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Detrimental Effect of Alprazolam on Sperm Viability of Mice (*Mus musculus*) Exposed to Chronic Unpredictable Mild Stress

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Abstract

Objective: This study aims to investigate the effect of alprazolam administration on sperm viability of mice (*Mus musculus*) strain Balb/C following CUMS for 53 days. **Method:** This study used a true experimental design through randomization. Control group kept undisturbed without treatment and therapy throughout 60 days. K1 and K2 were exposed to a 53 day period of CUMS along with 7-day pretreatment adaptation. K2 was adapted for a week, stressed for 53 days along with cotreatment of alprazolam (4 mg/kg BW). **Results:** K1 and K2 showed insignificant improvement of sperm viability ($p > 0.05$). Meanwhile, K0 and K1 showed significant value compared to K2 ($p < 0.05$). K2 showed little increase of sperm viability in CUMS group along with subsequent treatment of alprazolam. **Conclusion:** Overall, the results indicate an insignificant effect of alprazolam on sperm viability of CUMS-induced mice.

Keywords: Chronic Unpredictable Mild Stress, Alprazolam, Viability, Sperm

Introduction

Psychological stress like anxiety can be defined as an uncomfortable emotional experience due to the biochemical and psychological stress and behaviour. In dealing with stress, benzodiazepine drugs are often prescribed for hypnotic, sedative and anxiolytic symptoms at all ages in health services. As one of benzodiazepine group, alprazolam is ranked as 13th place among drugs that are commonly sold in 2012⁽¹⁾. Alprazolam is prescribed to treat panic disorder and anxiety. Further consumption in Indonesia during 2010-2013 increases with the average rate around 0.56 of Define Daily Doses for Statistical Purposes (S-DDD)⁽²⁾. One of the factors contributing into the increased consumption of drug is the pharmacological profile. On a pharmacodynamic property, alprazolam is more potent than that of diazepam with 1 mg of alprazolam relatively

equivalent to 10 mg of diazepam⁽⁴⁾. Additionally, it is well absorbed and metabolized extensively into the whole body after oral administration⁽⁵⁾. Thus, the pharmacological profile increases the prolonged drug use.

Few studies show that longer term use of alprazolam causes withdrawal syndrome, tolerance, and dependence⁽⁶⁾. Longer term use of alprazolam eventually disrupts the mechanism of chronic stress due to the activation of sympathetic adrenal system, hypothalamic-pituitary-gonadal (HPG) axis, dan hypothalamic pituitary adrenal (HPA) axis. Furthermore, alprazolam can lead to side effects on the testes of mice. Studies showed that injection of alprazolam in mice possess a deleterious effect on structural histopathology in the testes and infertility. Infertility is evident on late spermatids with the head deformities⁽⁷⁾. Also, administration of alprazolam limits the intratesticular vascularization effecting on sperm evaluation⁽⁸⁾.

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Chronic unpredictable mild stress (CUMS) has been used in the animal setting because it can potentially

stimulate depression⁽⁹⁾. Given the roles of HPA axis and HPG axis in the production of sperm under chronic stress conditions, the viable sperm quality in mice was evaluated in CUMS-induced mice in this present study.

Despite the scant research about alprazolam effect on male fertility, the chronic use of alprazolam still remains extensive. This present study was designed to investigate the effect of alprazolam administration on the sperm viability in CUMS-induced mice.

Materials and Method

Experimental Animals:

Thirty-three healthy male mice (*Mus musculus*) Balb/C, aged 6-8 week (25-30 gram) were used in this study and housed in animal laboratory, Faculty of Science and Technology, Universitas Airlangga under controlled conditions. Mice were acclimated for 7 days in prior to actual experiments. Each group consisting of 11 mice was obtained through randomization. Mice were maintained under a 12 hour light/dark cycle with 40-70% of relative humidity and housed per cage (40 cm length x 30 cm width x 18 cm height) with a close fitting wire, tap water and foods. The experiment protocols in this study have been approved by Ethical Eligibility and Research Committee (No. 296/EC/FKUA/2019, Faculty of Medicine, Universitas Airlangga).

Table 1. Time length of stressors in CUMS protocol for 53 days^{(10),(11)}

Types of Stressors	Description	Time
Bath	The cage is not provided with mat base and the water is poured into the cage with normal temperature of ± 2 cm	30 minutes – 1 hour
Damp bedding	The mat is placed in the wet cage	2.5 hours – 3 hours
No bedding	The cage is not provided with mat base to expose the mice on the plastic as a base of the cage	3.5 hours
Bedding change	Bed cages is replaced with the reused bedding from other species object such as rat	Change per 15 – 30 minutes for 1.5 hours – 3 hours
Cage tilt	The cages with no base are tilted at an angle of 45 degrees in the hope that the mice gather in the cage corner.	1 hour – 2.5 hours
Altered light cycle	Exposure to a 5 watt light, then continuous alteration on and off.	Altered per 15 minutes during 1 hour – 3 hours
Social stress	Mice are transferred to the cages of other mice in order to expose the mice on the new environment and leave the mice alone without any social interaction	30 minutes – 60 minutes

Table 2. Type of interventions in CUMS protocol^{(10),(11)}

Monday	Tuesday	Wednesday	Thursday	Friday
Cagetilt (2.5 h)	No bedding (3.5 h)	Cagetilt (2.5 h)	Bathing (1 hour)	No bedding (2 h)
Dampbedding (2.5 h)	Socialstress (2 h)	Bathing (30 min)	Beddingchanges (every 30 minutesfor 3 hours)	Dampbedding (2 h)
Bathing (30 min)	Dampbedding (2,5 h)	Dampbedding (3 h)	Socialstress (30 min)	Cagetilt (1 h)
Socialstress (1.5 hour)	Light/dark (every 15 minutes carried out simultaneouslywithdampbedding)	Light/dark (1 hour and carried out simultaneously withdampbedding)	Light/dark (3 hours and carried out simultaneously withsocialstress)	Light/dark (2 hours and simultaneously carried out withBathing)
		Socialstress (1 hour)		Bathing (2 h)

CUMS Procedure:

The experimental group (K1, K2) were exposed to various stressors of CUMS model for 53 days according to the protocols described by Zhu et al. and de Andrade et al.,^{(11),(10)}with some minor modifications. The stress groups were subdivided into 4 groups (K1-1; K1-2; K2-1; K2-2). Each stress protocols wasdone for 7 hours (8 a.m. to 3 p.m.) at one time per day to prevent the mice from adapting.

Preparation of alprazolam:

Alprazolam was obtained as tablets in maximum dose (4 mg/kg BW). The applied dose of alprazolam was prepared by diluting the drugs in distilled water to 0.025 mg/kg/daily (equivalent to the therapeutic dose calculation for humans by Nair and Jacob)⁽¹²⁾. Alprazolam was suspended with 2% sodium CMC. Suspension of the mixture (0.1 ml) wasadministered to CUMS-induced mice through oral gavage. Control group was administered withdistilled water.

Viability Test:

To determine whether spermatozoa was alive or dead, observation was conducted by mixing one drop of

fresh semen with HE 0.5% on an object glass with cover glass. Each mean of alive or dead sperm with absorption of eosin was observed and compared.

Statistical Analysis

The data results were statistically assessed using one way ANOVA, Lavene’s test and Least Significant Difference (LSD) Post Hoc test. Statistical analysis was constructed using SPSS 25. Data distribution with p-value <0.05 was considered statistically normal.

Results

Based on the statistical findings, tests of alprazolam effect on CUMS-induced group for the period of CUMS (day 7 to day 21) showed no significant effect of alprazolam among K0, K1 and K2 groups. To assess data distribution, Saphiro Wilk test was used. It resulted in normal distribution with the value of p> 0.05. The data homogeneity and variance, Lavene’s test, One Way Anova, and LSD Post Hoc test were used. The data was homogeneous with p> 0.05. Out of 33 samples, the table showed there was a significant difference in between K0, K1, and K2 with p = 0.971 (p > 0.05) using One Way AnovaLavene’s test. The LSD Post Hoc test was then performed to determine significant differences among

groups. The statistical data analysis showed that K0 was 75.09% higher than K1 and K2 groups (Table 3). As a result, no significant difference was seen in the sperm viability data at 65.73% and 65.91%.

Table 3. Mean, Distribution, and variance of sperm viability data

Sample groups	Mean	SD	Minimum	Maximum	Normality (Sapphiro-Wilk)
K0 (control group)	75.09	5.873	65	84	0.772
K1 (CUMS-induced group)	65.73	6.498	54	74	0.325
K2 (CUMS + alprazolam treated group)	65.91	6.818	57	80	0.527

Table 4. Variance of sperm viability data using One Way Anova

	P value	Result
Control, CUMS-induced, CUMS + alprazolam treated groups	0.971	Significant

Table 5. Results of difference in sperm viability among groups (K0, K1, and K2)

	K1(CUMS-induced group)	K2(CUMS + alprazolam group)
K0 (control group)	0.002	0.002
K1(CUMS-induced group)	-	0.947

Discussion

Stress disrupts the hormonal regulatory system through HPG axis pathway as a major influence on infertility. Our current study showed that 53 days exposure to CUMS is sufficient to induce significant chronic stress and decrease the mean of sperm viability. Intermittent chronic stress, heat stress, obesity, and social defeat stress have been shown to have a suppressive effect on spermatogenesis⁽¹³⁻¹⁶⁾. However, after 42 days of alprazolam administration (5 mg/kg BW) and exposure to stressors, the chronic moderate stress-induced mice were observed. It has been found to elicit a neutralizing response against the damaging effects of moderate chronic stress, restore antioxidant balance and

reduce serum cortisol⁽¹⁷⁾.

This current study also correlates with the study using 8 different stressors for 6 weeks, showing an increase in cycle cell arrest of spermatogonia and apoptosis of spermatid cells. There was also an increase of glucocorticoid expression in spermatogonia and spermatocytes due to the feedback mechanism of the HPA and HPG axis pathways⁽¹⁸⁾. The germ cells in the testes are particularly susceptible to ROS because of the high levels of polyunsaturated fatty acids and low antioxidant capacity⁽¹⁹⁻²¹⁾.

It is evident that ROS levels result in the formation of abnormal sperm morphology. ROS as a signal has the

potential to induce apoptosis^(22,23). The capacitation, acrosome reactions and binding of spermatozoa to zona pellucida can take place at low levels of ROS. The processes would not occur when the production of ROS exceeds normal limits, resulting in infertility. The male infertility is manifested in decreased sperm quality such as motility, viability, and defects in spermatozoa-oocyte fusion^(24,25).

Sperm viability is associated with the intact plasma membrane due to its interaction between spermatozoa and oocyte⁽²⁶⁾. Aside from that, this current study is in line with the previous study that observed the decreased sperm quality in diazepam-treated mice with various doses (2 mg/kg BW; 5 mg/kg BW; 10 mg/kg BW) for 8 weeks⁽²⁷⁾. In diazepam-treated mice (5 mg/kg BW), the significant decreased sperm was heightened as a consequence of antioxidant imbalance and lower level of testosterone.

To assess the drug toxicity, the morphology of spermatozoa can be an important aspect of consideration. Low testosterone levels disturb the differentiation of spermatids after spermiogenesis⁽²⁸⁻³⁰⁾. Meanwhile, oral administration of fluoxetine in CUMS-induced mice resulted in a decrease of sperm concentration, sperm motility and an increased number of abnormal spermatozoa⁽³¹⁾. Administration of fluoxetine (200 mg/kg) in male rats for 60 days resulted in a significant decrease in spermatogenesis, levels of FSH, the weight of testes, epididymides, ventral prostate and seminal vesicle⁽³²⁾. Other clinical study proved the effect of paroxetine administration in 35 healthy male volunteers for 5 weeks. The results showed that there was an increase in abnormal DNA fragmentation considerably⁽³³⁾.

On the other hand, oral administration of alprazolam (0.5 mg/kg BW, 1.5 mg/kg BW, 4.5 mg/kg BW) in mice for 3 months caused gradual decrease in serum testosterone, significantly at a dose of 4.5 mg/kg BW⁽⁷⁾. Corresponding to the previous studies, the dosage (4 mg/kg body weight) in this study was relatively maximum in humans.

Nevertheless, another study found out that alprazolam exerts a therapeutic effect on a 6-hour trial of acute immobilization stress-induced mice⁽³⁴⁾. Because of

HPA axis pathway activation, it has an impact on several biological effects either at the central or peripheral levels. In this case, pretreatment with alprazolam (0.25 and 0.5 mg/kg/ BW) elicits a reversible effect of anxiety, analgesia, and impaired locomotor activity. It was effective to produce neuroprotective effects⁽³⁴⁾. Positive effects in chronic moderate stress-induced mice within 21 days were against the damaging outcomes of moderate chronic stress, antioxidant balance restoration, and serum cortisol reduction after treated with alprazolam (5 mg/kg/ BW)⁽¹⁷⁾. On the contrary, K2 group in this current study had a progressive build-up of oxidative stress due to CUMS.

In fact, the present study in K2 group at the same dose (4 mg / kg BW) as in human doses has not been shown to be able to suppress viable sperm levels by <50%. The standard cut-off value for human semen viability should be at least 58% of alive sperm⁽³⁵⁾. Semen that contains alive sperm below 50% is identified as abnormal when observed and assessed. The alprazolam administration at maximum dose does not reflect that it should be considered as toxic. It is interesting to point that a change of antioxidant capacity was also observed in rats exposed to immobilization, cold and water immersion for 21 days^(36,37). Exposure to chronic stress for 40 days which increases lipid peroxidation and protein oxidation in the cortex area may also explain why alprazolam does not improve the sperm quality^(38,39).

Conclusion

Our findings suggest that alprazolam administration decreases sperm viability of CUMS-induced mice (*Mus musculus*). Further studies are necessary to investigate about the effect of alprazolam with various doses on sperm quality of mice (*Mus musculus*) following CUMS.

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