ORIGINAL ARTICLE

DO AROMATASE INHIBITORS REDUCE FERTILITY AND IMPAIR SEXUAL BEHAVIOUR IN AN ANDROGEN DOPING MODEL IN RATS?

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Abstract

The use of androgens and anabolic steroids as doping substances decreases fertility and influences social behaviour (addiction, aggression, changes in libido). Effects on fertility can be reduced with corrective medication ("post cycle therapy") that interferes with the hypothalamic-pituitary regulation of androgen synthesis (aromatase inhibitors, antiestrogens, gonadotropins). The purpose of this study was to evaluate fertility and sexual behavior in an androgen doping model in male rats under conditions of progressive effort (swimming) over a period of 8 weeks. Rats were divided into 4 groups (Group I control – C; Group II – testosterone undecanoate 1 g/ kg b.w. every 2 weeks, 3 doses i.m. - T; Group III – letrozole orally 2.5 mg/ kg b.w. daily - L; Group IV – combined therapy, letrozole and testosterone in the doses described above - T+L). Fertility was assessed by spermogram (number, viability, and spermatozoids morphology) and the capacity to fertilize the receptive female rat. Social behaviour was evaluated in the presence of female rats (interest, copulation), and in the presence of foreign males (aggression, dominance). Experimental findings: monotherapy (testosterone or letrozole) reduces fertility and produces oligo- or asthenospermia; aromatase inhibitors decrease aggression, but also diminish the sexual desire of the treated males; combined therapy effect on spermatogenesis is reduced compared to separate use, but with decreased libido produced by letrozole, and antagonizing aggressive behaviour caused by androgen doping.

Rezumat

Utilizarea androgenilor şi steroizilor anabolizanţi ca substanţe dopante diminuează fertilitatea şi afectează comportamentul social (adicţie, agresivitate, modificări ale *libido*-ului). Efectele asupra fertilităţii pot fi atenuate cu medicaţie corectivă ("*post cycle therapy*") care interferă cu reglarea sintezei androgenilor prin acţiune la nivel hipotalamo-hipofizar (inhibitori de aromatază, antiestrogeni, gonadotropine). Lucrarea de faţă are drept scop evaluarea fertilităţii şi a comportamentului sexual într-un model de dopaj androgenic la şobolanul mascul, în condiţii de efort progresiv (înot), cu o durată de 8 săptămâni. 4 loturi de şobolani (Lotul I (C) – control; Lotul II (T) – undecanoat de testosteron 1 g/kg corp la 2 săptămâni, 3 doze i.m.; Lotul III (L) – letrozol oral, 2,5 mg/kg corp, zilnic; Lotul IV (L+T) – terapie combinată, letrozol şi testosteron, în dozele descrise anterior). Fertilitatea a fost evaluată prin spermogramă (numărul, viabiliatatea şi morfologia spermatozoizilor) şi prin capacitatea de fecundare a femelelor receptive. Comportamentul social a fost evaluat faţă de femele (prospecţie, copulaţie) şi faţă de masculi străini (agresivitate, dominanţă). Concluziile experimentului: monoterapia (testosteron, respectiv letrozol) are efecte de diminuare a fertilităţii – oligo- şi asteno-spermie; inhibitorii de aromatază scad agresivitatea, dar diminuează totodată şi dorinţa sexuală a masculilor trataţi; terapia combinată are efecte atenuate asupra spermatogenezei faţă de utilizarea separată, dar cu păstrarea diminuării *libido*-ului produsă de letrozol şi antagonizarea comportamentului agresiv facilitat de dopajul androgenic.

Keywords: androgen-anabolic steroids, aromatase inhibitors, fertility, sexual behaviour

Introduction

Doping with androgens and androgen-anabolic steroids (AAS) is common among young men, not only in order to increase athletic performance, but also for aesthetic purposes ("*body image enhancing drugs"*) - caused by the media model of masculine beauty - a man with muscular hypertrophy and minimized adiposity.

AAS are administered in doping cycles, initial escalation dose and its subsequent decrease ("pyramiding cycle"), or androgen combination with different half-life based on additive properties ("stacking cycle") [6].

Doses used for doping are much higher than usual doses, serum androgens often exceeding 10 - 50 times the physiological testosterone levels [22, 32].

The direct consequence is the frequent occurrence of side effects, pharmacotoxicological tropism manifesting in the skin (acne, sometimes severe - cystic forms, conglobata acne, truncal location), cardiovascular system (cardiac hypertrophy favouring atherogenic process, salt and water retention), liver (cholestatic jaundice in case of AAS 17-alkylated, peliosis), blood (erythrocytosis, increased haematocrit) [19, 24, 31]. The most common side effect is reproductive toxicity - decreased spermatogenesis and testicular hypotrophy (by inhibiting the hypothalamic pituitary testicular axis), prostatic hypertrophy, gynecomastia (by oestrogen excess derived from AAS aromatization) [19, 24, 32]. Other side effects include priapism and aggravation of sexual dysfunction (premature ejaculation especially in young men - a condition for which only dapoxetine has been granted marketing authorization) [17].

To prevent or correct the reproductive toxic effects, androgens are often associated with aromatase inhibitors. They are used "*off label*" in male infertility caused by follicle - stimulating hormone (FSH) and luteinizing hormone (LH) deficiency in men with intact hypothalamic pituitary testicular axis (with severe obesity or after androgen abuse) [4, 14, 28], because regulating the endogenous secretion of testosterone by the feed-back loop is achieved, at least in part, through oestrogen. In doping cycles, AAS association with aromatase inhibitors (letrozole, anastrozole, exemestane) can also prevent/correct gynecomastia induced by oestrogen excess caused by peripheral conversion of exogenous AAS (as doping agents), by aromatase (and for keeping a balanced testosterone/oestradiol ratio specific to males, with the additional role of reducing the inhibition of hypothalamic pituitary testicular axis) [29, 32].

Information available on the Internet "offers" doping schedules, with doses much higher than those used for therapeutic purposes, but which are based on pharmacological fundamentals [35].

The present experiment aimed to develop a doping model with aromatase inhibitors or androgens associated with aromatase inhibitors in male rats, in terms of physical exercise with gradually increasing intensity. For ethical reasons, such studies cannot be performed on humans. The parameters evaluated in this study were fertility (assessed by analysing the spermatozoids and fertilization capacity), as well as highlighting some behavioural changes (presence/absence of male aggression toward foreign males, prospective/copulative conduct towards sexually receptive females).

Materials and Methods

Experimental animals. The experiment used four groups of 10 male Wistar rats housed in standard conditions (day/night cycles of 12 hours, free access to food and water). An additional group of untreated females was used to assess male fertility and sexual behaviour (to which sexual receptivity was evaluated by vaginal smears and lordosis - natural posture for facilitating copulation) [5, 19].

Substances and doses used, administration pathway and time are presented in Table I.

Table I

Experimental groups, treatments and procedures

Nr. crt.	Group	Treatment/dose	No. of animals	Gender	Procedure
1	**Group I** control	Saline solution	10	Male	Swimming
2	**Group II** testosterone	Testosterone undecanoate (Nebido®) 1 g/kg b.w. i.m., every two weeks, 3 doses	10	Male	Swimming
3	**Group III** letrozole	Letrozole 0,5 mg/kg b.w. p.o., every day	10	Male	Swimming
4	**Group IV** testosterone + letrozole	Testosterone undecanoate (Nebido®) 1 g/kg b.w. i.m., every two weeks, 3 doses Letrozole 0.5 mg/kg b.w. p.o., every day	10	Male	Swimming
5	**Total**	-	40	Female	Mating

Dose selection. Relevance of the doses used in animal experiments to human predictions can be attained by using the relative differences expressed in mg/kg or relative body surface [18, 23]. Treatment with letrozole 0.5 mg/kg b.w. in rats reflects a human equivalent dose twice the standard dose (2.5 mg/day) (Nair A.B., Jacob S.; Reagan-Shaw S. *et al.*) [18]. However, in doping athletes the AAS doses used are an order of magnitude higher than normal and in the case of delayed release formulations (such as testosterone undecanoate) the frequency of administration is 1 g i.m. every 2 - 3 weeks to get supra-physiological stationary plasma concentrations, much higher than the therapeutic range (in hypogonadism replacement therapy standard interval between doses being 3 months) [26, 34]. These considerations justify the dose of 1 g/kg b.w. i.m. used in rats, at a 2-week-interval, to achieve high *steady state* concentrations, stable at the end of the experiment, similar to the doses in a doping cycle in athletes (a human equivalent dose one order of magnitude higher than the standard dose in an adult of 60 - 70 kg, administered at a time interval to maintain stationary concentrations specific to androgen abuse).

Experimental Protocol. The animals were subjected to physical exercise with intensity gradually increasing, for 5 days/week (10 minutes on the first day, 15 minutes on the second day, until the limit of 30 minutes as an equivalent of a daily basis "training") for 8 weeks. This model is also validated for exercise-induced cardiac hypertrophy [13], in this case it is superposed with androgen doping (in order to present exercise/doping addictive behavioural patterns of athletes).

Sperm sampling and processing. At the end of the experiment, animals were euthanized by cervical dislocation, and left cauda epididymis was sampled. After epididymis incision, a dense tissue sample was taken and transferred to a 37ºC thermostat microscope slide in the presence of 1 - 2 drops of 2.9% sodium citrate. After mixing and liquefying, 10 μL of sperm were used for determinations by making dilutions if necessary. Spermatozoids count and motility assessment was performed using a Beerker camera. Viability was measured using eosin nigrosine staining (unviable spermatozoids are coloured in pink-red because the membrane pore allows dye penetration) [9], whereas morphology was determined by *Rose bengal* staining [8, 27] identifying the anomalies (head, tail, intermediate piece) in 200 sperm cells through a binocular microscope examination (Micros Petunia MCX50) with camera, using image analysis software Micro-visible and a 40x lens.

Social behaviour in the presence of a competitor (foreign male). Five rats from each experimental group (including the control group) were individually introduced in a plexiglass cage with a foreign male. Their behaviour (prospecting, social interaction) was filmed for 2 hours with a video camera equipped with motion sensor; the number of aggressive behaviour episodes was counted (domination, fight). The degree of aggressiveness was assessed using the following score: 0 – calm; 1 – disturbance, teasing; 2 – fight.

Evaluation of mating behaviour (in the presence of sexually receptive females). Fertility assessment. For studies of male reproductive behavioural pharmacology the method described by Yamamoto Y *et al.* was used, modified as follows: five male rats from each group were individually introduced in a plexiglass cage for 10 minutes-habituation [33]. Subsequently two young nulliparous but sexually receptive females were placed in the same cage and the prospective and copulative behaviour of males was evaluated; animals were motion sensor video monitored (infrared at night). The latency to the first pairing, the number of mattings in 2 hours, with or without ejaculation (highlighting characteristic female lordosis and *pelvic thrusting during intercourse* in males) were evaluated. The experiment took place in the latter part of the night (last third of the dark cycle) [33]. For the next 24 hours, the animals were kept together, subsequently females were separated, and pregnancy or parturition was assessed after 21 days. The number of litters born alive was quantified and fertile/ infertile status for each male was determined based on the occurrence of pregnancy in at least one female after mating [9, 33].

Statistical analysis. Depending on the results obtained from the normality test (Kolmogorov-Smirnov test), statistical analysis was performed by choosing a parametric test (ANOVA one way) or its nonparametric equivalent (Kruskal-Wallis). Thus, for the total spermatozoids count, viability, and morphologically abnormal forms the one-way ANOVA test has been applied. The same test was used to analyse the latency time until the first mating. Tukey-Kramer test was used as a post-hoc test for multiple comparisons between groups. The number of episodes and the aggression score were evaluated with the Kruskal-Wallis test. For multiple comparisons, the Dunn's test was used as a post-hoc test in this case. Threshold of statistical significance was set at $p < 0.05$.

Ethical considerations. All experimental procedures were performed according to Directive 2010/63/EU of the European Parliament and of the Council of September the 22nd, 2010 on the protection of animals used for scientific purposes. The experimental protocol was approved by the Research Ethics Committee of University of Medicine and Pharmacy Târgu Mureş, Romania (no. 62/ 20.05.2016).

Results and Discussion

Sperm analysis. The number, the percentage of viable sperm cells, as well as the percentage of abnormal morphological forms are presented in Table II and in Figures 1 - 3.

Table II

Sperm characteristics

Nr. crt.	Group	Sperm count[a] ($\cdot 10^6$/mL)	Viabilitity[b] (%)	Abnormal forms[c] (%)
1	**Group I** control **(C)**	79.4 ± 8.95	71.4 ± 8.16	17.2 ± 6.37
2	**Group II** testosterone **(T)**	54.9 ± 13.84[a]	57.2 ± 19.43	22.9 ± 8.71
3	**Group III** letrozole **(L)**	51.3 ± 11.42[a]	52.1 ± 16.38[b]	27.4 ± 8.54[c]
4	**Group IV** testosterone + letrozole **(L + T)**	55.1 ± 12.60[a]	60.1 ± 16.95	27.5 ± 8.26[c]

[a]**p < 0.001** (C *vs.* T; C *vs.* L; C *vs.* L+T); [b]**p < 0.05** (C *vs.* L); [c]**p < 0.05** (C *vs.* L; C *vs.* T+L);

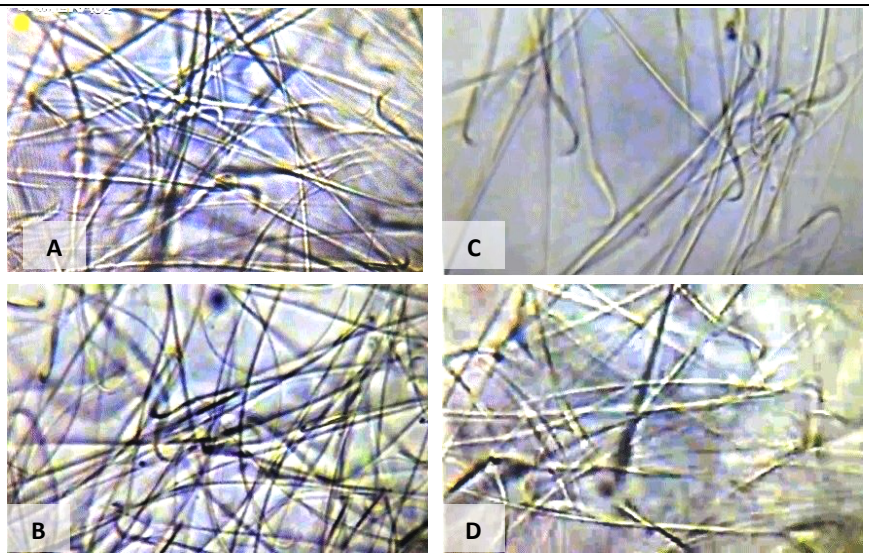

Figure 1.
Screenshots - semen from the control groups (A), and groups treated with testosterone (B), letrozole (C), letrozole + testosterone (D)

Figure 2.
Unviable sperm - eosin/nigrosin staining

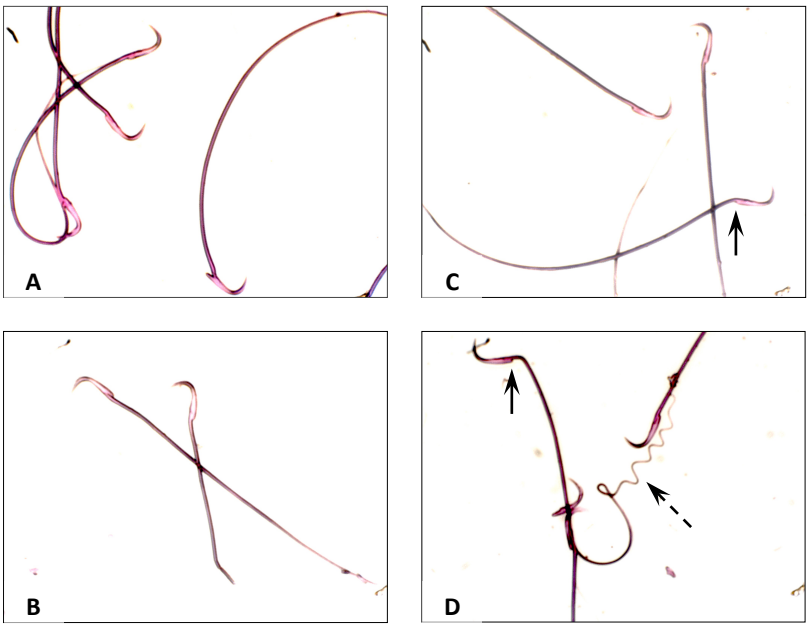

Figure 3.
Sperm morphology - rose Bengal staining - control groups (A), and groups treated with testosterone (B), letrozole (C), letrozole + testosterone (D)
(continuous arrow - abnormal implantation of the head; dashed arrow - tail abnormalities)

In all treated groups regardless of the type of treatment, both modification of motility (asthenospermia), number (oligospermia), and viability (necrospermia) of sperm was observed, as well as the emergence, in varying percentage of morphologically abnormal forms (especially abnormal implantation of the head or modified forms of the head or tail - coiled or shortened tails). All these changes are significant compared to the control group, the most important aspect of reproductive toxicology being the significant reduction in spermatozoids count. The combined treatment (testosterone associated with letrozole) has slightly reduced effects on spermatogenesis, oligospermia is less marked compared to treatment with aromatase inhibitors or androgen, but the values do not differ significantly from the monotherapy group because of the high variability.

Evaluation of social behaviour in the presence of a competitor (foreign male). The average number/ group of aggression episodes against an intruder (foreign male) is presented in Table III. Increased aggressiveness is evident in the groups treated with androgen (assessed by the aggression score; the number of aggressive episodes did not reach a threshold of statistical significance due to the high variability in outcomes); aromatase inhibitors alone produce a decrease in domination behaviour compared to the control group, but without statistical significance; androgenic doping associated with letrozole decreases androgen aggressiveness/ dominant behaviour.

Table III

Aggression evaluation in the presence of a same sex competitor

Nr. crt.	Group	Number of episodes of aggression/fighting for 2 hours*	Aggressive score**
1	**Group I** control **(C)**	1.6 ± 0.89	1.4 ± 0.54
2	**Group II** testosterone **(T)**	4.4 ± 1.67[a,b]	1.8 ± 0.44[c,d]
3	**Group III** letrozole **(L)**	0.8 ± 0.8	0.6 ± 0.54
4	**Group IV** testosterone + letrozole **(T+L)**	0.6 ± 0.54	0.8 ± 0.73

* [a]**p < 0.05** (T *vs.* L); [b]**p < 0.05** (T *vs.* T+L) ; ** [c]**p < 0.01** (T *vs.* L); [d]**p < 0.05** (T *vs.* T+L)

Evaluation of sexual behaviour and fertility. After introducing the sexually receptive females, they were quickly copulated by the males in the control group and, respectively, by the males in the androgenic doping group (less than a minute before the first mating). The results show a marked decrease in sexual behaviour (both prospective and copulative) in the group treated with letrozole alone or in combination with testosterone (in which case the initiative of belonging rather to females through scouting, smelling, and copulation facilitating position - lordosis) - see Table IV. The fertility rate does not differ between the treated groups. The only difference is the total number of offspring. All this shows the difference between the fertility rates (males' ability to fecundate the females - only 20% of the treated groups, compared to the intact fertility of the control group).

Table IV

Evaluation of sexual behaviour and fertility towards an opposite sex partner

Nr. crt.	Group	Latency time until first mount (s)*	No. of mounts/2 h**	Fertility rate (%)
1	**Group I** control	32.6 ± 13.64	5.0 ± 1.58	100
2	**Group II** testosterone	38.6 ± 15.14	4.8 ± 1.30	20
3	**Group III** letrozole	294.2 ± 165.12	1.2 ± 1.09	20
4	**Group IV** testosterone + letrozole	245 ± 121.88	1.4 ±1.14	20

* **p < 0.01** (C *vs.* L; T *vs.* L); **p < 0.05** (C *vs.* T+L; T *vs.* T+L); ** **p < 0.05** (C *vs.* L)

The effect of high doses of AAS on reproductive function is well known, caused by a decrease in LH and FSH secretion by inhibiting the hypothalamic - pituitary - testicular axis [19, 22, 24]. The consequence is severe oligospermia, reduced fertility and, in the long term, testicular atrophy. In order to avoid this, doping cycles associate chorionic gonadotropin (HCG) or anti-oestrogen doping substances (either directly at pituitary level - clomiphene, or indirectly, by inhibiting oestrogen synthesis - aromatase inhibitors).

There are many such successful therapeutic cases in treating male infertility caused by hypogonadotropic hypogonadism or ASIH (*anabolic steroid-induced hypogonadism*) [4]. However, there are many negative examples - HCG association with anabolic steroids is directly correlated with the number of abnormal spermatozoids forms in athletes (Karila T. *et al*.) [101]. Finkelstein J.S. *et al.* demonstrated the involvement of oestrogens (comparing replacement therapy with androgens in the presence/absence of an aromatase inhibitor in male hypogonadism) in the regulation of sexual function, bone metabolism, and in the relative proportion of body fat [7]. Preclinical studies in animal models (rats) show harmful effects on testicular morphology and fertility of aromatase inhibitors [12] and substances with anti-estrogenic effects - tamoxifen [25]. On the other hand, *in vitro* studies incriminate the oestrogen

excess derived by androgens aromatization into oestrogen to determine Leydig tumour cell proliferation [1]. Clinical data promote an optimal testosterone/ oestradiol ratio for health status in adult men, not only from reproductive perspective; both excess and deficiency of oestrogen are incriminated in the occurrence of pathological changes - the optimum level of oestradiol is correlated with the protective HDL level and bone anabolism, the excess is involved in metabolic syndrome and infertility while oestrogen deficiency is incriminated in osteoporosis and decreased libido [7, 29, 32].

Regarding social behaviour, androgen doping in sports is linked to increased aggression [20-22]. During sports competitions this may be an advantage, but sometimes it may progress to severe forms ("roid rage"). This effect has also been described in experimental animal models [16, 22].

Decreased oestrogen through preventing aromatisation of androgens has been described as being the disadvantage of reproductive function, independent of favourable effects on fertility via LH release. In hypogonadal men undergoing androgen replacement therapy, serum oestradiol is directly correlated with libido (at the same serum testosterone concentration, serum levels of oestradiol less than 10 pg/mL decrease sexual desire by almost a third (Finkelstein *et al*.) [7]. Trainor *et al.* show that the paternal behaviour in monogamous mammals is directly related to oestrogen derived from aromatisation of androgens [30], while data presented by Cushing *et al*. suggest that reducing the expression of oestrogen receptors ERα in *bed nucleus* of the *stria terminalis* and *medial amygdala* favours developing a prosocial behaviour (monogamous social organization) [2, 3]. These data can be correlated with our results, the mating male sexual behaviour is clearly decreased by oestrogen suppression and aggression is facilitated by the high, supra-physiological testosterone level.

Conclusions

In an animal model of doping, androgens present reproductive toxicity (oligospermia, infertility, they maintain libido and mating behaviour, but with increased aggressiveness against same-sex competitors). Aromatase inhibitors reduce fertility in eugonadism (despite facilitating the release of LH), decrease aggression, and strongly modify mating behaviour by decreasing sexual desire. A combination of aromatase inhibitors and androgens decreases the androgen potential to produce oligospermia, but increases the number of abnormal germ cell forms, while reducing aggressive behaviour and libido.

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