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The adverse effects of exercise and supraphysiological dose of testosterone-enanthate (TE) on exploratory activity in elevated plus maze (EPM) test – indications for using total exploratory activity (TEA) as a new parameter for exploratory activity estimation in EPM

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Abstract

OBJECTIVES: The aim of this study was the estimation of effects induced by chronic administration of supraphysiological dose of TE and prolonged exercise in male rats on anxiety levels (alterations in exploratory activity patterns in EPM).
MATERIAL AND METHODS: Two sedentary (control – C and testosterone-enanthate – T) and two exercise (exercise – E and testosterone-enanthate plus exercise – T+E) groups (n=32) underwent adequate protocols – the swimming protocol (1 h/day) and TE (20 mg/kg/w, s.c.) for six weeks. Testing was performed in EPM.
RESULTS: Anxiolytic effects of exercise were manifested as increased exploratory activity in EPM – increase in cumulative duration in open arms, the number of rearings and head-dippings, and TEA. Supraphysiological dose of TE decreased the number of rearings and head-dippings, cumulative duration in open arms and TEA compared to the control group, while this effect of TE was more pronounced compared to the exercise group. The applied dose of TE attenuated beneficial effects of exercise by means of all estimated parameters.
CONSLUSIONS: Our results confirmed the beneficial effect of exercise on anxiety levels observed in EPM by means of parameters considering the alterations in exploratory activity. Supraphysiological dose of TE resulted in anxiogenic-like behavior in EPM. The effect of TE was so pronounced that the beneficial effect of exercise was reversed to the control values (or even below them). Based on the results of this trial, we propose that use of the TEA (a new parameter for overall exploratory activity) can improve the evaluation of EPM test results.

ORIGINAL ARTICLE

Abbreviations
TE – testosterone-enanthate
EPM – elevated plus maze
TEA – total exploratory activity
AAS – anabolic androgenic steroid

INTRODUCTION

Anabolic androgenic steroids (AASs), synthetic compounds derived from testosterone, have been widely used for therapeutic purposes, such as androgen replacement therapy (van Amsterdam *et al.* 2010), chemotherapy (Langer *et al.* 2001), in AIDS-associated malnutrition (Basaria *et al.* 2001) and in inflammatory pulmonary diseases (Ferreira *et al.* 2001). Parallel with medical indications, the prevalence in non-medical AASs abuse has been observed in adolescents and adults, typically in athletes and individuals seeking physical strength and enhanced appearance (Ambar & Chiavegatto 2009). Beside various adverse effects (including disorders of cardiovascular and reproductive systems, hepatic and renal failure, etc.), AASs abuse is associated with a wide range of behavioral alterations (in animal models) and psychiatric disorders (in humans). Thus, prolonged use of supraphysiological doses of AASs represents a potential danger for: increased anxiety, aggression, euphoria, extreme irritability, paranoid jealousy, extreme mood swings and violent murders (van Amsterdam *et al.* 2010, Oberlander & Henderson 2012, Wood *et al.* 2013), etc. All mentioned disorders induced by AASs abuse give the image of "roid rage" – a sudden and exaggerated aggressive response to a minimal provocation (Wood *et al.* 2013). The wide range of altered behaviors has also been observed in animals subjected to AASs abuse, the neurobiochemical mechanisms that underlie behavioral changes suggest widespread involvement of different signaling systems in the brain (Zotti *et al.* 2013). One of the most prominent adverse effects observed in studies performed in animal models has been related to increased anxiety levels (Olivares *et al.* 2014, Rosic *et al.* 2014) induced by AASs abuse (when applied in doses that mimic human abuse). Considering specific behavioral effects of testosterone-enanthate (one of commonly used synthetic androgen receptor agonists), it should be noticed that TE was commonly used in combination with other AASs ("AASs stacking") in behavioral studies, while it was rarely used as the single AAS. However, it has been reported that a supraphysiological dose of TE did not induce significant changes by means of angry behavior in humans (Tricker *et al.* 1996). On the other hand, intrahippocampal TE application resulted in spatial learning and memory impairment in rats (Naghdi *et al.* 2005).

It has been reported that nearly all AASs abusers follow intense exercise programs (Ip *et al.* 2011). Beneficial effects of physical exercise by means of alterations in anxiety level and depressive state (Strohle 2009) have been shown. Studies performed on animal models confirmed that voluntary exercise induced anxiolytic effects in mice (Santos-Soto *et al.* 2013) and rats (Fulk *et al.* 2004). Nevertheless, reported effects of combined protocols (simultaneous administration of supraphysiological doses of AASs and a prolonged exercise protocol) have remained very contradictory in literature. The diversity of both exercise protocols (by means of different load, duration, type…) and AASs treatment (dose, duration…) may be the explanation for different behavioral consequences observed in numerous studies.

Since TE is a representative of one of the most frequently used classes of AASs (applied alone or in combination with other classes of AASs in human abuse), the aim of this study was to estimate the certain behavioral effects induced by chronic TE administration in supraphysiological dose (in order to mimic heavy human abuse). As the abuse of AASs is commonly associated with intense exercise protocols, it was of our interest to evaluate the effects of prolonged exercise in male rats, on anxiety levels and exploratory activity patterns in EPM, as well as resulting behavioral effects of simultaneous TE administration along with an exercise protocol.

MATERIALS AND METHODS

A total of 32 male rats (three months old Wistar albino rats, 350–400 g) were housed in groups of four in polycarbonate cages in standard environmental conditions (temperature was maintained at 23±1 °C, 12/12h light/dark cycle). The animals were fed a standard pellet diet and had free access to food and water.

Two sedentary (control – C and testosterone-enanthate – T) and two exercise (exercise – E and testosterone-enanthate plus exercise – T+E) groups underwent adequate protocols – swimming protocol (1 h/day, for 5 consecutive days, 2 days break) and testosterone-enanthate – TE protocol (Galenika a.d., Serbia, 20 mg/kg/w, s.c.) for six weeks. There were 8 animals in each group. The swimming protocol was performed in a heated (32 ± 1 °C) glass swimming pool (60 × 75 × 100 cm) in a group of four animals. In order to reduce water-induced stress (Contarteze *et al.* 2008), the exercise protocol was performed following the adaptive period (20 minutes of swimming per day for one week). The swimming protocol was defined on the basis of a previous report for the duration of swimming trial that was sufficient to induce alterations in certain brain regions in rats (Liu *et al.* 2010). All rats were able to swim for the whole 60 minutes. The supraphysiological dose of TE was equivalent to the doses for heavy human AAS abusers (Long *et al.* 1996, Kurling *et al.* 2005). T+E group performed the same swimming protocol as the exercise group and simultaneously received 20 mg/kg of TE (s.c.) weekly for six weeks. The control and exercise groups received approximately the same amount of sterilized olive oil in the same manner as T group received TE. The rats from control and T group were placed in water for 2

minutes each day of swimming protocol to eliminate the difference between exercise and sedentary groups caused by water immersion. The experimenter was present throughout the swimming protocol monitoring the rats. Rats were towel dried and placed in a clean cage following each swimming trial.

Two days after the protocols were completed, the rats were placed in the testing room for 1–2 h to accommodate before testing in EPM. Our apparatus was made of wood, painted black and consisted of two opposite open (50 × 20 cm) and two opposite enclosed arms (50 × 20 × 30 cm), extended horizontally at right angles from a central area (20 × 20 cm). EPM was elevated 100 cm from the floor and placed in the centre of testing room. Each rat was placed in the centre of the elevated plus maze facing the open arm, and allowed 5 minutes for free exploration. The activity of the rats was recorded by digital video camera placed approximately 2.5 m above the maze. Video files were analyzed using Ethovision software [version XT 10 base], an integrating video tracking system for automation of behavioral experiments [Noldus Information Technology, the Netherlands]. The maze was cleaned following the trial for each animal with water and ethanol (70%) to remove possible interfering scents. The following parameters obtained in EPM were estimated: the cumulative duration in open arms, the number of rearings, the number of head-dippings and total exploratory activity (TEA), expressed as the number of TEA episodes. The number of TEA episodes was calculated as the sum of patterns of exploratory activity observed in closed arms (number of rearings) and in open arms (number of head-dippings), representing the overall exploratory activity in EPM. The total duration in open arms was considered as the key indicator for anxiogenic/anxiolytic effects of any applied protocol by means of EPM test, while the number of rearings and head-dippings, as behavioral patterns of exploratory activity, were also considered as parameters for testing the anxiety level in EPM since exploratory activity in EPM was directly affected by changes in anxiety (Pellow *et al.* 1985, Pellow & File 1986).

All research procedures were carried out in accordance with European Directive for welfare of laboratory animals N° 86/609/EEC and principles of Good Laboratory Practice (GLP), approved by Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

STATISTICAL ANALYSIS

Results expressed as mean ± SE, and p values <0.05 were considered significant. Variables were checked for normal distributions of the data with the Shapiro-Wilk test. Parameters obtained in EPM test for different groups of animals were compared using unpaired student t test. Pearson's correlation was used to determine if there was any association between the cumulative duration in open arms and the parameters for exploratory activity in EPM. A confidence level of 95% was accepted as significant. Statistical analysis was performed by using SPSS version 20.0 statistical package (IBM SPSS Statistics 20).

RESULTS

Prolonged exercise protocol induced significant increase in cumulative duration in open arms of EPM, while chronic TE treatment also resulted in significant decrease in time spent in open arms comparing to all groups (*Fig. 1A*). This anxiogenic effect of TE was also observed in the combined group (when compared to control and exercise groups).

Similar effects of two applied protocols were manifested in the number of rearings in close arms EPM, since this behavioral pattern was not observed in open arms. The swimming protocol induced significant increase in the number of rearings compared to all groups (*Fig. 1B*). On the other hand, supraphysiological dose of TE produced significant decrease of rearing compared to the control and exercise groups. The number of rearings observed in the combined group was significantly lower compared to the exercise group.

The number of head-dippings also varied depending on the applied protocol. While TE administration significantly decreased this behavioral pattern comparing all investigated groups, the swimming protocol resulted in a significant increase in the number of head-dippings when compared to both T and the control groups (*Fig. 1C*).

The number of TEA episodes, as a new parameter for overall exploratory activity in EPM, the most clearly confirmed the opposite effects of applied protocols on alteration in anxiety level. So, the number of TEA episodes was significantly increased in the exercise group, while chronic treatment with TE resulted in a significant decrease compared to all groups (*Fig. 1D*). Furthermore, simultaneous administration of TE, along with swimming training, significantly decreased anxiolytic effect observed in exercise group by means of the number of TEA episodes.

As shown in Figure 2, simple regression analysis revealed that both the number of rearings and the number of head-dippings (*Fig. 2A, B*) were significantly positively correlated with the cumulative duration in open arms (Pearson's r=0.60, r=0.62, p<0.001, respectively). The regression analysis also confirmed that the number of TEA episodes was significantly positively correlated (*Fig. 2C*) with the cumulative duration in open arms (Pearson's r=0.70, p<0.000001).

DISCUSSION

The results of our study confirmed anxiolytic effects of exercise manifested as increased exploratory activity in EPM – the increase in cumulative duration in open

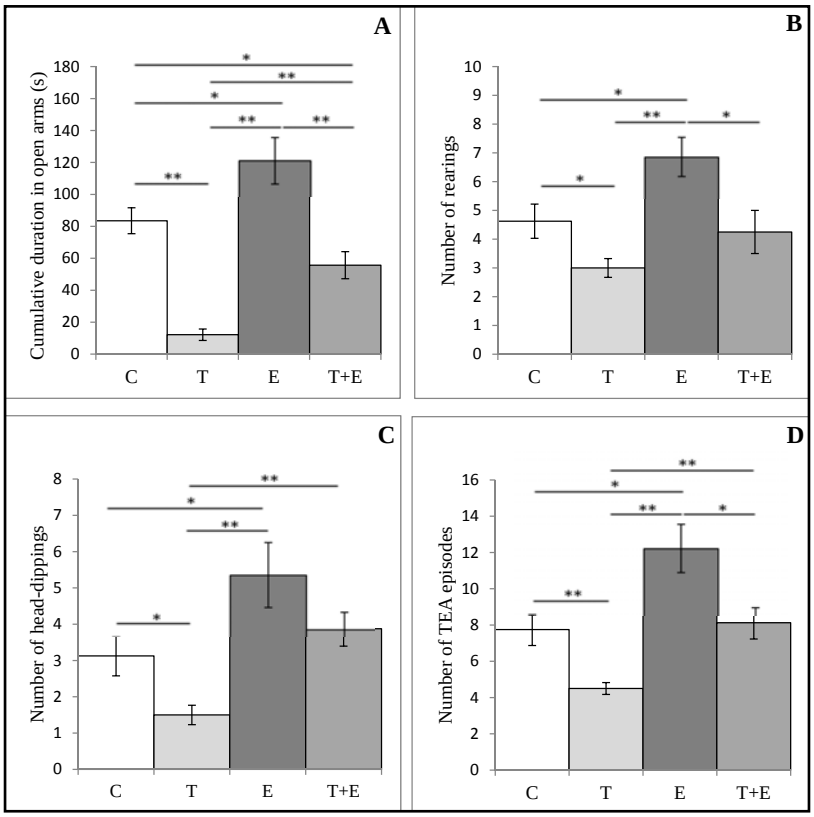

Fig. 1. Parameters calculated from the elevated plus maze test: A - the cumulative duration in open arms, B - the number of rearings, C - the number of head-dippings, D - the number of TEA episodes (Mean ± SEM, *denotes a significant difference $p < 0.05$, **denotes a significant difference $p < 0.01$)). C - control group, T – testosterone-enanthate group, E - exercise group, T+E - testosterone-enanthate plus exercise group.

arms, the number of rearings and head-dippings, and TEA (45%, 49%, 72% and 58%, $p<0.05$, respectively) comparing to the control group. Our results are in accordance with previous reports confirming the beneficial effects of exercise by means of decreased anxiety level following various exercise protocols in mice (Santos-Soto *et al.* 2013) and rats (Fulk *et al.* 2004) by means of alterations in parameters of explorative activity. Interestingly, it has recently been shown that only physical activity that results in low and mild stress may induce anxiolytic effect, while high-intensity exercise that exerts high-stress reaction produced anxiety-like behavior in EPM (Otsuka *et al.* 2016). Therefore, it seems that exercise could be included in mediation of anxiety level in a manner that depends on the exercise intensity.

Supraphysiological dose of TE induced decrease in the number of rearings and head-dippings, cumulative duration in open arms and TEA (35%, 52% – $p<0.05$, 85% and 41%, $p<0.001$, respectively) comparing to the control group, while this effect of TE was even more pronounced when compared to the exercise group ($p<0.001$ for all parameters). The diversity of AASs actions on the anxiety levels in rodents is likely to be attributed to interactions of age, sex, type(s) of AAS, the regimen of administration of AAS and environmental context (Oberlander & Henderson 2012). Physiological doses of AAS (usually used as supplementation for therapeutic purposes) commonly produced anxiolytic-like behavior (Frye & Seliga 2001). Decreased anxiety following a single dose of AAS in rats was observed in a study of Hodosy and coauthors (Hodosy *et al.* 2012). On the other hand, chronic administration of AAS in supraphysiological doses that mimic heavy human abuse induced clear anxiogenic effect in mice (Ambar & Chiavegatto 2009) and rats (Olivares *et al.* 2014).

Also, the applied dose of TE was sufficient to attenuate beneficial effects of exercise following a combined protocol by means of all estimated parameters in EPM. The values obtained in the combined group were similar to the control group, except for the cumulative duration in open arms (decrease by 33%, $p<0.05$). Our results correlate to a previous report of simultaneous administration of AAS along with exercise protocol by means of reduction of explorative activity obtained in open field tests (Bento-Silva *et al.* 2010).

Observed behavioral alterations following both exercise and TE protocol could be related to various biochemical, functional and morphological changes in certain brain regions that are involved in modulation of anxiety level. It has been reported that the hippocampus has the main role in both cognitive and emotional

processes. Hippocampal GABAergic interneurons are attributed for local connections within the hippocampus (El Falougy *et al.* 2008). Since GABA is the major inhibitory neurotransmitter in the rodent brain, GABAergic system is suggested to be the crucial factor in modulating local noradrenergic, dopaminergic, serotonergic and glutamatergic neuronal circuitry that are all included in the regulation of anxiety levels. It has been reported that beneficial effect of exercise on hippocampal GABAergic system has been attributed to specific cell proliferation and neurogenesis (Arida *et al.* 2004). At the same time, the adverse effect of supraphysiological doses of AASs has been confirmed by means of alterations in hippocampal plasticity (Clark *et al.* 1995) and neurogenesis (Brannvall *et al.* 2005). Taken together, it seems that the effects of exercise and TE protocols on anxiety levels observed in this study may be connected to the alterations of hippocampal function, as its impact on anxiety has already been confirmed in numerous studies (Bannerman *et al.* 2014).

Since the cumulative duration in open arms is considered as the strongest indicator of anxiety level in EPM, we estimated correlations between this parameter and two commonly used parameters of exploratory activity (number of rearings and head-dippings), that count for estimation of anxiety level, as well as the correlation between the cumulative duration in open arms and the number TEA episodes as a new parameter for overall exploratory activity. Although rearing is the most frequently used as a parameter from the open field test, we propose that it should be taken into consideration for analysis of EPM test as well. As exploratory activity in EPM test is usually based almost exclusively on the estimation of the number of head-dippings, which can only take place in the open arms, we consider the number of rearings that can be observed in both open and closed arms as an additional parameter that can improve the estimation of exploratory activity during the whole EPM test. Comparison of statistical analysis results for the correlation between parameters for exploratory activity – the number of TEA episodes, the number of rearings and the number of head-dippings with the main indicator of anxiety in EPM – the cumulative duration in open arms confirmed significantly stronger connection for TEA episodes comparing to two other parameters of exploratory activity (49% vs. 36% and 38%, respectively). That led us to the conclusion that we should introduce a new parameter for exploratory activity measurement in EPM – TEA that includes both the number of rearings and head-dippings, and therefore represents the overall exploratory activity in EPM.

In conclusion, our results confirmed beneficial effects of exercise on anxiety levels observed in EPM by means of parameters that are connected to alteration in exploratory activity. Supraphysiological dose of TE resulted in anxiogenic-like behavior in EPM. The effect of TE was so pronounced that beneficial effect of exercise was reversed to the control values (or even below them). Also, based on the results obtained in this trial, we propose the TEA as a new parameter for overall exploratory activity that can be helpful in evaluation of EPM test results.

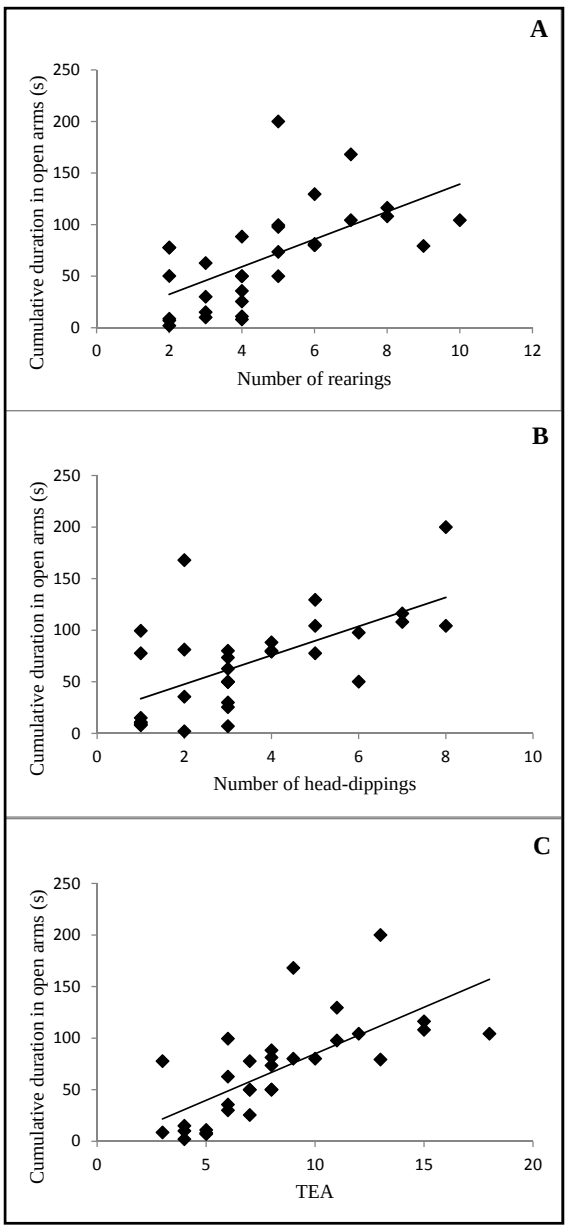

Fig. 2 Relationship between exploratory activity alterations and the cumulative duration in open arms observed in the EPM test for all investigated groups (n=32). Simple regression analysis indicated that the cumulative duration in open arms was significantly and positively correlated with the number of rearings (A, $p<0.001$), the number of head-dippings (B, $p<0.001$) and the number of TEA episodes (C, $p<0.000001$).

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