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# Assessment of *in-utero* venlafaxine induced, ROS-mediated, apoptotic neurodegeneration in fetal neocortex and neurobehavioral sequelae in rat offspring

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ABSTRACT

Venlafaxine (VEN), a serotonin and noradrenaline reuptake inhibitor is being used as a drug of choice for treating clinical depression even during pregnancy. It is an important therapeutic option in the treatment of perinatal depression, but the effects of VEN on fetus and the newborn are uncertain. Therefore, present study was undertaken to investigate the safety of *in-utero* exposure to VEN in terms of developmental neurotoxicity and neurodegenerative potential by using prenatal rat model. The selected doses of VEN (25, 40 and 50 mg/kg) were administered to pregnant rats from GD 5 to 19 through oral gavage. The fetal brains were dissected and processed for histopathological measurements of neocortical thickness that showed significant reduction. Considering vulnerability of immature brain to free radical injury, VEN exposed neocortices were tested for reactive oxygen species (ROS) levels which were significantly increased. As ROS play important role in the initiation of apoptotic mechanisms, we explored for *in situ* detection of apoptosis by confocal microscopy that showed enhanced apoptosis including chromatin condensation which was further reconfirmed by electron microscopy. Substantially increased levels of pro-apoptotic protein Bax and decreased levels of anti-apoptotic protein Bcl2 as shown by western blotting also supported the increased neuro-apoptotic degeneration. For further correlation of these findings, prenatally VEN exposed young-adult rat offspring were assessed for open field exploratory behavior that showed increased anxiety-like and stereotypic responses indicating disturbed neurobehavioral pattern. The study concludes that prenatal VEN exposure may primarily enhance ROS generation that plays a key role in regulating release of proapoptotic factors from mitochondria and thereby enhancing apoptotic neurodegeneration that affect proliferation, migration and differentiation of cells, resulting in neuronal deficits manifested as long term neurobehavioral impairments.



## 1. Introduction

Venlafaxine (VEN), a newer antidepressant (AD), is fast becoming a drug of choice for the treatment of clinical depression, even during pregnancy (Pakalapati et al., 2006; Huntington and Zantop, 2004), because of its peculiar pharmacokinetic drug profile and dual mode of action (Benkert et al., 2006; Preskorn, 1997; Redrobe et al., 1998). VEN is the first drug of a new class of ADs *i.e.* SNRIs (serotonin and noradrenaline reuptake inhibitors) that selectively blocks the serotonin and noradrenaline uptake pumps without blocking muscarinic, histaminergic and adrenergic receptors or inhibiting sodium fast channels. Due to these properties, VEN has a wide therapeutic index when compared to other ADs (Preskorn, 1997; Rolla et al., 2014). Though, it has been considered to be an important therapeutic option in the treatment of pre and perinatal depression, yet its effects on fetus and newborn are uncertain (Pakalapati et al., 2006; Nulman et al., 2012; Kieviet et al., 2013). The effects of the drug are of concern as most of the ADs have the ability to cross the placental barrier in varying and unpredictable quantities (Hostetter et al., 2000; Dalmizrak et al., 2012). There are some clinical studies reporting maternal/neonatal toxicity and withdrawal

*Abbreviations:* VEN, venlafaxine; AD(s), antidepressant(s); SNRI(s), serotonin and noradrenaline reuptake inhibitor(s); GD, gestation day; DCFDA, 2,7-dichloro fluorescein diacetate; ROS, reactive oxygen species; PS, phosphatidyl serine; FITC, fluorescein isothiocyanate; PI, propidium iodide; Bcl-2, B-cell lymphoma-2 protein (anti-apoptotic); Bax, Bcl-2-associated X protein (pro-apoptotic); 5HT, serotonin; NA, noradrenaline; OFT, open-field test.

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symptoms attributed to maternal VEN exposures (Broy and Berard, 2010; Nakhai-Pour et al., 2010; Oberlander et al., 2004; Laine et al., 2003; De Moor et al., 2003; Einarson et al., 2001), but these reports are sketchy and inconclusive in nature and the safety of *in-utero* exposure to VEN especially in terms of fetal/neonatal neurotoxic and neurodegenerative potential has not been established so far. There are evidences that several teratogens (drugs and/or environmental factors) affect the embryo-fetal development by increasing its free radical load and oxidative stress by producing excessive reactive oxygen species (ROS) due to bioactivation (Ornoy, 2007). It has been reported that developing fetus in general and immature brain in particular are highly vulnerable to ROS and free radical injury because of their poorly developed scavenging systems (Ikonomidou and Kaindl, 2011). ROS play important role in the initiation of apoptotic mechanisms and in mitochondrial permeability transition (Brodská and Holoubek, 2011). ROS regulated release of proapoptotic factors from mitochondria appears to play a much more important role in the immature brain (Blomgren and Hagberg, 2006). It is, therefore, not surprising that any environmental agents, chemicals and/or drugs that produce increased levels of free radicals might affect the developing fetus by increasing protein oxidation, DNA fragmentation, chromatin condensation and apoptotic cell death which ultimately might induce growth retardation, congenital anomalies and, in severe cases, embryonic death (Ornoy, 2007; Kohen and Nyska, 2002; Zaken et al., 2000; Ornoy et al., 1999). There are plenty of studies demonstrating apoptotic neurodegeneration in the developing brain under the influence of compounds such as sedatives, anesthetics or anticonvulsants (Jevtovic-Todorovic et al., 2003; Bittigau et al., 2002; Ikonomidou et al., 2000; Pulera et al., 1998); but surprisingly, there is no such study evaluating the effects of intrauterine exposure to ADs including VEN on the aspects like neurocyto-architecture of neocortex, levels of ROS, levels of apoptosis related proteins, apoptotic neurodegeneration and long term behavioral consequences. Therefore, considering the necessity to render some conclusive information about the effects of *in-utero* exposure to VEN, this study has been planned to investigate developmental neurotoxic and neurodegenerative potential of VEN at therapeutically relevant doses in prenatal rat model.

## 2. Materials and methods

### 2.1. Animals

Laboratory inbred nulliparous female Charles-Foster (CF) rats (180 ± 10 g) were used for the experimental procedures. Animals were housed in plastic cages with rice bran as bedding material at standard laboratory environment (24 ± 2 °C, 12/12 h light/dark cycle and 60% RH). Standard rat food and tap water were made available *ad libitum*. Animals were maintained and used in accordance with the Animal Welfare Act and the protocol for experimental use of rats was approved by Institutional Animal Ethics Committee (IAEC), University of Allahabad, Allahabad, India.

### 2.2. Determination of pregnancy

Female rats were first allowed to mate with males overnight (ratio 2♂:1♀) and then were checked for the presence of sperms in vaginal smears for determining the onset of gestation (GD-0). Such sperm positive females were used further for the experimental procedures.

### 2.3. Drug doses and rationale for dose selection

VEN was purchased from the pharmaceutical market manufactured by Cipla, India. The human therapeutic recommended dose range of VEN is 75–375 mg/day [6.25 mg/kg body weight (BW)/day as maximum human recommended doses (MHRD)] (Harrisson, 2008; Preskorn, 1999). The experimental doses of the drug were calculated as 25 mg/kg BW (4× MHRD), 40 mg/kg BW (6.5× MHRD) and 50 mg/kg BW (8× MHRD), on the basis of 'mg/kg BW/day'; and its suitability to the animal model, rat. The rationale for selection of three doses of VEN was as per MHRD for dose translation from animal to human studies (Reagan-Shaw et al., 2007) and considering the higher metabolic rate (4–6 times faster than humans) of rats (Kapur et al., 2003). Therefore, 25, 40 and 50 mg/kg doses of VEN were selected and calculated per day according to animal body weight to mimic the therapeutic dose range.

### 2.4. Experimental design

Four groups of six sperm positive female rats ($n = 6$) in each group, were formed. All the control and experimental rats were exposed from gestation day 5–19 (GD 5–19) either with the drug or without the drug (vehicle). In this study, selected doses of VEN (25, 40 and 50 mg/kg) were dissolved in distilled water and gavaged to sperm positive dams once daily (at 09.00 hour) from GD 5 to 19 orally with the help of cannula. According to the experimental protocol, half of controls and half of VEN treated rats of each experimental group were sacrificed under halothane anesthesia on GD-19, and fetuses were collected by uterectomy and weighed. The brains of fetuses were immediately dissected on ice. The remaining 50% of the dams were survived and allowed to deliver normally. Their pups were culled ($n = 6$ per group) and reared with their biological mothers up to postnatal day (PND) 56 to record neurobehavioral observations in an open-field arena (maze).

### 2.5. Measurement of thickness of neocortical layers

Six fetuses (two from each dam) were selected randomly from each group ($n = 6$) and their brains were dissected, weighed and then processed for histological procedures as per standard protocol (Singh and Tripathi, 2014). In brief, brains were fixed in 10% neutral formalin, dehydrated in ascending grades of alcohol, incubated in molten paraffin wax (58 °C), and finally embedded in paraffin blocks which were serially sectioned at 8 μm by rotary microtome. Sections were transferred on egg albumin coated glass slides and finally stained with Hematoxylin and Eosin (H&E). The relevant neocortical area in coronal brain sections of control and VEN exposed fetuses were identified with the help of atlas (Altman and Bayer, 1994) and imaged under Eclipse CCD camera of Nikon 831 light microscope. These photomicrographs were used for measuring the thickness in the medial frontal cortex of the fetal brain sections ($n = 6$ per group) at different neuronal layers (I to VI) by using Image-J software. For the measurements, first of all the scale was set in the software according to scale bar of the photomicrographs then line tool was selected for measurement of total cortical thickness, from outer surface of the cortex (molecular layer) to inner surface of the neuroepithelium. Likewise, measurements of different layers (I to VI) were also done separately as shown in Fig. 1. In each experiment, six sections of medial frontal cortex of fetal brains of each group were selected for the measurement of neocortical thickness (Singh and Tripathi, 2014; Singh and Gupta, 2014).

### 2.6. Estimation of reactive oxygen species (ROS)

ROS was estimated by fluorimetric method using the oxidation sensitive fluorescent probe 2,7-dichloro fluorescein diacetate (DCFDA) (Tota et al., 2009). Fetal brain tissues ($n = 6$ per group) were homogenized with 0.5 ml HEPES–Tyrode solution (145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES, pH 7.4) and incubated with 10 μl DCFDA (5 μM) for 15 min at 37 °C and the DCF fluorescence was measured by using a "fluorescence multi-well plate reader" with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

### 2.7. In situ detection of apoptosis by confocal microscopy

In the present study Annexin V-FITC apoptosis detection kit (Sigma–Aldrich) has been used to detect drug induced apoptosis in rat brain cells by using confocal laser scanning microscopy. The kit is designed to target one of the first identifiable events during apoptosis which is an 'eat me' signal involving the 'flipping' of phosphatidyl serine (PS) on the cell membrane, resulting in exposure of PS on the cell surface (Verhoven et al., 1995). This translocation of PS to the external membrane serves as an 'eat me' or recognition signal for neighboring phagocytes. Since Annexins specifically bind to PS in the presence of calcium ($Ca^{2+}$), fluorescein isothiocyanate (FITC), a fluorescent marker, has been coupled with Annexin-V to prepare Annexin-V–FITC conjugates which were used for the detection of apoptosis. FITC has excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) spectrum peak wavelengths of approximately 495 nm/521 nm (Green, 1990). Propidium iodide (PI), a cell-impermeable fluorescent DNA stain ($\lambda_{ex}$ – 535 nm and $\lambda_{em}$ – 617 nm), has been used to differentiate among necrotic, apoptotic and normal cells (Lecoeur, 2002). Hoechst-33342, a cell-permeable DNA stain ($\lambda_{ex}$ – 350 nm and $\lambda_{em}$ – 461 nm), has been used to distinguish the DNA condensation, a characteristic sign of apoptotic nuclei (Latt et al., 1975). These triple labeled slides, prepared as per manufacturer's manual (Sigma–Aldrich-Annexin V–FITC apoptosis detection kit), were imaged and counted for apoptotic cells under confocal microscope (Carl Zeiss' LSM 510 META) simultaneously using sequential mode of imaging. For counting of apoptotic cells, 3 fetal brains per group and 3 consecutive sections per brain were used.

### 2.8. In situ detection of apoptosis by electron microscopy

Ultrastructural apoptotic neurodegenerative changes in neocortex region of the brains of control and prenatally VEN exposed rat fetuses were observed by using transmission electron microscopy. Immediately after uterectomy, fetuses were anesthetized by halothane and perfused transcardially with PBS followed by