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ORIGINAL ARTICLE

Limb Defects in Developing Chick Embryos after Administration of Glucose

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ABSTRACT

Objective: To assess the role of administered glucose on gross development of limbs of the chick embryos, in comparison with age matched controls.

Materials and Methods: Fertilized eggs of Egyptian Fayyumi breed were injected with glucose (5% weight/volume solution) into egg albumen. The eggs were put in the incubator under standard conditions of temperature and humidity. Eggs were divided in two groups, control (A) and experimental (B). Each group is subdivided in two subgroups comprising of 30 eggs each. Eggs were opened on day 10 of incubation in subgroup (A1, B1) while eggs from subgroup (A2, B2) were allowed to hatch on day 21 or day 22 of incubation. The exposed embryos were compared with age matched control subgroups. Effects of glucose were assessed by observing limb defects in the embryos from two groups.

Results: The experimental animals had limb defects manifested as absent, one hind limb in 5 out of 53 (9.433%). Both hind limbs were absent in 6/53 (11.32%). In turned phalanges with inability to stand and limping gait was found in 8/53 (13.79%). No embryo of the control group demonstrated such findings.

Conclusion: Administration of glucose resulted malformation such as limb defects and turned digits in growing chick embryos.

Key words: Glucose, Chick embryos, Limb defects.

Author`s Contribution
[1] Conception, synthesis, planning of research and manuscript writing Interpretation and discussion
[2] Data analysis, interpretation and manuscript writing, [3] Active participation in data collection.

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Introduction

Congenital malformations occur when the development of a structure is arrested, delayed or misdirected in fetal life. Evidence that maternal diabetes significantly increases the risk for congenital malformations has accumulated over the last several decades.[1] The total number of people with diabetes is projected to rise from 382 million in 2013 to 592 million in 2035 as mentioned in a study estimating the prevalence of this disorder. The same study indicates that in Pakistan this number will rise from 6,713,000 in 2013 to 12,798,000 in 2035.[2] Hyperglycemia, is a constant feature of diabetes mellitus [3] and a positive correlation is present between hyperglycemia during embryogenesis and congenital anomalies as evidenced by clinical and experimental data.[4,5]

Chicken (*gallus gallus domesticus*) and its eggs is a good comparative model for basic sciences because of easy availability, and low cost. Chicken genome is found to have significant similarities with that of humans.[6] This animal model can be used to explore the adverse effects of glucose on development and contribute in avoidance of

unfavorable consequences of pregnancy in humans. In chick embryos limb development is indicated as a small "bud," protruding from the body and comprised of lateral plate mesoderm (LPM) cells and the overlying surface ectoderm. The mesenchymal cells in this bud give rise to the skeletal elements while the limb muscles arise from cells that migrate into the limb bud from the somites. Limb development depends upon the maintenance of three signaling centers within the limb bud: the apical ectodermal ridge (AER), at the distal margin of the bud; the zone of polarizing activity (ZPA) in the mesenchyme at the posterior margin of the bud and the non-ridge ectoderm of the bud.[7] There are different signaling molecules for the development to proceed smoothly. Sonic hedgehog (Shh) is produced in the zone of polarizing activity (ZPA) of the limb bud and it specifies muscle pattern in the chick limb.[8] In addition to Shh Fibroblast growth factor-8 (FGF8) is another morphogen expressed in the AER responsible for maintaining the outgrowth and differentiation of the mesenchymal cells making up the limb bud.[9] The current study was planned to investigate the outcome of exogenous glucose on gross limb development in chick embryos keeping in mind the increasing prevalence of diabetes, particularly among the women in their reproductive years.[10] The administration of glucose provides an opportunity to access the direct effects of this individual metabolite.

Materials and Methods

This randomized control trial was carried out at Anatomy Department, Regional Centre, College of Physicians and Surgeons Pakistan, Islamabad from January 2013 to January 2014. A total of 120 eggs belonging to Egyptian Fayoumi breed of *Gallus domesticus*, were obtained from Poultry Research Institute, Punjab, Rawalpindi. The cracked eggs and those stored in the refrigerator were excluded from the study. All selected eggs were counted and numbered starting from 1 to total count and were given labels depicting the sample number, group, subgroup and date of injection. After marking, the eggs were randomly allocated into control group (A) and experimental group (B) of 60 eggs each using the random selection table. Each group was again randomly divided into two subgroups 1 and 2 comprising of 30 eggs. The day on which eggs were put in the incubator was taken as day 1. Eggs from subgroup (A1 and B1) were opened on day 10 of incubation while those from subgroup (A2 and B2) were allowed to hatch on day 21 or 22. The experimental group was injected 15mg of 5% weight/ volume solution of glucose into egg albumen while the control group was injected with same volume of normal saline before putting into incubator. The glucose used for injection was 5% weight/ volume solution.

Un-incubated egg of day 0 was sponged with sterile cotton gauze moistened with 70% ethanol. Each egg was kept vertically with blunt pole upwards for sometimes so that the blastoderm floats up. Then a hole was drilled into its upper pole where the air sac is located. Another hole was drilled at a point about a finger span above the lower pole with a thumb pin. Only the shell was punctured. Next, the shell membrane of the upper pole was pierced through hole already drilled with an empty insulin syringe with needle size 30 gauges' x 8mm.This was to release air from the air sac. Another insulin syringe having measured dose of glucose was used to inject the contents into the albumen through the lower hole. The two holes were sealed soon after injecting the dose with melted paraffin and the eggs were placed in the incubator (manufactured by Memmert Electric Company Germany) after thoroughly cleaning the incubator. Incubation was under standard monitoring with temperature maintained at 38 ^{0}C and relative humidity was kept between 60-70%. An uninterrupted electric supply was maintained to the incubator. Eggs were rotated ½ turn twice daily. This change in position aids in gaseous exchange between the embryo and outside air and adequate nutrient distribution to the developing embryo.[11]

The embryos from subgroup A1 and B1 were dissected out of the eggs on day 10 of incubation. After breaking the shell from the broader end in a bowl of water, embryos were cleanly extracted avoiding trauma. Those from subgroup A2 and B2 were allowed to hatch. The number and percentages of embryos with gross limb defects were recorded. Data were entered on SPSS version 14 for analysis. Fisher's exact test was applied and p-value < 0.05 was considered statistically significant.

Results

There were 120 eggs in the study that were divided equally into 4 groups of 30 each. The number of alive

chicks in experimental group (B1 and B2) was 53/60 (88.33%) while in control group (A1 and A2) this number was 60/60 (100%). The experimental animals had limb defects manifested as absent one hind limb in 5 out of 53 (Table 1; Figure 1).

Table 1: Comparison of limb defects between experimental and control groups

Anomalies	Groups; n(%)		p-value
	Control (n=60)	Experimental (n=53)	
Absent one lower limb	0	5 (9.43)	0.026
Absent both lower limbs	0	6 (11.32)	0.013
In turned phalanges with difficulty to stand	0	8 (13.79)	0.006

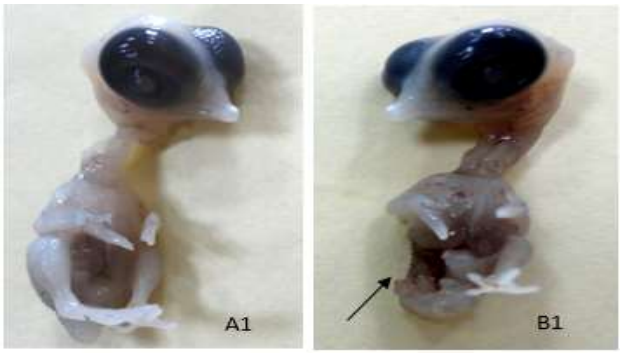

Figure 1: Photograph of day 10 chick embryo belonging to experimental group B1 with one lower limb missing (Arrow). Compare with age matched control A1

Figure 2: Day 10 chick embryo belonging to experimental group B1 with both hind limbs missing along with protruded abdominal contents

Both hind limbs were absent in 6/53 (Figure 2). In turned phalanges with inability to stand and limping gait was found in 8/58 (Figure 3). No embryo of the control group demonstrated such findings (Table1).

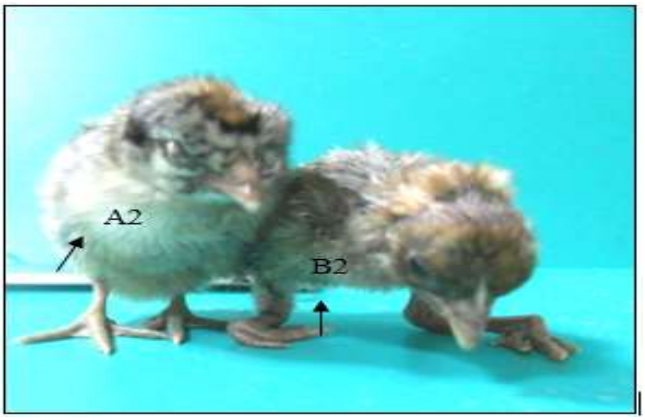

Figure 3: Photograph of newly hatched chicks. The glucose exposed subgroup B2 showing in-turned fingers (arrow) with inability to stand properly while A2 belongs to control subgroup.

Discussion

Embryogenesis is a complex process, which can be altered by genetic and environmental factors causing birth defects. One of the environmental factors having strong influence on developmental process and increasing the risk of congenital malformations is high blood glucose level, as a result of maternal diabetes. The offspring of mothers with unrecognized type 1, type 2, or gestational diabetes have a high risk for being born with congenital anomalies, macrosomia, and neonatal, childhood, and adult complications.[12] Although these complications are known, few models exist to determine the effects of glycemic stress on the development of congenital malformations during organogenesis. We administered glucose in fertilized chick eggs before incubation to mimic embryonic conditions during maternal hyperglycemia. In our study, high glucose abridged the development of limbs. Our observations are consistent with the findings of the research done by Yao Chen and colleagues, which revealed that increased glucose concentration caused decreased number and changed morphology of somites accompanied by alteration in the limb bud development in chick embryos. The study also reported inhibition of Shh by treating the chick embryos with high gucose.[13] Since cells from somites give origin to limb muscles and Shh

specifies the muscle pattern[8], alteration in these two factors might be responsible for limb defects seen in our work.

The same study reported that in the presence of high glucose, FGF8 expression was significantly repressed in the AER of the limb buds. As FGF8 is essential for limb growth, and for the induction of Shh expression, this would explain why limb buds did not develop in our embryos treated with glucose. Another study reported complete arrest of hind limb development in mice in which FGF 8 was conditionally inactivated.[14] Exogenous glucose also resulted in impaired cartilage development in chick hind-limbs resulting from repressed formation of the myotome by disrupting Shh signaling.[13] This might be an underlying mechanism which in turn effected the development of the skeleton and musculature of limbs in our study. One possible reason might be the disruption of Wnt signaling pathway by glucose as it is proposed that Wnt signaling is involved in the AER formation in chick limb bud via β catenin pathway.[13],[15] Inactivation of β catenin pathway leads to degeneration of AER and limb truncation.[16]

The development of cartilage and ossicles present in the sclera of chick was also disrupted by glucose as revealed by a research work done already. The study showed decreased number of chondrocytes in addition to disordered growth of bones in sclera in experimental animals.[17] This abnormal limb phenotype has also been reported in infants whose mothers had gestational diabetes, or preexisting diabetes. Caudal regression syndrome is particularly strongly associated with diabetes mellitus. It is a condition in which agenesis or hypoplasia of the femorae occurs in conjunction with agenesis of the lower vertebrae.[18,19]

One of our experimental animal had protruded abdominal contents along with absent hind limbs. The combination of two abnormalities necessitates further studies to guarantee better understanding of the extent of birth defects caused by increased concentration of glucose.

Conclusion

Exposure of developing chick embryos to glucose resulted in absent lower limbs along with in turned fingers and protruded abdominal contents.

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