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Teratological Effects of High Dose Progesterone on Neural Tube Development in Chick Embryos

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ABSTRACT

During the embryonic development, a complex program defines the normal neural tube development, alteration in this process, results in Neural Tube Defects (NTD). The present study found the effects of progesterone, more than the physiological value in neural tube development of chick embryos. 240 fertile, pathogen-free eggs, incubated at 37.5^{0}C and 75% relative humidity until the embryos reached stage ten of development. The eggs were divided into five equal groups. Statistical Analysis Used- None. Our study showed that progesterone at the ten and twenty times more than its physiological values caused NTDs.

Introduction

The congenital anomalies are the most important cause of death in infants under one year of age. Neural tube defects (NTDs) are one of the commonest birth defects causing significant morbidity and mortality. Children born with NTDs often have associated physical and mental handicap. Congenital central nervous system anomalies are the second most common anomalies following the cardiovascular anomalies [1]. In the early embryonic life, neural tube is the embryo precursor of central nervous system. The neural plate gradually deepens to form neural groove and neural folds; the folds become elevated and ultimately close in the mid-line to convert the groove into a closed tube. When the neural tube does not close completely, the neural tube defects (NTD) developed. Progesterone is a 21-Carbon (C_{21} H_{30} O_2) steroid hormone that is associated with pregnancy. The exogenous progesterone is commonly used in "in-vitro fertilization" or "intracytoplasmic sperm injection" therapies, repeated abortions or luteal phase defects [2]. The progesterone has its own beneficial effects on the development of embryo in pregnancy. Only few evidences are available on the adverse effects of these drugs, when it is consumed in overdose especially during pregnancy.

Progesterone administration is widely used in the in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) therapies to prolong the luteal phase in the pregnancy [3, 4, 5, 6, 7, 8, 9]. In IVF, the luteal phase has been defined as, the between the transfer of embryo to the secretion of endogenous hCG (human Chorionic Gonadotropin). Luteinizing hormone (LH) surge, may fail to reverse back during the initial luteal phase. The progesterone supplement is ultimately essential to maintain the luteal phase from the disappearance of exogenous hCG to the secretion of endogenous hCG in the early implantation period [10]. The embryo developed through IVF or ICSI are more likely affected with congenital anomalies, like cleft palate to spina bifida[11].

The reasons behind these congenital defects are not certain; however, the possible explanations may include the methodology of the procedure or how the egg, sperm and embryos are manipulated or the medications used to induce the ovulation or to sustain the pregnancy [11].

Materials & methods

Chick Embryos: The chick embryo has been routinely used to observe the teratological studies, because embryos are available in large numbers. Even though the eggs of hen and mammals contain different amounts of yolk, the similarities of the post-blastula chick embryo to the mammalian embryo are similar, and thus the chick embryo is a reasonably good model for studying vertebrate embryonic development. All aspects of animal care compiled with the ethical guidelines and technical requirements were approved by the Institutional Animal Ethics Committee (IAEC) and Institutional Review Board (IRB).

240 fertile, pathogen free eggs (White Leghorn, Gallus gallus) incubated at 37.50C and 75% relative humidity until the embryos reached stage ten of development according to Hamburger and Hamilton [12]. All the eggs were labelled and divided into five groups consisting of forty eight (48 nos.) eggs per group. The Group one (G1), Normal (uninjected) eggs; Group two (G2), Sham-operated eggs, injected with physiological saline; Group three (G3), injected with the Normal dose of progesterone; Group four (G4), injected with ten times the normal dose of progesterone and group five (G5), injected with high dose, twenty times more than physiological level of progesterone.

Dosage of Progesterone

Normal Progesterone level in the chick embryo was found to be 0.823 ± 0.035 nanogram /ml [13]. Ten and twenty times of the equal doses of progesterone excess (water soluble progesterone (C21H30O2), Sigma Aldrich) per egg will be calculated. The calculated dose of progesterone will be diluted in 0.1 ml physiological saline (0.9% NaCl).

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Table 1. Experimental design of Chick embryos

Hamburger & Hamilton Stage of Chick Embryo Development	Experimental Model				
	Normal Group (G1)	Sham-Operated Group (G2)	Normal Dosage Group (G3)	10 Times of Normal Dosage (G4)	20 Times of Normal Dosage (G5)
Stage – 0 (0 hrs)	12	12	12	12	12
Stage – 4 (18- 19 hrs)	12	12	12	12	12
Stage – 8 (26 – 29 hrs)	12	12	12	12	12
Stage –12 (45 – 49 hrs)	12	12	12	12	12

Method of injection

All the series of developmental stages mentioned in Table-1 (Hamburger & Hamilton), the eggs will be washed with 70% alcohol and properly labeled on the outer shell. A hole will be made on the blunt pole of the egg with a sharp and thick needle under aseptic condition. Using a sterile 28-guage needle and a tuberculin syringe, 0.1 ml of saline, Normal dosage,10 times and 20 times of progesterone excess will be injected to the corresponding groups of eggs. The gap created in the eggs, will be sealed [13].

Observations

The optimal stage of development and closure of neural tube in chick embryo models [12, 14] (Fig-1 & 2) are observed through the following parameters.

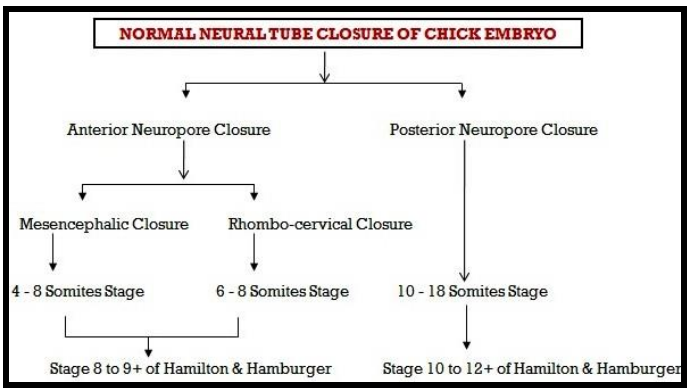

Figure 1. Normal stages of Neural tube development in chick embryos (Hamilton & Hamburger)

A. Gross observation of normal development:

In each group, to determine the optimal stage in the neural tube development of the chick embryos, the eggs will be removed from the incubator on Stage 14 / 15 (50-55hrs) of Hamburger & Hamilton. The eggs will be cracked open and the outer shell will be chipped out to create a large opening to see the embryo. The viability of the embryos will be assessed by the heart beat under ova view with ova scope. All the chick embryos will be transferred to a petri dish by the careful sterile dissection.

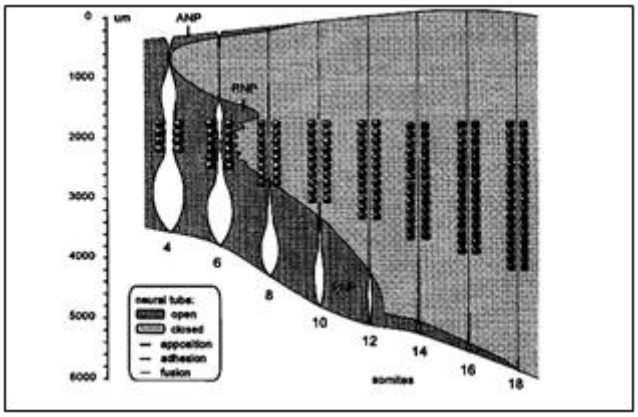

Figure 2. Multiphasic closure of Neural tube in chick embryos (Henry W.M.et al, 1996)

Stage – 0 (0 hrs): On examination it revealed that, 4/60, which is 1/12 embryo from G2, 2/12 embryos from each G4 and 1/12 embryo from G5 showed no development. Out of 42/60 live embryos 12/12 embryos of the G1, 11/12 embryos G2 and G3, 5/12 embryos from each G4 and 3/12 embryo from G5 are passed the characteristics of Stage 12+ development (18 somites stage). Whereas, in G3 1/12, in G4 5/12 and in G5 8/12 were underdeveloped after the 50-55hrs of incubation period (Table-2).

Stage – 4 (18-19 hrs): Under the dissected microscope examination it revealed that, 5/60, which is 1/12 embryo from G1, 1/12 embryo from G3, 2/12 embryos from each G4 and 1/12 embryo from G5 showed no development. Out of 39/60 live embryos 11/12 embryos of the G1, 12/12 embryos G2, 10/12 embryos of the G3, 4/12 embryos from each G4 and 2/12 embryo from G5 are passed the characteristics of Stage 12+ development (18 somites stage). Whereas, in G3 1/12, in G4 6/12 and in G5 9/12 were underdeveloped after the 50-55hrs of incubation period (Table-3).

Table 2. Experimental design of Chick embryos in Stage – 0 (0 hrs) groups

Hamburger & Hamilton Stage – 0 (0 hrs)	Experimental Model					
	Normal Group (G1)	Sham-Operated Group (G2)	Normal Dosage Group (G3)	10 Times of Normal Dosage (G4)	20 Times of Normal Dosage (G5)	TOTAL
Normal Development	12	11	11	5	3	42/60
No Development	0	1	0	2	1	4/60
Under Development	0	0	1	5	8	14/60

Table 3. Experimental design of Chick embryos in Stage – 4 (18 - 19 hrs) groups

Hamburger & Hamilton Stage – 0 (0 hrs)	Experimental Model					
	Normal Group (G1)	Sham-Operated Group (G2)	Normal Dosage Group (G3)	10 Times of Normal Dosage (G4)	20 Times of Normal Dosage (G5)	TOTAL
Normal Development	11	12	10	4	2	39/60
No Development	1	0	1	2	1	5/60
Under Development	0	0	1	6	9	16/60

Stage – 8 (26 - 29 hrs): On observation it revealed that, 6/60, which is 1/12 embryo from G1, 2/12 embryos from each G4 and 3/12 embryo from G5 showed no development. Out of 46/60 live embryos 11/12 embryos of the G1, 12/12 embryos G2 and G3, 07/12 embryos of the G4, and 4/12 embryo from G5 are passed the characteristics of Stage 12+ development (18 somites stage). Whereas, in G4 3/12 and in G5 5/12 were underdeveloped after the 50-55hrs of incubation period (Table-4).

Stage – 12 (45 - 49 hrs): On gross observations of experimental chick embryos, 4/60, which is 2/12 embryo from G2, 1/12 embryos from each G3 and G5 showed no development. Out of 53/60 live embryos 12/12 embryos of the G1, 10/12 embryos in G2 G3& G5 and 11/12 embryos of the G4 are passed the characteristics of Stage 12+ development (18 somites stage). Whereas, in G3, G4 and in G51/12 in each, were underdeveloped after the 50-55hrs of incubation period (Table-5).

B. Quantitative Observations:

The development of chick embryos in all the stages groups are 180/240, the embryos are properly developed according to Hamburger & Hamilton stage of development and their neural tubes were closed normally (Fig-3). All these embryos showed the following features of Stage-14, after the 50-55hrs of incubation period. Twenty-two somites: (50-53 hrs.). Flexures and rotation: Cranial flexure: axes of forebrain and hindbrain form about a right angle, cervical flexure a broad curve, rotation of body back as far as somites 7-9 (behind this level, a slight flexure makes its appearance which will be referred to as "trunk-flexure"), Visceral arches 1 and 2, and clefts 1 and 2 are distinct (posterior arches not distinct). Primary optic vesicle begins to invaginate and the lens-placodes were formed. Opening of auditory pit constricted. Rathke's pouch can be recognized and the ventricular loop of heart now ventral to atrio-ventricular canal.

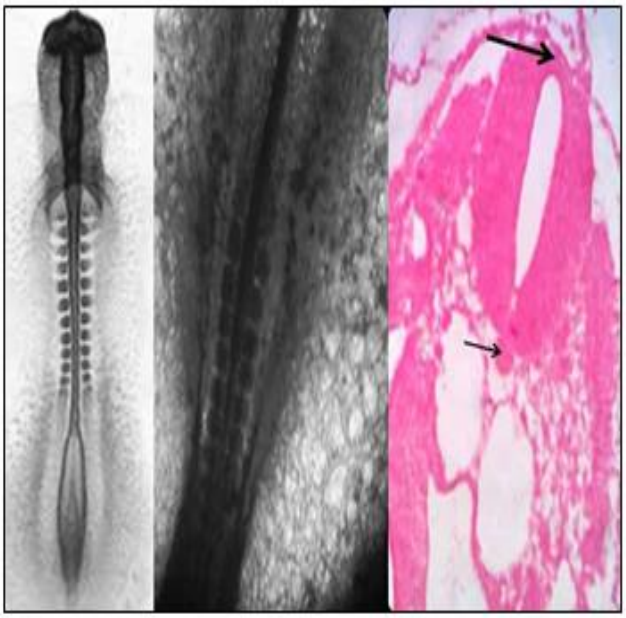

Figure 3. The neural tube in the chick embryo opened at the 50-53th hour, appeared to be closed in the complete. The thick arrow points at the neural tube that was closed, while the thin arrow points at the notochord (x40 magnification).

The embryos in Stage - 8, received with ten and twenty times more than physiological level of progesterone in both G4 (3/60) and G5 (5/60), predominantly shows the opening of

Table 4. Experimental design of Chick embryos in Stage – 8 (26 - 29 hrs) groups

Hamburger & Hamilton Stage – 0 (0 hrs)	Experimental Model					
	Normal Group (G1)	Sham-Operated Group (G2)	Normal Dosage Group (G3)	10 Times of Normal Dosage (G4)	20 Times of Normal Dosage (G5)	TOTAL
Normal Development	11	12	12	7	4	46/60
No Development	1	0	0	2	3	6/60
Under Development	0	0	0	3	5	8/60

Table 4. Experimental design of Chick embryos in Stage – 12 (45 - 49 hrs) groups

Hamburger & Hamilton Stage – 0 (0 hrs)	Experimental Model					
	Normal Group (G1)	Sham-Operated Group (G2)	Normal Dosage Group (G3)	10 Times of Normal Dosage (G4)	20 Times of Normal Dosage (G5)	TOTAL
Normal Development	12	10	10	11	10	53/60
No Development	0	2	1	0	1	4/60
Under Development	0	0	1	1	1	3/60

posterior neuropores in lumbo-sacral region. In stage – 4 embryos, those who received with ten and twenty times more than physiological level of progesterone in G4 (6/60) and G5 (9/60), reveals that failure in closure of both anterior and posterior neuropores. In the underdeveloped chick embryos, instead of 18 somites, 4 to 12 somites were seen.

C. **Qualitative Observations**

E**mbryo Collection and tissue preparation for microscopical observations:** To determine the optimal stage in neural tube development of chicks for histological observation, total six eggs (Table-1) in each group will be removed from the incubator on Stage 14 / 15 (50-55hrs) of Hamburger & Hamilton. All the chick embryos will be transferred to a petri dish by the careful sterile dissection. Then it will be fixed with 10% Neutral buffer formalin, embedded with paraffin, five microns (5m) thick paraffin sections will be cut and staining will be done with hematoxylin and eosin for microscopic examination.

On examination, under the 40X light microscope, the defective regions revealed that the neural folds usually heaped up normally, but fusion often failed to occur. In many of the embryos with neural tube defects, the elevated neural folds actually moved away, deviated laterally. The embryos treated with ten and twenty times more than physiological level of progesterone were showed NTD, due to failure in the elevated neural folds to fuse in the midline. F or stage – 8 treated groups, fusion occasionally failed to occur at various levels along the length of the neural tube, but frequently inhibited in the area of posterior neuropore (lumbo-sacral) region (Fig-4). But, stage – 4 treated groups who received the ten and twenty times more than physiological level of progesterone, reveals the failure of closure of neural tube in both anterior and posterior neuropore levels (Fig-5 & 6).

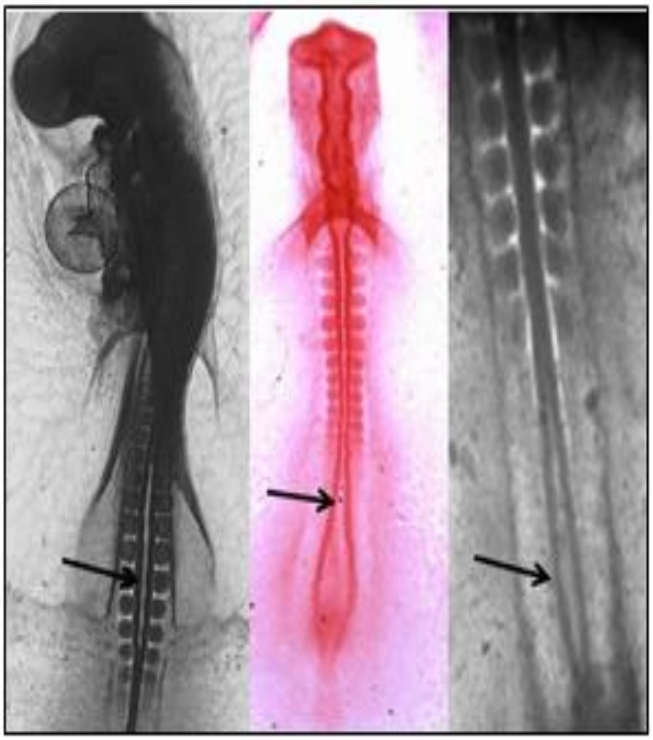

Figure 4. The neural tube in the stage-8 group chick embryos, treated with ten and twenty times more than physiological level of progesterone, appeared to be incomplete closure. The arrow points at the neural tube indicate the failure of closure of posterior neuropores (x40 magnification).

Figure 5. The neural tube in the stage-4 group chick embryos, treated with ten and twenty times more than physiological level of progesterone, appeared to be incomplete closure. The arrow points at the neural tube indicate the fusion occurs only at Rhombo-cervical and Mid-spinal region (x40 magnification).

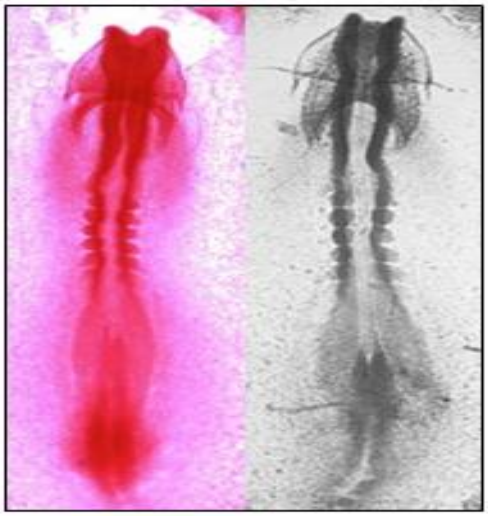

Figure 6. The neural tube in the stage-4 group chick embryos, treated with ten and twenty times more than physiological level of progesterone, shows the complete failure in closure.

Discussion

During the embryonic development, a complex program defines the differentiation & completion of the normal neural tube development. Any alterations in this normal process, results in NTD. The present study designed to observe the effects of ten and twenty times' more than physiological value of progesterone in neural tube development of chick embryos. The proposition under consideration, the high dose progesterone causes the neural tube developmental defects in the early chick embryo models. The analysis made through the hematoxylin and eosin (H&E) stained microscopical observations. The chemical agents such as Caffeine, Phenytoin, Diazepam and local anesthetics are known teratogens for the neural tube defects in chick embryos [15]. Stage zero and twelve chick embryos [12] were chosen in this study for the investigations. The developing neural tube tissue exhibits gradual variations in the degree of openings along its length, which provides an exceptional opportunity to study the effects of chemical agents on the closure of neural tube [16].

The present study observations on the chick embryos, indicated that high dose of progesterone causes an inhibitory effect on the closure of neural tube. Previous studies showed that high dose extraneous progesterone is a known teratogenic agent in certain animal models. In rabbit embryos, the high dose progesterone administration during the period of development, cause neural tube defects [17]. In chick embryos, exposure to the high dose of progesterone causes neural tube defects. Until recent, in human embryos, the progesterone was frequently used in repeated abortions or luteal phase defects.

Majorities of the previous workers failed to explain that, the progesterone can be a human teratogen [6, 18, 19]. All the previous studies failed to explain, that the dose dependent response relationship with high dose progesterone administration during the period of organogenesis. Eventually, the IVF or ICSI were only the large scale uses of exogenous progesterone in the teratogenically potential period of pregnancy. In IVF and ICSI the exogenous progesterone were administered during the organogenesis period of the embryo. According to the international guidelines, maximum daily dose of progesterone is 600 mg as vaginal gel or pessaries, or 100 mg as intramuscular and the average serum levels of progesterone achieved at this dose would be around 50- 60 ng /ml ^{2}. The normal ranges of plasma levels progesterone were identified as 9-45 ng/ml in first trimester. However, in an anecdotal evidence from an IVF specialists suggested in clinical practice, the doses have been known to up to 1600 mg for three times daily (in case of multiple pregnancies), and plasma levels reaching over 250 ng/ml , on an average of 7-30 times. The serum level corresponds to the 20 times physiological levels of progesterone, which was instinct to the present experiment.

Conclusion

Progesterone acts as an agonist to GABA-A receptors. GABA-A receptor agonists increase the occurrence of neural tube defects, especially spina bifida [20]. The anti-epileptic drug like Valproic acid were known for its teratogenic effects, mainly acts on GABA-A receptor [20, 21]. It would be more significant to experiment this hypothesis in future and explain the mechanisms of this teratogenic effect, which would be essential in clinical use to women undergoing IVF or ICSI treatment.

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