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Allopregnanolone, the active metabolite of progesterone protects against neuronal damage in picrotoxin-induced seizure model in mice

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ABSTRACT

Progesterone exerts anti-seizure effect against several chemoconvulsants. However, there is no published report on the interaction between progesterone and picrotoxin (PTX). The present study evaluated the effects of progesterone and its active metabolite, allopregnanolone against PTX-induced seizures, brain lipid peroxidation and DNA fragmentation in male mice. Finasteride, a 5α-reductase inhibitor and indomethacin, an inhibitor of 3∞-hydroxysteroid dehydrogenase were assessed against progesterone's effects on PTX-induced seizures, brain lipid peroxidation and DNA fragmentation. PTX produced clonic–tonic seizures in mice with CD50 and CD97 of 2.4 and 4.0 mg/kg, i.p. respectively. Progesterone significantly countered PTX-induced seizures, with ED50 of 78.30 mg/kg and ED97 of 200 mg/kg. Progesterone antagonized PTX-induced DNA fragmentation. Finasteride (200 mg/kg) and indomethacin (1 mg/kg) reversed the anti-seizure and anti-DNA fragmentation effects of progesterone. Allopregnanolone, also protected against PTX-induced seizures and DNA fragmentation. There was no significant change in the brain lipid peroxidation parameters in any of the treatment groups. It may be concluded that progesterone protects against PTX-induced seizures and DNA fragmentation through its active metabolites allopregnanolone and 5α-pregnan-3,20-dione. However, it appears from the present study that, the neuroprotection with progesterone is primarily on account of allopregnalone. The therapeutic potential of allopregnanolone deserves to be evaluated clinically.

1. Introduction

Picrotoxin (PTX), an alkaloid is obtained from cocculus indicus, the dry fruit of the oriental plant *Anamirta paniculata*. PTX acts as a non-competitive antagonist at a specific site on $GABA_A$ receptor in the brain thus producing CNS disinhibition, leading to seizures having similar functional, biochemical and behavioral changes as is observed in human absence seizure. Picrotoxin as a chemoconvulsant has the advantage over bicuculine in terms of very specific mechanism of action and good solubility in saline. Picrotoxin acts specifically by blocking $GABA_A$ receptor chloride channel (Velisek, 2006). However, the seizure and EEG pattern by picrotoxin resemble those of pentylenetetrazole and bicuculine (Mackenzie et al., 2002). Studies in experimental models suggest that seizure-induced neuronal death have similar biochemical and morphologic characteristics of apoptosis or programmed cell death (Zhang et al., 1998). DNA fragmentation in the brain has been reported following kainic acid-induced seizures in rat (Filipkowski et al., 1994) while recurrent seizures can cause neuronal death by necrosis or apoptosis (Sloviter et al., 1996).

It has been found that female sex hormones especially estrogen and progesterone possess neuro-active properties that can modulate seizures. Progesterone has been reported to depress cerebellar neuronal firing to amino acid neurotransmitters (Smith et al., 1987); to depress cortical interictal epileptiform discharges in the cat (Landgren et al., 1987) and to elevate seizure threshold against kainic acid in female rats (Nicoletti et al., 1985). It is also reported to prevent secondary neuronal loss following traumatic brain injury (He et al., 2003) and also both progesterone and allopregnanolone are reported to decrease mono and oligonucleosomal DNA fragmentation in traumatic brain injury in rats (Djebaili et al., 2005). Further, progesterone is reported to promote recovery following CNS injury (Gibson and Murphy, 2004).

Extensive study of the literature could not reveal any report on the effect of progesterone or its metabolites on PTX-induced seizure and subsequent biochemical and genetic changes, occurring in the brain of animals. Hence, the present study was planned to evaluate the effect of progesterone on PTX-induced seizures, changes in the oxidative stress and DNA in the brain of male mice.

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2. Materials and methods

2.1. Experimental animals

Young adult male Swiss strain albino mice weighing between 20 and 30 g were procured from the central animal house of the institute and maintained under standard laboratory conditions (temperature of 25 ± 1 °C, humidity of 60 ± 2% and 12 h light:dark cycle). Mice had free access to standard chow diet and water and were acclimatized to the laboratory conditions for at least one week prior to experimentation. All the experiments were conducted between 09:00 and 15:00 h. The study protocol was approved by the Institute animal ethics committee prior to initiation and all procedures were carried out in accordance with animal ethical guideline.

2.2. Drugs and chemicals

PTX and allopregnanolone were sourced from Sigma-Aldrich (MO, USA). Gift samples of pure powders of progesterone and indomethacin were obtained from Sun Pharmaceuticals Ltd (Baroda, India) and finasteride was sourced from Dr. Reddy's Laboratories Ltd. (Hyderabad, India) respectively. Tetramethoxyprapane (TMP), NADPH, glutathione reductase, lysis buffer, sodium dedecyl sulphate, proteinase K, agarose, Tris acetate EDTA (TAE), TAE buffer, bromophenol blue and ethidium bromide were sourced from Sigma-Aldrich (MO, USA). All other chemicals and reagents used in the study were of AR (analytical reagent) grade. The doses for progesterone, finasteride and allopregnanolone used in the study were selected from published literature. (Lonsdale and Burnham, 2003; Reddy et al., 2004).

Stock solutions of progesterone, indomethacin and finasteride were made in 30% hydroxypropyl-β-cyclodextrin (β-cyclodextrin) in distilled water. PTX and allopregnanolone were dissolved in normal saline and the solution was made just before use. All drug solutions were injected intraperitoneally (i.p.) to the animals in a volume not exceeding 10 ml/kg. Pretreatment time for vehicle (30% β-cyclodextrin in distilled water), progesterone and allopregnanolone was 30 min while that of finasteride and indomethacin was 1 h prior to PTX administration.

3. Methods

3.1. PTX-induced seizure

The study was undertaken in six groups of mice ($n = 6$ in each group). The first group received normal saline. PTX in the doses of 1, 2, 3, 3.5 and 4 mg/kg was injected to the remaining five groups. The animals were individually placed in perspex cages and observed for 1 h for the recording of time to onset of seizure and its severity. The severity of seizure was scored according to the scale as: 0, no response; 1, ear and facial twitching; 2, one to 20 myoclonic body jerks in 10 min; 3, more than 20 body jerks in 10 min; 4, clonic forelimb convulsions; 5, generalized clonic convulsions with rearing and falling down episodes; 6, generalized convulsions with tonic extension episodes. The response to PTX was considered positive when the seizure score was ≥ 3 (Giorgi et al., 1991). If no seizure was observed in an animal, then the time to onset of seizure was recorded as 60 min. The results for PTX were compared with that of saline (0.2 ml, i.p.) treated control group.

From the above dose–response study, the convulsive dose (CD) 50 and CD 97 (doses that produced positive seizure score in 50% and 97% of animals respectively) of PTX were determined. The CD97 of PTX was found to be 4 mg/kg and was used in subsequent experiments.

3.2. Effect of progesterone on PTX-induced seizure

The study was undertaken in six groups of mice ($n = 6$ in each group). Graded doses of progesterone in the doses of nil (0.2 ml vehicle pretreated), 20, 50, 100, 150 and 200 mg/kg were administered to different groups of mice at 30 min prior to administration of CD97 (4 mg/kg, i.p.) of PTX. The animals were observed for 1 h for any change in the seizure behaviour as described above. The results for the graded doses of progesterone against PTX-induced seizures were compared with that of the vehicle treated group. From the dose–response study, the effective dose (ED) 50 and ED97 of progesterone (doses that protect 50% and 97% of animals respectively against ED97 of PTX) were determined.

3.3. Effect of combined progesterone and finasteride on PTX-induced seizure

Six mice were pretreated with both finasteride (200 mg/kg, pretreatment time of 1 h) and progesterone (200 mg/kg, pretreatment time of 30 min) followed by CD97 of PTX and was observed for 1 h for seizure onset and severity as described above. The results were compared with those of progesterone (200 mg/kg) pretreated animals.

3.4. Effect of indomethacin, allopregnanolone and combined indomethacin and progesterone on PTX inducd seizure

Three groups of mice were pretreated with indomethacin (1 mg/kg), allopregnanolone (10 mg/kg) or indomethacin (1 mg/kg) plus progesterone (200 mg/kg) followed by CD 97 dose of PTX (4 mg/kg) and were observed for 1 h for seizures onset and severity as described above.

3.5. Biochemical parameters

Biochemical parameters were estimated in the whole brain of nine groups of mice ($n = 6$ in each group) namely saline control; vehicle control; PTX (CD97); vehicle + PTX (CD97); progesterone (200 mg/kg) + PTX (CD97); finasteride (200 mg/kg) + progesterone (200 mg/kg) + PTX (CD97); indomethacin (1 mg/kg) + PTX (CD97); indomethacin (1 mg/kg) + progesterone (200 mg/kg) + PTX (CD97); and allopregnanolone (10 mg/kg) + PTX (CD97) treated groups. Under ether anesthesia, the mice were sacrificed by decapitation at 1 h after saline, vehicle or PTX administration. The whole brains were carefully dissected out and placed in ice-chilled Tris–HCl buffer, pH 7.4. Each brain was subjected to the estimation of following biochemical parameters.

3.5.1. Brain protein

Brain protein assay was done according to method of Lowry et al. (1951).

3.5.2. Lipid peroxidation

Tissue lipid peroxidation was evaluated by measurement of thiobarbituric acid-reactive substances. Malondialdehyde (MDA) has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give red light absorbency at 532 nm at an optimum pH 3.5. TMP was used as an external standard and the level of lipid peroxidation was expressed as nmole MDA (mg protein)$^{-1}$ (Ohkawa et al., 1979).

3.5.3. Superoxide dismutase (SOD)

The assay is based on the principle of the inhibitory effect of SOD on reduction of nitroblue tetrazolium (NBT) dye by superoxide anions generated by the photo oxidation of hydroxylamine hydrochloride (NH_2OH. HCL). Percentage inhibition in the rate of NBT reduction was noted at 560 nm. One unit of enzyme was expressed as inverse of the amount of PMS (postmitochondrial supernatant of whole brain

homogenate) protein (mg) required to inhibit the reduction rate of NBT by 50% (Kono, 1978).

3.5.4. Catalase

Activity of catalase was measured by adopting the method of Luck (1963).

The reaction mixture contains 3 ml of 0.66 M phosphate buffer, pH 7.0 and 1.25×10^{-2} M H_2O_2 in the sample cuvette. Reference cuvette contains only 3 ml of 0.66 M phosphate buffer, pH 7.0. The reaction was started by adding PMS of whole brain homogenate to the sample as well as reference cuvettes. The rate of elimination of hydrogen peroxide by catalase was measure by recording the time (in s) required for 0.05 decline of absorbance at 240 nm. Catalase activity in International Units (IU) was calculated by the following formula and was expressed in terms of nmol H_2O_2 consumed min^{-1} $(mg\ protein)^{-1}$.

$$IU = [17/\text{time(s)for 0.05 absorbance change}) \text{ x } (1/\text{weight of homogenate})] \text{ x } 13$$

3.5.5. Glutathione peroxidase (GPx)

GPx was estimated using PMS of whole brain homogenate by the method of Paglia and Valentine (1967). Aliquots of PMS was diluted with 50 mM phosphate buffer at pH 7.4 and were added to a reaction mixture consisting of 5 mM EDTA, 0.01 ml of 1.125 M sodium azide, 0.1 ml of 0.15 M GSH, 2.4 U of glutathione reductase (10 µl) and 0.1 ml of 8.4 mM NADPH to make a final volume of 2.9 ml. Reaction mixture was incubated at 37 °C for 10 min. Reference cuvette contains 100 µl of distilled water instead of PMS. Reaction was started by adding 0.1 ml of 2.2 mM hydrogen peroxide solution in both the test and reference cuvettes. The decrease in absorbance at 340 nm was recorded for 3–4 min. The enzyme activity was expressed in terms of NADPH consumed min^{-1} (mg of protein)$^{-1}$ using extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

3.5.6. DNA fragmentation

The study was undertaken in nine groups of mice ($n=6$ in each group): namely saline control; vehicle control; PTX (CD97); vehicle + PTX (CD97); progesterone (200 mg/kg) + PTX (CD97); finasteride (200 mg/kg) + progesterone (200 mg/kg) + PTX (CD97); indomethacin (1 mg/kg) + PTX (CD97); indomethacin (1 mg/kg) + progesterone (200 mg/kg) + PTX (CD97) and allopregnanolone (10 mg/kg) + PTX (CD97) treated groups. Mice were sacrificed by decapitation under ether anesthesia at 1 h following saline, vehicle or PTX administration. Whole brain was carefully dissected out, placed in ice-chilled container containing normal saline and subjected to DNA fragmentation study (Sambrook and Russel, 2003) as described below.

3.5.7. DNA extraction

DNA was isolated from cortical brain specimens of male mice by phenol–chloroform extraction method. Briefly, 0.1–0.3 g of tissue was suspended in microfuge tube. Added 0.5 ml of lysis buffer containing 1% SDS and 0.01% proteinase K in Tris–EDTA (TE) buffer (pH 8.0). The tissue was homogenized and incubated overnight at 55 ° C. After phenol–chloroform extraction, DNA was precipitated in chilled isopropanol. The pellet containing DNA was air dried for 10–15 min and resuspended in 100 µl TE buffer and incubated at 65 °C for 15 min to dissolve DNA which was then subjected to agarose gel electrophoresis.

3.5.8. Agarose gel electrophoresis

Fifteen µl of extracted DNA in TE buffer and 3 µl of gel loading buffer (6×) was mixed in an eppendorf tube and loaded in each well of the agarose tray containing 0.8% agarose in TAE buffer (1×) mixed with 0.5 µg/ml of ethidium bromide. The electrophoresis was run at 55 mA, 140 V for 1 h. The gel was DNA viewed using a trans-illuminator under ultraviolet light, to identify the typical "ladder pattern" of fragmented DNA specific to apoptosis. The photographs of ethidium bromide stained DNA in the gel were captured using a gel documentation system (Image master, Pharmacia Biotech).

3.6. Statistical analysis

Data for seizure-score, time to onset of seizures and lipid peroxidation parameters were expressed as mean ± SEM. Seizure score data between the groups were compared with non-parametric Kruskall–Wallis test followed by Mann–Whitney *U* test. Data for time to onset of seizures were compared by one-way ANOVA followed by Tukey's test. Lipid peroxidation parameters were compared among groups using one-way ANOVA. Data for percentage of positive responders to PTX-induced seizures and percentage of animals showing DNA fragmentation were compared by Fisher's exact probability test. The CD50 [95% confidence limits (CL)] and CD97 values for PTX and ED50 [95% confidence limits (CL)] and ED97 values for progesterone were determined by log-probit analysis using Litchfield and Wilcoxon (1949) procedure. In all tests, the criterion for statistical significance was $p<0.05$. Statistical analysis was carried out with SPSS version 10.0.

4. Results

4.1. Effect of PTX on seizures (Table 1, Fig. 1)

PTX produced dose-dependent increase in the mean seizure score and percentage of animals showing positive seizure response with concomitant decrease in the time to onset of seizures. However, the statistical significance for mean seizure score and percentage of positive responder were achieved at doses of 3, 3.5 and 4 mg/kg, while that for the seizure onset time, it was achieved at 2 mg/kg and above dose levels of PTX compared to the saline control group (Table 1). From the dose–response relationship for percentage of positive responders, the CD50 (95% CL) and CD97 values for PTX were determined at 2.40 mg/kg (CL 2.10–2.80 mg/kg), i.p. and 3.91 mg/kg, i.p. respectively (Fig. 1).

Table 1
Effect of Picrotoxin (PTX) on seizure score, percentage of + ve responders and time to onset of seizures in mice.

Dose of PTX (mg/kg, i.p.)	Seizure score (mean ± SEM)	Number of + ve responder/total (% of + ve responders)	Time to onset of seizures (min)(mean ± SEM)	CD50 (95% CI) (mg/kg, i.p)
Nil (saline control)	0	0/6 (0)	60.0 ± 0	
1	0.7 ± 1.0	0/6 (0)	43.3 ± 28.9	
2	2.0 ± 1.1	2/6 (33.3)	15.0 ± 1.9***	2.4 (2.1-2.8)
3	2.7 ± 0.5	4/6 (66.7)*	9.0 ± 3.2***	
3.5	3.8 ± 1.2**	5/6 (83.3)*	5.2 ± 1.7***	
4	4.0 ± 0.9**	6/6 (100)***	3.5 ± 0.6***	

$n=6$ in each group; *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compares PTX treated vs. saline control group for the corresponding parameters; CD50 (95% CL) value was obtained from log-probit fits to the data presented in Fig. 1.

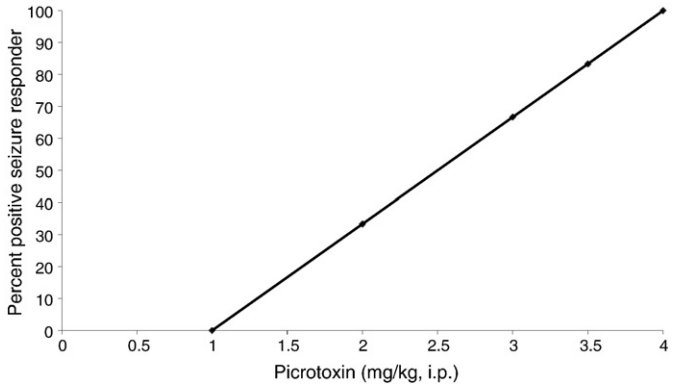

Fig. 1. Dose–response curve for PTX-induced seizures in male, Swiss albino mice. Five doses of PTX were given in the dose range of 1–4 mg/kg, i.p. Positive responder refers to animals having seizure score ≥ 3. Each point represents data from six animals. CD50 (95% confidence limits) value is given in Table 1.

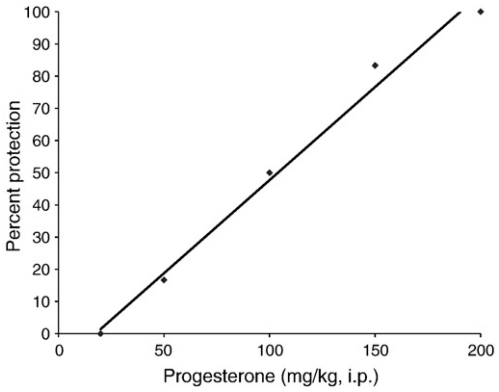

Fig. 2. Dose–response curve for protection against PTX-induced seizures by progesterone in male Swiss albino mice. Progesterone in 30% β-cyclodextrin was administered 30 min before CD97 of PTX (4 mg/kg, i.p.) seizure test. Each point represents data from six animals. ED50 (95% confidence limits) value is given in Table 2.

4.2. Effect of progesterone, allopregnanolone, indomethacin and finasteride on PTX-induced seizures (Table 2, Fig. 2)

Progesterone pretreatment offered dose-dependent protection to the animals against CD97 of PTX-induced seizures. There was progressive decline in the mean seizure score and percentage of animals showing positive seizure score, with concomitant increase in the seizure onset time with increasing doses of progesterone pretreatment. However, significant decrease in the mean seizure score was observed at 200 mg/kg, i.p. dose while significant decrease in the percentage of positive responder was observed at 150 and 200 mg/kg, i.p. doses of progesterone compared to the vehicle treated group of animals. Finasteride pretreatment at 200 mg/kg followed by pretreatment with 200 mg/kg dose of progesterone resulted in significant increase in the mean seizure score and percentage of positive responder with concomitant decrease in the seizure onset time compared to progesterone alone pretreated groups of animals at doses of 150 mg/kg and 200 mg/kg suggesting reversal of the protective efficacy of progesterone against PTX-induced seizures. Indomethacin (1 mg/kg) pretreatment did not show significant difference on any of the seizure parameters compared to the vehicle treated group. However, indomethacin (1 mg/kg) had significantly antagonized the protective effect of progesterone (200 mg/kg) on all seizure parameters. Allopregnanolone (10 mg/kg) significantly countered all seizure parameters compared to vehicle treated group against CD 97 of PTX-induced seizure. The degree of protection offered by allopregnanolone was found to be less as compared to that of progesterone (200 mg/kg) but the difference was not statistically significant (Table 2).

From the dose–response relationship for percentage of protected animals, the ED50 (95% CL) and ED97 values for progesterone were determined at 78.3 mg/kg (CL 65.7–91.0 mg/kg), i.p. and 185.4 mg/kg, i.p. respectively (Fig. 2).

4.3. Lipid peroxidation and antioxidant enzymes

Data for MDA, SOD, CAT and GPx for the nine groups of animals showed no significant difference for any of the parameters amongst the various groups of animals (Table 3).

4.4. DNA fragmentation

Fig. 3A shows the whole brain DNA electrophoresis pattern in six groups of animals. Ladder pattern was not observed in the saline control (3A-A), vehicle control (3A-B) and progesterone + PTX (3A-C). However, ladder pattern was observed in PTX-treated (3A-D), vehicle + PTX (3A-E) and finasteride + PTX + progesterone treated groups (3A-F).

Fig. 3B shows ladder pattern in the whole brain DNA electrophoresis in the PTX (3B-B), PTX + indomethacin (3B-C) and PTX + progesterone + indomethacin (3B-D) treated groups of animals.

Table 2
Effect of progesterone, allopregnanolone, indomethacin and finasteride on picrotoxin (CD97)-induced seizures in mice.

Group	Dose of Progesterone (Prog) (mg/kg, i.p.)	Seizure score (mean ± SEM)	Number of +ve responders/total (% of +ve responders)	Time to onset of seizures (mean ± SEM)	ED50 (95% CI) (mg/kg, i.p.)
1	nil (vehicle treated)	4.0 ± 0.8	6/6 (100)	3.2 ± 0.6	
2	Prog 20	3.8 ± 0.7	6/6 (100)	4.0 ± 1.7	
3	Prog 50	3.0 ± 0.6	5/6 (83.33)	6.3 ± 2.7	78.3 (65.7–91.0)
4	Prog 100	2.0 ± 1.7	3/6 (50)	7.7 ± 25.2	
5	Prog 150	1.5 ± 2.0	1/6 (16.7)***	33.4 ± 28.2*	
6	Prog 200	1.0 ± 1.1***	0/6 (0)***	1.1 ± 20.9**	
7	Prog 200+Finasteride (200 mg/kg)	3.8 ± 1.0$^{\#\#}$	6/6 (100%)$^{\#\#,\$}$	4.0 ± 2.1$^{\#,\$}$	
8.	Indomethacin (1 mg/kg)	4.5 ± 0.6	6/6 (100)	3.8 ± 0.7	
9.	Prog 200 + Indomethacin (1 mg/kg)0	3.1 ± 0.8^{+}	4/6 (66.7)$^{+}$	5.6 ± 0.7^{+}	
10.	Allopregnanolone (10 mg/kg)	2.7 ± 0.8$^{@}$	3/6 (50)$^{@}$	44.8 ± 16.6$^{@}$	

$n = 6$ in each group; PTX CD 97 (4 mg/kg) was administered to all the groups.
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compares group 5 or 6 vs. 1 for the corresponding parameters.
$^{\$}p < 0.05$ compares group 7 vs. 5 for the corresponding parameters.
$^{\#}p < 0.01$, ## $p < 0.001$ compares group 7 vs. 6 for the corresponding parameters.
$^{+}p < 0.05$ compares Sr. no. 9 with Sr no. 6.
$^{@}p < 0.05$ compares Sr. No. 10 with Sr. No. 1.
ED50 (95% CL) value was obtained from log-probit fits to the data presented in Fig. 2.

Table 3
Effect of progesterone, finasteride, indomethacin and allopregnanolone on brain lipid peroxidation parameters in picrotoxin-induced seizure in mice.

Treatment groups	MDA [nmol (mg protein)$^{-1}$]	SOD [unit (mg protein)$^{-1}$]	Catalase [unit (mg protein)$^{-1}$]	GPx [unit (mg protein)$^{-1}$]
Saline control	29.9 ± 5.5	6.8 ± 0.5	1.3 ± 0.3	6.4 ± 0.6
Vehicle control	29.7 ± 5.5	6.7 ± 0.5	1.2 ± 0.2	6.2 ± 0.7
PTX	35.8 ± 5.6	6.4 ± 0.2	1.2 ± 0.2	5.9 ± 0.3
Vehicle + PTX	36.2 ± 5.8	6.5 ± 0.3	1.2 ± 0.2	5.9 ± 0.4
Prog (200 mg/kg) + PTX	32.8 ± 5.3	6.8 ± 0.6	1.4 ± 0.3	6.1 ± 0.7
Finasteride (200 mg/kg) + Prog (200 mg/kg) + PTX	29.7 ± 5.4	6.5 ± 0.6	1.1 ± 0.3	5.7 ± 0.5
Indomethacin (1 mg/kg) + PTX	24.9 ± 3.6	5.5 ± 0.6	1.4 ± 0.2	5.7 ± 0.7
Indomethacin (1 mg/kg) + Prog (200 mg/kg) + PTX	26.7 ± 8.0	6.0 ± 1.2	1.7 ± 0.4	6.9 ± 0.7
Allopregnanolone (10 mg/kg) + PTX	30.2 ± 7.7	6.4 ± 0.7	1.7 ± 0.3	7.1 ± 0.3

$n = 6$ in each group; MDA; malondialdehyde, SOD; superoxide dismutase; GPx; glutathione peroxidase.
Dose of PTX was 4 mg/kg (CD 97).
Vehicle, progesterone and allopregnanolone pretreatment time was 30 min; finasteride and indomethacin pretreatmaent time was 1 h.
The vehicle for both progesterone and finasteride was 30% β-cyclodextrin in distilled waterValues are expressed as mean ± SEM.

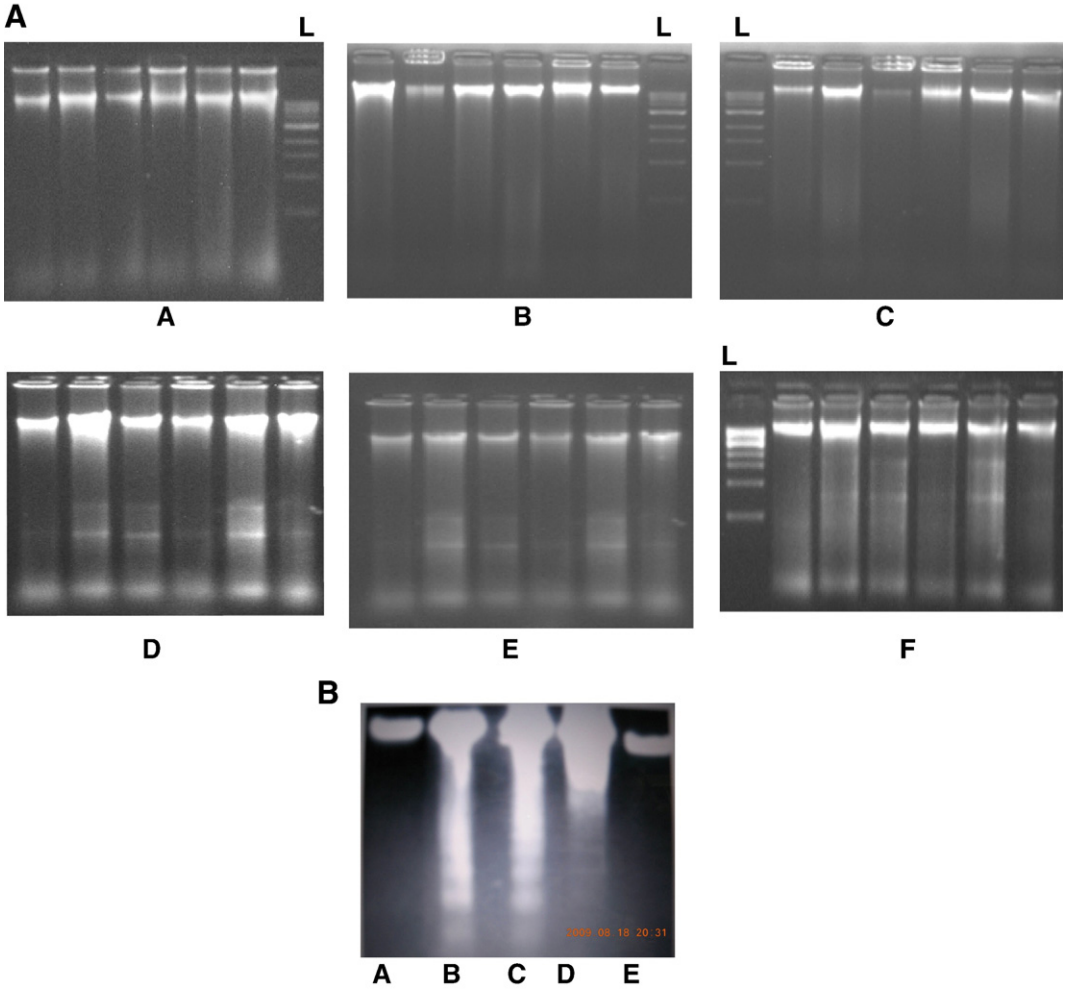

Fig. 3. A. Photograph of whole moue brain DNA electrophoretic pattern in 0.8% agarose gel in six groups of mice ($n = 6$ in each group). Ladder pattern suggestive of DNA fragmentation was observed in groups D, E and F while no such was seen in groups A, B and C. A: saline control group; B: Vehicle control group; C: progesterone (200 mg/kg) + PTX (4 mg/kg) group; D: PTX (4 mg/kg) group; E: vehicle + PTX (4 mg/kg) group; and F: finasteride (200 mg/kg) + progesterone (200 mg/kg) + PTX (4 mg/kg) group. B. Photograph of mouse whole brain DNA electrophoretic pattern in 0.8% agarose gel in five groups of mice ($n = 6$ in each group, however representative data from one mouse per group has been shown in the figure). Ladder pattern suggestive of DNA fragmentation was observed in groups B, C and D, while no such pattern was seen in groups A and E. (A: saline control group; B: PTX (4 mg/kg) group; C: PTX (4 mg/kg) + indomethacin group; D: PTX (4 mg/kg) + progesterone (200 mg/kg) + indomethacin (1 mg/kg). E: PTX (4 mg/kg) + allopregnanolone (10 mg/kg) group).

However, no ladder pattern was observed in the saline control (3B-A) and PTX + allopregnanolone (3B-E) treated groups of animals.

4. Discussion

The present study highlighted the anticonvulsant activity of progesterone in PTX-induced seizure model in male mice. Progesterone also countered PTX-induced DNA fragmentation. The protective effect of progesterone, on both the counts i.e. seizures and DNA fragmentation were reversed by finasteride and indomethacin. There are no reports available in the literature on the effects of progesterone against PTX-induced seizures and DNA fragmentation in the brain of male mice. Progesterone is synthesized in the adipose tissue and adrenal cortex of male mice. The hormone is present in the blood in the male, albeit in lower concentrations and does not show cyclical changes as observed in the female animals. Further progesterone is also present in the brain and functions as a neurosteroid in both the genders (Ibanez et al., 2003). Progesterone exerts both genomic and non-genomic effects on neuronal activity in the central nervous system (Leonhardt et al., 2003).The genomic actions of progesterone are mediated by progesterone receptors (PR), a member of the nuclear receptor superfamily and transcription factors, that exist in two isoforms, PR-A and PR-B (Guerra-Araiza et al., 2003). Both of the isoforms of PR have been documented in male, rodent brain (Auger and De Vries, 2002). Unlike several genomic effects of progesterone in discrete regions of female brain to regulate ovulation, pregnancy and other aspects of reproduction (Clarke and Sutherland, 1990), progesterone is also reported to affect CNS function independent of the PR through its reduced metabolites, 5α-pregnan-3,20-dione and 3α-hydroxy-5α-pregnan-20-one (allopregnanolone; Pistritto et al., 2009). The metabolites act as positive modulators of $GABA_A$ receptors at both synaptic and extrasynaptic sites thereby augmenting inhibitory chloride conductance, resulting in decreased neuronal excitability (Lambert et al., 2003; Rogawski and Reddy, 2004; Rhodes and Frye, 2005). PTX, on the other hand, induces seizures by acting as a non-competitive antagonist at $GABA_A$ receptor. It binds to specific PTX site on chloride channel of the GABA/benzodiazepine/chloride ionophore receptor complex and blocks GABA mediated chloride conductance thus causing disinhibition of neurons (Velisek, 2006).

In the present study, both finasteride and indomethacin antagonized the anti-seizure and neuroprotective effects of exogenously administered progesterone. Finasteride acts by inhibiting the enzyme 5α-reductase (Ciriza et al., 2004) which catalyse the rate limiting step in the conversion of progesterone to its metabolite 5-α-pregnane-3,20-dione while indomethacin inhibits the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD) and thereby blocks the conversion of 5-α-pregnane-3,20-dione to allopregnanolone. Thus, finasteride pretreatment is associated with significant reduction in the brain and circulating levels of both the metabolites while indomethacin pretreatment is associated with reduction of allopregnanolone levels (Brinton et al., 2008). Finasteride *per se*, does not influence seizure susceptibility (Kokate et al., 1999). In the present study, it was observed that indomethacin *per se* did not influence the seizure susceptibility. Thus, the observations support the fact that the neuroprotective effect of progesterone in the PTX model depends on its conversion to its metabolites i.e. 5-α-pregnane-3,20-dione and allopregnanolone. Both progesterone and allopregnanolone significantly blocked PTX-induced seizures but the seizure score was less in progesterone treated animals as compared to that of allopregnanolone treated ones suggesting the protective role of the intermediate 5-α-pregnane-3,20-dione metabolite. This was further supported by the fact that the seizure score obtained by combined finasteride and progesterone pretreatment was more as compared to that of combined progesterone and indomethacin pretreated animals.

It was earlier reported that kainate-induced excitotoxicity results in neurodegeneration (Hoffman et al., 2003); apoptotic cell loss in hippocampal neurons (Pollard et al., 1994) and DNA fragmentation (Filipkowski et al., 1994) in rat brain. Internucleosomal DNA fragmentation is considered to be the biochemical hallmark of apoptosis (Willingham, 1999). In the present study, PTX-induced excitotoxicity resulted in ladder pattern on agarose gel electrophoresis of DNA extracted from mice brain suggestive of DNA fragmentation. There are no reports in the published literature on the effect of PTX on the viability of mouse brain DNA. The precise mechanism for PTX-induced DNA damage is unclear.

It was observed in the present study that, PTX had no significant effect on lipid peroxidation or changes in the antioxidant enzyme system, thus, ruling out the possibility of oxyfree radical-induced DNA damage. However, it was reported earlier, that chronic treatment with PTX for 20 days resulted in significant changes in the activity of free lysosomal enzymes in the brain namely acid phosphatase, cathepsin-D, RNAse II and DNAse II (Acharya et al., 2005). Elevated cathepsin-D expression was reported in kainate-evoked rat brain neurodegeneration (Hetman et al., 1995). Further studies are necessary to elucidate a possible linkage between PTX-induced DNA damage and neurodegeneration to changes in lysosomal enzyme activity. Protective efficacy of progesterone against PTX-induced DNA damage was antagonized by both finasteride and indomethacin further suggesting that the protective effect of progesterone is mediated through its metabolites. However, further studies are necessary to elucidate the relative contribution of the two metabolites in the neuroprotective effect of progesterone.

The present study highlights two distinct non-genomic effects of progesterone i.e. anti-seizure and anti-DNA fragmentation in PTX-induced seizure model in male mice. Both the effects are independent of cytosolic PR binding of the hormone and its consequent genomic events since, the effects of progesterone were rapid in onset. It was recently reported that progesterone offers neuroprotection in an ischemic model in mice (Aggarwal et al., 2008). From the evidences provided in the study, it would be worthwhile to evaluate progesterone and its major active metabolite allopregnanolone in the clinical settings of seizures and cerebral ischemia as a possible neuroprotective agent, since the neurosteroids have advantages of low levels of toxicity, easy to administer in emergency situations and possess long lasting effects (Djebaili et al., 2005).

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