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Research Article

Studies on the Evaluation of Teratogenic Potential of SSRI (Escitalopram) on Chick Embryogenesis (*Gallus gallus domesticus*)

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Abstract

Escitalopram is a selective serotonin reuptake inhibitor (SSRI) used for the treatment of depression, anxiety and panic disorders. The purpose of this study is to examine the effect of Escitalopram on the early developmental stages of chick embryo *Gallus gallus*. Escitalopram's teratogenic potential was further assessed using YSM analysis and protein biochemistry. The treated embryos showed mild to severe teratogenic effects at 5mM and 10mM dose respectively. Enzyme Assay was conducted to see whether the drug treatment interfered with the cellular pathways. The levels of Alkaline phosphatase (ALP) and Acetylcholinesterase (AChase) were found to be reduced in the treated embryos. YSM Assay reveals that *in ovo* drug administration has an impact on the angiogenesis of the embryo. In depth further studies are needed to understand the long-term effects of Escitalopram on the developing embryos at cellular and molecular level so as to take caution while prescribing the drug during pregnancy.

Keywords: *Gallus gallus domesticus,* Enzyme Assay, Escitalopram, Teratogenesis, Yolk Sac Membrane (YSM) Assay, Protein Biochemistry.

INTRODUCTION

Serotonin has an important modulatory role in the brain which includes regulation of sleep, mood, behaviour, cognition as well as the development of central nervous system (CNS) in early stages [1]. Escitalopram is the S-enantiomer of Citalopram and has its effect on the postsynaptic reuptake of serotonin at the terminals and cell bodies of serotonergic neurons through the serotonin transporter (SERT) and is one of the most widely used antidepressants during pregnancy. It exerts minimal effects on norepinephrine and dopamine neuronal reuptake [2]. Escitalopram is mainly metabolised in the liver using hepatic enzymes - CYP isoenzymes (CYP3A4, CYP2C19 and CYP2D6). Monoamine oxidases A and B (MAO-A and MAO-B) and aldehyde oxidase (AO) may mediate metabolism of Escitalopram in the brain [3].

Previous studies on B104, Kelly, SH-SY5Y and IMR32 cell lines, showed significant decrease in cell viability after treatment of Escitalopram [4]. Pre and post treatment Escitalopram raises BDNF levels, reduces oxidative stress and lowers microglia activation to prevent ischemia-induced neuronal death in the CA1 due to temporary cerebral ischemia damage [5]. Treatment with Escitalopram (10 mg/kg) increased the expression of BDNF through the activation of the CREB/CRE transcription pathway in the prefrontal cortex[6].

Fertilized chicken eggs are a model system in developmental biology research as an alternative model to study drug distribution and pharmacokinetics and pharmacodynamics on developing embryos [2,7]. The chicken genome has been sequenced and genomic data is available for further research [5]. Having a convenient size and short incubation period adds as an advantage for using chicken embryos as a model. Being physiologically similar to humans, they can be used for preclinical neurotoxicological studies as valuable information about the potential of neurodevelopmental impact of antidepressants may be found [2].

The Chorioallantoic membrane (CAM) is a highly vascularised outermost extra-embryonic membrane in the developing avian embryos. Drug kinetics, metabolism, distribution, and effects of drugs on the developing foetus can be studied using CAM assay [2]. The *in ovo* Yolk Sac Membrane (YSM) model is now used for toxicity and angiogenesis studies for testing a wide variety of xenobiotics and drugs. When compared to CAM

Assay, YSM Assay can be performed on early-stage embryos [8].

We hypothesise that Escitalopram induces malformations in the developing embryos and we assess its safety for gestational use.

MATERIALS AND METHODS

Preparation of stock and working solutions of test compound

Stock solution of Escitalopram oxalate (24.13mM) was prepared in sterile 1X Phosphate Buffer Saline (PBS- pH 7.4) and further used to prepare working solutions of desired concentration of 5mM and 10mM.

Chick embryo culture and drug treatment

HH stage 20-21 fertilised eggs [9] were collected from Venkateshwara Hatcheries Pvt Ltd. Pune. Eggs were separated into control and treated groups (n = 10, * 3 sets) and later were used for treatment of the test drug. Working solutions of Escitalopram were administered by *in ovo* technique by air sac route under sterile conditions. The treated and control eggs were incubated in the BOD incubator for 24 hrs at 37ºC and a relative humidity of 70-80%.

Protein and Enzyme Biochemistry

Control and treated embryos were harvested in 1X PBS at 4^0C. Protein was extracted using 1X Protein Extraction Buffer (PEB) and then quantified using Bradford Assay [10]. Acetylcholinesterase (AChase) assay was done using autoanalyzer by DELTA^R Cholinesterase Kit (Butryl Thiocholine method). Alkaline Phosphatase enzyme assay was performed using Merilyzer Instrument using CliniQuant^R Kit.

In ovo Yolk Sac Membrane (YSM) assay

Gallus domesticus eggs of HH stage 20-21 were kept upright with the blunt end facing upwards to allow embryo alignment with the air sac. With the help of pointed forceps, a small window of 3cm X 3cm was made and pieces of egg shells were carefully removed. The inner shell membrane was cut resulting in the exposure of YSM vasculature of the embryo on which Escitalopram treatment was administered. The window was sealed and eggs were kept for incubation for 24 hours in a BOD incubator at 37.8 °C. After incubation, the embryos were harvested and images were captured using Magnus HDR camera fitted to a stereo-zoom binocular dissecting microscope and later analysed using Wimasis Image Analysis software (https://www.wimasis.com) [8].

RESULTS AND DISCUSSIONS

A) **Teratogenic studies-** Escitalopram-treated embryos showed teratogenesis at 5mM and 10mM dose treatment.

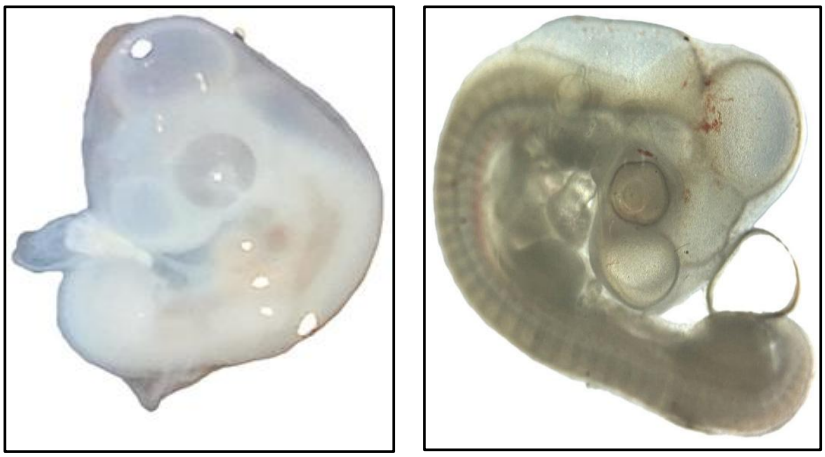


Figure 1: Control Embryo (HH 20 - 21)

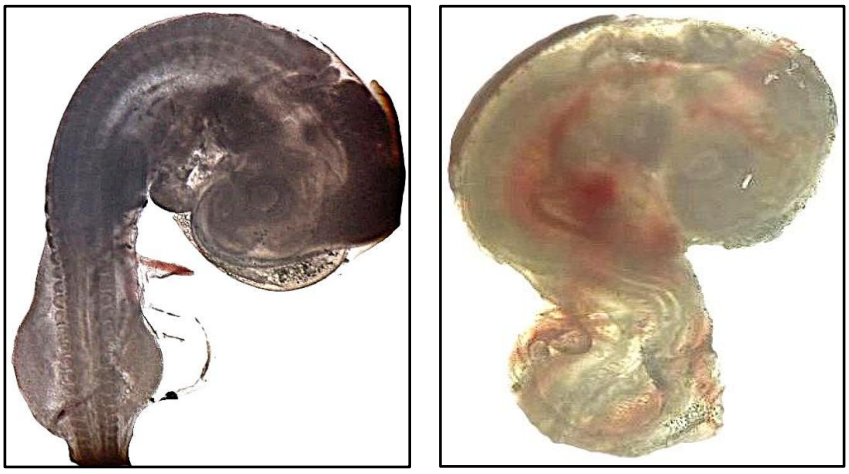


Figure 2: Escitalopram (5 mM) induced teratogenesis in chick embryos (HH Stage 20 - 21)

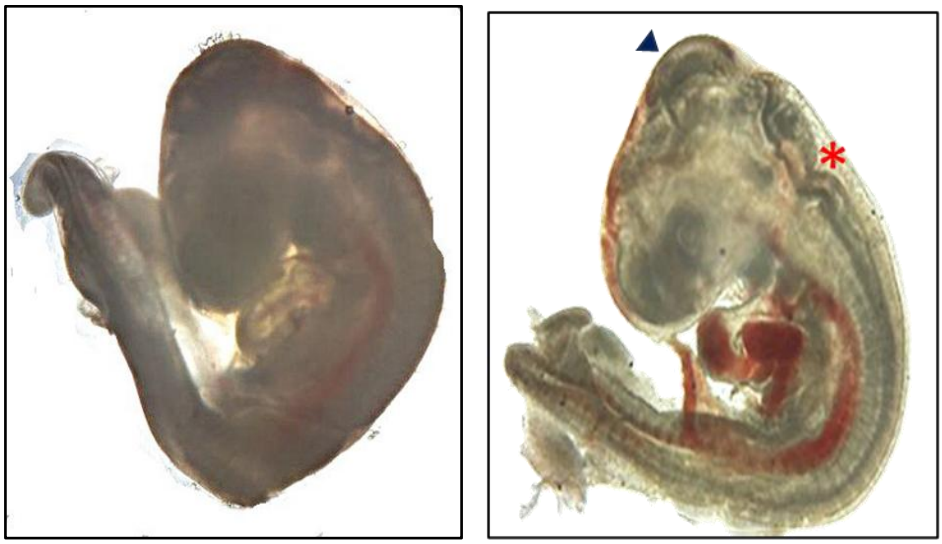


Figure 3: Escitalopram (10mM) induced teratogenesis in chick embryos (HH Stage 20 - 21)

B) Protein and Enzyme Biochemistry – The results of the Bradford Assay and Enzyme Analysis showed decrease in the Protein and Enzyme Content respectively.

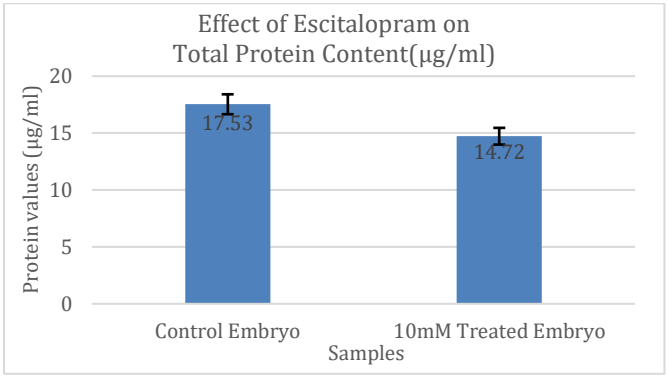


Figure 4: Protein content of Escitalopram treated chick embryos (HH stage 20-21) by Bradford assay.

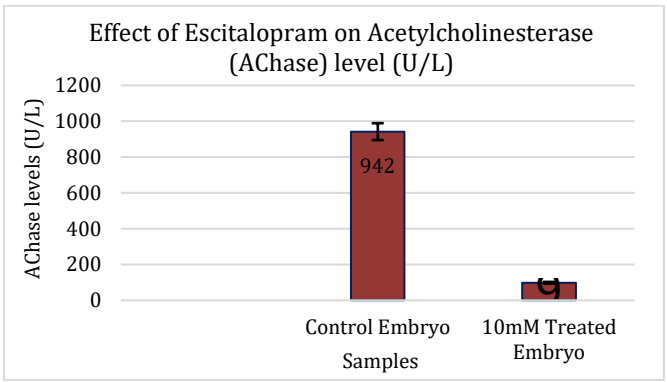


Figure 5: Acetylcholinesterase (AChase) assay for Escitalopram treated chick embryos (HH Stage 20-21)

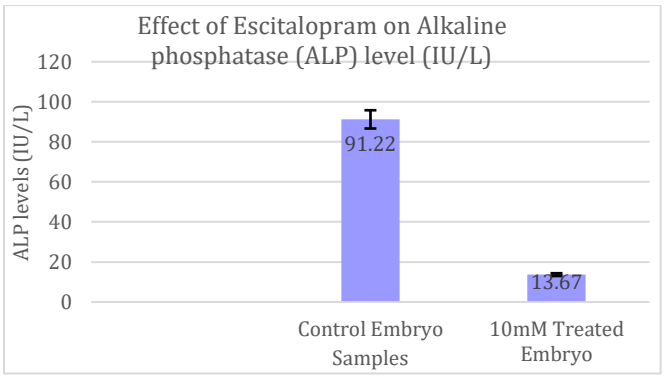


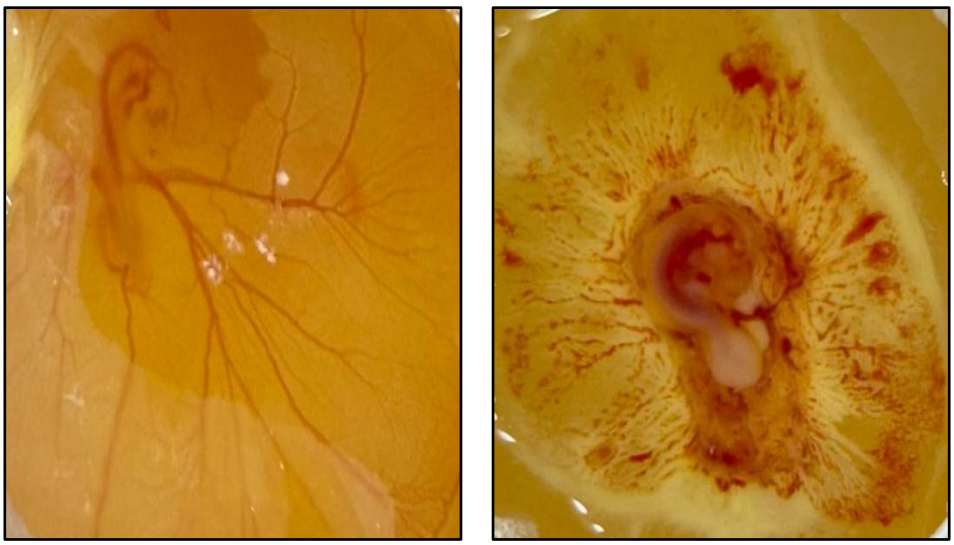
Figure 6: ALP enzyme assay for escitalopram treated chick embryos (HH Stage 20-21)

C) YSM Assay – The Effect of Escitalopram on angiogenesis of embryo.



Pre-incubated Post-incubated (24 hours)

Figure 7: YSM Assay of Control Embryos (HH stage 20-21)



Pre-treatment Post-treatment (24 hours)

Figure 8: YSM Assay of 5mM Escitalopram treated embryo (HH stage 20-21)



Pre-treatment Post-treatment (24 hours)

Figure 9: YSM Assay of 10mM Escitalopram treated embryo (HH Stage 20–21)

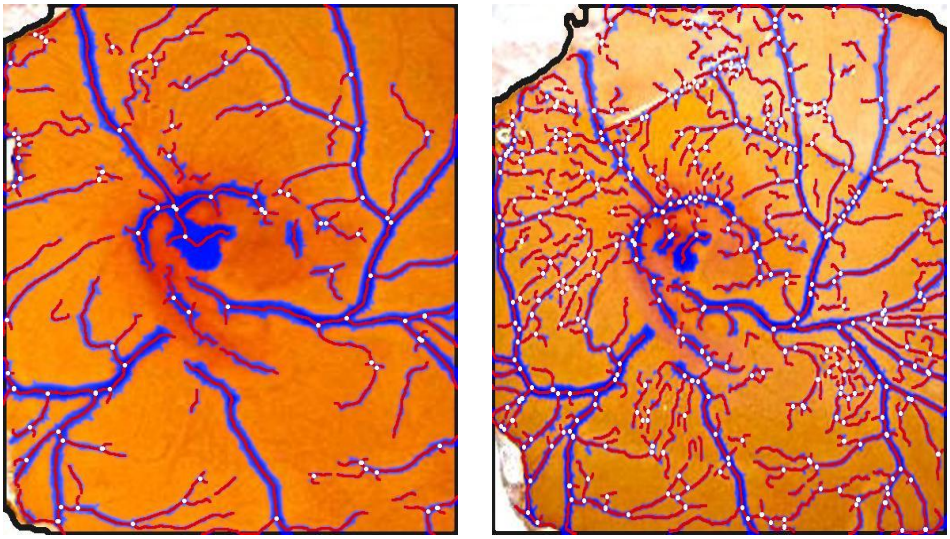
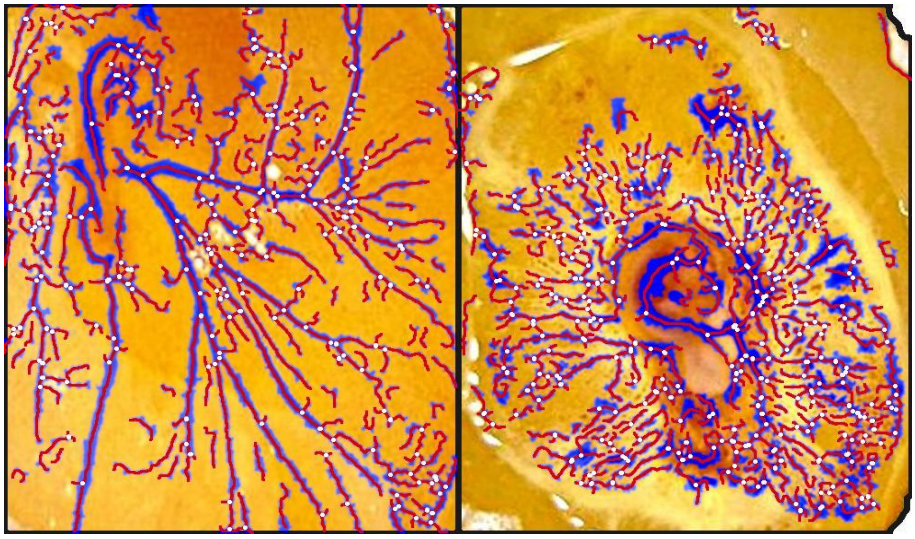
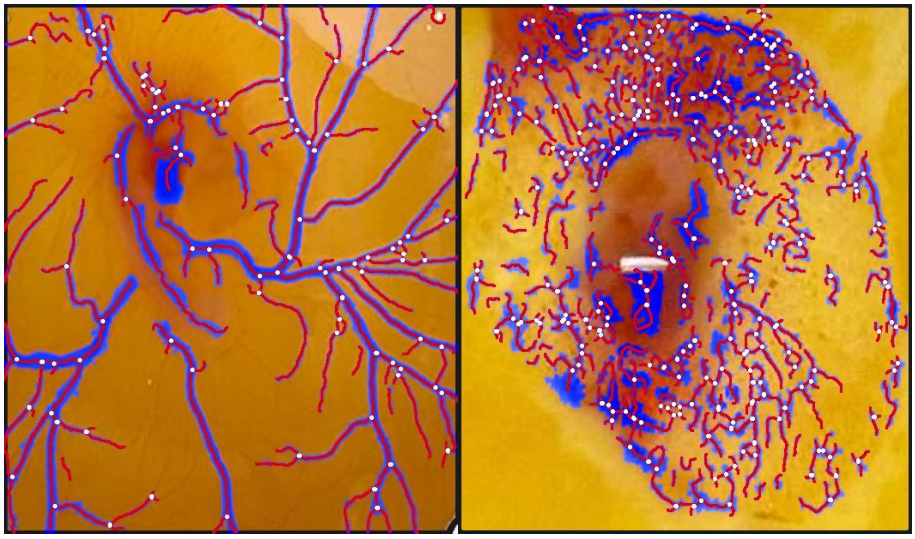


Figure 10: YSM Analysis of Control Embryos (HH stage 20-21)



Pre-Treatment Post-Treatment

Figure 11: YSM Analysis of 5mM Escitalopram treated embryo (HH stage 20 –21)



Pre-Treatment Post-Treatment

Figure 12: YSM Analysis of 10mM Escitalopram treated embryo (HH Stage 20-21)

D) Graphical Analysis of YSM for Escitalopram

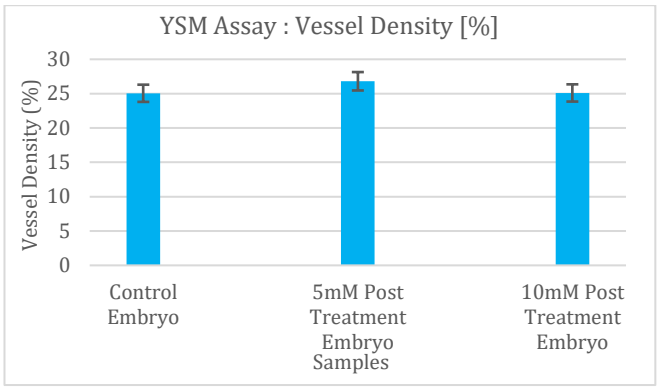


Figure 13: Vessel Density Analysis using YSM Assay

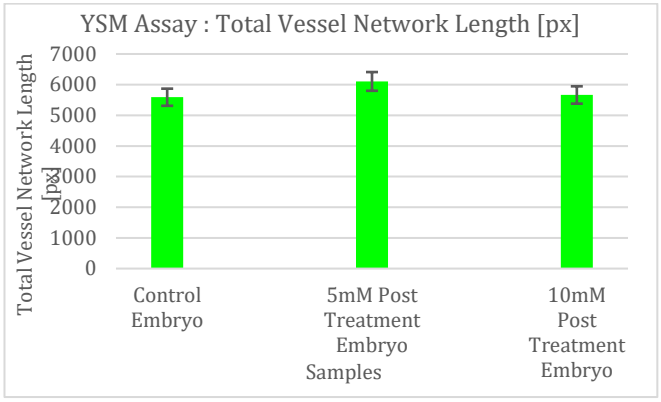


Figure 14: Total Vessel Network Length Analysis using YSM Assay

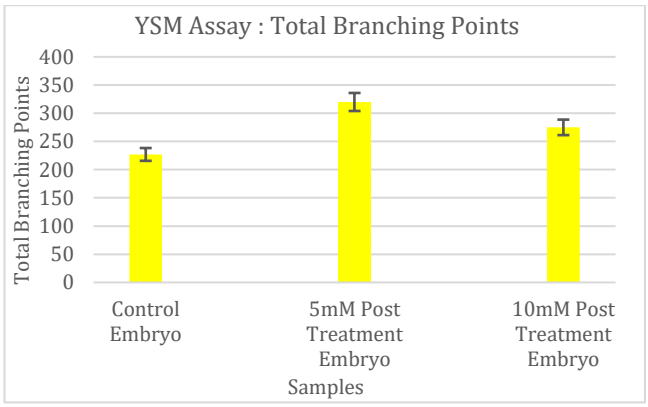


Figure 15: Total Branching Points Analysis using YSM Assay

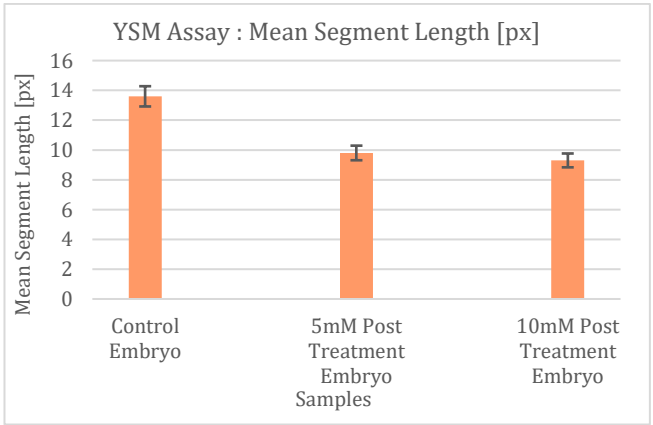


Figure 16: Mean Segment Length Analysis using YSM Assay

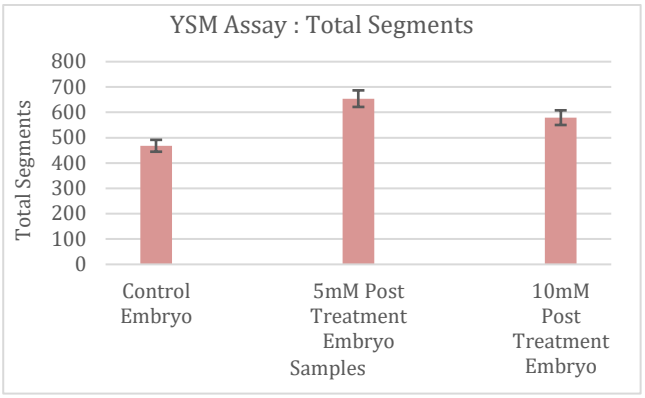


Figure 17: Total Segments Analysis using YSM Assay

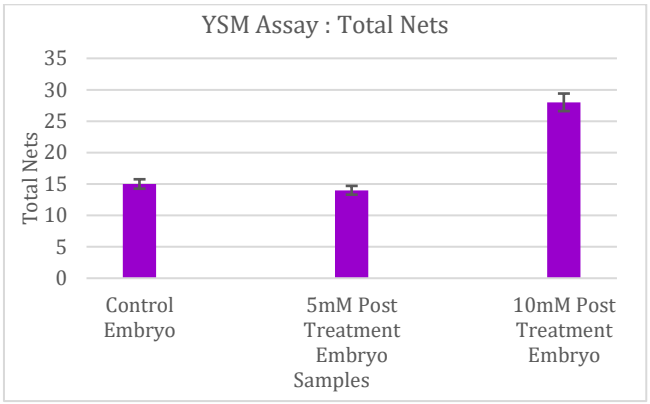


Figure 18: Total Nets Analysis using YSM Assay

DISCUSSION

Chick Embryos (HH stage 20-21) when treated with Escitalopram showed teratogenic effects. Abnormal torsion and flexion in the lumbar region were observed at 5mM and 10mM concentrations. 5mM escitalopram treated embryos (fig.2) exhibited neural tube defects with significant enlargement in anterior (forebrain) and posterior (hindbrain) structures. 10mM treated Escitalopram embryos showed a wavy neural tube (*) (fig.3) and severe malformation in the embryonic axis. Hematoma in the cardiovascular and cardiothoracic region, and reduction in Mesencephalic bulge (◢) (fig.3) was also noted.

10mM Escitalopram treated embryos (fig.4) showed decreased protein content (14.72 µg /ml) compared to the control embryos (17.53 µg /ml) while 5mM Escitalopram treated embryos showed protein content higher than both control and 10mM treated embryos. Escitalopram treatment also affected the Acetylcholinesterase (AChE) levels (fig.5) when compared to the control embryos (control AChE levels = 942 U/L vs 10mM Escitalopram treated embryo = 97.9 U/L). Alkaline Phosphatase levels (ALP) (fig.6) also decreased in the treated embryos (from 91.22 IU /L to 13.67 IU/L) which may indicate that Escitalopram not only modulates neurotransmitter levels in the prefrontal cortex (CNS) but also interferes with cellular metabolic pathways. Previous studies involved acute and long-term effects of escitalopram and citalopram on rat brain 5-HT neurotransmission using electrophysiological techniques, which suggested that there was a delayed effect of SSRI on 5-HT serotonin transmission [1]. Chronic

administration of escitalopram decreased the activities of complexes I and II–III in cerebellum, hippocampus, striatum and posterior cortex whereas prefrontal cortex was not affected. However, chronic administration of escitalopram did not affect complex IV and enzymes of Krebs cycle activities as well as creatine kinase [11].

To observe the effect of Escitalopram on angiogenesis, Yolk Sac Membrane (YSM) (refer figs. 7 to 12) assay was performed and it was observed that there was no change in the vessel density of control (25.05%) and treated embryos (5mM= 26.8%, 10mM = 25.1%) (fig.13). However, there was a significant increase in the total vessel network length (fig.14) in 5mM treated embryos (6106.2 px) compared to control (5589.85 px), while there was a slight increase by 73.95 px in the 10mM treated embryo. Total branching points (fig.15) increased in both 5mM (320) and 10mM (275) treated embryos when compared with control (227). Reduction in Mean segment length (fig.16) was seen in treated embryos (Control= 13.6px, 5mM= 9.8px and 10mM= 9.3px) and rise in total segments (fig.17) (Control= 468, 5mM= 654 and 10mM= 579). 5mM Escitalopram treated chick embryos showed no effect in YSM vasculature while there was an increase in total YSM nets (fig.18) in the 10mM treated embryos (control = 15 and 10mM = 28). Escitalopram affects angiogenesis in the chicken YSM and VEGF being a marker for angiogenesis, it may point to the fact that Escitalopram modulates VEGF expression in the developing embryo. Previous studies have also shown that in the Escitalopram intervention group, VEGF expression in ischemic brain tissue was higher compared to the saline control group. This was done using immunohistochemical analysis and western blotting. Thus, using VEGF mediated angiogenesis, Escitalopram may have a role in reducing neurological deficit in cerebral ischemia in rats [12]. Alexander Havardstan performed Kinetic studies to assess the effect of Escitalopram on chicken embryonic CAM and it was found that 0.3mM escitalopram when applied on the CAM, reached the brain within 30 min and Cmax was achieved between 30 minutes and 2 hours 2. It was also observed that Escitalopram suppressed the effects of increased oxidative stress and enhanced the antioxidant protection in the hippocampus and frontal cortex, along with alleviating stress-induced depressive and anxious behaviours [13]. Previous studies have also shown that the chicken embryo can metabolise the Escitalopram to demethylescitalopram which when injected onto CAM reaches the brain in concentrations relevant to human use. Chicken Cerebellum Granule Neurons (CGNs) have shown that 72 hours exposure to (1-100 μM) Escitalopram, induce a significant reduction in cell viability with inhibitors P75 and TrkB [2].

Study on male Wistar rats suggested that Escitalopram has a beneficial effect on endothelial function. Its antioxidant effect is mediated through activation of enzymes like superoxide dismutase-1, catalase, and glutathione peroxidase. Vascular cell adhesion protein-1 (V-CAM-1) expression also reduced due to production of transcription factors NF-κB and activator protein-1, which lead to less severe aortic atherosclerosis lesions [1].

Previous studies on treatment with Escitalopram showed that IMR32 cells were most drug sensitive, showing decreased viability with a starting dose of 25 μM of Escitalopram, and showed 0% viability at 150 μM concentration. SH-SY5Y cell line exhibited a similar profile, with no significant difference with IMR32 at higher concentrations of Escitalopram. The viability of B104 and Kelly cell lines was decreased at concentrations of Escitalopram above 100 μM [4]. The study investigated the protective effects of long-term escitalopram administration on memory, as well as on hippocampal *BDNF* and *BCL-2* gene expressions in rats exposed to predictable and unpredictable chronic mild stress, increased hippocampal *BDNF* gene expression in the PCMS and UCMS subjects [15]. There was a significant decrease in serum levels of total cholesterol, triglycerides, low density lipoproteins, very low-density lipoproteins, and serum malondialdehyde, and a significant increase in high density lipoproteins in high fat diet atherosclerosis induced rats compared with the atherosclerosis model group [16]. Similar studies were conducted for another antipsychotic drug Venlafaxine on chick embryo and similar results were obtained like in our studies [17].

CONCLUSION

Escitalopram, being a highly selective SSRI, is the most widely used antidepressant for psychotic disorders (anxiety and depression), as well as during pregnancy. The action of antidepressants might differ in the developing embryos than in adults since the developing brain has a different array of gene expressions. Hence, to understand the potential threats of SSRIs and SNRIs, these must be investigated in developing neurological systems.

From the above results obtained, our proposed hypothesis was found to be true: that Escitalopram indeed induces malformations in the developing embryos. This study thus provides insight into the molecular biology of SSRIs and may help in the selection and dosage of safer antidepressants during gestation. However, further studies are needed to understand escitalopram-modulated gene expression and gene networks involved in developing embryos to reduce the undesirable side effects of this drug.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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