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**Title:** Valproic Acid (VPA) Disrupts Maternal Behavior and Induces Cannibalism: An  
Unexplored Insight

**Running Title:** VPA Evokes Cannibalism in Rat Dams

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To appear in: **Basic and Clinical Neuroscience**

**Received date:** 2022/06/30

**Revised date:** 2022/08/04

**Accepted date:** 2022/08/09

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**Please cite this article as:**

Jain, A., Dhir, N., Singh, H., Sharma, A. R., Medhi, B., Prakash, A. (In Press). Valproic Acid (VPA) Disrupts Maternal Behavior and Induces Cannibalism: An Unexplored Insight. *Basic and Clinical Neuroscience*. Just Accepted publication Aug. 15, 2022. Doi: <http://dx.doi.org/10.32598/bcn.2022.4410.1>

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## Abstract

**Background:** Valproic acid is the most widely used chemical to develop the preclinical model of autism spectrum disorder (ASD). However, in addition to autism, it has different teratogenic effects like teeth malformation, tail kink, and abnormal body growth in offspring. However, no study has explored VPA-induced maternal behavior, miscarriage, and maternal Cannibalism. We aim to determine the cannibalistic effects of VPA in pregnant female Wistar rats and VPA's influence to cause miscarriage frequency.

**Methods:** Our study used pregnant Wistar rats. On GD 12.5, they were treated with VPA at 600 mg/kg i.p., dissolved in saline at 250 mg/ml concentration. The observations were mean litter size, mean male/female pups, mean mortality, maternal Cannibalism, mean pups alive, Cannibalism of malformed pups, miscarriage, survival analysis of pups, and odds and risk ratio were calculated for deaths observed in both (control & VPA-treated) the groups. The study was conducted till the weaning period.

**Results:** VPA-exposed pregnant females portrayed significantly decreased litter size ( $p < 0.0001$ ), significantly higher cannibalistic behavior ( $p = 0.0023$ ), and significantly higher cannibalism of malformed pups ( $p = 0.0484$ ) than the control group. VPA had caused complete pregnancy loss (miscarriage) in pregnant females ( $n = 5$ ). Moreover, the VPA group's mortality percentage ( $p = 0.0019$ ) was significantly higher than the control group.

**Conclusion:** Overall, VPA has marked teratogenic effects (anatomical and morphological changes in offspring) with maternal behavior disruption, which causes Cannibalism in Wistar female rats. The current manuscript findings can aid in investigating the novel mechanisms involved in maternal behavior disruption during the development of the VPA autism model.

**Keywords:** Cannibalism, Maternal behavior, Miscarriage, Wistar rat dams, Valproic acid (VPA).

## Highlights

- Effect of VPA on maternal Cannibalism in Wistar rats
- Effect of VPA on maternal Cannibalism of malformed litter
- Effect of VPA on Wistar rats' pregnancy.

Teratogenicity and Maternal behavior disruption ability of VPA at 600 mg/kg, i.p.

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## 1. Introduction

Autism spectrum disorder, which leads to abnormal neuro-developmental manifestation in affected individuals, requires various preclinical and clinical studies to understand the disease pathology and discover a new drug to manage the symptoms.(Al Dera & Pharmacotherapy, 2022; Gileadi et al., 2021; Jain et al., 2019; Rylaarsdam & Guemez-Gamboa, 2019; Wang & Doering, 2015; Won et al., 2013) The ASD model can be developed with different chemicals like propionic acid, 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), chlorpyrifos, Polyinosinic: polycytidylic acid (poly I: C), and valproic acid (VPA).(Guo et al., 2018; Lan et al., 2017; MacFabe et al., 2007; Reisinger et al., 2015; Schneider & Przewłocki, 2005) Among these, VPA is predominantly used due to its action by inhibiting histone deacetylase and thereby causing neural tube defects.(Kawanai et al., 2016) VPA is an antiepileptic agent with varied adverse effects in the pregnant population.(Alsdorf & Wyszynski, 2005) VPA causes behavioral abnormalities, delays neuronal development, and alters various gene expressions, leading to autism.(Kawanai et al., 2016) In pregnant women, following VPA's oral administration, it becomes readily available in the intestine. It crosses the placental barrier from the apical (maternal interfacing) syncytiotrophoblast plasma membrane to the circulation of basal (foetal facing). As a result, VPA accumulates, and the concentration is three-fold in the embryonic circulation resulting in teratogenic effects.(Lloyd, 2013; Vajda, 2012) The teratogenic effects are cleft lip and palate, fetal valproate syndrome, genitourinary defects, autism, etc.(Alsdorf & Wyszynski, 2005) However, apart from its teratogenic effects, the effect of VPA on the mother has not been established to date.(Lloyd, 2013)

VPA can disrupt maternal behavior during pregnancy or post-birth. VPA's potential to cause autism in rats was first reported by Schneider *et al.*, in 2004.(Schneider & Przewłocki, 2005) However, VPA's mechanism for causing autism is unclear. Nevertheless, a study hypothesises that VPA inhibits histone deacetylase post-administration in rodents.(Kawanai et al., 2016) VPA causes teratogenic effects in rodents and causes teeth malformation, tail kink, delayed/abnormal growth, autism etc.(Ruhela et al., 2017)

The current study was an observation during the development and validation of an experimental model of VPA in Wistar rats. Apart from teratogenicity, the behavioral alterations among the dams are still unexplored and under-reported in the VPA model. Cannibalism is a behavioral abnormality common among animals but more evident in laboratory rodents.(Lane-Petter, 1968) Cannibalism is massacring and ingesting an organism's specific part or

whole.(Fouilloux et al., 2019) Various reasons which could lead to cannibalistic activity among the rodents are human handling, malnutrition, infant care, environmental conditions, noise decibels, home cage conditions, and other factors.(Lane-Petter, 1968)

Additionally, specific drugs administered during pregnancy can induce cannibalism post-birth.(J. L. Schardein et al., 1978) Even though much research focuses on different types of autism models, it is necessary to report the VPA's cannibalistic effects in rodent models. The effect of VPA in inducing maternal cannibalism has not yet been fully established. VPA can cause total loss of pregnancy/miscarriage in rodents. The extent of the case is that the total number of pups cannibalised by dams is unreported. Hence, the observational study aims to estimate maternal cannibalistic behavior post-VPA induced ASD model development and validation in Wistar rats. Additionally, VPA's potency to cause miscarriage (total/complete loss of pregnancy) in pregnant Wistar rats is also reported in this manuscript.

## **2. Materials and Methods**

### **2.1 Animals & Housing conditions**

Male and non-pregnant female Wistar rats around 8-10 weeks of age, weighing 180 to 280 gm, were used in this study. Animals (3-4) were randomly housed in individually ventilated propylene cages (30 x 18 cm) under a 12-hour light: dark cycle. The temperature and humidity were maintained at  $21\pm 2^{\circ}\text{C}$  and  $55\pm 2\%$ . They had access to food and water *ad libitum* throughout the experiment. The hygiene conditions of the animal vivarium were maintained according to the CPCSEA guidelines. The experiments were conducted in the experimental pharmacology laboratory (EPL), neurobehavioral research laboratory (NBRL), PGIMER, Chandigarh, India. All procedures were conducted according to ARRIVE guidelines.(Percie du Sert et al., 2020)

### **2.2 Acclimatization & mating**

All animals were acclimatised for a week. All-female rats (25) were observed for the estrus phase and allowed to mate in a 1:3 ratio (Figure I).

### **2.3 Blinding & Randomization**

The female Wistar rats post copulation was randomised into two groups (control & VPA). On GD 12.5, both groups received their respective injectables (Figure I). Blinded investigators performed the treatment, behavioral observation, and data analysis in all analysis steps.

## **2.4 Pregnancy determination**

Initially, the female rat was handheld by an experimenter, and another individual using a 200  $\mu$ l pipette, drew inside-out in the female rat's vagina about 60-100  $\mu$ l of 0.9% saline. The vaginal fluid (vaginal smear) with 0.9% saline was placed on the glass slide and observed under the microscope at 40x magnification. If the smear depicted both nucleated epithelial & cornified cells (estrus phase) with spermatozoa (sperm cells), the pregnancy was confirmed as the gestational day (Elnahas et al.) 0.5/1 day varied from animal to animal (Figure I, II).(Marcondes et al., 2002)

## **2.5 VPA administration**

Post-pregnancy female Wistar rats (n=10) at GD 12.5 days received Valproic acid sodium salt (CAS: 1069-66-5) at 600 mg/kg, i.p., dissolved in 0.9% saline. The volume was prepared as 250 mg/ml. Similarly, control dams (n=10) received an equal volume of saline at GD 12.5 days.(Ruhela et al., 2017) Administration and route of VPA at 600 mg/kg, i.p., was chosen as it has shown to cause higher significance of ASD symptoms than other doses (300mg, 400mg, 500mg/kg).(Chaliha et al., 2020) The Wistar female rats (n=5) were excluded from the cannibalism observation as they underwent miscarriage (complete loss of pregnancy) post-VPA administration.

## **2.6 Weight observation:**

The Weight of all pregnant female rats was observed from day 1 of copulation till day 20. Post-VPA administration rats with weight reduction were observed until GD day 20 for any delivery of offspring. If no pups were delivered, then they were excluded from the study.

## **2.7 Miscarriage observation**

Miscarriage was considered when there was a complete loss of pregnancy. All females post-mating and post-VPA administration were examined for any miscarriage. Miscarriage was confirmed by substantial weight reduction, and no pups were delivered till GD 21-23. Furthermore, blind investigators held the animal post-VPA administration and palpated the rat's abdomen to check for any pup's sensation. The investigation was performed only in animals undergoing immediate weight loss post-VPA administration.

## **2.8 Total pups' delivery observation**

The no. of pups delivered to each parental female rat was observed. All delivered pups were housed with their respective maternal, and the total count of male/female pups was also observed during the study.

## **2.9 Mortality observation**

All delivered pups were observed for mortality (death by Cannibalism or death by birth or death post-birth) until PND 21.

## **3.0 Natural Death**

The death of all delivered pups, apart from maternal Cannibalism, was considered a natural death (death on delivery/death post-delivery but not cannibalised).

## **3.1 Mortality percentage**

The mortality (including cannibalism and natural death) percentage was calculated as (No. of animals died/Total no. of delivered pups) \*100.

## **3.2 Cannibalism observation**

Dams were observed for cannibalistic behavior until weaning (PND 21). During the late gestation period, we increased our visits (06:00 to 22.30 hrs at every 15 min intervals) to the animal house (central unit for animals in PGIMER) to observe the delivery, litter count respective to each dam, male/female ratio, aggressive behavior (Davis & Psychology, 1933), and cannibalistic behavior of the maternal. No video recordings were done during this study. We tried to minimise human handling during the delivery and post-delivery. Only one maternal cannibalisation (VPA-treated) was recorded with a video camera, and the representative image adheres in this manuscript.

## **3.3 Cannibalism observation on malformed pups'**

Maternal Cannibalism was observed depending on the pups' malformation. Post-delivery malformation was immediately observed.

## **3.4 Timeline of the experiment**

Figure I experimental setup and timeline. Female rats were mated with male rats (1:3 ratio). After microscopic examination, the vaginal plug was confirmed as GD 0.5/1 day. On GD 12.5, VPA was administered to pregnant Wistar rats at 600 mg/kg i.p., and control pregnant rats

received an equal volume of saline. Until GD 21, miscarriage (complete pregnancy loss) was observed, and miscarriage females were excluded. The first day of pups' delivery was considered as PND 1. A female rat cannibalising its pup was video recorded with a smartphone. All study observations were carried out until weaning, and post-completion data analysis was performed.

### **3. Statistical analysis**

GraphPad Prism (v 9) was used for data analysis. Numerical data were expressed as the standard error of the mean (mean  $\pm$  SEM/SEp). Student t-test or t-test with Welch's correction was conducted for parametric observations to compare two groups/within-group means. The Mann-Whitney test was conducted for non-parametric data. Two-sided Fisher's exact test, odds ratio, and relative risk were calculated for pup's death/cannibalised in both control and VPA groups.

Additionally, Fisher's exact test was used for miscarriage analysis. The relative risk of 95% CI was calculated using Koopman asymptomatic score, and the odds ratio of 95% CI was calculated using the Baptista-Pike method. Survival analysis was performed using the Mantel-Cox test. The Kaplan-Meier curve depicted the results and calculated the day-wise mortality/cannibalisation for both control and VPA groups. The Standard p-value  $<0.05$  was considered statistically significant.

### **4. Results**

#### **4.2 Pregnancy determination/Vaginal plug observation**

All-female rats were observed for the presence of sperm cells in the vagina. As shown in Figure II, a) proestrus phase, b) estrus phase, c) metestrus phase, d) diestrus phase and e) confirmation of sperm cells in the vaginal plug. Pregnancy can be confirmed only during the estrus phase (epithelial and cornified cells with spermatozoa – sperm cells), as shown in Figure II e.

#### **4.3 Weight observation**

All females' body weight post-copulation was monitored from GD 1 to GD 20 (Figure III). No significant differences were observed between VPA and control groups.

#### **4.4 Effect of VPA on miscarriage**

We examined the effect of VPA on miscarriage from GD 1 to GD 21 by weight reduction. Prenatally injected VPA pregnant female (33.33%) rats were subjected to miscarriage. No miscarriage was observed in the control groups

#### **4.5 VPA-treated rats exhibited reduced litter size**

To observe the effect of VPA on litter size, the VPA treated and control dams' litter size was calculated. Table III shows that VPA-treated female dams delivered reduced litter size ( $p < 0.0001$ ) compared to their female control counterparts, and the data were statistically significant. We also observed the birth rate of male and female pups in control and prenatally VPA-treated pups. Table III shows that VPA-treated dams delivered significantly reduced male ( $p = 0.0025$ )/female pups ( $p < 0.0001$ ) compared to control dams.

#### **4.6 Prenatal VPA-treated pups showed a substantial increase in mortality**

We observed that no. of mortality significantly increased in the prenatally VPA-treated pups ( $p = 0.0077$ ) compared to control pups (Table III). Moreover, no significant difference was observed in natural death among the prenatal VPA-treated and control pups. However, the mortality percentage increased significantly in the prenatally VPA-treated ( $p = 0.0019$ ) group compared to the control pups (Table III).

#### **4.7 VPA-treated maternal rats manifested increased cannibalistic behavior**

We examined the cannibalistic behavior under the influence of VPA postnatally. As illustrated in Figure I, Table III, VPA-treated maternal rats ( $p = 0.0023$ ) exhibited significantly increased acts of Cannibalism in comparison to control females. Similarly, we examined the effect of maternal Cannibalism in the VPA-treated group on healthy vs malformed pups. Table III shows that malformed pups ( $p = 0.0484$ ) were subjected to significantly increased Cannibalism by maternal females compared to healthy pups.

#### **4.8 VPA depicted significant differences in alive pups**

Prenatal VPA dams ( $p < 0.0001$ ) had significantly reduced lively pups (Table III) compared to control.

#### **4.9 VPA causes malformation in offspring**

Prenatal exposure to VPA showed different malformations (tail kink, paw malformation and short tail) post-birth (Table I).

#### **5.0 Survival analysis**

Survival analysis showed that prenatal administration of VPA ( $p=0.0288$ ) causes decreased litter survival rate compared to control pups (Figure IV a, Table II & III). The difference was found to be statistically significant. These results indicate that prenatal VPA decreases the survival rate of delivered pups either by Cannibalism or by natural death.

#### **5.1 The risk associated with Prenatal VPA administration**

The outcome of events (death) was calculated. Fisher's exact test revealed that the mortality associated with prenatal VPA pups ( $p<0.0001$ ) was much more significant than control pups (Figure IV b, Table II, & Table III).

### **5. Discussion**

The current study is the first to explore the effect of VPA on maternal Cannibalism and observe male/female and malformed litter cannibalism.

Different studies have reported rodent cannibalism, and some have reported it for possible reasons.(Abel & biology, 1979; Buntin et al., 1984) Additionally, Lane-Petter W in 1968 reported that Cannibalism is more common in rats and mice. This same study stated that genetic factors might contribute to this cannibalistic behavior.(Lane-Petter, 1968) Schardein and colleagues 1978 reported that maternal rats treated with a teratogen during gestation exhibited cannibalistic behavior. Moreover, these maternal rats cannibalise malformed pups rather than normal pups.(J. Schardein et al., 1978) Corresponding to this, D.F. Wyszynski *et al.*, observed that human pregnant females exposed to valproate cause an increased risk of malformations in their offspring.(Wyszynski et al., 2005)

On the other hand, analogous to the previous study, Ruhela RK *et al.*, 2017, observed anatomical malformations in prenatally VPA-administered Wistar rats.(Ruhela et al., 2017) Compared to the above studies, our findings reported similar observations that female rats cannibalise malformed pups compared to healthy pups (Figure I, Table III). However, a study has reported that the chemical modification of VPA can prevent malformation in the murine model. Okada A *et al.*, have reported the teratogenic effects of VPA in clinical aspects and

different preclinical models.(Jazayeri et al., 2020) Besides, no information was mentioned about Cannibalism.(Okada et al., 2004)

Cannibalism can be caused due to the disruption in maternal behavior. Gaffori O and M Le Moal, 1979 stated that thiamine induces disruption in normal maternal behavior and causes spontaneous abortion in pregnant female rats.(Ba, 2013) Another study observed that ventral mesencephalic tegmentum lesions cause disruption in maternal behavior, which results in Cannibalism. The study also observed that the maternal did not nurse their litter.(Gaffori & Le Moal, 1979)

Additionally, Perez-Laso *et al.*, (2008) investigated that olfactory bulbectomy causes maternal behavior disruption, which is relatable to previous findings.(Schwartz et al., 1976) Apart from this, another study stated that during gestation, if female rats are exposed to extreme environmental stress conditions can cause maternal behavior disruption.(Pérez-Laso et al., 2008) Libbin and Person 1979 portrayed that Cannibalism can be avoided by minimal human handling, limiting the change of beddings, and constantly acclimatising the pregnant rats to human touch during the gestation period.(Libbin & Person, 1979) Another study stated that Cannibalism could be prevented if maternal rats had not fasted and freely accessible to food and water during gestation.(Fox & systematics, 1975) However, our results were contradictory as we did not abstain from food and water for any female rats during gestation. However, it still resulted in Cannibalism (Figure I, Table III). Serrano *et al.*, (1991) explained the prevention of Cannibalism by performing cesarean sectioning in pregnant rats on GD 21.(Serrano et al., 1991) Helander and Bergh (1980) investigated the prevention of Cannibalism post-neonatal surgery. They described that litter post-surgeries must be cleaned for blood spots or clots and suture off open wounds. It has prevented Cannibalism successfully.(Helander & Bergh, 1980)

Mohan C 1974, observed the effect of starvation on age-dependent Cannibalism at Bangalore university. His findings were that at 3-3.9 months, female rats delivered a mean litter size ( $11.3 \pm 1.40$ ).(Mohan, 1974) However, our mean litter size ( $16.50 \pm 0.7782$ ) was significantly higher as there was no starvation (Table III). Nevertheless, we observed lesser Cannibalism in our control group, contrasting the above study. The probable reason for lesser Cannibalism is because no VPA was administered in the control rats. Jullie and colleagues have observed infanticide in rats. They stated that females synthesise low volatility chemo-signal during pregnancy, protecting males from cannibalising the delivered pups.(Helander & Bergh, 1980)

Another study noted that infanticide could be due to genetic, developmental, and hormonal imbalances.(Svare et al., 1981) Discordant to the above study, we separated males and females post copulation, so we did not observe any of these findings.

Porter G, 1968 reported a high chance of pre-weaning loss in laboratory animals. They observed the benefits of separating the mother and its litter or placing individual mothers in a separate cage. It resulted in preventing Cannibalism.(Porter, 1968) Supporting this study, Darlene and colleagues observed that the pre-weaning loss was around 33%, depending upon the intervention. However, they also observed that their maternal provided survival pups with good care and nursing.(DeSantis & Schmaltz, 1984) Our findings were similar to the above study; the survived pups were provided adequate care and nursing regardless of malformation or healthy pups in control and prenatally VPA treated groups (Table II – no. of alive pups).

K Komariah and colleagues observed the teratogenic effect of VPA at 250 mg/kg p.o (Abel & biology) on GD 10, 13, and 16 in Sprague Dawley rats. They reported that administering VPA on GD 10 and 13 showed decreased mean litter size compared to GD 16. In addition, they also observed birth weight, body weight, and growth rate, and the data were statistically significant.(Komariah et al.) Another study examined the effects of VPA at different doses at 0, 150, 200, 300, 400, and 600 mg/kg p.o. on GD 7-18. This study concluded that 600 mg/kg was significantly toxic and resulted in pups' mortality.(Vorhees, 1987) Our study had different observations as the route of administration was i.p., (intraperitoneal) on GD 12.5 at a 600 mg/kg dose. Our study reported reduced litter size (Table III) and a higher incidence of mortality (Table III) in the prenatally VPA-treated group.

Evidently, to the best of our knowledge, no study has dug deep into concepts of Cannibalism associated with VPA exposure in preclinical aspects. The study is the first to report the potential of VPA to cause maternal behavior disruption, thereby causing aggressive behavior towards cage-mates and its litter. Finally, miscarriage and Cannibalism are significant drawbacks in the preclinical model. We have reported the litter size, VPA effect on mortality, maternal cannibalistic behavior, average pups' survival, the difference among maternal cannibalistic behavior on malformed vs healthy pups, amount of miscarriage, and survival analysis of pups. In addition, we have reported the odds and risk ratios and percent of deaths associated with VPA post-pregnancy.

## 6. Conclusion

To conclude, valproic acid at a dose of 600 mg/kg i.p., on GD 12.5 has a marked cannibalistic effect on pregnant rat dams' post-parturition. Apart from this, VPA significantly reduced the litter size, increased Cannibalism of malformed pups, decreased pups' survival rate, and increased the chances of miscarriage upon administration. The mechanism behind Cannibalism could be the disruption of maternal behavior. The cause might be histone deacetylase (HDAC) inhibition, resulting in downregulation of the NF $\kappa$ B signaling pathway. Besides, serotonin and GABA have played a significant role in the aggressive behavior of the female rat. The rationale behind maternal behavior disruption and Cannibalism is yet to be explored.

Moreover, the relationship between Cannibalism and maternal behavior disruption is still an enigma. The current study raises some queries which could be explored shortly. Is the VPA preclinical model a gold standard model for ASD? Does it possess face validity, construct validity, and predictive validity? *Perse*, the VPA model, is the most widely used model for ASD. Still, many insights are yet to be scrutinised as many observations are unreported.

## 7. Summary

The current study primarily focused on the teratogenic effects of VPA in an experimental model and described the maternal behavior disruption associated with VPA. The maternal behavior disruption has resulted in maternal Cannibalism of offspring. The survival rate of offspring has been an issue in our study with the VPA model. Apart from this, VPA significantly impacted mean litter size compared to control rats. VPA has advantages but with many side effects too. However, it is widely used to develop the preclinical model of ASD.

## 8. Translational outcome

Valproic acid has been a significant concern for pregnant females resistant to other anti-epileptic drugs. The current study outcome highlights that VPA is responsible for inducing maternal behavior disruption in rodents when administered during gestation. Similarly, few case reports depict patients' abnormal behavior/irritability/agitation due to valproic acid administration. Thus, VPA should be the least preferred drug or should not be used in pregnant females suffering from seizures as it might be responsible for causing aggressive behavior in such cases. However, the current study is a pilot and needs to be explored in a large sample size.

## **9. Limitations**

Some studies have reported the role of neurotransmitters (serotonin, dopamine, GABA) in male/female behavior disruption or aggressive behavior associated with neurotransmitters. However, we are currently delineating the levels of different neurotransmitters in the maternal rat brain on molecular aspects. It may provide novel insights behind Cannibalism.

## **Acknowledgement**

The corresponding author acknowledges the Indian Council of Medical Research (ICMR) for providing manpower (Ashish Jain). The authors acknowledge the Postgraduate Institute of Medical Education and Research (PGIMER) for providing infrastructures and laboratory facilities within the institute.

## **Author contributions**

AJ: conception, data extraction and validation, analysis, initial draft, manuscript review, and approval. HS: data analysis and validation, manuscript review, and approval. ND: manuscript review and approval. ARS: manuscript review and approval. BM: important intellectual content, manuscript review, and approval. AP: conception, data validation, important intellectual content, manuscript review, and approval.

## **Disclosures**

## **Funding**

Indian Council of Medical Research (ICMR-SRF) provided a Senior Research Fellowship (SRF) to Ashish Jain via letter no: 45/45/2019-PHA/BMS, dated: 23/07/2019.

## **Conflict of interest**

Ashish Jain, Neha Dhir, Harvinder Singh, Amit Raj Sharma, Bikash Medhi, and Ajay Prakash declare no conflict of interest.

## **Data availability statement**

No additional data is required as everything adheres to this manuscript. However, raw data will be available from the corresponding author with valid justification.

## **Ethical care statement**

All animals used in this study were provided proper care in compliance with ARRIVE guidelines.

## **Ethical approval**

The Institutional ethics committee (IAEC), PGIMER, Chandigarh, India, approved all experiments (IAEC no: 106/IAEC/727, dated: 4/02/2020). The central small animal research facility, PGIMER, Chandigarh, provided the animals used in this study (approved by CPCSEA – Reg. No.: 47/GO/Re-SL/Bi-S/99/CPCSEA, Dated: 31/05/2016).

## **Abbreviations**

AED – Anti-epileptic drug

ASD – Autism Spectrum Disorder

CI – Confidence Interval

EPL – Experimental Pharmacology Laboratory

GABA – Gamma-aminobutyric acid.

GD – Gestation Day

HDAC – Histone Deacetylase

IAEC – Institutional Animal Ethics Committee

NBRL – Neurobehavioral Research Laboratory

PGIMER – Postgraduate Institute of Medical Education & Research

PND – Post-natal day

SEM – Standard error of the mean

SEp – Standard error of a proportion

VPA – Valproic acid

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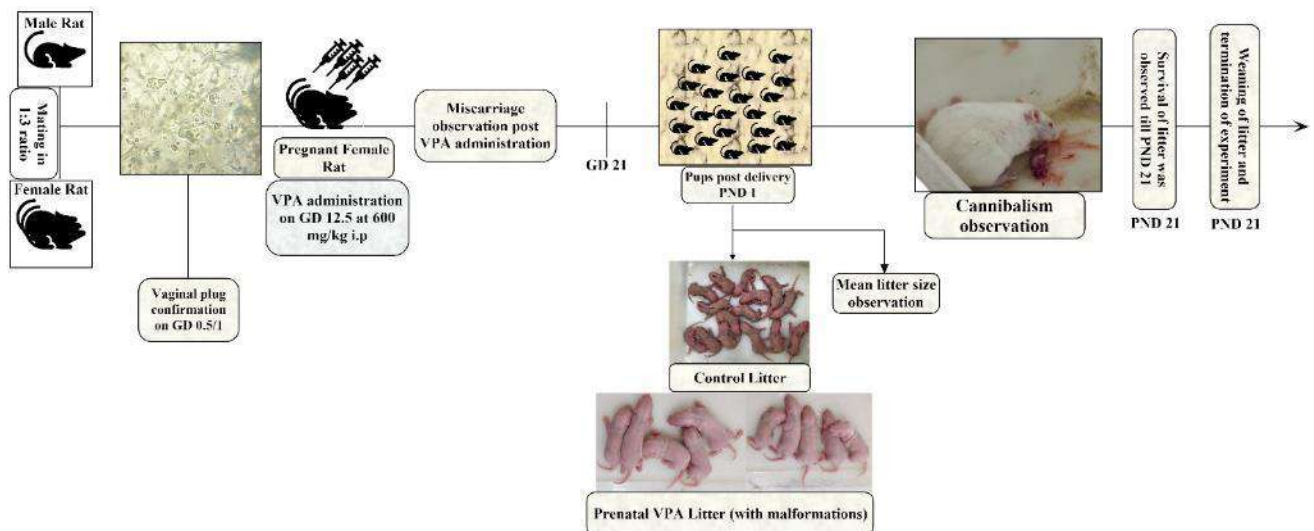
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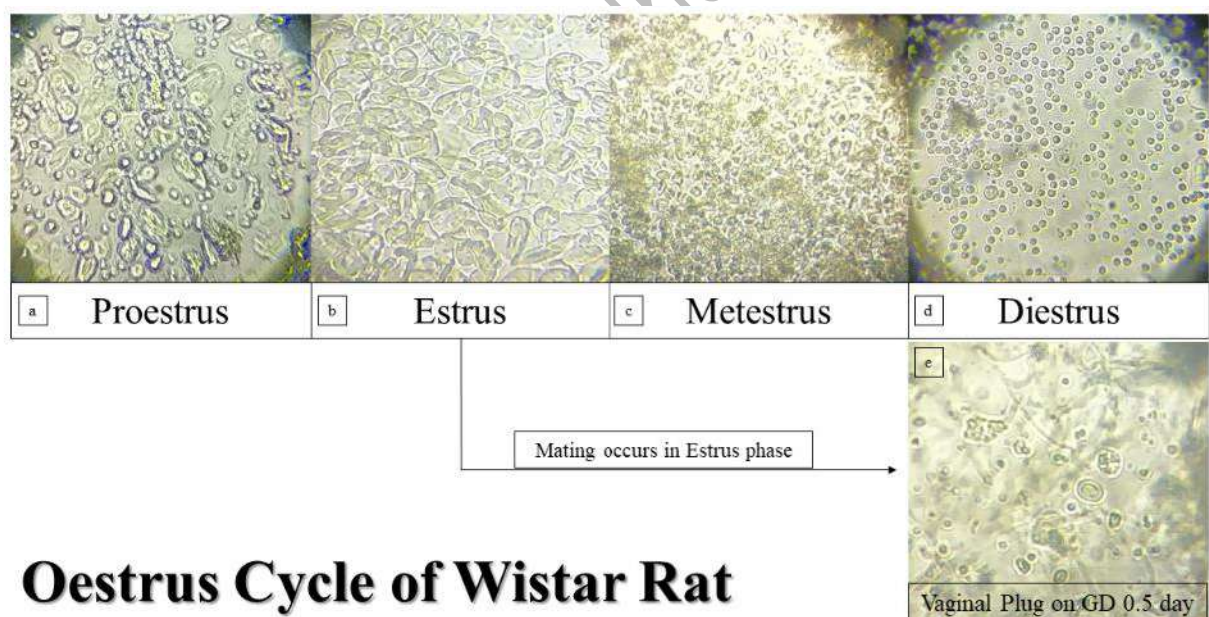
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## Figures

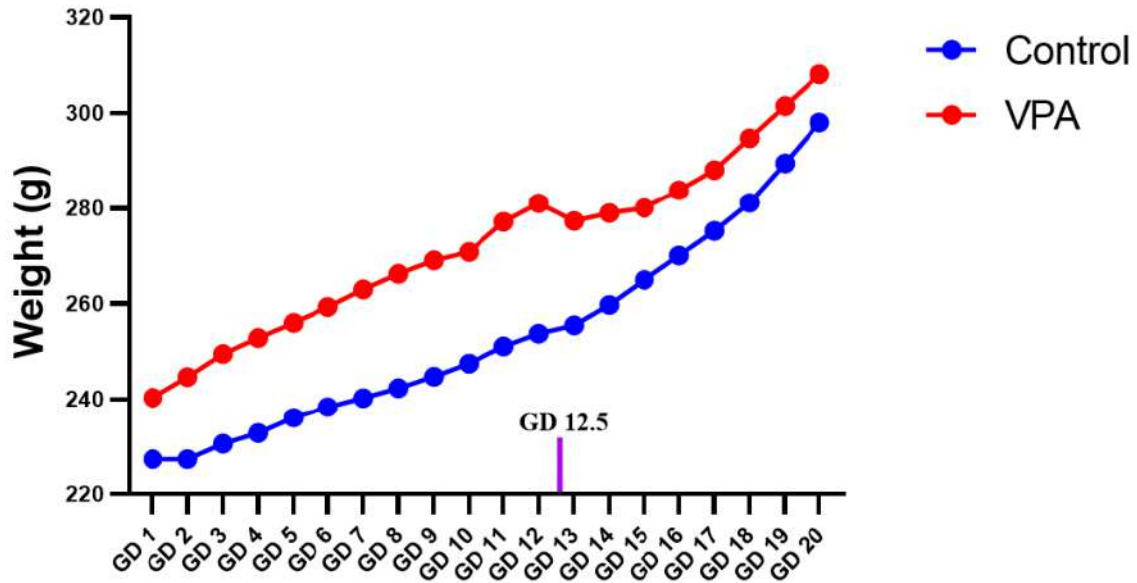


**Figure I | Pregnancy determination/Vaginal plug observation.** Experimental timeline. A pictorial representation of pregnancy determination, mortality observation, and Cannibalism during the experiment.

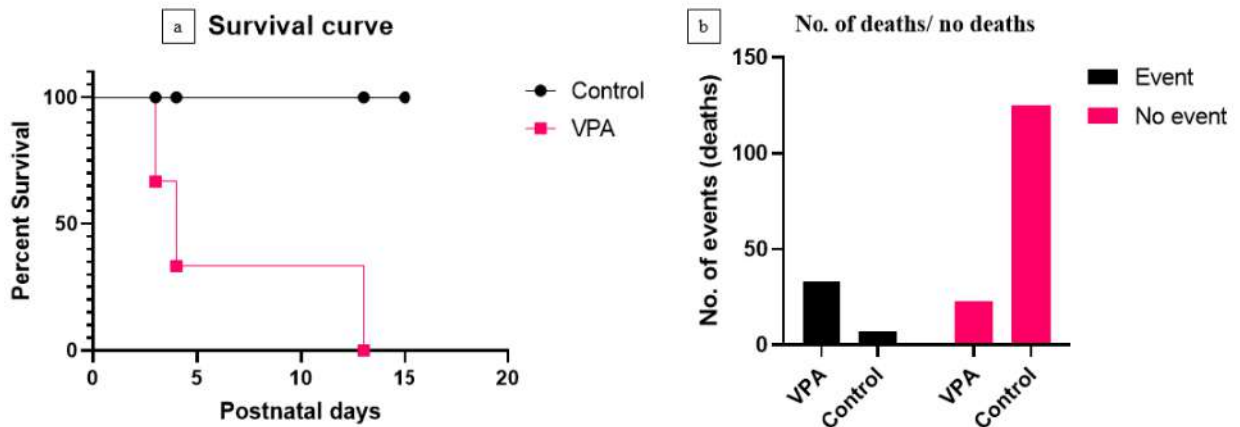


## Oestrus Cycle of Wistar Rat

**Figure II | Oestrus cycle was observed on different days (a) Proestrus phase, (a) Estrus phase, (c) Metestrus phase, (d) Diestrus phase, (e) Vaginal plug was confirmed post-mating as sperm cells within estrus phase were visible under the microscope. Magnification 40x.**



**Figure III | Weight of pregnant rats during gestation.** Post-copulation, the weight of pregnant female rats was observed from GD 1 to GD 20. VPA was administered on GD 12.5 (magenta colored line) at 600 mg/kg, i.p. in the VPA group. Equal volume of normal saline was injected into the control group on GD 12.5, i.p.



**Figure IV | Survival analysis.** (a) Prenatally VPA exposed litter had significantly decreased percent survival ( $*p < 0.0288$ ,  $df = 1$ ,  $\chi^2 = 4.778$ , Mantel-Cox test;  $*p < 0.0442$ ,  $df = 1$ ,  $\chi^2 = 4.050$ , Gehan-Breslow-Wilcoxon test). (b) Fisher exact test, odds ratio, risk ratio revealed that VPA exposed pups had decreased survival and increased risk associated with VPA administration ( $***p < 0.0001$ , relative risk = 11.11, 95% CI = 5.383 to 23.38, odds ratio = 25.62, 95% CI = 9.908 to 62.02). Events (deaths), no events (no deaths).

## Tables

**Table I:** Effect of VPA on pups' physical appearance

Type of malformation			
Dams	Tail Kink (n)	Paw malformation (n)	Short tail (n)
Dam 1	2	1	1
Dam 2	5	-	-
Dam 3	3	-	-
Dam 4	1	-	-
Dam 5	2	1	-
Dam 6	5	-	-
Dam 7	0	-	-
Dam 8	2	-	-
Dam 9	2	1	-
Dam 10	4	-	1
Mean±SEM	2.6 ± 0.520683	1 <sup>#</sup>	1 <sup>#</sup>

n = no. of pups

# Represents median

**Table II:** Postnatal day-wise mortality of pups in control vs VPA-treated groups

Postnatal day	Pups died (n)	
	Control	VPA
<b>PND 1</b>	4	14
<b>PND 2</b>	3	14
<b>PND 3</b>	0	1
<b>PND 4</b>	0	1
<b>PND 13</b>	0	1
<b>PND 15</b>	0	2
<b>Mean <math>\pm</math> SEM</b>	0.7 $\pm$ 0.2134	3.30 $\pm$ 0.7608**

**Note:** The mean  $\pm$  SEM values correspond to the no. of dams but do not depend upon the no. of PNDs.

\* Represents VPA vs control (\*\* p<0.01)

**Table III:** Tabular representation of the type of comparison with data distribution and respective p-value and confidence interval

S.no	Parameter type	Group Comparison	Data Distribution	Statistical Tests applied	Control	VPA	P-value	95% CI
1	Litter size (mean $\pm$ SEM)	Control vs Prenatal VPA	Parametric	Unpaired t-test with Welch's correction	13.2 $\pm$ 0.6633	5.60 $\pm$ 0.7630***	<0.0001	-
2	No. of male pups (mean $\pm$ SEM)	Control vs Prenatal VPA	Parametric	Unpaired t-test with Welch's correction	7.3 $\pm$ 3.6	3.6 $\pm$ 0.4761**	0.0025	-
3	No. of female pups (mean $\pm$ SEM)	Control vs Prenatal VPA	Parametric	Unpaired t-test with Welch's correction	5.9 $\pm$ 0.4583	2.0 $\pm$ 0.3651***	<0.0001	-
4	No. of cannibalised pups (median)	Control vs Prenatal VPA	Non- Parametric	Mann Whitney test	0	3**	U=13, 0.0023	-
5	Natural death (median)	Control vs Prenatal VPA	Non- Parametric	Mann Whitney test	0.5	0	U=45, 0.6499	-
6	No. of total pups died (mean $\pm$ SEM)	Control vs VPA	Parametric	Unpaired t-test	0.7 $\pm$ 0.2134	3.30 $\pm$ 0.7608**	0.0077	-

7	Mortality percentage (mean±SEp)	Control vs Prenatal VPA	Parametric	Unpaired t-test with Welch's correction	5.512 ± 1.598	60.56 ± 12.78***	0.0019	-
	No. of pups malformed (sum)	Prenatal VPA			-	31		-
8	No. of pups cannibalised (healthy vs malformed) (mean±SEM)	Prenatal VPA (healthy vs malformed pups)	Parametric	Paired t-test	-	0.8 ± 0.2494 vs 1.9 ± 0.5044*	0.0484	-
9	No. of pups alive (mean±SEM)	Control vs Prenatal VPA	Parametric	Unpaired t-test with Welch's correction	12.5 ± 0.7188	2.3 ± 0.7753***	<0.0001	-

\* Represents VPA (prenatal VPA) vs control (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ )