Research Article

Progesterone in High Doses Causes Neural Tube Defects in Early Chick Embryo Model

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Summary

Introduction: Progesterone is an essential hormone for the establishment and maintenance of pregnancy by inducing secretary changes in the lining of the uterus, which are important for implantation of the fertilized ovum. During pregnancy in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are the clinical context in which natural, exogenous progesterone is administered during the period of organogenesis of the embryo. IVF & ICSI are associated with increased incidence of birth defects.

Objective: To describe the effect of high dose progesterone on occurrence of neural tube defects in chick embryos.

Material and Methods: 45 Fertile, specific pathogen free eggs of Fyoumi species of chick were selected at zero hour of incubation. They were incubated at 37.5°C and 75% relative humidity until the embryos reached stage eight of development. At this stage the eggs were divided into three groups consisting of 15 eggs per group. The first group was incubated without any operation. The second group was injected with physiological saline and third group was injected with High dose progesterone. After 48 hours of incubation, all embryos were reviewed for the presence of Neural tube defects under light microscopy.

Results: None of the eggs in Group1 & Group2, showed NTDs, whereas 75% (9/12) of the embryos in the Group 3 (high dose progesterone injected) showed NTDs.

Conclusion: Exogenous progesterone at levels twenty times above its physiologic range in chick embryos causes NTDs. Further studies are needed to explain the mechanisms of this teratogenic effect, its clinical significance, and prevention.

Key words: Neural tube defects, progesterone, chick embryo, neural tube closure

Yüksek Dozdaki Progesteron Erken Civciv Embryo Modelinde Nöral Tüp Defektlerine Neden Olur

Özet

Giriş: Progesteron, hamileliğin oluşması ve devamlığı olayında döllenmiş yumurtanın implantasyonu için önemli olan uterusun iç katmanında sekretuar değişiklikleri sağlayan asal bir hormondur. İn-vitro Döllenme (IVF) ve Sıtoplazma İçine Sperm Enjeksiyonu (ICSI) ile hamilelik sırasında embryonun organogenezi sürecinde ekojen progesteron verilmesi doğaldır. IVF ve ICSI yükse k oranda görülen doğum defektleri ile birliktedir.

Amaç: Bu çalışmanın amacı civciv embryolardaki yüksek doz kullanılan progesteronun nöral tüp defektlerinin görülmesine olan etkisini tanımlamaktır.

Materyal ve Yöntem: İnkübasyonun 0 zamanında olan Fyoumi cinsi civcivlerinin 45 döllenmiş sağlıklı yumurtaları seçildi. Gelişimlerinin 8.evrelerine değin 37,5°C ve %75 oransal nem ortamında inkübe edildiler. Bu evrede yumurtalar 15'erli olmak üzere 3 ayrı gruba ayrıldı. İlk grup hiç bir işleme tabi tutulmadan inkübe edildi. İkinci gruba fizyolojik...
sonuçlar: İlk 2 gruptaki (Grup 1 ve Grup 2) hiçbir yumurtada NTD'i gözlemendi. Buna karşılık yüksek doz progesteron uygulanan grupta (Grup 3) %75 (9/12) oranında NTD olduğu gözlandı. Yargı Fizyolojik sınırların 20 misli üstünde ekzojen olarak verilen progesteron civciv embriolarında nöral tüb defektlerine neden olur. Bu teratojenik etkinin mekanizmasına, klinik önemini ve önlenmesini ortaya çıkartacak ileri çalışmalara gereksinim vardır.

Anahtar Kelimeler: Nöral tüb defektler, progesteron, civciv embriyo, nöral tüb kapanması

INTRODUCTION

Although it is known that folic acid deficiency is one of the factors which can cause spina bifida and other NTD but the exact pathophysiology is still not clear. Progestogens are a group of hormones, which bind to the progesterone receptors; they include both the natural female sex hormone progesterone and the synthetic forms. Progesterone is secreted during early pregnancy from the ovary by corpus luteum. It is an essential hormone for the establishment and maintenance of pregnancy by inducing secretory changes in the lining of the uterus, which are important for implantation of the fertilized ovum. Progesterone is secreted during early pregnancy from the ovary by corpus luteum. It is an essential hormone for the establishment and maintenance of pregnancy by inducing secretory changes in the lining of the uterus, which are important for implantation of the fertilized ovum. Progesterone modulates the immune response of the mother to prevent rejection of the embryo and it enhances uterine quiescence and suppresses uterine contractions.

Progesterone is often given to women whose corpus luteums do not produce enough progesterone. When this happens, the endometrial lining does not thicken enough to allow for implantation. Progesterone is administered to increase the length of the luteal phase, helping to allow for pregnancy.

Progesterone is also widely used in in-vitro fertilization (IVF) therapies. In earlier meta-analysis indicated that after IVF it is of value to give luteal support, using either HCG or Progesterone during the luteal phase itself. The luteal phase has been defined as the time span from the day of transfer of embryos until measurement of HCG 2 weeks later. Using the long protocol and down regulation with GnRH agonists. LH secretion may not have completely recovered during luteal phase .therefore progesterone supplement could be of benefit in order to, cover the gap, between the disappearance of exogenous HCG and the rise of endogenous HCG during early implantation.

Throughout the world, clinicians prescribe progesterone to women who achieve a pregnancy after either IVF or ICSI. Babies born through IVF are up to 4% more likely to suffer from birth defects that range from relatively minor problems like cleft palate to severe ones such as spina bifida. The cause of the defects is not certain; however, possible explanations include the methodology of the procedure itself, how the egg, sperm or embryo are manipulated, or the medications that are given to induce ovulation or to sustain pregnancy.

Around the world, there are hundreds of thousands of pregnancies affected each year by an NTD, with some fetal demise through spontaneous or induced losses. Seven percent of infant deaths from birth defects are a result of NTDs. Most of the studies on the teratogenic effects of progesterone have dealt mostly with the possible masculinising effect on the female fetus. Other abnormalities that have been described with the use of progesterone are: possible cardiac malformations, the effects on the central nervous system, development of amelia.
Although maternal ingestion of folic acid during preconception and early gestation prevents the occurrence of NTD in about 50-70% of the cases according to recent epidemiological studies\(^{(23)}\).

The present study was designed to describe the effect of high dose progesterone in neural tube development of chick embryo. The hypothesis under consideration was that high dose progesterone causes neural tube developmental defect in early chick embryo models.

**MATERIAL AND METHODS**

**DESCRIPTION OF CHICK EMBRYOS**

30 Fertile, specific pathogen free eggs of Fyoumi species of chick were selected and obtained from Poultry Research Institute Punjab, Rawalpindi at zero hour of incubation. The eggs were incubated at 37.5\(^\circ\) C and 75% relative humidity until the embryos reached stage eight of development according to Hamburger and Hamilton\(^{(9)}\). At this stage the eggs were labelled and divided into three groups consisting of 15 eggs per group. These groups were:

- G1, uninjected eggs;
- G2, injected with N/S;
- G3, injected with HDP

**DOSAGE OF PROGESTERONE**

The normal progesterone level that a chick embryo is exposed to is found to be 0.823 ± 0.035 ng/ml\(^{(21)}\). The calculated dose of progesterone, 157 nanograms, (Water Soluble Progesterone, Sigma - Aldrich Comp. code: P7556, St. Louis, Missouri, USA) was diluted in 0.1 ml of physiological saline (0.9% NaCl) for G3.

**METHOD OF INJECTION**

At stage eight of development, (26 to 29 hrs) the eggs from G2, and G3, were washed with 70% alcohol and properly labelled on the outer shell. A hole was made on the blunt pole of the eggs with a sharp and thick needle under laminar flow. Using a sterile 28-gauge needle and a tuberculin syringe, 0.1 ml of the fluid was injected from the blunt end under the embryonic disc. The holes were sealed with paraffin\(^{(21)}\). The eggs were then being placed again in the hatchery.

**EMBRYO COLLECTION**

The eggs were opened at 48 hours of incubation. The eggs were cracked open and the outer shells were chipped out to create a large opening to see the embryo. The viability of the embryos was assessed by the heartbeat. The embryos were transferred to a Petri dish by careful dissection along the allantoic stalk and other embryonic structures. All the embryos were fixed with Carnoy’s fluid, stained with HCl–carmine and examined under stereomicroscope to assess any gross developmental abnormalities. Then, embryos which were passed Hamburger Hamilton stage 12 were embedded into paraffin and seven microns thick paraffin sections were cut for light microscopic examination.

**RESULTS**

**Development**

Under dissecting microscopic examination it was revealed that, 8/45, that is 2 embryos from G1, 3 embryos from each G2 and G3 showed no development. Out of 37 live embryos 13/13 embryos of the G1, 12/12 embryos of the G2, 9/12 embryos of the G3, passed characteristics of Stage 12 development, while 3 embryos from G3 were under developed after 48 hours of incubation.

**Quantitative Observations**

Development of all the embryos from G1 and G2 were according to their stage of development, and their neural tubes were closed.

In the underdeveloped embryos of G3. Instead of 16 somites, 10 to 12 somites were seen; anterior neuropores were open.
Three primary brain-vesicles were clearly visible. Optic vesicles were not constricted at bases and hearts were bent slightly to right.

There was Neural tube development defect in nine embryos (9/12) of G3 and it was mostly in the region of lumbosacral region.

**Qualitative Observations**

Treatment of embryos with HDP resulted in a high percentage of embryos exhibiting non-closure-type neural tube defect. Examination under 4X objective of dissecting microscope of defective regions revealed that the neural folds usually elevated normally, but convergence often failed to occur (Fig. 3). In many of the embryos with neural tube defects, the elevated neural folds actually diverged, flaring laterally (Fig. 5). The formation of neural tube defects in embryos treated with HDP was due principally to a failure of the elevated neural folds to converge toward the dorsal midline. Fusion occasionally failed to occur at various levels along the length of the spinal cord, but much more frequently fusion was inhibited only in the area of the posterior neuropore (Fig. 3,5)

**Figure 1:** Examination of 48 hours embryo of group 1, (G1)under dissecting microscope. Embryo is fully developed according to its stage of development. Neural tube is closed throughout its length.(Examination under 40X)

**Figure 2:** Transverse section through the spinal cord region of a group1(G1) embryo. The neural tube is closed.(under 400X)
Figure 3: Examination of 48 hours embryo of experimental group three (G3), under dissecting microscope. Embryo is under developed according to its stage of development. Anterior and Posterior neuropores are open. (Examination under 40X)

Figure 4: Examination of 48 hours embryo of experimental group three (G3), under dissecting microscope. Embryo is fully developed according to its stage of development, neural tube is opened in lumbosacral region. (Examination under 40X)

Figure 5: Transverse section through the spinal cord region of a group3(G3) embryo. The neural tube is opened. (under 100X)
Table 1: Development of embryos of each group after 48 hours of incubation and the rate of neural tube defects

<table>
<thead>
<tr>
<th>Groups</th>
<th>No/insufficient development (%)</th>
<th>Normal development</th>
<th></th>
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<tr>
<td></td>
<td>Dead embryos</td>
<td>Underdeveloped embryos</td>
<td>Neural tube defect observed (%)</td>
</tr>
<tr>
<td>G1</td>
<td>15 2 0</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>G2</td>
<td>15 3 0</td>
<td></td>
<td>0%</td>
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<tr>
<td>G3</td>
<td>15 3 3</td>
<td></td>
<td>9(75%)</td>
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DISCUSSION

The major finding of this study is that in chick embryos high dose progesterone causes neural tube defects.

The strongest evidence for drugs causing neural tube defects exists for valproic acid and carbamazepine. Besides these antiepileptics, drugs that act as folate antagonists are also known to cause teratogenicity in the form of NTDs. Such drugs include aminopterin, methotrexate, and possibly trimethoprim, which is one of two drugs that constitute the widely used antibiotic trimethoprim-sulfamethoxazole (Septran)\(^{18}\).

It would be pertinent to briefly review the various effects of progesterone in the human body others than its principal role – preparation of the uterus to support and maintain pregnancy. Progesterone provides negative feedback at the level of hypothalamus and pituitary, and at midcycle, it acts with estrogen to promote the pre-ovulatory LH surge. Progesterone is also an extremely potent respiratory stimulant, and is thermogenic, causing approximately a 1° C increase in temperature at the time of ovulation. It has central nervous system depressive effects and at extremely high doses, it has anesthetic properties. Metabolic effects of progesterone include a decrease in serum high density lipoprotein level\(^{4}\).

Previous studies show that high dose extraneous progesterone is a known teratogen in certain animal models. In rabbit embryos, when progesterone is introduced at the appropriate time of embryonic development, neural tube defects result frequently\(^{(1)}\). In chick embryos, exposure to high dose, natural progesterone has been shown to cause neural tube defects\(^{(21)}\). In human embryos, natural progesterone is frequently used in threatened abortion, luteal phase defect and as part of in-vitro fertilization (IVF) therapy and ICSI. The use is primarily intended to supplement the endogenous production of progesterone by the corpus luteum, and later the placenta, during pregnancy. IVF and (intracytoplasmic sperm injection) ICSI are the clinical context in which natural, exogenous progesterone is administered during the period of organogenesis of the embryo. Thus, the findings of the present study are particularly relevant to IVF, which is being performed with increasing popularity all around the world. Theoretically, the dosage of exogenous progesterone administered during in-vitro fertilization should be small, as it is intended to make up for the iatrogenically induced deficiency of endogenous progesterone caused by the
use of GnRH agonists or antagonists during the procedure of IVF. International guidelines state a maximum daily dose of 600 mg progesterone as vaginal gel or pessaries, or 100 mg intramuscular(26) and the average serum levels of progesterone achieved at this dose would be around 50-60 ng/ml(26) however, anecdotal evidence from Pakistani IVF specialists suggests that in clinical practice, the doses have been known to go up to 1600 mg three times daily (in case of multiple pregnancies), and plasma levels reaching over 250 ng/ml 7-30 times the normal range of 9-45 ng/ml in first trimester. Serum levels this high correspond to the 20 times physiological levels of progesterone that was introduced in the present experiment.

Although a large number of studies have failed to show progesterone as a human teratogen(12,24,28). None of the studies explored a dose-response relationship with high dose progesterone administered in the period of organogenesis. Finally, IVF, the only large scale use of natural progesterone in the teratogenically relevant period of pregnancy, has been shown to increases the risk of neural tube defects(11). Whether it is due to the use of exogenous progesterone or not is not known, but, the discussion on the role of progesterone in occurrence of neural tube defects during IVF is far from conclusive.

One such mechanism is inhibition of folate transporters at the cellular level, which can lead to folate deficiency and predispose to neural tube defects. Indeed it has also been noted in in-vitro studies that progesterone inhibits folic acid uptake and efflux in human trophoblasts by its action on the folic acid transporter RFC (Reduced Folate Carrier)(13). Whether this potential mechanism has a significant role in production of neural tube defects in humans or animal models of neural tube defects remains to be elucidated.

Modification of activity on GABA-A receptor in neural embryonal neural tissue is the key to understanding the effects of progesterone in the present study. GABA-A receptor agonists increase the frequency of neural tube defects, especially spina bifida(2), the anti-epileptic drug Valproic acid, which is notorious for its teratogenic effects, and has important effects on the GABA-A receptor, may act through this pathway(2,20). Progesterone acts as an agonist at GABA-A receptors. It would be pertinent to test this hypothesis in future experimental studies, that would be of clinical use to women undergoing IVF treatment.

The strongest evidence for drugs causing neural tube defects exists for valproic acid and carbamazepine. Besides these anti-epileptics, drugs that act as folate antagonists are also known to cause teratogenicity in the form of NTDs. Such drugs include aminopterin, methotrexate, and possibly trimethoprim, one of two drugs that constitute the widely used antibiotic trimethoprim-sulfamethoxazole (Septran)(18). Diazepam and Cotinine, a metabolite of nicotine, have been shown to cause neural tube defect in chick embryo models(6,7).

In relation to this study, there are many unanswered questions that can be addressed in future research work. It is not clearly known whether the progesterone is related to increased risk of neural tube defects in babies born through IVF. Progesterone dosage regimens in IVF are non-standardized and multiple additional factors such as absorption and metabolism of various dosing forms used in IVF need to be evaluated in detail. Large, follow-up studies on babies born through IVF would be able to settle this question in future research.

Further research is required to explain the mechanisms of this teratogenic effect and to explore whether a dose-response mechanism exists. Only then, definite recommendations can be made on the need for reduction of use of progesterone, or preferential use of a specific preparation or
route, or supplementation that would decrease the risk for neural tube defects caused by progesterone in babies born through IVF and ICSI.

CONCLUSIN

In conclusion, the present study found that exposure to high dose progesterone greatly increases the incidence of neural tube defects in chick embryos. Further research is required to explore the relationship between natural progesterone and neural tube defects in humans.

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