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EFFECT OF NEWLY SYNTHESIZED METHYLXANTHINES ON MOTILITY, FERTILIZATION CAPACITY OF MALE GAMETES AND EMBRYO QUALITY

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Abstract

Male factor infertility affects 30–40% of the subfertile couples worldwide and that poses a significant medical and social issue. Different strategies are developed to increase the fertilization potential of male gametes during procedures of assisted reproduction. One of these approaches is the addition of biologically active molecules, which help to stimulate the motility of spermatozoa. In the current investigation, we analysed the effect of two newly synthesized original methylxanthines (N-61 and T-1) on the kinetic parameters of male gametes and compared it to the action of pentoxifylline (methylxanthine approved for utilization in clinical practice). It has been found that N-61 and T-1 increase the motility and the biological viability of spermatozoa, but do not influence their fertilization capacity and the quality of the obtained embryos.

Key words: methylxanthines, spermatozoa, in vitro fertilisation

Introduction. Infertility is an important medico-biological and social issue, affecting 10–15% of the couples in reproductive age. In large part of the cases, the cause is male related infertility. Different medical implications and exogenous factors may influence the process of spermatogenesis and lead to suboptimal sperm parameters, such as low concentration and motility of the gametes. The use of assisted reproductive technologies for the treatment of infertility (intrauterine

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inseminations and in vitro fertilisation) makes possible the application of compounds, which stimulate the spermatozoa motility during sperm processing. This is required most often when in the in vitro fertilisation procedures are involved patients with asthenozoospermia, cryopreserved semen (in the process of cryopreservation part of the gametes are damaged or lose their motility), or testicular spermatozoa, which have been obtained with microsurgical techniques [1].

In clinical practice for stimulation of motility are usually used the methylxanthines pentoxifylline and theophylline [purine alkaloids, which on cellular level inhibit the cyclic nucleotide phosphodiesterase, leading to increase in the levels of cyclic adenosine monophosphate (cAMP)] [2]. Data exists, that pentoxifylline apart from stimulating the motility, may induce preliminary acrosome reaction and have a negative impact on the development of the embryos [3]. Those findings suggest that new compounds for the stimulation of motility should be put to trial.

The aim of the current investigation is to compare the influence of two original newly synthesized methylxanthines on the functional parameters of male gametes, in order to explore the possibility for them to be used in the programmes of assisted reproduction.

Materials and methods. The investigations have been carried out on the premises of the Institute of Biology and Immunology of Reproduction – Bulgarian Academy of Sciences and In Vitro Medical Centre "Dimitrov". Human ejaculates, collected from patients in the centre for assisted reproduction and donor oocytes have been used as experimental material (permission from the local Medical Ethics Committee and informed consent from the patients have been obtained prior to performing the investigations).

The evaluation of the sperm kinetic parameters was carried out in compliance with the WHO criteria [4], with the use of CASA. To assess the fertilization capacity of the gametes conventional in vitro fertilization and ICSI (injection of a single sperm in the oocyte cytoplasm with the help of micromanipulator) techniques [5] have been applied. The obtained embryos have been incubated for 6 days and their quality was evaluated daily [6].

Media produced by Vitrolife (Sweden) have been used for sperm processing, fertilization and embryo culture and for the preparation of the working dilutions of methylxanthines.

Three compounds from the methylxanthines class of drugs have been used in the experiments – pentoxifylline and two original, newly synthesized (in the department of Inorganic Chemistry of the Faculty of Pharmacy, Medical University, Sofia) under code names N-61 and T-1 in 3.6 mM concentration (Fig. 1).

Two series of experiments have been performed. In the first one, the effect of the methylxanthines on spermatozoa in native and processed semen has been investigated. After the collection and evaluation of the ejaculates, they were diluted with culture medium, containing equimolar concentrations (3.6 mM) of

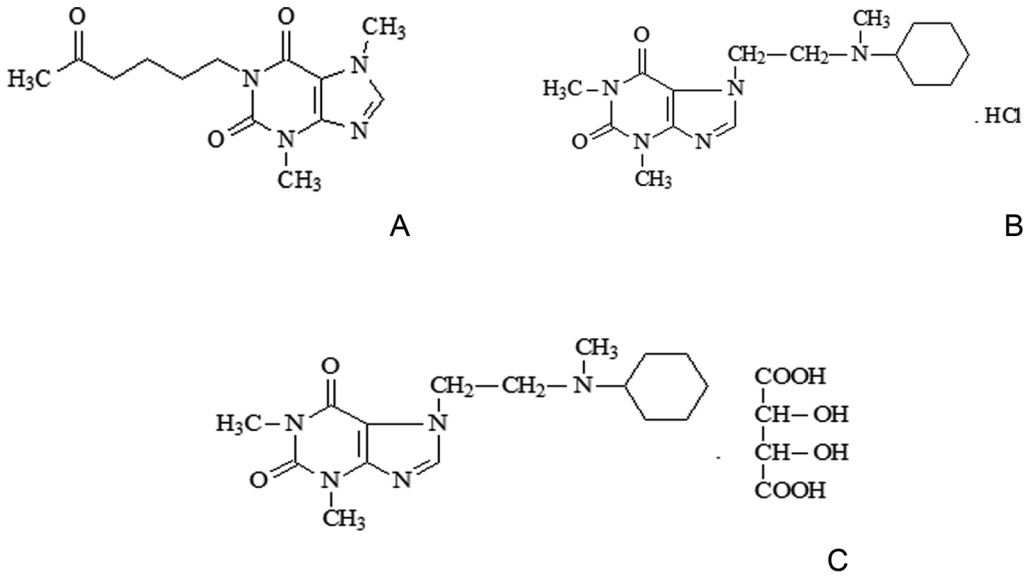


Fig. 1. Structural chemical formulae of the investigated methylxanthines. A: pentoxifylline (1-(5-oxohexyl) theobromine); B: T-1 (7-[(N-methyl-N-cyclohexyl)aminoethyl]-1,3-dimethylxanthine hydrochloride); C: N-61 (7-[(N-methyl-N-cyclohexyl)aminoethyl]-1,3-dimethylxanthine tartrate)

the investigated compounds. Then, they were incubated at 37 °C in 6% CO_2 and high humidity. The motility of the gametes was assessed every 120 min. In part of the experiments the ejaculates were processed with gradient centrifugation prior to the addition of the methylxanthines to separate a fraction of normokinetic spermatozoa [4]. Diluted in culture media semen was used as a control. In the second set of experiments, the fertilization capacity of the gametes treated with N-61 and T-1 and the quality of the in vitro obtained embryos was assessed.

Results. For the purpose of the current investigation 32 ejaculates were collected from males aged 23 to 42 years. The average semen volume was 2.9 ml (1.5–4.6 ml). The concentration of spermatozoa ranged from 21 to 105 mln/ml. After initial evaluation, the ejaculates were classified as: 14 with normozoospermia; 12 with asthenozoospermia; in six ejaculates increased number of leukocytes (leukocytospermia) was observed and therefore they have been excluded from the investigation.

Spermatozoa motility. The effect of the investigated methylxanthines on the motility of the gametes from native (normo- and asthenozoospermia) and processed semen is presented in Fig. 2. Statistically significant difference with the control was found after 2 h of incubation of the samples with asthenozoospermia and after 6 h in normozoospermic semen. Single viable gametes have been detected up to about 24 h of incubation in ejaculates with normozoospermia and up to 18 h in those with asthenozoospermia. In processed semen, after 2 h the results pointed out slight increase of the motility in both the experimental and

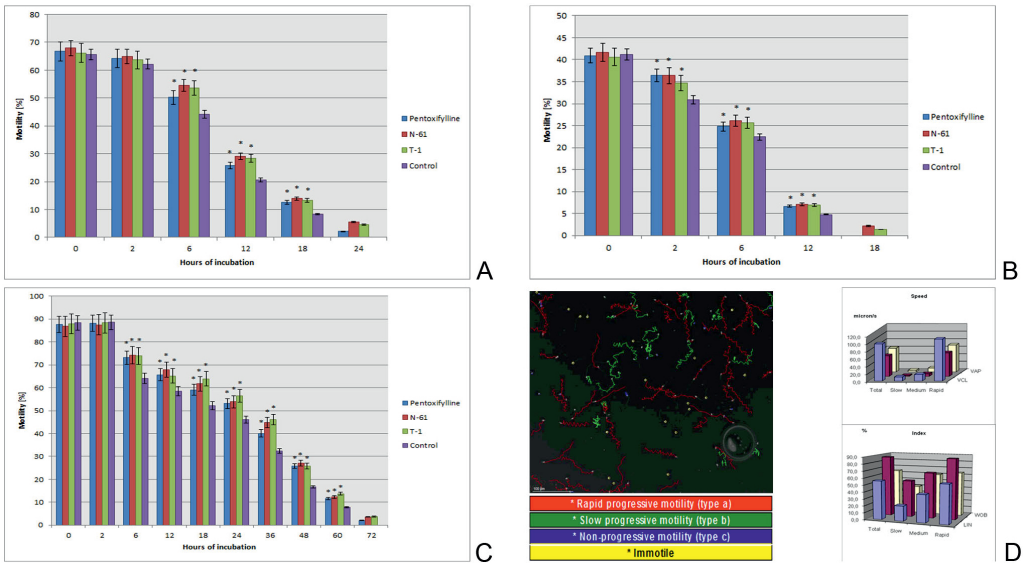


Fig. 2. A: Mean percentage spermatozoa motility from native normozoospermic semen samples incubated with pentoxiphylline, N-61 and T-1; B: Mean percentage spermatozoa motility from native semen samples with asthenozoospermia incubated with pentoxiphylline, N-61 and T-1; C: Mean percentage spermatozoa motility from processed normozoospermic semen samples incubated with pentoxiphylline, N-61 and T-1; D: Representative figures from CASA analysis of the samples. $^{*}p \leq 0.05$

the control groups. Statistically significant difference was detected after 6 h of incubation. After 24 h, it was observed that N-61 and T-1 retain spermatozoa motility better than pentoxifylline. In some cases, there were changes in the parameters of the movement of the gametes (larger amplitude of the deviation of the tail from the head). Viable reproductive cells from processed semen samples with normozoospermia were noticed for about 72 h of incubation.

In vitro fertilization. During the investigation, we fertilized 27 donor oocytes (obtained from two women), which were randomly divided into four groups: the first one included six oocytes, subjected to conventional IVF with N-61 treated spermatozoa; the second eight oocytes fertilized with ICSI, again with spermatozoa incubated with N-61; the third and the fourth group of oocytes underwent IVF ($n = 7$) and ICSI ($n = 6$), respectively, with T-1 treated spermatozoa. The obtained results showed that in group one four out of six oocytes were successfully fertilized (66.7%). One of them stopped its development on fourth blastomeres stage, three formed expanded blastocysts on day 5 (75%). Spontaneous hatching was noticed in two of them. In the second group, eight out of eight oocytes were fertilized, six reached to the stage of expanded blastocyst on day 5, again two spontaneously hatched. In the groups including spermatozoa treated with T-1, with conventional IVF six of the seven oocytes were fertilized (85.7%), four of which formed expanded blastocysts (66.7%), three hatched spon-

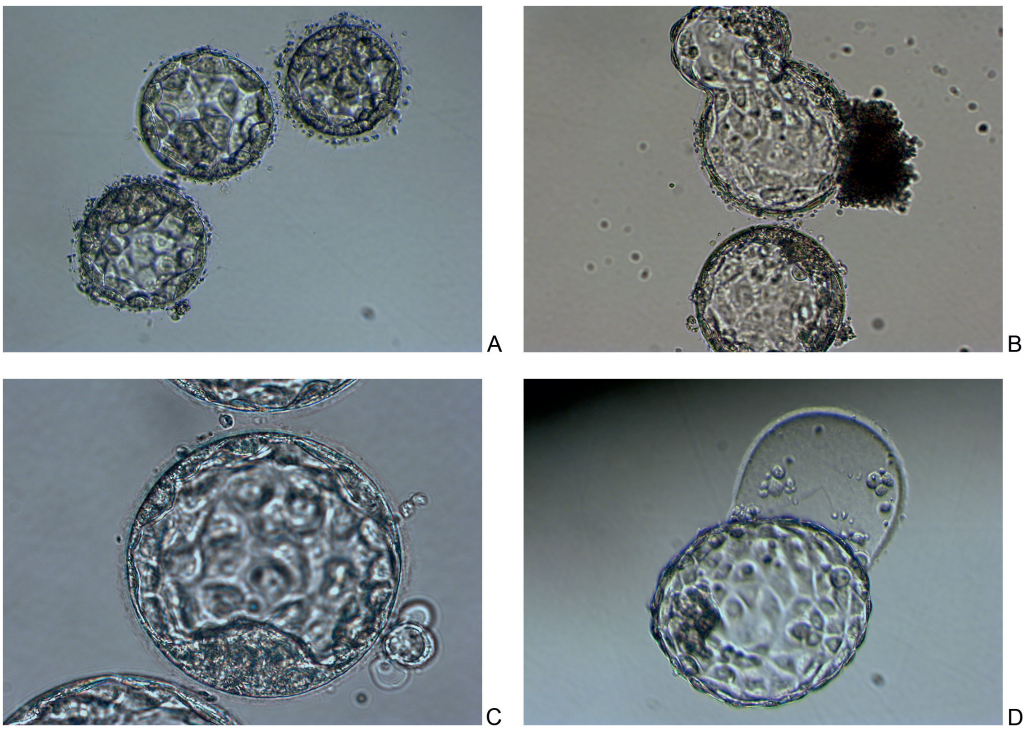


Fig. 3. Embryos obtained after fertilization with N-61 incubated spermatozoa: A: blastocysts after conventional IVF (×100); B: hatching blastocyst after IV (×100); C: blastocyst after ICSI (×200); D: hatched blastocyst after ICSI (×150)

taneously. In the last group, five out of six oocytes were successfully fertilized (83.3%), three reached the expanded blastocyst stage (60%) and two hatched spontaneously (Fig. 3, 4).

Discussion. Infertility is not only a significant social and economic, but also clinical implication with a substantial psychological impact on the patients. As considerable part of the cases has been established to be due to male factors, the optimization of the protocols for sperm motility stimulation during assisted reproduction procedures remains an important aspect that needs to be addressed. Up until now, in clinical practice well established is the use of pentoxifylline as media additive to increase the male gametes kinetic parameters prior to it vitro fertilisation.

Pentoxyphiline is dimethylxanthine derivative, with a similar structure as theophylline, caffeine and theobromine. It possesses various intracellular effects, including the downregulation of TNFa and some adhesion molecules (ICAM1 and VCAM1) and the decrease in the phosphorylation of p38 MAPK and ERK [7]. In spermatozoa, it acts as a competitive non-selective inhibitor of some enzymes from the superfamily of phosphodiesterases [8], leading to elevated levels of cyclic

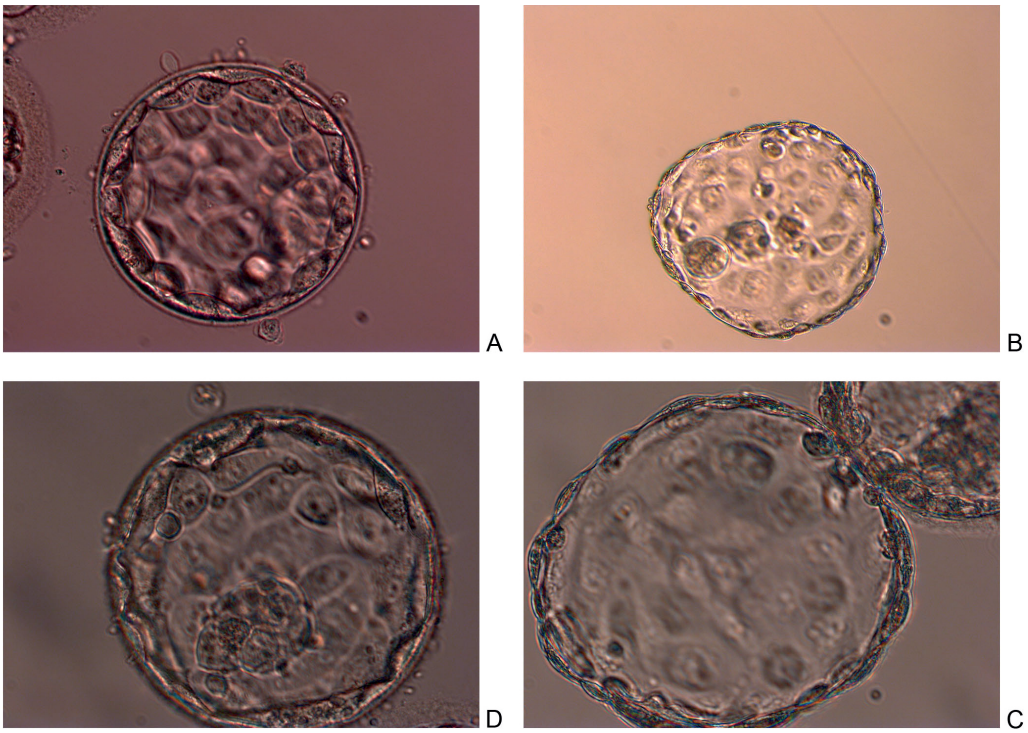


Fig. 4. Embryos obtained after fertilization with T-1 incubated spermatozoa. A: blastocysts after conventional IVF (×200); B: hatched blastocyst after IVF (×100); C: blastocyst after ICSI (×200); D: hatching blastocyst after ICSI (×200)

adenosine monophosphate (cAMP). The second messenger, in turn, activates various downstream enzyme cascades involved in capacitation, increased motility, initiation of the acrosome reaction. The enhanced motility of the male gametes is a result of the activation of protein kinase A, which leads to dephosphorylation of the dynein arms in the spermatozoon's axoneme. On the other hand, a number of studies have shown that pentoxifylline induces adverse effects on certain cell functions as DNA repair [9], may cause premature acrosome reaction, exert oocyte toxicity and impair embryo development [10]. These observations leave open the question for the identification of alternative drugs with the potential to stimulate spermatozoa motility.

In our study, we demonstrated that the originally synthesised xanthine derivatives N-61 and T-1 increased sperm motility and their effect exceeded that of pentoxifylline. The induced effect of pentoxifylline is dose-dependent. In clinical practice and literature it has been shown that the most effective concentration is 3.6 mM, therefore we chose to use it in our experiments.

Regarding the influence of the investigated substances on the oocyte function and embryo quality, our results show that from fertilisation up to blastocyst stage,

there is no significant difference from the clinical data in the medical centre in the last years. All of the obtained embryos demonstrated good pronuclear and embryo score [6] Pentoxifylline was not assessed during that part of investigation, because there is plenty of information about its influence on the fertilisation ability and subsequent embryo development [11].

Conclusions. The newly synthesised original methylxanthines N-61 and T-1 increase the motility and viability of male gametes and their stimulating effect was higher than that of pentoxifylline. There is no influence of the use of spermatozoa pretreated with N-61 and T-1 for in vitro fertilisation on the quality of the obtained embryos.

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