Diazepam: a peripheral benzodiazepine receptor ligand, inhibits mitochondrial F1-F0 ATPase and induces oxidative stress in goat epididymal sperm in vitro

G. Mohana Krishna, M. Asha Kiran and P.P. Mathur

Abstract:

Benzodiazepines are the most preferred anxiolytics. Their activity as anxiolytics is mediated through central – type benzodiazepine receptor (CBR), which is associated with GABA_A receptors and enhances the inhibitory effect of GABA. Another type of benzodiazepine receptors called peripheral-type benzodiazepine receptors (PBR) expressed exclusively on the outer membrane of mitochondria and functions mainly with steroidogenic machinery. Besides, PBR is involved in many other functions. The main theme of the present work has emanated from recent research that PBR ligand has another target distinct from PBR, which binds to oligomycine-sensitivity conferring Protein (OSCP) of mitochondrial F1-F0 ATPase and reduces its activity, so that increasing the cellular free radicals thus acts as an anti-proliferative and pro-apoptotic. In the present work we evaluated the effect of diazepam (Valium, a PBR ligand) on goat epididymal spermatozoa taking ROS enhancement and diminution of F1-F0 ATPase activity in vitro as conclusive points. Results evince that diazepam decreases motility and viability of sperm, increases oxidative stress and disturbs F1-F0 ATPase function. Increased oxidative stress is due to rise in ROS, which is imputed to defunct F1-F0 ATPase. We are first to report the effect of diazepam on goat epididymal spermatozoa. The work concludes the deteriorating effect of diazepam on goat epididymal sperm and the results also imply that this particular action of diazepam is through binding to OSCP of sperm mitochondria. We suggest that in male diazepam users infertility could be a complication of concern. Dichotomously this effect of diazepam could be a valuable consideration in developing new contraceptive strategy.

Key words: Benzodiazepines; diazepam; peripheral-type benzodiazepine receptor (PBR); oligomycine-sensitivity conferring Protein (OSCP); F1-F0 ATPase; spermatozoa; reactive oxygen species.

INTRODUCTION

Sperm is an intricate motile cell, rich in DNA with a head that is made up mostly of chromosomal material. Covering the head like a cap is the acrosome, a lysosome-like organelle rich in enzymes involved in sperm penetration of the ovum and other events involved in fertilization.

A reap from exhaustive research in sperm biology has concluded the presence of number of neuronal receptors on sperm. Those include GABA_A receptor (Erdo & Wekerele., 1990; Ritta, Calamera & Bas., 1998), metabotropic GABA_B receptors (He et al., 2001), glycine receptors (Melendrez & Meizel., 1996; sato, son & Meizel., 2000), nicotinic acetyl choline receptors (Baccetti et al., 1995), muscarinic acetyl choline receptor (Young & Laing., 1991), metabotropic glutamate receptor (Shorto et al., 2001), purinergic receptors (purinoreceptors) (Glass et al., 2001), angiotensin II receptors (Vinson et al., 1995; Wernemuth, Babcock & Hille, 1999), cannabionoid receptors (Gerard et al., 1991; Galiegue et al., 1995; Brown, Wager-Miller & Mackie., 2002) and cocaine receptors (Yazigi, Odem & Polakoski., 1991). These neuronal receptors tend to assist in sperm-unique functions and the ligands for these receptors are present either in semen or reproductive tract.

Benzodiazepines are the most commonly prescribed anxiolytics. Their activity as anxiolytics is mediated through central – type benzodiazepine receptor (CBR), which is exclusively expressed in CNS. CBR is associated with GABA_A and upon binding with benzodiazepines enhance the inhibitory effect of GABA by neuronal hyperpolarization through activation of chloride currents (Dingemanse and Breimer., 1984). In 1977 a report from Braestrup and Squires has kept another target of benzodiazepines at the forefront, called peripheral-type benzodiazepine receptor (PBR). Glandular and secretory tissues such as pineal gland, adrenal glands, salivary glands, olfactory epithelium, ependyma and gonads are particularly rich in PBR. Majority of PBR are located in the outer membranes of mitochondria (Basile and Skolnick., 1986; Anholt et al., 1986). Despite their primary localization PBRs have also been found on the plasma membranes of...
erythrocytes (Oslon et al., 1988; O’Beirne et al., 1990) and around the nucleus of cells from human breast tumor biopsies and breast tumor cell lines (Hardwick et al., 1999). The PBR is a functional component of the steroidogenic machinery mediating cholesterol delivery from the outer to the inner mitochondrial membrane (Besman et al., 1989; Papadopoulos et al., 1997). More direct studies on PBR function have yielded a diverse list of activities involving the PBR including the regulation of cellular proliferation (Alho et al., 1994), immunomodulation (Zavala., 1997), porphyrin transport and heme biosynthesis (Taketani et al., 1995), anion transport (Basile et al., 1988), steroidogenesis (Besman et al., 1989) and apoptosis (Hirsch et al., 1998). In addition PBR has intimate role in reduction of Reactive oxygen species (ROS) and apoptosis (J.E. Stoebner., 1999; Kosenko et al., 1998).

Owing to Bz-423 (A benzodiazepine) role in oxidative stress, suppression of ATP synthesis and consequent redox-regulated apoptosis, a new target of benzodiazepines distinct from CBR and PBR has come in to knowledge. Affinity based screening of a phage-display human C-DNA expression library identified the oligomycine-sensitivity conferring Protein OSCP a component of the mitochondrial F1-F0 ATPase as the molecular target for Bz-423 (Johnson et al., 2005). Binding of Bz-423 to the OSCP in the context of intact enzyme inhibits both synthesis and hydrolysis of ATP (Johnson et al., 2005 & 2006).

There has been an increasing concern regarding the infertility in humans these days so even a small factor contributing to this must not be treated trivial. Infertility primarily refers to the biological inability of a person to contribute to fertilization. Subsumed among the causes are abnormal motility and viability of male spermatooza. Infertility is one among the complications associated with usage of anxiolytic drugs. Though no previous studies reported infertility among side effects in diazepam (Valium) users, we speculate the problem is concern worthy. In the present study we addressed the effect of diazepam on goat epididymal spermatooza, which could be imputed to the effect of benzodiazepines on new target of PBR ligands that inhibit Fke-Fk ATPase.

**MATERIALS AND METHODS**

**Chemicals**

Diazepam was purchased from TOCRIS Bioscience and dissolved in DMSO and used for incubating samples. All other chemicals were of analytical grade and purchased from local commercial sources.

**Sperm Motility**

Sperm motility was evaluated immediately after scarifying the animals. A drop of sperm suspension was placed on a clean glass slide and covered with a cover slip and allowed to stand for about a minute. The slide was examined under the microscope at 40X and motility was scored from different fields. Spermatooza showing any degree of movement are considered motile. Around hundred spermatooza was scored, which includes motile as well as immotile. Sperm motility was calculated in percent by using the formula as below,

\[ \text{Motility} \% = \frac{\text{Number of motile sperm}}{\text{Total number of sperm}} \times 100 \]

**Sperm Viability**

Sperm viability was assessed using supra vital staining tub. All glassware as well as eosin stain were maintained at 37°C. A drop of sperm suspension was placed on a clean glass slide and a drop of eosin stain (5% eosin) was added and then mixed thoroughly with the help of a fine glass rod. A portion of the mixture was transferred to second slide and a thin film was prepared. The slide was examined under the microscope at 40X and around hundred spermatooza were scored, which includes viable and as well as dead sperms, from different fields of slide. Spermatooza appearing pinkish (stained) are considered dead. While those appearing colorless (unstained) as viable. Sperm viability was calculated in percent by using the formula as below,

\[ \text{Viability} \% = \frac{\text{Number of viable}}{\text{Total number of sperm}} \times 100 \]

**Concentration of sperm**

\[ C = N \times 10,000 \times 20 \text{ (or) } C = N \times 0.2 \times 10^6 \text{ sperm/ml} \]

**Catalase Assay**

Catalase was assayed by the method of claiborn., 1985. To 2.40 ml of phosphate buffer (50mM, pH 7.0), 10 µ hydrogen peroxide (19mM) is added then to this 50 µ sonicated sperm sample added. The decrease in the absorbance is measured at 240 nm against blank containing all the components except hydrogen peroxide and enzyme source at 10 seconds interval for 3 min on Shimadzu UV spectrophotometer. The activity of the enzyme was expressed in µM of hydrogen peroxide consumed/min/mg of protein.
Superoxide Dismutase Assay

Superoxide dismutase was assayed by the method of Marklund and Marklund., 1974. The assay mixture contained 2.4 ml of 50mM Tris-HCl buffer containing 1 mM EDTA (pH 7.6), 300 µl of 0.2 mM pyrogallol and 300 µl enzyme source. The increase in absorbance is measured at 420 nm against blank containing all the components except enzyme and pyrogallol at 10 seconds interval for 3 min on Shimadzu UV spectrophotometer. The activity of the enzyme was expressed in µM of pyrogallol oxidized/min per mg of protein.

Lipid Peroxidation

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) was measured by the method of Ohkawa et al., 1979. This TBA test involves the reaction of aldehydes with TBA at 100°C under acidic condition to produce a pink colour, which strongly absorbs light at wave length of 535 nm. 100 µl of sonicated sperm sample was added in 900 µl Tris buffer (pH 7.5) then 2 ml of TCA-TBA-HCl reagent was added to this and mixed thoroughly. The solution was incubated for 15 minutes in a boiling water bath. The flocculent precipitate was removed by centrifuging at 1000X g for 10 minutes. The absorbance of sample was measured at 535 nm against blank that contain no sample. A standard curve was constructed by plotting the amounts of commercially bought product malondialdehyde to the measured absorbance. The TBARS (Thio Barbutiric Acid Reactive Substance) was expressed as µM of malondialdehyde produced/minute/mg of protein.

Subcellular Fractionation

Mitochondrial fractionation was prepared by differential centrifugation method. 1 ml of sperm sample is centrifuged at 1000X g for 10 min and the pellet was suspended in 0.25M ice cold sucrose solution and sonicated at 50% amplitude on ice bucket. This sonicated sample was the centrifuged at 1000x g for 10 min at 4°C and the supernatant was taken in the fresh vial. This supernatant was again centrifuged at 12000X g for 10 min at 4°C supernatant was discarded and the brown coloured pellet was stored in TAE buffer (50 mM EDTA) pH 8.5 which seems to stabilize the ATPase permitting them to be stored at 4°C for several weeks without significant loss of activity.

Total ATPase Assay

Ascorbate - ammonium molybdate assay was used to measure inorganic phosphate by the modified method of Monk and Kurtz., 1991. The ATP hydrolysis assay was conducted in TM buffer (100 mM Tris buffer, 10 mM Mgcl) pH 8.5 for F1F0-ATPase. TM buffer was adjusted appropriately higher than its final pH to neglect the pH drop caused by 10 mM ATP. The linear range of phosphate detected was 1 to 40 nM. The compound is prepared in different concentrations with TM buffer (final volume 1 ml) and to this 100 µl of mitochondrial fraction is added and the reaction is carried out 30°C for 15 min, at which point it was stopped with 300 µl of solution containing sodium dodecyl sulfate (SDS), HCl, and ammonium molybdate (0.05%) and incubated for 10 min. Absorbance is measured at 750 nm against blank containing all the components except enzyme source.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple range tests. Differences were considered to be significant at p< 0.05 against control group. Data were presented as mean ± SD.

RESULTS

In diazepam incubated sperm, the epididymal sperm motility and viability showed significant changes at 200µM and 400 µM concentrations as compared to the corresponding control sperm samples (Figure: 1). The specific activities of superoxide dismutase and catalase were found to be decreased while the level of lipid peroxidation was increased in diazepam incubated sperm samples in dose dependent manner when compared to the corresponding controls (Figure: 2). When subcellular mitochondrial fraction was incubated with diazepam the specific activity of F1-F0 ATPase was significantly decreased in a dose dependent manner when compared to the corresponding controls (Figure: 3).
Figure: 1. Effect of incubation of sperm with graded doses of diazepam on goat epididymal sperm motility (A) and viability (B). The values are expressed as mean ± SD. The data were expressed in percentage of total sperm.

DISCUSSION

Bz-423 is a pro-apoptotic agent with efficacy against autoimmune diseases in several murine models (Blatt et al., 2002; Bednarski et al., 2003). An intimate activity of PBR ligand Bz-423 in suppressing keratinocyte proliferation could be attributed to its ability to bind OSCP and inhibit F₁-F₀ ATPase (Narasimharao Bhagavathula et al., 2008). Report from Miltyk et al., 2006 infers antimitotic activity of high affinity PBR ligand PK-11195, which as well could be
attributed to the action of PBR ligand on F\textsubscript{1}-F\textsubscript{0} ATPase. Inhibition of F\textsubscript{1}-F\textsubscript{0} ATPase would lead to increased superoxide, resulting in cell growth arrest and redox regulated apoptosis (Joanne cleary et al., 2007), consistent with the reports mentioned above.

ROS causes destruction of all cellular structures including membrane lipid (Ichikawa et al., 1999) and has been shown to damage macromolecules, including membrane bound polyunsaturated fatty acids (PUFA) causing impairment of cellular function (Lenzi et al., 2000). Spermatozoa are rich in PUFA and therefore could be highly susceptible to oxidative stress. Several lines of evidence exist for the adverse effects of ROS on male reproduction (Ichikawa et al., 1999). In spermatozoa several antioxidant defense systems, namely glutathione peroxidase (Alvarez et al., 1989), superoxide dismutase (Mennela et al., 1980) and catalase (Jeulin et al., 1989) known to protect against ROS. Superoxide dismutase generally dismutates superoxide anion radical into hydrogen peroxide (Nissen et al., 1983). The hydrogen peroxide thus produced will turn in to non-reactive forms by the action of catalase or glutathione peroxidase/ reductase system (Alvarez et al., 1989). Obviously when the level of stress is beyond the protectable level of antioxidant system, stress induced aberrations in cellular structure and function would prevail.

Reports impeccably suggest that benzodiazepines other than Bz-423 and PK-11195 also function through binding to the OSCP (Johnson., 2005). With this view in mind the present work was planned to evaluate diazepam’s (PBR ligand) place in disturbing the oxidative stress balance in goat epididymal spermatozoa. The results show that incubation with diazepam has increased oxidative stress and diminished mitochondrial F\textsubscript{1}-F\textsubscript{0} ATPase function in subcellular fractions of mitochondria suggesting the significant role of diazepam in disturbing the balance between the oxidative stress and antioxidant system. Results also show that the viability and motility of spermatozoa was significantly affected and the effect of diazepam was concomitant with the graded doses. We are first to report the effect of diazepam on goat epididymal spermatozoa. Although further research that men on diazepam suffer infertility or semen abnormalities and that diazepam can enter the male tract is required for detailed perception it is well substantiated at this point to suggest usage of diazepam could be a considerable factor in causing infertility in humans (diazepam users).

CONCLUSION

Diazepam induces oxidative stress in goat epididymal sperms and also affects the motility and viability of it. Consistent with the previous reports is its ability to diminish the activity of mitochondrial F\textsubscript{1}-F\textsubscript{0}ATPase thus increases free radical stress. This function of diazepam could be attributed to its ability to bind OSCP. We are further researching in to binding details of this drug. However this concludes the use of diazepam could be a considerable factor in causing infertility in human males. Dichotomously further research could embrace this effect of diazepam in developing new contraceptive strategy.

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