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Inhibition of Alkaline Phosphatase Activity of Boar Semen by Pentoxifylline, Caffeine, and Theophylline

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ABSTRACT: Methyl xanthines have been used frequently as additives to sperm suspensions in order to improve sperm characteristics. The mechanism of action on spermatozoa is generally assumed to be inhibition of sperm phosphodiesterase activity, resulting in elevation of complementary adenosine monophosphate levels in spermatozoa. The present study was designed to examine the effect of methyl xanthines (pentoxifylline, caffeine, and theophylline) on another important enzyme system, alkaline phosphatase, in boar seminal plasma and spermatozoa. Inhibition of sperm alkaline phosphatase could be distinguished from that of seminal plasma by a paradoxical stimulation by pentoxifylline at lower pH values in spermatozoa. Among the three methyl xanthines, theophylline exhibited the most dramatic inhibition of alkaline phosphatase activity and substrate inhibition was observed with increasing concentrations. Each methyl xanthine had a different action on alkaline phosphatase activity at lower pH; theophylline showed the highest inhibition, caffeine inhibition was not related to pH, and pentoxifylline did not inhibit alkaline phosphatase of seminal plasma and, in fact, it stimulated its activity (or that of a phosphatase with lower pH optimum) in spermatozoa. These results indicate another possible mechanism of action of methyl xanthines on sperm and are in agreement with data indicating that methyl xanthines are not specific inhibitors of sperm phosphodiesterase, because clearly, they inhibit alkaline phosphatase activity as well.

Key words: Methyl xanthines, seminal plasma, spermatozoa.
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Methyl xanthines are frequently used as additives to sperm suspensions in order to improve sperm characteristics. Among methyl xanthines, pentoxifylline (3,7-dimethyl-1-(5-oxo-hexyl)-xanthine), caffeine (1,3,7-trimethylxanthine), and theophylline (1,3-dimethylxanthine) have been employed most often. In numerous studies, methyl xanthine supplementation resulted in better motility characteristics in fresh and cryopreserved spermatozoa. These include a greater percentage of motile cells and greater velocity of sperm movement, including curvilinear velocity, which often leads to a higher percentage of nonhyperactive sperm shifting to either a transitional or hyperactivated state (Tash and Means, 1983; Schoff and Lardy, 1987; Rees et al, 1990; Sikka and Hellstrom 1991; Cowart et al, 1994; Lewis et al, 1994; Koutsarova et al, 1997; Merino et al, 1997; Sharma and Agarwal, 1997; Nassar et al, 1999a; Ponce et al, 1999).

In immature epididymal or testicular spermatozoa, addition of methyl xanthine is crucial for acquisition or improvement of sperm motility and fertilizing ability (Gould et al, 1988; Jaiswal and Majumder, 1996; Mahony et al, 1996; Angelopoulos et al, 1999). Stimulatory effects of methyl xanthines on capacitation and the acrosome reaction have also been demonstrated (Tesarik et al, 1992; Tesarik and Mendoza, 1993; DasGupta et al, 1994; Jayaprakash et al, 1997; Esteves et al, 1998; Ain et al, 1999). Apart from modulation of sperm function, a protective role on sperm membranes by pentoxifylline has been described (Ponce et al, 1999). This effect may be ascribed to neutralization of reactive oxygen species and a reduction of lipid peroxidation (Bell et al, 1993; McKinney et al, 1996; Okada et al, 1997). Pentoxifylline has been proposed as a cryoprotectant owing to its protective action (Wang et al, 1993; Brennen and Holden, 1995). Overall, the addition of methyl xanthines to sperm suspensions seems to improve sperm function, leading to better sperm fertilizing capacity (Fraser, 1979; Louglin and Agarwal, 1992; Nagai et al, 1994; Negri et al, 1996; Abeydeera and Day, 1997; Chauhan et al, 1998; Nassar et al, 1999a). However, the beneficial effects of methyl xanthine on sperm quality have been questioned (Lewis et al, 1993, 1994; Mathieu et al, 1994; Tournaye et al, 1994; Dimitriadou et al, 1995; Paul et al, 1996).

The generally accepted mechanism of methyl xanthines is their inhibition of sperm cyclic nucleotide phosphodiesterase. This reduces destruction of cyclic adenosine 3′,5′ monophosphate (cAMP), which results in elevation of cyclic adenosine monophosphate levels in spermatozoa

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(Tash and Means, 1983). Indeed, a rise in cAMP levels in spermatozoa after methyl xanthine treatment has been consistently demonstrated (Garbers et al, 1971; Hoskins et al, 1975; Wang et al, 1993). Elevated levels of cAMP enhance cAMP-dependent processes of spermatozoa, including motility, capacitation, and acrosome reaction (Tash and Means, 1983; Monks and Fraser, 1987; Fraser and Monks, 1990; Armstrong et al, 1994; Aitken, 1997). The molecular basis of cAMP regulation of these processes is based on protein phosphorylation, especially for capacitation (Duncan and Fraser, 1993; Galantino-Homer et al, 1997; Flesch et al, 1999; Nassar et al, 1999b). An alternate way for methyl xanthines to elevate cAMP levels is through the modulation of adenosine receptors (Vijayaraghavan and Hoskins, 1986; Louglin and Agarwal, 1992). Methyl xanthines may also affect translocation of intracellular calcium (Louglin and Agarwal, 1992; Nagai et al, 1994), neutralization of reactive oxygen species, and reduced lipid peroxidation. These observations support the hypothesis that non–cAMP-mediated events in spermatozoa may also be modulated by methyl xanthines (Vandevoort et al, 1994).

It is well known that methyl xanthines can inhibit alkaline phosphatase of somatic origin (Vinet et al, 1978; Dai and Snow, 1991; Wang and Gilles-Baillien, 1992; Rezende et al, 1998). This effect on somatic alkaline phosphatase is employed in determining theophylline in serum (Jourquin and Kauffman, 1998). These data raise the question of whether methyl xanthines may also inhibit alkaline phosphatase in mammalian semen, where it is universally present (Bell and Lake, 1962; Jones, 1978; Glogowski, 1988; Tang 1998). If they do, their effects exerted on sperm functions may also be mediated through modulation of alkaline phosphatase activity. In this study, we tested the effects of pentoxifylline, caffeine, and theophylline on the alkaline phosphatase activity of boar seminal plasma and spermatozoa.

Materials and Methods

Animals, Preparation of Seminal Plasma and Sperm Extracts, and Alkaline Phosphatase Assay

Semen was collected from adult boars housed in the Animal Breeding and Insemination Station in Olsztyn, Poland. Semen was collected manually into a cup with a filter, and the gel fraction was discarded. Seminal plasma was obtained by centrifugation (10 000 × *g*). Plasma was diluted 100–150 times before alkaline phosphatase assay. Sperm extracts were obtained as follows: semen (8 mL) was washed 3 times with 0.055 M Hepes-NaOH, 0.7% NaCl pH 7.4, and then 2.5 mL of cold Hepes buffer (without NaCl) was added. Sperm suspensions were stored on ice for 30 minutes, sonicated for 25 seconds, and centrifuged (10 000 × *g*). Pellets were discarded and supernatants were diluted 1:1 with Hepes buffer containing 1.5% NaCl. Extracts were frozen and stored for up to 3 days at −20°C and were not further diluted for alkaline phosphatase assay. Alkaline phosphatase activity was measured with a colorimetric assay with 4-nitrophenylphosphate (Merck & Company, Whitehouse Station, NJ) as a substrate according to the method described by Bessey et al (1946). The incubation mixture contained a 1.1 mL final volume of 50 mM glycine buffer and 7.5 mM 4-nitrophenylphosphate (disodium salt). Incubations were carried out at 37°C for 30 minutes, and the reaction was terminated by adding 10 mL of 0.02 N NaOH. Amounts of liberated 4-nitrophenol were calculated from standard curves. Alkaline phosphatase activity was expressed as micromolar units of 4-nitrophenol liberated per minute. In total, seminal plasma and sperm extracts from 5 different boars were used.

Effects of Methyl Xanthines on Alkaline Phosphatase Activity of Seminal Plasma and Spermatozoa

Caffeine and pentoxifylline (Sigma Chemical Company, St Louis, Mo) effects were tested in the concentration range of 0–20 mM and theophylline (Sigma) in the range of 0–2.5 mM. Substrate and inhibitors were added first and preincubated at 37°C for 5 minutes. Reactions were started by adding an enzyme source (either seminal plasma or sperm extract).

Effect of pH on Inhibition of Seminal Plasma and Sperm Alkaline Phosphatase Activity by Methyl Xanthines

Alkaline phosphatase activities were tested in the presence of 10 mM caffeine and pentoxifylline, and 0.625 mM of theophylline using 0.05 M (final concentration) glycine-NaOH buffer in the pH range of 8.6–11.0.

Effects of Substrate Concentration on Inhibition of Seminal Plasma and Sperm Alkaline Phosphatase Activity by Methyl Xanthines

A 4-nitrophenylphosphate concentration range of 0–5 mM was used. Alkaline phosphatase activities were measured in the presence of 10 mM caffeine and pentoxifylline, and 0.625 mM of theophylline. The pH of the reaction mixture was 10.6 for variants with caffeine and pentoxifylline, and 10.2 for theophylline (owing to greater inhibition at pH 10.2 than 10.6).

Statistical Analysis

Data are expressed as means ± SEM (n = 5). One-way analysis of variance (ANOVA) was used for evaluating different concentrations of methyl xanthines on alkaline phosphatase activities and two-way ANOVA for evaluation inhibition at different pHs. A Tukey test was used for post hoc comparisons. Concentrations of theophylline for 50% inhibition of alkaline phosphatase (IC_{50}) and regressions of Lineweaer-Burk plots were calculated using the GraphPad PRISM statistical package (GraphPad Software, San Diego, Calif).

Results

Effects of Methyl Xanthines on Alkaline Phosphatase Activity of Seminal Plasma and Spermatozoa

All methyl xanthines inhibited alkaline phosphatase activity, whether they were derived from seminal plasma or

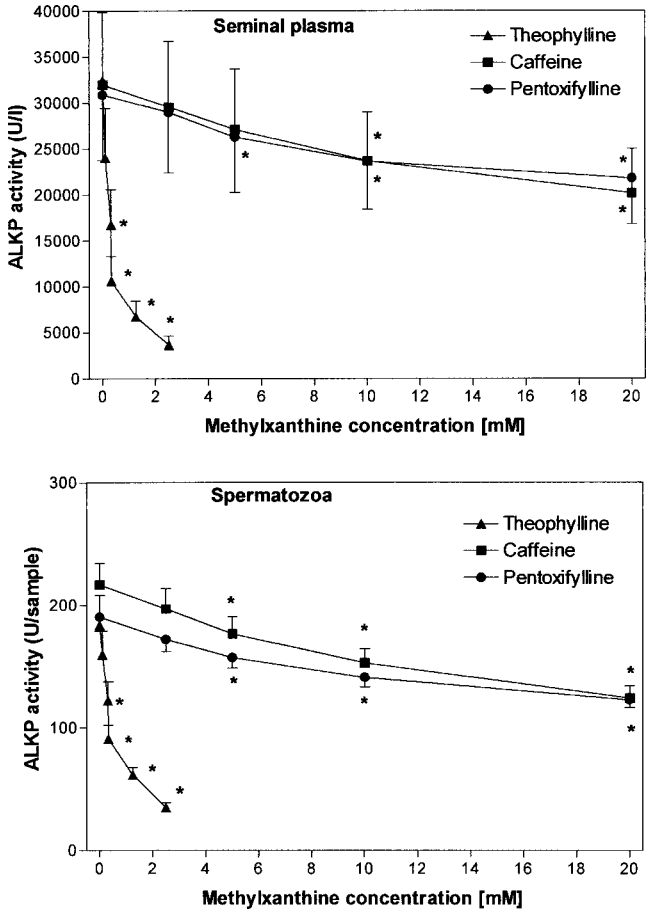

Figure 1. Effects of caffeine (0–20 mM), pentoxifylline (0–20 mM), and theophylline (0–2.5 mM) on alkaline phosphatase activity of boar seminal plasma and spermatozoa (n = 5, *$P < .01$).

spermatozoa, in a concentration-dependent manner (Figure 1). Theophylline was more effective than pentoxifylline and caffeine; the dose response line for theophylline was much steeper than it was for other methyl xanthines. Alkaline phosphatase inhibition was 27% and 37% for seminal plasma and 35% and 43% for spermatozoa, at 20 mM pentoxifylline and 20 mM caffeine, respectively. In contrast, 50% alkaline phosphatase inhibition was observed at theophylline concentrations of less than 1 mM. This inhibition was more effective for seminal plasma alkaline phosphatase than it was for spermatozoa alkaline phosphatase activities. The IC_{50} for seminal plasma alkaline phosphatase activity was 0.34 ± 0.02 mM, and for sperm alkaline phosphatase it was 0.62 ± 0.05 mM.

Effect of pH on Inhibition of Seminal Plasma and Sperm Alkaline Phosphatase Activity by Methyl Xanthines

Inhibition of both seminal plasma and sperm alkaline phosphatase activities was affected by pH (Figures 2 and 3), however, the effects were different for particular methyl xanthines (Figures 2D and 3D), especially at lower pH. Inhibition of alkaline phosphatase by theophylline increased with decreasing pH. In contrast, inhibition by caffeine was not affected by pH, and pentoxifylline inhibition was greatest at high pH (>9.6 for seminal plasma and >10.3 for spermatozoa). At a lower pH, pentoxifylline did not inhibit seminal plasma alkaline phosphatase activity, but it stimulated sperm alkaline phosphatase activity.

Effects of Substrate Concentration on Inhibition of Seminal Plasma and Sperm Alkaline Phosphatase Activity by Methyl Xanthines

Pentoxifylline and caffeine inhibited alkaline phosphatase activities of seminal plasma and spermatozoa in a noncompetitive manner (Figures 4 and 5). In contrast to theophylline, a substrate inhibition of alkaline phosphatase was observed.

Discussion

This study demonstrates that methyl xanthines can inhibit alkaline phosphatase activity in seminal plasma and spermatozoa. Our study revealed differences among inhibition modes of 2 sources of alkaline phosphatase (seminal plasma and spermatozoa) as well as among 3 methyl xanthines. Inhibition of sperm alkaline phosphatase could be distinguished from that of seminal plasma by stimulating the activity with pentoxifylline at lower pH values. Among the 3 methyl xanthines, theophylline exhibited the most dramatic inhibition of alkaline phosphatase activity, and substrate inhibition was observed with increasing concentration. Each methyl xanthine had a different action at lower pH: theophylline showed highest inhibition, caffeine inhibition was not related to pH, and pentoxifylline did not inhibit seminal plasma alkaline phosphatase, but it stimulated spermatozoa alkaline phosphatase activity. These results indicate another possible action of methyl xanthines on sperm and are in agreement with previous studies indicating that methyl xanthines are not pure inhibitors of phosphodiesterase.

There are some similarities between semen alkaline phosphatase and phosphodiesterase. Like alkaline phosphatase, phosphodiesterase is present in spermatozoa and seminal plasma, it occurs in multiple molecular forms, and it is differentially inhibited by methyl xanthines (Tash, 1976). Vijayaraghavan and Hoskins (1986) found that the theophylline IC_{50} for inhibiting bovine sperm phosphodiesterase was 0.7 mM, which is within the same range as inhibition obtained in this work for boar alkaline phosphatase. As with boar sperm alkaline phosphatase inhibition, theophylline is a more effective inhibitor of phosphodiesterase found in the sperm of sea urchins (Wells and Garbers, 1976) and buffalo (Bhatangar et al,

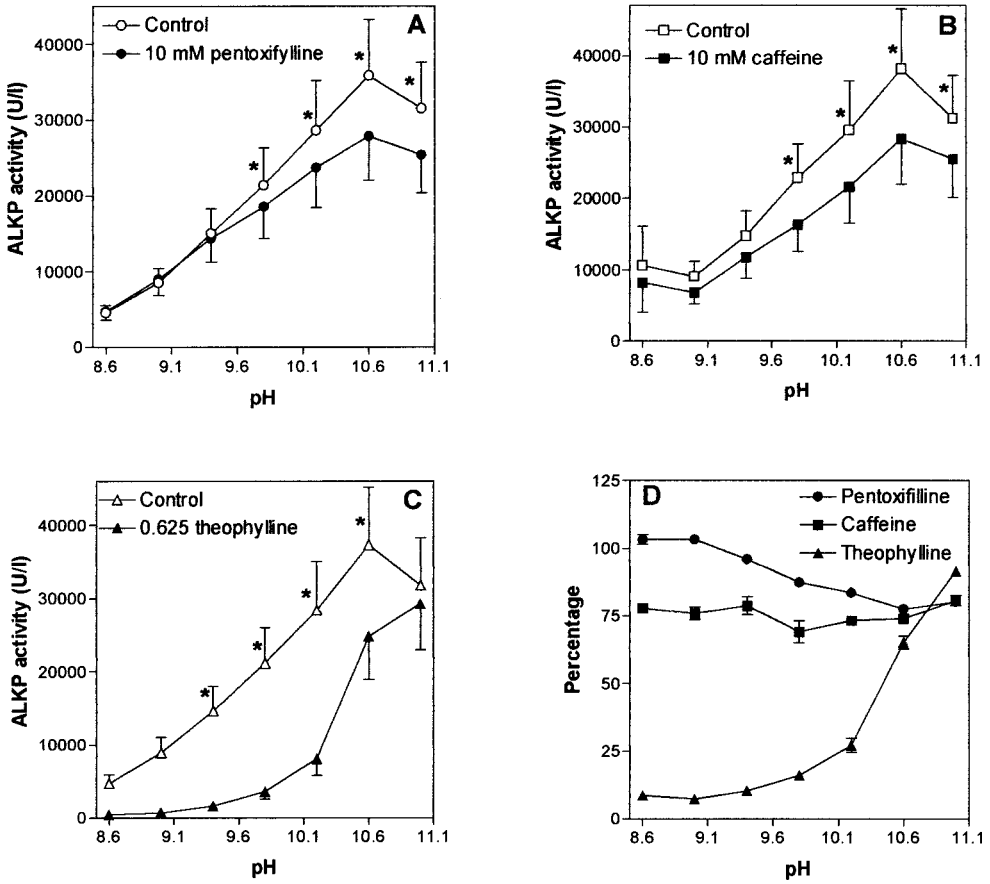

Figure 2. Effects of pentoxifylline (**A**, 10 mM), caffeine (**B**, 10 mM), and theophylline (**C**, 0.625 mM) on alkaline phosphatase activity of boar seminal plasma, measured at the pH range of 8.6–11.0. Percentage of inhibition at different pHs are presented in **(D)**; n = 5, *$P < .01$.

1979). On the other hand, some species-specific differences were reported: in ram semen, for example, caffeine was a more potent inhibitor of phosphodiesterase than theophylline, and both methyl xanthines inhibited phosphodiesterase by 33%–79% at concentrations of 5 mM (Tash, 1976). Therefore, interspecies comparisons of the effects of methyl xanthine on phosphodiesterase and alkaline phosphatase activity should be interpreted cautiously.

It is difficult to translate the alkaline phosphatase inhibition by methyl xanthines described here into specific clinical applications. Concentrations of methyl xanthines reported in our work were similar to those used for enhancement of sperm quality. For example, enhancement of semen characteristics has been reported at concentrations of caffeine and pentoxifylline up to 20 mM (Stachecki et al, 1994), and theophylline has been used at concentrations of 10 mM and 20 mM (Loughlin and Agarwal, 1992; Cowart et al, 1994) or even 30 mM (Jaiswal and Majumder, 1996). If sperm alkaline phosphatase of other mammalian species is similarly inhibited by methyl xanthines, as we have demonstrated for boar alkaline phosphatase in the present study, these data suggest that beneficial effects of these substances may be partially related to their action on sperm alkaline phosphatase. This inhibition may not always produce better sperm characteristics because a decrease in sperm quality at high concentrations of methyl xanthine has been reported (Brennan and Holden, 1995). These authors reported the detrimental effect of a high dose of pentoxifylline (10 mM) on acrosome morphology. Also, Armstrong et al (1994) found that 5 mM caffeine or pentoxifylline stimulated the motility of rat sperm more than 10 mM did. In contrast, improvement of sperm characteristics has been reported at concentrations of methyl xanthines that, in the present study, do not significantly affect boar sperm alkaline phosphatase. For example, common doses of pentoxifylline used for supplementation of human sperm suspensions are 1 mM or 3–4 mM (Wang et al, 1993; Lewis et al, 1994; Brennan and Holden, 1995; McKinney et al, 1996) doses lower than those that were inhibitory in the present study. In such cases, inhibition of alkaline phosphatase may not be involved in the beneficial effects of pentoxifylline.

We recognize that the experiments included in the present study were performed at artificially elevated (non-

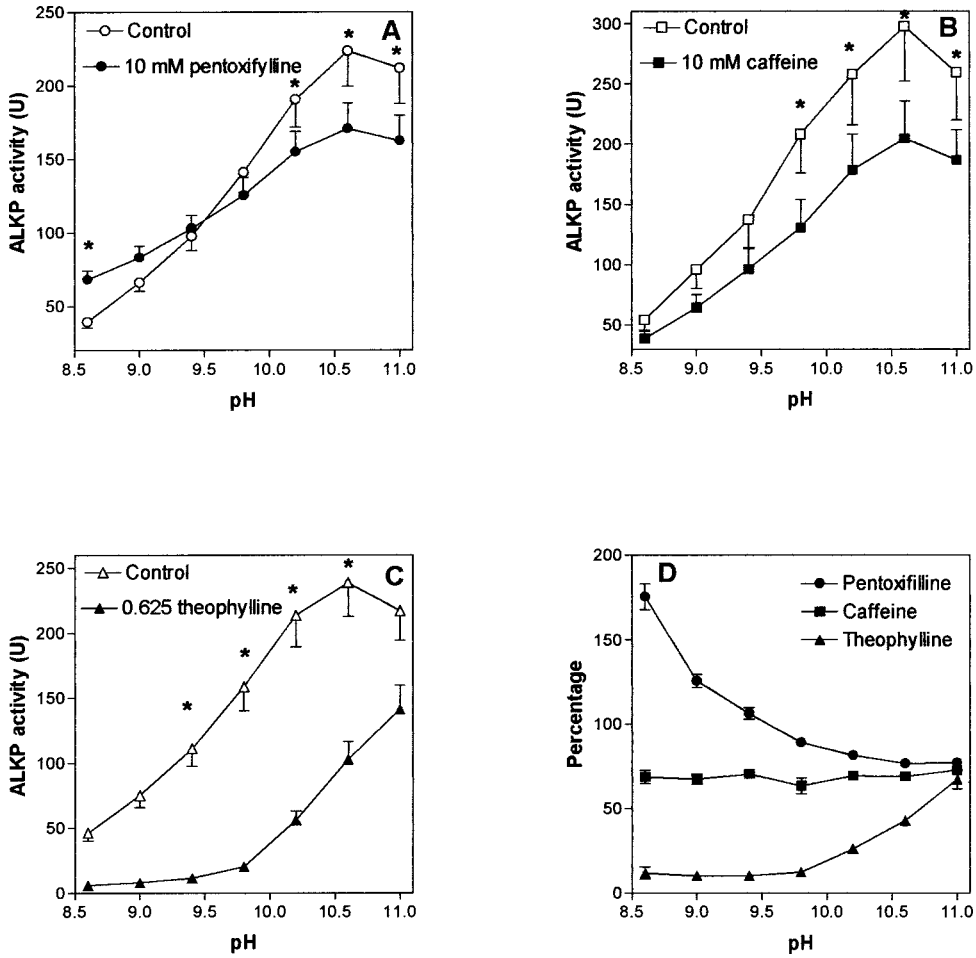

Figure 3. Effects of pentoxifylline (**A**, 10 mM), caffeine (**B**, 10 mM), and theophylline (**C**, 0.625 mM) on alkaline phosphatase activity of boar spermatozoa measured at the pH range of 8.6–11.0. Percentage of inhibition at different pHs are presented in **(D)**; n = 5, $*P < .01$.

physiological) pH values owing to the alkaline optimum of alkaline phosphatase activity. For this reason, extrapolation of alkaline phosphatase inhibition at more physiological (neutral or slightly alkaline) pHs requires caution. Our data indicate that theophylline is the most effective inhibitor of alkaline phosphatase activity at lower pH values, whereas pentoxifylline stimulates alkaline phosphatase activity (or a different phosphatase with an optimum pH near neutral pH [see below]). Most experiments investigating methyl xanthines have been performed using human sperm, and there are substantial differences in seminal plasma alkaline phosphatase activities among mammals. The highest levels of alkaline phosphatase enzyme activities are found in boars and the lowest are found in humans (Bell and Lake, 1962; Jones, 1978). Thus, if methyl xanthines act on sperm function through modulation of alkaline phosphatase, effects on human sperm may not be as profound as those observed in the present study using boar sperm.

Despite extensive research on alkaline phosphatases in the male reproductive tract, their role in reproductive physiology is not clear. Difficulties in understanding the role of alkaline phosphatase originate from the universal presence of alkaline phosphatase in reproductive tissue and its wide substrate specificity. Alkaline phosphatase is present in both seminal plasma and spermatozoa. In seminal plasma, it is present in multiple forms that share similar kinetic properties, and it generally originates from epididymal fluid (Strzezek and Glogowski, 1979; Glogowski and Strzezek, 1980; Frenette et al, 1986; Glogowski, 1988; Iyer et al, 1988; Tang, 1998). Alkaline phosphatase is present in prostasomes (Fabiani and Ronquist, 1995) and is a component of sperm plasma membranes (Soucek and Vary, 1984; Parks et al, 1987). In addition, it is associated with cytoplasmic droplets and acrosomes (Moniem and Glover, 1972; Bavdek and Glover, 1970; Yuan et al, 1995). Alkaline phosphatase derived from semen can hydrolyze phosphate esters of various mononucleotides, sugars, glycerophosphate (Glogowski, 1988), and pyridoxal 5′-phosphate (Glogowski, 1988; Ciereszko et al, 1994), as well as adenosine triphosphate (Glogowski, 1988). Minelli et al (1995) suggest a possible

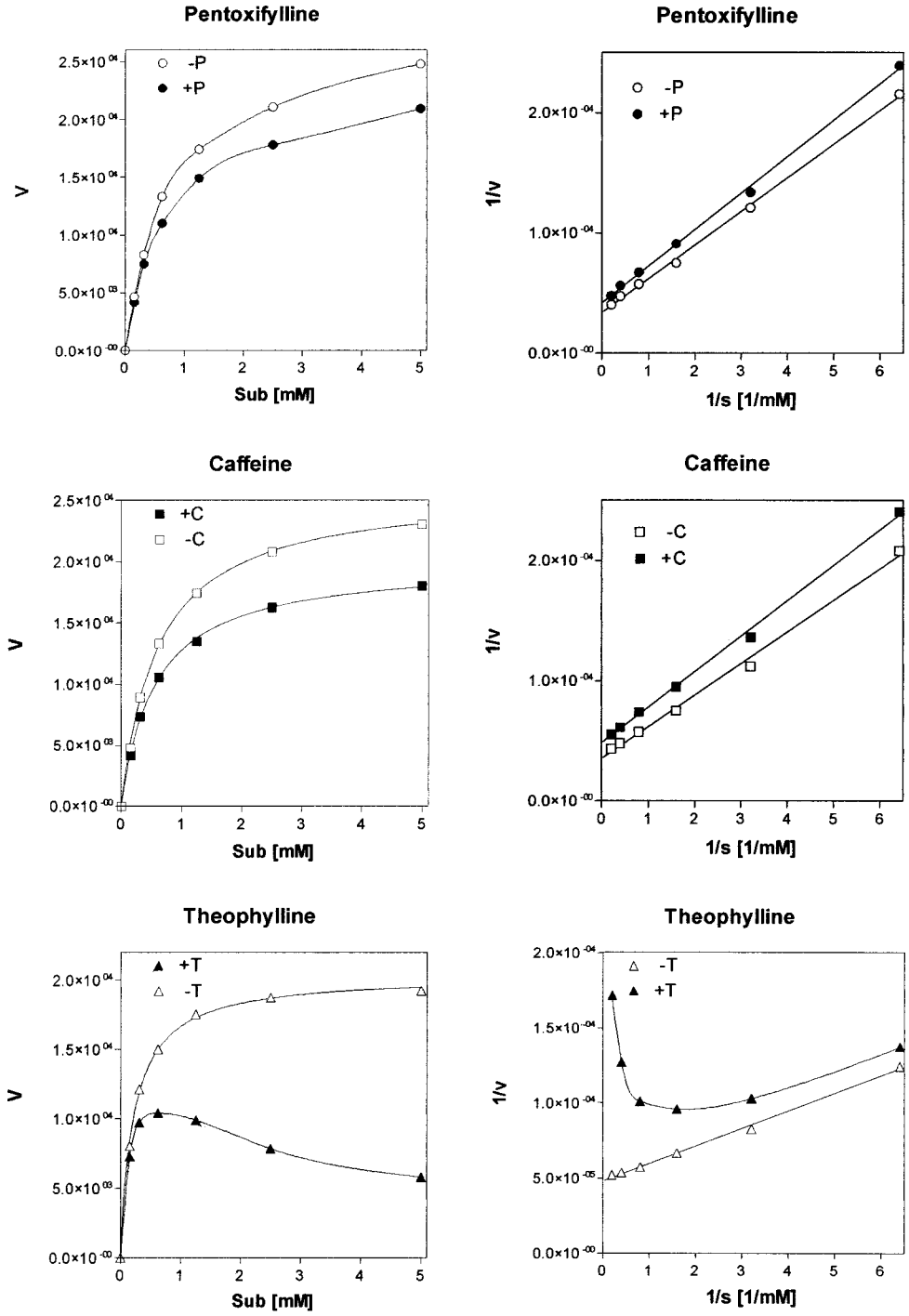

Figure 4. Effects of 4-nitrophenylophosphate concentrations (range 0–5 mM) in the presence of caffeine (10 mM), pentoxifylline (10 mM), and theophylline (0.625 mM) on alkaline phosphatase activity of boar seminal plasma. Double reciprocal plots are presented in right panels; n = 5.

role for alkaline phosphatase in the dephosphorylation of adenosine monophosphate. Tang (1998) suggested that alkaline phosphatase may inhibit the glycosylation of sperm surface glycoproteins through the hydrolysis of nucleotide sugars. The existence of multiple possible modes of alkaline phosphatase enzymatic actions makes it difficult to identify the specific functions of alkaline phosphatase in semen and to relate inhibition by methyl xanthine to specific sperm functions. In other tissues, membrane-bound alkaline phosphatase has been associated with chloride channels (Becq et al, 1993). Such channels are present in spermatozoa and are presumably involved in the acro-

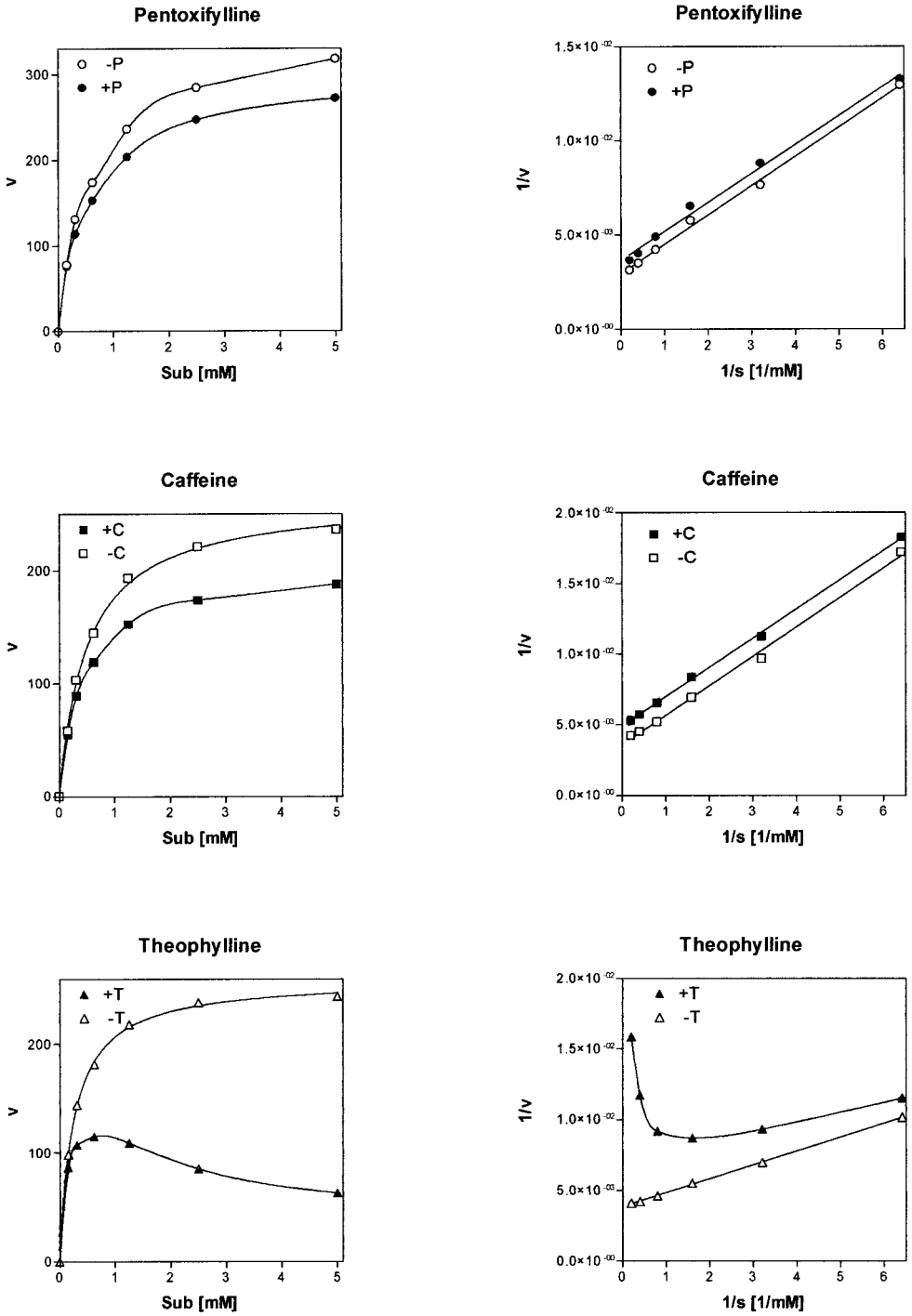

Figure 5. Effects of 4-nitrophenylophosphate concentrations (range 0–5 mM) in the presence of caffeine (10 mM), pentoxifylline (10 mM), and theophylline (0.625 mM) on alkaline phosphatase activity of boar spermatozoa. Double reciprocal plots are presented in right panels; n = 5.

some reaction (Meizel, 1997). The possible link between sperm alkaline phosphatase and chloride channels deserves further study.

Our study revealed considerable differences between theophylline and 2 other methyl xanthines in their ability to inhibit alkaline phosphatase. This finding suggests that if inhibition of alkaline phosphatase is important for normal boar sperm physiology, a difference in mechanism of action on sperm should be seen between theophylline and caffeine or pentoxifylline. Such differences have not been reported for other species. However, to our knowledge, no comparative studies have been performed on the ef-

fects of all 3 methyl xanthines on boar sperm physiology. For this reason, it is difficult at present to relate inhibition of alkaline phosphatase to sperm function in the boar.

Differences in methyl xanthine action may be related to differences in the structures of a particular methyl xanthine. Theophylline lacks a methyl group at carbon 7, and this absence may play a critical role in its having the highest effectiveness among methyl xanthines in inhibiting alkaline phosphatase. On the other hand, it is possible that methyl xanthine in sperm suspensions may induce changes in membrane structure that may stimulate conformational changes of membrane proteins and possibly by modulating their function. Sato et al (1991) indicated that pentoxifylline can change the fluidity of erythrocyte membranes. It is interesting that caffeine and theophylline did not induce such fluidity changes. Because of the critical role of changes in sperm membranes in sperm functions, it needs to be established whether pentoxifylline may act by inducing changes in the fluidity of sperm membranes, and whether these changes may modulate sperm alkaline phosphatase. We used unpurified material for our analysis, and it is possible that stimulation by pentoxifylline might be due to the presence of a different phosphatase with a pH maximum near neutral pH, and that differences between sperm and seminal plasma could thus be explained by differing amounts of this phosphatase. For this reason, further studies using a purified preparation of phosphatases are necessary to define the target phosphatase for pentoxifylline. Such studies would also further our understanding of the mechanisms of alkaline phosphatase inhibition by methyl xanthines, especially theophylline.

Our data demonstrate that methyl xanthines can inhibit alkaline phosphatase activity of seminal plasma and spermatozoa. Methyl xanthine inhibition of alkaline phosphatase activity was specific to pH, substrate, and tissue. These data suggest that, in addition to the classical effect on phosphodiesterase, methyl xanthines may also modulate alkaline phosphatase activity in spermatozoa and seminal plasma.

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