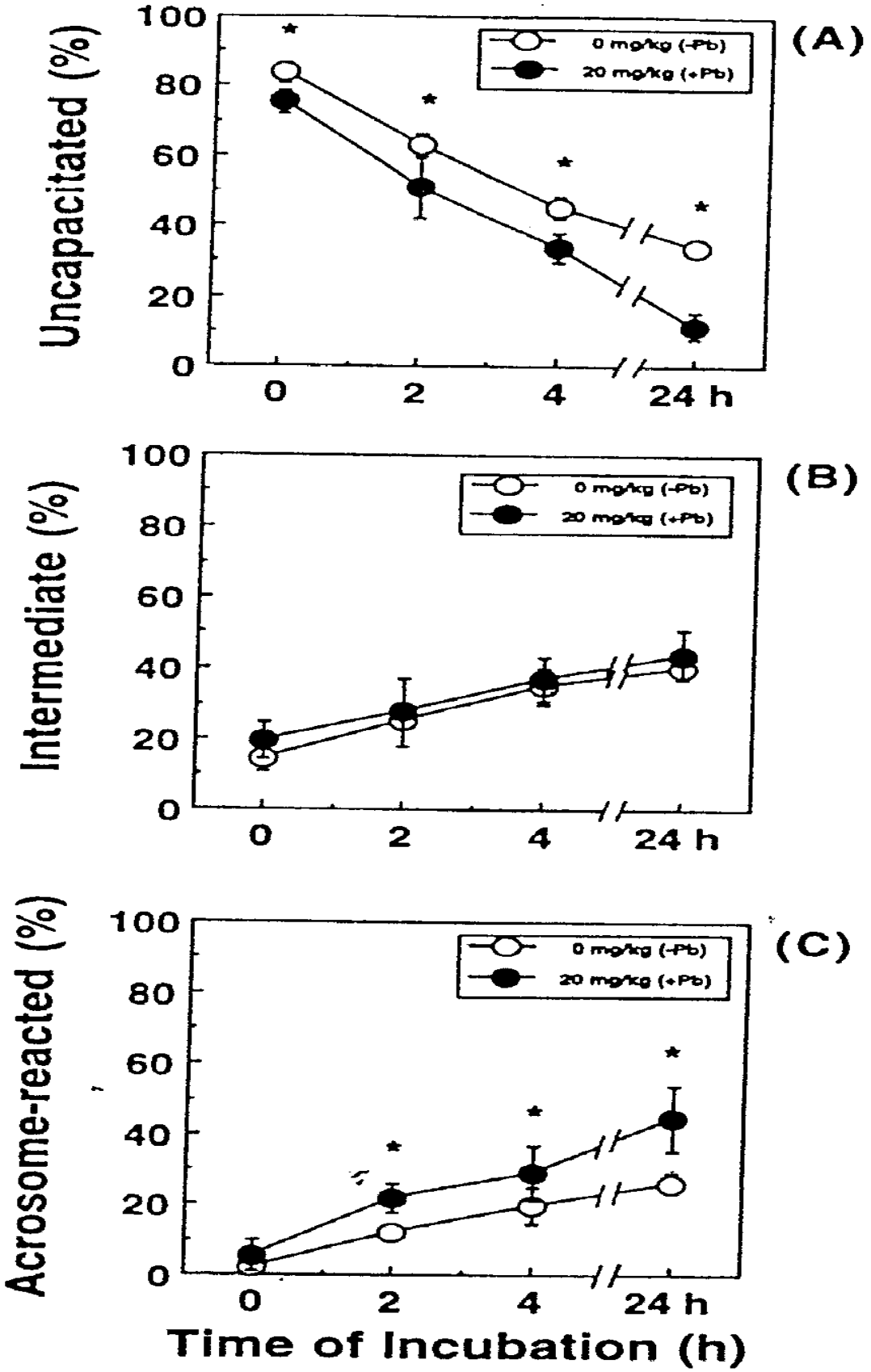


FIGURE 1. (*Continued*) Fluorescence patterns of rats sperm heads after staining with chlortetracycline during capacitation. (B) Intermediate spermatozoa, with a fluorescence-free, dark band (arrow) in the postacrosomal region of the sperm head. (C) Acrosome-reacted spermatozoa, with dark head except for the tip (arrow), which retained some fluorescence.



FI[illegible] intermediate, and (C) acrosome-reacted pattern of chlortetracycline fluorescence over 24 h of incubation in 20mg/kg lead-exposed(+Pb) and control (-Pb) rats. All values are expressed as mean±SD, n=6 rats for each group. Asterisk indicates $p<.05$compared with their respective control group at different time points of incubation.

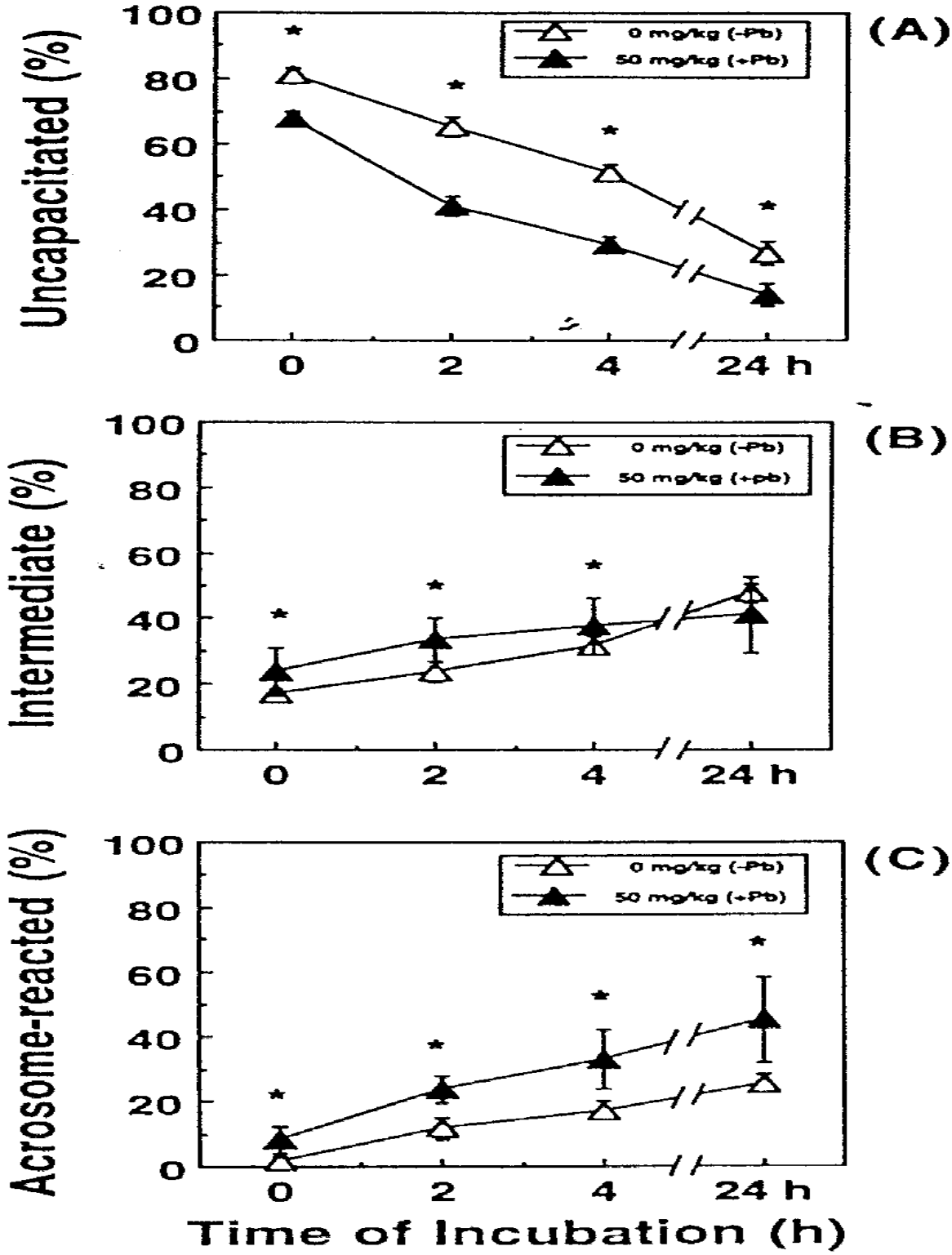
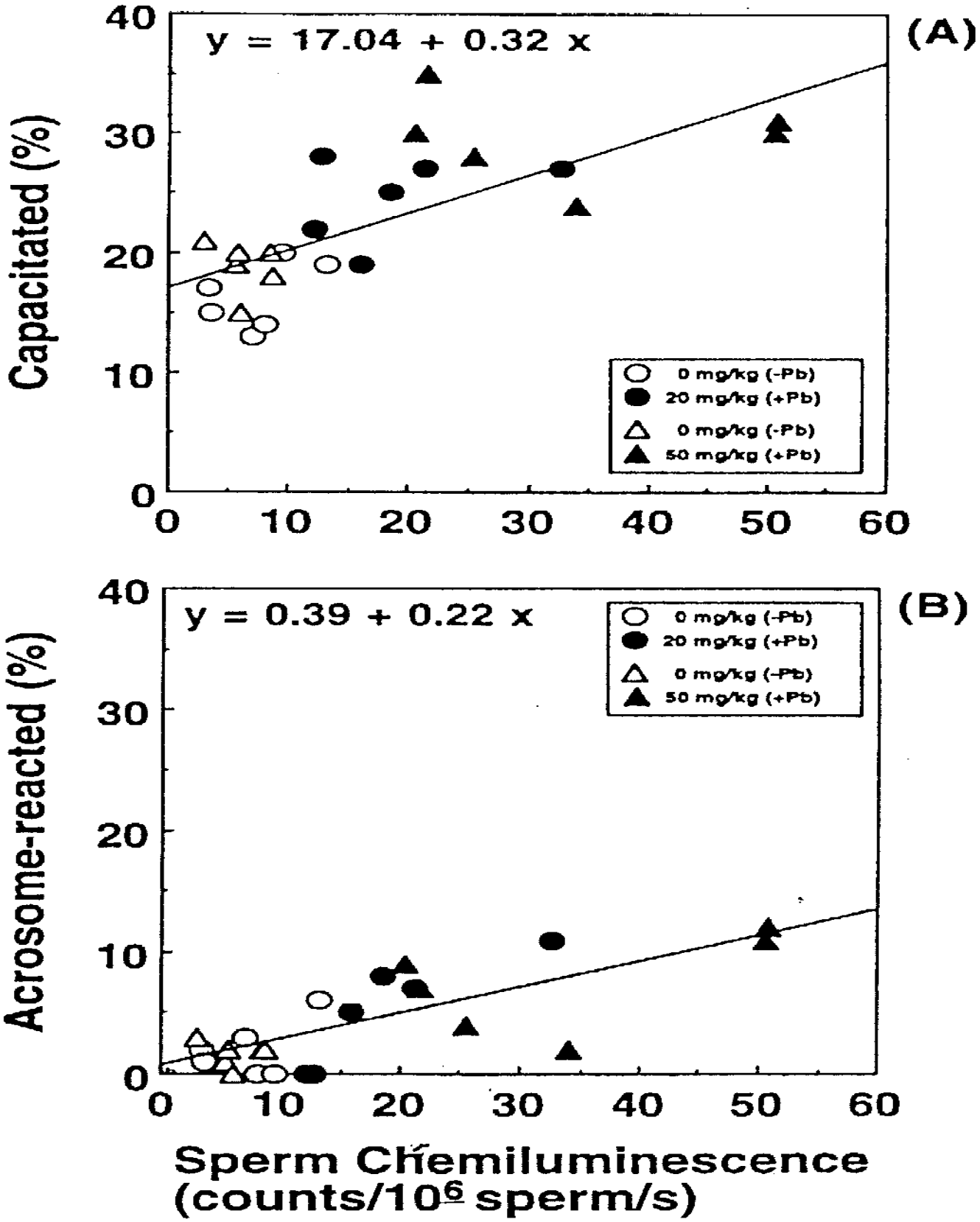
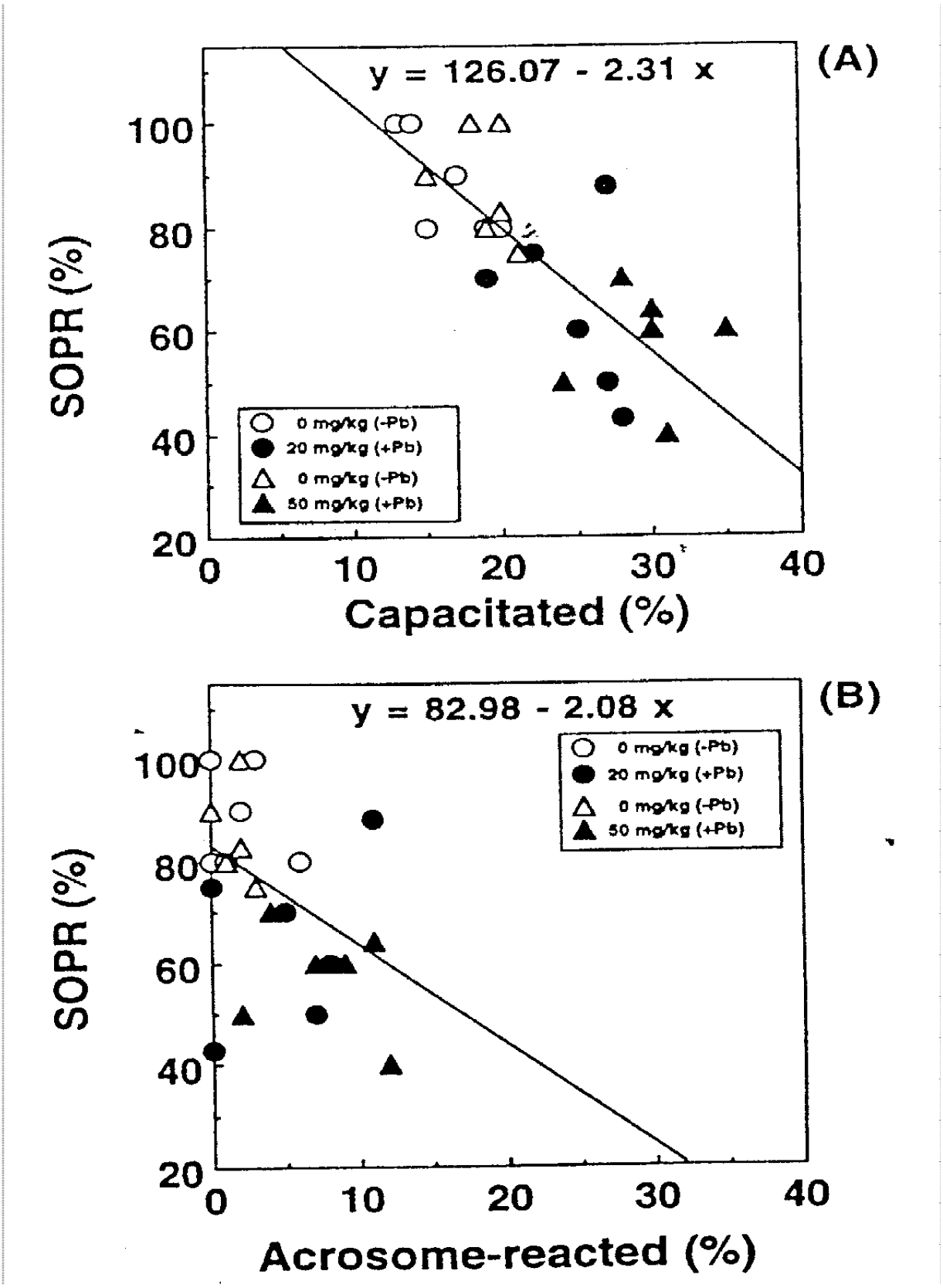


FIGURE 3. Effects of lead exposure on cauda epididymal spermatozoa following the development of (A) uncapacitated, (B) intermediate, and (C) acrosome-reacted pattern of chlortetracycline fluorescence over 24 h of incubation in 50mg/kg lead-exposed(+Pb) and control (-Pb) rats. All values are expressed as mean±SD, n=6 rats for each group. Asterisk indicates p<.05compared with their respective control group at different time points of incubation.



FlGURE 4. Relationship between sperm chemiluminescence, a direct indicator for reactive oxygen species, and percentage of (A) capacitated and (B) acrosome-reacted spermatozoa(r^2=.529 and .608 ; p<.05respectively) in the 20 mg/kg and 50 mg/kg lead-exposed rats (+Pb) and their respective controls(-Pb).



FICURE 5. Relationship between the sperm-oocyte penetration rate (SOPR), and percentage of (A) capacitated and (B) acrbsome-reacted spermatozoa (r^2= .581 and .196;p<.05, respectively) in 20 mg/kg and 5O mg/kg lead-exposed rats (+Pb) and their respective controls (-Pb).