Poor sperm motility is an important factor in male infertility. Preliminary results in our laboratory on a group of 19 men (10 suspected infertile men and 9 fertile donors) showed stimulation of sperm fertilizing ability after sperm washing with theophylline as demonstrated by zona free hamster egg penetration test. The egg penetration rate for the control spermatozoa samples from subfertile men was 16%. Incubation with theophylline (10 mM) increased the penetration rate to 46%, whereas semen incubation with theophylline (20 mM) increased the penetration rate to 51%. A similar twofold increase in egg penetration was observed in the semen of fertile men incubated with theophylline of similar concentrations. Subfertile patients with ejaculate volumes of \( \leq 1 \) ml or total motile sperm count of \( \leq 10 \times 10^6 \)/mL or increased semen viscosity did not exhibit beneficial effects with theophylline washing as measured by hamster egg penetration test score. The increase in percentage of penetrated eggs with theophylline use in both fertile and subfertile men was significant at 10 mM concentration \( (p < .001) \) and 20 mM \( (p < .001) \) when compared to control (untreated) samples. No significant difference in penetration rate was seen between 10 and 20 mM theophylline concentrations. It appears that theophylline may be useful in improving the fertilizing capacity of selected human semen samples with poor motility and poor penetration ability under artificial insemination conditions.

Key Words: Sperm washing, Theophylline, Hamster test, Motility.

INTRODUCTION

The etiology of infertility in most men remains idiopathic [11]. Much effort has been directed to identifying substances that can improve sperm function. Caffeine [10, 15], pentoxifylline [6], platelet activating factor [14], relaxin [9], kinins [16], calcium [3], creatinine phosphate [3], prostaglandins [2], and 2-deoxyadenosine [1] have all been utilized to "wash" sperm in an attempt to enhance sperm performance. Most studies have used sperm motility as the endpoint to ascertain whether there has been benefit from a specific sperm washing technique.

Theophylline is a methyl xanthine derivative that inhibits phosphodiesterase activity, thereby increasing intracellular cyclic AMP. The purpose of this study was to evaluate the
effect of different concentrations of theophylline on the fertilizing capacity of suspected subfertile men and fertile controls as measured by the hamster egg penetration test.

MATERIALS AND METHODS

**Semen Collection and Analyses.** Semen samples were obtained by masturbation from nine proven fertile donors and ten suspected infertile patients, after 48 h of sexual abstinence, into sterile wide-mouth plastic containers. The specimens were allowed to liquefy at 37 °C for 30 min. The suspected subfertile male population was randomly recruited from the Urology Division and was defined as three consecutive (biweekly or monthly) abnormal semen analyses that showed one or more abnormalities in the following three parameters: sperm count < 20 million/mL; motility at 2 h after collection < 40%; and morphology < 60% normal forms.

Semen analyses were performed, using an automated, computerized system, Cell Soft 3000 (Cryo Resources, New York), by analyzing a drop of semen (5 μL) placed on a Makler chamber (Sefi Medical, Haifa, Israel) using phase contrast microscopy (Olympus, BH-2).

**Sperm Washing and Incubation with Theophylline.** After liquefaction, semen specimens were equally aliquoted into three polystyrene (15-mL) conical centrifuge tubes. Each aliquot was mixed in a 1 : 3 ratio with Biggers, Whitten and Whittingham medium (BWW) supplemented with 0.3% (w/v) bovine serum albumin (Fraction V, Sigma) and pH adjusted to 7.4 before use. The tubes were centrifuged at 600g for 5 min in a tabletop centrifuge and the supernatant was discarded. The spermatozoa pellets were then resuspended in BWW medium containing different concentrations of theophylline (Sigma; 0, 10, and 20 mM) and the final spermatozoal concentration was adjusted to 10 × 10⁶/mL. Sperm aliquots without theophylline served as controls. Spermatozoa suspensions were then incubated at 37 °C in horizontal position under the air atmosphere for 5 h.

**Hamster Egg Penetration Test.** The zona-free hamster egg penetration experiments were conducted according to the method of Yanagimachi et al. [17]. The zona-free ova were washed three times in fresh BWW medium before incubation with the treated spermatozoa. At the end of the 5-h incubation period, spermatozoa suspensions were diluted with equal volumes of BWW medium (37 °C) and centrifuged at 300g for 3 min to remove the theophylline. The control tubes received the same treatment. The supernatants were aspirated and the spermatozoa pellets were resuspended in fresh BWW medium to give a final concentration of 10 × 10⁶ spermatozoa/mL. Duplicates of 0.1-mL aliquots of the spermatozoa suspension were placed in sterile petri dishes and covered with mineral oil (Sigma). Twenty-five zona-free ova were added to each droplet and the mixture was incubated in 5% CO₂ in air at 37 °C. After 6 h incubation, the ova were removed and washed in BWW medium twice. The ova were examined with a phase-contrast microscope and were recorded as penetrated when swollen human spermatozoal heads were discernible within the cytoplasm. The penetration rate was defined as the number of ova penetrated/number of ova incubated × 100.

**Statistical Analysis.** The results were statistically analyzed by using the paired Student t test.

RESULTS

A hamster egg penetration score of 25% eggs penetrated is considered to be in the fertile range in our laboratory. The egg penetration rate for the control spermatozoa samples (0 mM) from subfertile men was 16%, whereas the control penetration rate for the fertile donors was 30%. Incubation of the subfertile samples with theophylline (10 mM) increased
FIGURE 1 Effect of sperm washing with theophylline on the fertilizing ability of human sperm. The mean percent penetration rate of spermatozoa from fertile donors and subfertile patients in the hamster egg penetration test showed a significant increase at a concentration of 10 mM theophylline when compared to untreated samples.

<table>
<thead>
<tr>
<th>Group</th>
<th>Theophylline Concentration mM</th>
<th>Mean Percentage of Penetration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n = 10)</td>
<td>0</td>
<td>16*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>46a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>51b</td>
</tr>
<tr>
<td>Donors (n = 9)</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>52b</td>
</tr>
</tbody>
</table>

*Egg penetration rates <25% were considered in the infertile range in this study.

*a p < .001 when compared to control untreated (0 mM) samples.

*b p < .001 when compared to control untreated (0 mM) samples.
DISCUSSION

Our results have shown that theophylline incubation of human semen appears to have a beneficial effect on the fertilizing capacity of the sperm of both fertile and suspected infertile men as measured by the hamster egg penetration test, and no significant differences between 10 and 20 mM theophylline concentrations were seen. Theophylline, caffeine, and theobromine are three closely related alkaloids that occur naturally in plants that have a wide geographic distribution [7]. Structurally they are methylated xanthines and are referred to as xanthine derivatives. Methylxanthines appear to have three basic cellular mechanisms of action [5, 7]: (1) translocation of intracellular calcium; (2) inhibition of phosphodiesterase, which causes an accumulation and higher intracellular concentration of cyclic AMP; and (3) mediation of receptor blockade of adenosine receptors.

The exact mechanisms by which theophylline may enhance sperm function remain unknown, although several other agents that have demonstrated a beneficial effect on sperm function have been thought to increase intracellular cyclic AMP [2, 6, 9, 10, 15]. There is some concern that methylxanthines may have mutagenic potential in animals [4, 12, 13], but at present there is no convincing evidence that this occurs in humans. Theophylline and other methylxanthines offer the potential to augment sperm function. The preliminary experience with theophylline washing of sperm in our laboratory has been encouraging and further investigation with this class of compounds seems warranted.

REFERENCES