RESEARCH ARTICLE

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Study on Potential Teratogenic Effects of Antidepressant Drugs on the Development of Chick Embryos

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ABSTRACT

Background:
The use of antidepressant drugs has been on a rise. The aim of this study is to reveal the harmful effects; these drugs can have on developing fetuses, when taken by pregnant women. Thus, Chick embryos are used as developmental model system to study the potential teratogenic effects of antidepressants.

Materials & methods:
Two antidepressant drugs namely Etizolam and Fluoxetine (Prozac) at 3 doses (low, medium, high) were injected into 48 hours fertilized eggs through air-sac route. A control group of eggs was injected with normal saline and maintained. The effect of drugs on early development (after 11 days incubation), late development (after 18 days incubation) and hatchability characteristics were studied. Further biochemical analysis of treated tissues was carried out. This included Agarose gel electrophoresis, quantification of DNA (Deoxyribonucleic acid) by DPA (Diphenylamine) method, SDS-PAGE and protein quantification by Bradford assay.

Results:
The effect of drugs on early development resulted in no growth and death of embryos however effect on late development caused malformations and reduced size compared to control embryos. Both drugs damaged DNA to different extents and protein concentration was also greatly reduced.

Conclusion:
However Etizolam proved to be more toxic as it engendered more DNA damage than Fluoxetine. Further, a difference in their mechanism of teratogenicity was also inferred as response of cells to both the drugs was different. Thus, the use of these antidepressant drugs should be avoided during pregnancy especially during the first trimester and third trimester.

Keywords: antidepressants, teratogenic drug, Etizolam, Fluoxetine, fetal malformations, chick embryos.

Introduction

Depression affects an estimated 121 million people worldwide. The World Health Organization forecasts that depression will displace heart disease as the heaviest disease burden by 2020. About one in ten Americans aged 12 and over takes antidepressant medication.(National centre for health statistics, October 2011) Rising cases of depression in India has been accompanied by the abuse of anti-depressants to keep up with the stress and anxiety. Women as a group have also received considerable attention with regard to risk factors for development of depressive disorders (Patel V *et al.* 2006)[19].Antidepressants are drugs used to treat depression and mental illness. They express their own side effects in patients taking them. However, Women who take anti-depressants during pregnancy are endangering their unborn babies, experts have warned, as medications increase the risk of a miscarriage by 17 %(Sophie Borland ,October 2012)[22].The two commonly prescribed antidepressant drugs Etizolam and Fluoxetine are used in this study to test for their teratogenic effect. Etizolam belongs to a new class of diazepines, thienotriazolodiazepines. It posses amnesic, anxiolytic,

anticonvulsant, sedative, skeletal and muscle relaxant properties (Badria Fathy *et al.* 2008)[1]. At higher doses it acts like a sleeping pill. It is used to treat anxiety and panic disorders. It has been reported that Etizolam is a powerful teratogen (Shweta P. Alai, 2012)[21]. An overdose of Etizolam can prove to be fatal (J. Francis 1953)[11]. On the other hand, Fluoxetine belongs to the class called selective serotonin reuptake inhibitors-SSRIs. As the name suggest this drug blocks the reuptake of serotonin into neurons. Serotonin is the 1st neurotransmitter expressed in developing embryo & plays crucial role in brain development (Bonnin A, 2011)[2]. It has been reported in a study that, Fluoxetine causes an acute increase in plasma serotonin levels, leading to transient reduction in uterine blood flow. This in turn reduces the delivery of oxygen and nutrients to the fetus; there by presenting a mechanism for reduced growth (Morrison JL *et al.*, 2005)[17].It also affects lungs, bones, heart. It was also reported that women of childbearing age make up a large percentage of SSRI users so the issue of pregnancy exposure is important. Estimates are that up to 13% of US pregnancies are exposed (Cooper WO *et al.*, 2007)[5]. Moreover, Between 2 and 3 per cent of pregnant women in the UK are thought to be on these drugs – up to 19,500 every year. Since the use of these drugs has increased, the purpose of this study was therefore to evaluate the potential teratogenic effects on development of chick embryos, which are used as model systems.

Materials and Methods:

Drugs

The Antidepressants drugs used were: 1) Fluoxetine (Prozac): It is Available in brand names-Fludac, Exiten, Fludeep, Fluox in the form of capsules of varying dosage such as10mg, 20 mg & 40mg. It is administered by oral route. 2) Etizolam: Available in brand names Etizola, Sedekopan, Pasaden or Depas, in the form of tablets of 5 mg. Both drugs we easily purchased from a medical store in the market.

Animals

Chick embryo was used as a model system for this study. As they present us with the following advantages: 1) they are vertebrates and their developmental process has a great deal in common with human embryo development 2) Like humans they have 4 chambered heart and our warm blooded animals.3) The eggs are self-contained and the embryos develop autonomously.4) Most importantly, the effects of various chemicals applied can be studied directly at any given time of its development without having to stop the process. About 60 fertilized 0 hours eggs were purchased from Shri Venkatesh hatchery [Pune], and incubated at 37 degree Celsius for 48 hours. The eggs were divided into groups of 5 for all doses of each drug, and 5 were kept as controls for each group.

Treatment

The average dose of Fluoxetine prescribed by a medical practioner to a person is 20-60mg per day, where as for Etizolam it is maximum 3 mg per day. Taking these into consideration, three doses (low, medium, high) of each drug were prepared. By using n1v1=n2v2 the below concentrations were prepared by dissolving the drug powder in sterile distilled water. Each group [containing 5 eggs] was inoculated with each dose of drug solution in sterile conditions by air-sac route; while the control group was inoculated with 1 ml of sterile distil water using a hypodermic syringe. They were kept for incubation to study the effects.

DRUG /DOSE	Low Dose (mg/ml)	Medium Dose (mg/ml)	High dose (mg/ml)
ETIZOLAM	0.1	0.5	1
FLUOXETINE	5	10	20

Table 1: various concentrations of each drug prepared.

Assessment of effects

According to hamburger and Hamilton it takes 21 days for a chick to hatc0h from 0 hour stage. During this process it undergoes various developmental stages. Thus, In order to understand effect of drugs on overall development, the following four characteristics: Morphology, Hatching ability, DNA damage and protein concentration of treated embryos were studied.

Morphology: Effect of drugs on morphology was studied at 2 stages: early developmental stage and late developmental stage. For early development, the incubated eggs were broken open on the 11th day to asses treated embryos. For late development, the 0 hours fertilized eggs were 1st allowed to develop normally in absence of drug application until the 11th day. Then these eggs were inoculated with 1ml of drug solution [low dose] and opened on the 18th day of incubation to study the effects. The chick embryo was separated from the yolk sac and placed on petriplates; they were weighed and examined for visible physical abnormalities.

Hatching: To study hatching ability, 11th day chick embryos were injected with low dose of Fluoxetine and Etizolam and control with distilled water. They were kept incubated till the 21stday and observed for hatching.

DNA studies: Tissue from treated embryos was used to isolate DNA. Isolation of genomic DNA was carried out by using phenol-chloroform extraction in presence of proteinase k (S Janarthanan and S Vincent's Practical biotechnology: methods and protocols, 2007)[20]. DNA

samples were subjected to Agarose gel electrophoresis, to observe banding pattern. Further DNA extracted was stored in TE buffer and was used for quantification by DPA [diphenylamine] Assay (S Janarthanan and S Vincent's Practical biotechnology: methods and protocols, 2007)[20]. For standard curve, 100 ug/ml stock solution of DNA [from herring sperm] was prepared using distil water.

Protein studies: Tissue from dissected embryos were used to extract proteins by using Phosphate buffered saline and centrifugation(S Janarthanan and S Vincent's Practical biotechnology: methods and protocols, 2007)[20]. Protein samples from treated and control was subjected to SDS-PAGE for a qualitative assay. Quantification of proteins was carried out by using Bradford assay. The Bradford dye was freshly prepared just before use. A stock of 100 ug/ml of BSA [Bovine serum albumin] was prepared using Phosphate buffered saline.

RESULTS:

Effect of drugs on early development of chick embryos results in arrested growth or reduced size.

Drug/Dose	Low	Medium	High
Etizolam	• No further growth of embryo after 24 hours stage. • Absence of blood plexus.	• No further growth of embryo after 24 hours stage. • Absence of blood plexus.	• Peculiar lump like structure was observed in the centre in place of embryo.
Fluoxetine	• Embryo shows reduced size compared to control.	• Complete growth retardation, embryo underdeveloped. • Clumped and scarcely placed blood vessels.	• Growth arrested at 24 hour stage embryo.
Control	Healthy embryo, with well-developed blood system.		

Table 2: Effects of Etizolam and Fluoxetine on early development of chick embryos

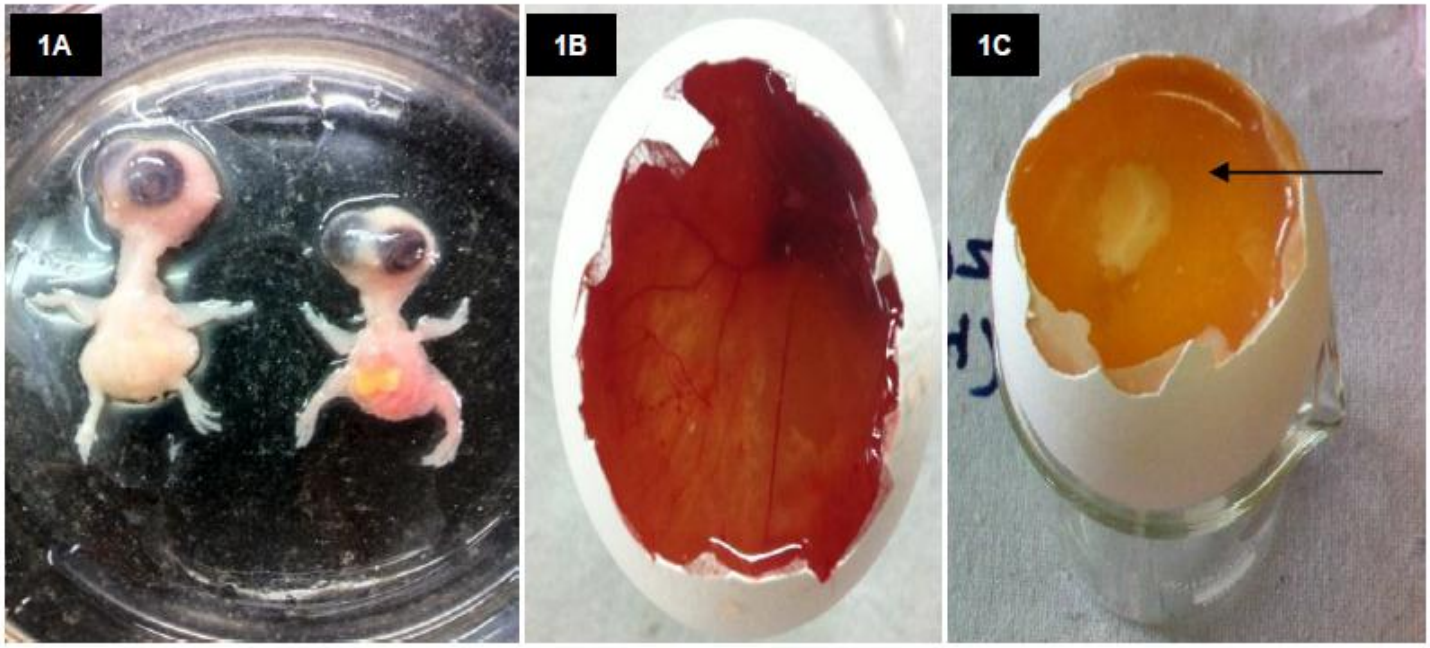

Figure 1: 1A- Photo showing comparison of 11th day control embryo (left) and reduced size Fluoxetine low dose (5mg/ml) treated embryo (right). 1B- control egg opened on 11th day, showing healthy blood plexus. 1C-Arrow indicates whitish lump like structure due to treatment with high dose (1mg/ml) of Etizolam

1) Effect of drugs on late development.

CHARACTERS (of embryo under study)	CONTROL	FLUOXETINE TREATED	ETIZOLAM TREATED
1. Size of embryo	8cm	6cm	5.9cm
2. Size of feather	3cm	2cm	1.1
3. Size of forelimb	5cm	3.2cm	2.5cm
4. Diameter of head	1.7cm	1.5cm	1.4cm
5. Diameter of eye vesicle	1.2cm	1cm	1cm
6. Beak size	1.1 cm	0.9cm	0.6cm
7. Feather development	Highly dev.	Moderately developed	Hardly developed
8. size of neck	1.7cm	1.4cm	0.9cm
9. Size and bending of neck	1.7cm Distinct	1.4cm Slight bending	0.9cm Very little bending
10. Weight of embryo	**13.39 grams**	**9.075 grams**	**7.53 grams**

Table 3: shows a comparative study of effect of both drugs on late development. The data collected indicates that Etizolam causes more physical abnormalities and reduced growth in terms of weight, size and other developmental characters.

Figure 2: 2A- Shows 18th day control embryo with well developed, head, feather and limbs. 2B-shows Etizolam treated 18th day embryo with stunted growth and under developed body compared to control. 2C-Fluoxetine treated 18th day embryo, showing moderate development compared to control. The arrow indicates heart malformation.

2) Effects of drugs on Hatching.

Control chicks hatched after three weeks of incubation (23rd day), and were able to stand on their own feet. However, the Etizolam treated embryo did not hatch; all embryos were dead, with blackened body, tumor like structures with foul smell. Further Fluoxetine treated embryo did not hatch until the 4th week of incubation, hence, assisted hatching (Kiran Kamran, April 2011)[8] were given.

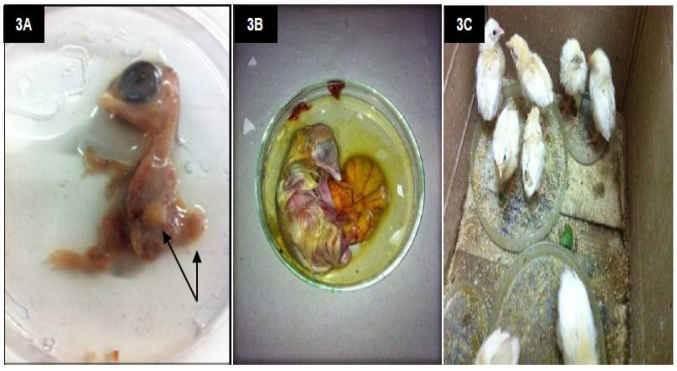

Figure 3: 3A- Etizolam treated embryo (28 days old), arrows indicate tumor blobs. 3B-Fluoxetine treated embryos (28 days old) showing incomplete retraction of yolk sac. 3C-control embryos hatched into healthy chicks.

3) Effect of drugs on DNA and Proteins.

Agarose gel electrophoresis showed two clear bands of control DNA (well2).Fluoxetine treatment caused slight damage to DNA, as reduced intensity of DNA bands (well 3) was observed. Etizolam treatment caused great damage to DNA, which was reflected by the absence of the formation of 1st DNA band. Moreover from the position of the second DNA band, DNA smearing was observed. This is indicative of random cleavage of DNA, which is also a characteristic of necrosis. Coherent results were obtained for quantitative analysis of DNA by DPA method. When DPA is added to DNA sample blue color is obtained, the deeper the color the greater is the concentration of DNA. Inspection by simple visualization yields that DNA concentration is less in Fluoxetine (light blue) compared to control (bright blue), and DNA concentration is least in Etizolam (no blue color). This is further verified by plotting standard curve and deriving concentrations in ug/ml.

SDS-PAGE analysis revealed that Etizolam banding (well 3) was very faint compared to control (well1), moreover distinct bands are not seen indicating that the drug has affected protein synthesis. In case of Fluoxetine (well 2), the banding pattern is similar to that of control, however an extra band (indicated by the arrow) was observed, indicating the synthesis of a new protein which is not synthesized in the control. The protein concentration in 1ml sample of Etizolam (19.2 ug/ml) was lesser then in 1ml of control sample (23.76 ug/ml) and the protein oncentration of Fluoxetine sample was more or less the same as control. Hence it could be inferred that Etizolam reduces protein synthesis and Fluoxetine results in synthesis of another protein which is absent in control.

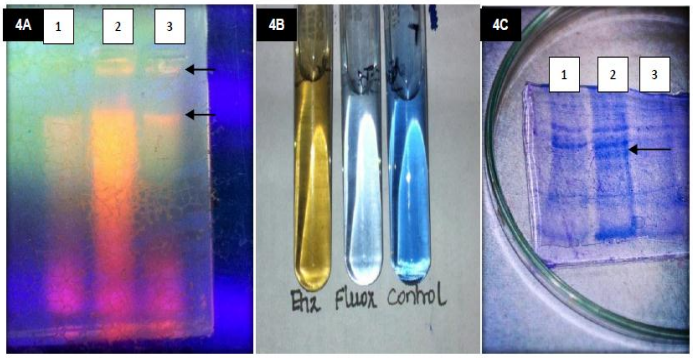

Figure 4: 4A- results of Agarose gel electrophoresis under ultraviolet, the arrows indicate band positions. 4B-color gradient after adding DPA during quantification of DNA, indicates DNA damage follows Etizolam>Fluoxetine>Control. 4C- SDS-PAGE, well 2 shows extra protein band which is absent in control sample (well3).

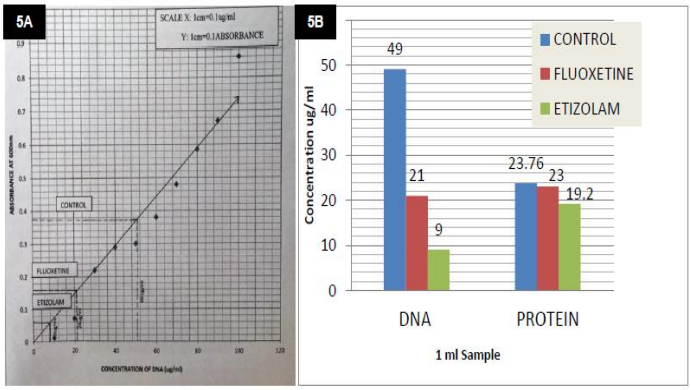

Figure 5: 5A- Graph showing standard curve for DNA concentration, and calculation of unknown concentration of samples by extrapolation. 5B- A comprehensive illustration of affected DNA and Protein concentrations in comparison to control, clearly shows that Etizolam causes more damage than Fluoxetine.

4) Incomplete apoptosis:

One of the effects of Etizolam on late development was incomplete apoptosis. The digits of limb Etizolam treated embryo were not completely separated, indicating hindrance to normal rate of apoptosis as compared to control.

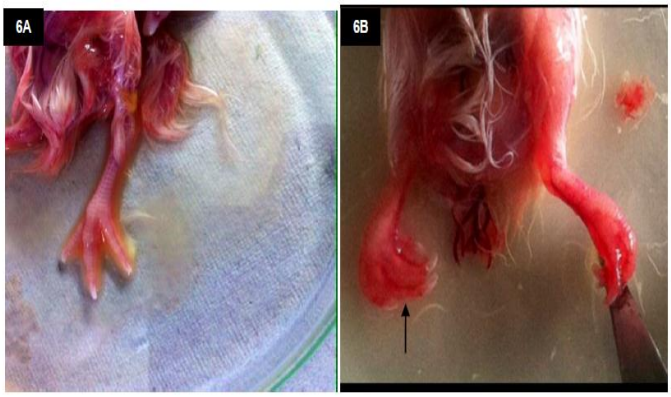

Figure 6: 6A- shows completely separated digits of control chick embryos. 6B- Shows incompletely cleaved digits in Etizolam treated embryo

DISCUSSION

The most sensitive time of pregnancy is usually during the first three months which is called the trimester. It is during this period that the formation of major organ systems of the body occurs. The growing fetus is supported by the growth of the amniotic sac. This is the substance that surrounds and protects the fetus for the entire duration of pregnancy. Oxygen and nutrients are carried from the mother to the fetus via the placenta and umbilical cord.

The antidepressant drugs have been reported to readily cross the placenta (Hendrick V, May 2003)[9]. As a result, there is direct exposure of the growing fetus to these drugs when ingested by the mother.

Fluoxetine and Etizolam have been classified as pregnancy category C drug, which means the drug is not safe for use during pregnancy as animal reproduction studies have shown an adverse effect on the fetus but there are no adequate and well-controlled studies in humans. The purpose of this project was to create an awareness of the teratogenic effects of these antidepressants on the developing fetus. The above study was carried out using chick embryos which is a model system for vertebrate development; hence we can co-relate the results to human embryos. We saw the effect of Etizolam on development of chick embryos, and it can be concluded that administration of this drug in early stages at concentrations of 0.1 & 0.5 mg/ml proves to be fatal by arresting growth. At a higher concentration that is 1mg/ml it results in tumor like lump in place of embryo. However in late development Etizolam causes reduced size and incomplete rather slow rate of apoptosis which was indicated by the not properly separated digits.

From the biochemical assays, it is clearly seen that the drug has not only caused phenotypic changes but also damaged DNA, and reduced its concentration. Smearing of DNA was observed on Agarose gel under ultraviolet, which is a characteristic of random cleavage during necrosis.

Necrosis and apoptosis are 2 ways in which a cell responds to toxins. Necrosis is caused by catastrophic toxic or traumatic events in contrast apoptosis is programmed cell death which is required for normal development.

In necrosis cells, leakage of cellular contents causes pro inflammatory response in neighboring cells, and they either inhibit or enhance apoptotic rates (Thompson 1995)[15]. In carcinogenesis inhibition of apoptosis has been correlated with tumor promotion (W. Bursch et al. 1992)[24]. Thus Etizolam may have caused necrosis of embryo cells which resulted in death of embryo and at

higher concentration it has also promoted formation of tumor by inhibiting apoptotic rates. Further, in late development, again the drug has thought to reduce apoptotic rates as separated digits were not observed.

As per central dogma of life, genetic information encoded by DNA is transcribed to RNA and then translated into proteins. Thus DNA damage will invariably affect protein synthesis. Etizolam has affected protein synthesis significantly, which was confirmed by SDS-page and Bradford assay. The reduction in protein concentration is precisely why embryos were reduced in their size.

Some clinical studies suggest poor neonatal outcome after exposure to Fluoxetine in uterus (Morrison JL, 2005)[17]. This was observed in the above study as well. After studying morphological effects it can be stated that Fluoxetine at low concentrations causes slow and retarded growth, however it can be detrimental at higher concentrations.

Women who take Prozac during the last trimester of their pregnancy may be lowering the birth weight of their babies and increasing the risk of preterm delivery and other complications California researchers have warned (Boston, Oct. 2, 1996). This warning stands true as a similar result was obtained in our study, where late ingestion of Fluoxetine (Prozac) not only caused reduced size and low birth weight but also showed malformed heart, thus acting as a teratogen. Fluoxetine also showed brittleness in bones indicating bone metabolism was affected. As a result of which assisted hatching was given (Kiran Kamran, April 2011)[12].

The biochemical assay revealed Fluoxetine damaged DNA to a small extent which was reflected by the reduced DNA concentration. However protein studies showed varied results.

Although there was DNA damage, protein synthesis was not affected in the sense the protein concentration was same as that of the control. In SDS-page gel analysis an additional band was observed, indicating synthesis of a new protein which was not synthesized in the control. Carolyn and Nicole[4] (1984) showed in their study that certain drugs that act as teratogen induce a subset of small heat shock proteins. In another study, it was stated that, Induction of heat shock proteins is closely correlated with cytotoxicity and lipophilicity of the substances given to the cell (Neuhaus-Steinmetz, 1997)[18]. That means when cells are under stress, heat shock proteins are synthesized. Thus it can be said that, treatment with Fluoxetine caused stress in the developing cells, as a result of which a new band of protein was synthesized that could be heat shock protein.

CONCLUSION:

From the above study it can be concluded that both drugs Etizolam and Fluoxetine act as a teratogen and affect the normal development of chick embryos. The response of embryo cells to both drugs is different. Etizolam causes necrosis which eventually leads to cell death, where as Fluoxetine treatment induces synthesis of another protein (such as heat shock) as a means to combat cell stress. However in early and late development Etizolam acts as a more powerful teratogen than Fluoxetine. The use of these antidepressant drugs should be avoided during pregnancy especially during the first trimester and third trimester. These drugs should be considered if and only if the benefits outweigh the number of risks.

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