

Effects of Glucose Administration on Development of Sclera in Chick Embryos

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ABSTRACT

Objective: To determine the effect of glucose administration on the development of sclera in the chick embryo *Gallus domesticus*.

Study Design: Experimental study.

Place and Duration of Study: Anatomy Department, CPSP Regional Centre, Islamabad, from January 2013 to January 2014.

Methodology: The study was carried out in two main groups, control A and experimental B, which were subdivided into three subgroups comprising 30 eggs each. The group A was injected with normal saline (0.3 ml) in the egg albumen. The group B was injected with 0.3 ml of 5% w/v solution of glucose equivalent to 15 mg of glucose. Subgroups A1 and B1 were opened on day 10 of incubation. Subgroups A2 and B2 were sacrificed on day 12 of incubation. Eggs from subgroups A3 and B3 were opened on day 15 of incubation. Experimental subgroups were compared with matched control subgroups and quantitative data was analysed statistically.

Results: Administration of glucose resulted in changes in thickness of sclera. The mean thickness (μm) of sclera at day 10 of incubation was 43.54 ± 2.45 in control subgroup and 43.03 ± 5.86 in the experimental subgroup ($p=0.673$). The mean thickness (μm) of sclera at day 15 of incubation 77.48 ± 8.32 in control subgroup and 73.99 ± 8.62 in experimental subgroup ($p=0.145$). The mean number of chondrocytes/unit area of hyaline cartilage of sclera in day 10 was 17.40 ± 1.44 control subgroup and 14.57 ± 1.87 in the experimental subgroup ($p < 0.001$). The mean number of chondrocytes/unit area of hyaline cartilage of sclera on day 15 was 10.02 ± 0.86 in the control subgroup and 9.54 ± 0.59 in the experimental subgroup ($p=0.025$). There was disrupted ossicular formation indicating adverse effects on the development of bony sclera as well.

Conclusion: Administration of glucose caused alteration in the histology of sclera in developing chick embryos.

Key Words: Sclera. Hyaline cartilage. Ossicular ring. Intraembryonic glucose.

INTRODUCTION

Glucose, also known as D glucose or dextrose, acts as a fuel which powers the cellular machinery. Prenatal exposure to increased level of glucose has long lasting consequences for the offsprings of diabetic mothers,¹ as chronic and persistent elevated levels of glucose in blood (hyperglycemia) is a feature of diabetes mellitus.²

The teratogenic potential of hyperglycemia is well documented by research done on experimental animals.^{3,4} The dysmorphogenic effects seen in laboratory animals are mainly in the tissues and the organs derived from the neural crest cells. Elevated levels of glucose inhibit the survival of cranial neural crest cells (NCC) which established it as a target for teratogenic actions of glucose.⁵ Epidemiological studies have demonstrated abnormal development of neural crest-derived structures in children born to diabetic mothers.⁶

The role of NCC in ocular development has been studied by making quail-chick chimera. The perocular mesenchyme (POM) receives contribution from NCC and these cells contribute to the development of the sclera (cartilaginous and bony).⁷ Distinct transcription factors are required for the determination, development and maintenance of POM. The transforming growth factor-beta (TGF- β) is present in POM.⁸ It regulates an important transcription factor pituitary homeobox 2 (*Pitx2*) also present in POM. The TGF- β dependant expression of *Pitx2* is required for NCC to adapt scleral fate.⁹ The chick sclera has a hyaline cartilage layer and a fibrous layer along with a ring of scleral ossicles which are overlapping plates of membrane bone, 14 to 16 in number, encircling the cornea.¹⁰

The teratogenic mechanism involved in glucose toxicity is found to be oxidative stress.¹² NCC is a particularly vulnerable cell population to oxidative stress. A reason for this is that they do not have super oxide dismutase (SOD) and catalase enzymes, which scavenge free radicals.¹¹

As excess glucose causes deranged development of the structures derived from NCC, it is likely to have an effect on the development of the chick sclera. The purpose of this study was to evaluate the effects of elevated glucose on the developing sclera in the chick embryo.

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METHODOLOGY

This experimental study was carried out at the Department of Anatomy, College of Physicians and Surgeons Pakistan (CPSP), Regional Centre, Islamabad, from January 2013 to January 2014. The study was carried out in two main groups, each comprising 90 eggs. Group A was the control group and group B was the experimental group. Group A was subdivided into three subgroups, A1, A2 and A3, each comprising 30 eggs. This control group was injected with normal saline (0.3 ml) in egg albumen.

Similarly, group B was subdivided into three subgroups B1, B2 and B3, each comprising 30 eggs. This group was injected with 0.3 ml of 5% w/v solution of glucose (5% dextrose injection) equivalent to 15 mg of glucose into albumen of the egg. Both groups were injected just before incubation. Un-incubated egg of day 0 was wiped with a sterile cotton wool pad moistened with 70% ethanol. Each egg was held vertically with blunt pole upwards for 5 minutes so that the blastoderm floated up, and a hole was drilled into its upper blunt pole where the air sac was located. Another hole was drilled at a point about a fingerbreadth above the lower pole with a thumb pin. Only the shell was punctured. Next, the shell membrane of the upper pole was pierced with an empty insulin syringe with needle size of 30 gauges x 8 mm. This was done to release air from the air sac. Lastly, an insulin syringe containing the glucose or saline was used to inject the contents into the albumen through the lower hole after complete insertion of its needle. This 5% solution is isotonic and this was to prevent any teratogenic effects that may result from change in the osmolarity.

Eggs from the subgroup A1 and B1 were opened on day 10 of incubation. Eggs from the subgroup A2 and B2 were opened on day 12 of incubation to see all scleral ossicles,¹² and eggs of the subgroup A3 and B3 were opened on day 15 of incubation to see the teratogenic impact in the chick eye.¹³

The eggs were placed in the incubator (Memmert Electric Company). The day on which eggs were placed in the incubator was taken as day 0. Eggs were rotated 1/2 turn twice daily.¹⁴ Incubation was under careful and standard monitoring. The temperature was maintained at $38 \pm 0.50^\circ\text{C}$, the relative humidity kept between 60 and 70%, and fresh air ventilation was ensured. Sufficient measures were taken to maintain a continuous electric supply. On the scheduled day of sacrifice, eggs were taken out of the incubator and allowed to lie horizontally on table for about 10 minutes so the embryos can float up above the yolk sac. On breaking the shell from the broader end in a bowl of water, embryos were cleanly extracted without any traction and avoiding trauma. Survivability was noticed and only alive embryos were included in the study. The embryos were fixed in 10%

neutral buffered formalin solution for 24 hours. The fixed embryos were decapitated and the heads were bisected in the mid sagittal plane. The right eyes were dissected out, and then the anterior half of the eyeball was cut off. This again was bisected at the meridian plane and one half of this was processed for paraffin embedding from the embryos of subgroup A1 and B1 (day 10 of incubation) and subgroup A3 and B3 (day 15 of incubation). The sections cut at 7 μm thickness were stained with haematoxylin and eosin (H&E) for routine examination and Alcian Blue and Alizarin Red stains were used for hyaline cartilage and ossicles of sclera, respectively. The anterior half of the right eyes from embryos of subgroup A2 and B2 (day 12 of incubation) was stained as whole with Alizarin Red.

Statistical analysis of all data of quantitative parameters was done with Statistical Package for Social Sciences (SPSS) version 10. Student's t-test was applied to detect any significant difference in the weight of eyeballs, thickness of cartilaginous sclera in μm and the number of chondrocytes per unit area of cartilaginous sclera between control and experimental groups. P-value of equal to or less than 0.05 was considered statistically significant. Quantitative data is summarised along with mean and standard deviation.

RESULTS

On gross examination, the eye defects were manifested by anophthalmia and microphthalmia in 6.75% of alive experimental embryos (Figure 1). After dissecting out, the weight of eyeball in embryos exposed to glucose was less as compared to that of control subgroup, although this difference was statistically insignificant (for A1, B1: $p=0.698$, for A3, B3: $p=0.392$).

In histological sections in day 10 embryos, the sclera from control subgroup showed two layers, an inner cartilaginous layer and an outer fibrous layer (Figure 2). The fibrous layer was thinner than underlying cartilaginous layer and consisted of closely packed collagen fibers. The cartilaginous layer was made of hyaline cartilage with chondrocytes surrounded by the matrix. The chondrocytes were small and rounded, individually placed inside the lacunae. An intensely staining ring was seen around the lacunae, which was territorial matrix that reflected the increased density of glycosaminoglycans. The chondrocytes near the perichondrium were flat and became rounded as they moved inside. In glucose-exposed embryos, the thickness of cartilaginous sclera was the same but chondrocytes were less in number than those of control subgroup (Figure 3, Table I).

The sclera of day 12 embryos showed 14 small ossicles at sclerocorneal junction in both control and experimental subgroups (Figure 4).

Table I: Comparison of sclera among day 10 control and experimental (A1 and B1) and day 15 control and experimental (A3 and B3) subgroups.

Parameter	A1 N=28 Mean ±SD	B1 N=25 Mean ±SD	p-value	A3 N=28 Mean ±SD	B3 N=24 Mean ±SD	p-value
Thickness of cartilaginous sclera (µm)	43.54 ±2.45	43.03 ±5.86	0.673	77.48 ±8.32	73.99 ±8.62	0.145
Number of chondrocytes/ UA of cartilaginous sclera(625µ ²)	17.40 ±1.44	14.5 ±1.87	< 0.001	10.02 ±0.86	9.54 ±0.59	0.025

N=Number of specimens; UA=Unit area; SD=Standard Deviation.

The sclera of day 15 embryos had the similar structure as that of day 10 with scleral ossicles apparent in histological sections, as thin bony lamina with a single layer of osteocytes (Figure 5). The glucose-treated subgroup at this age had less developed ossicles with no osteocytes (Figure 6).

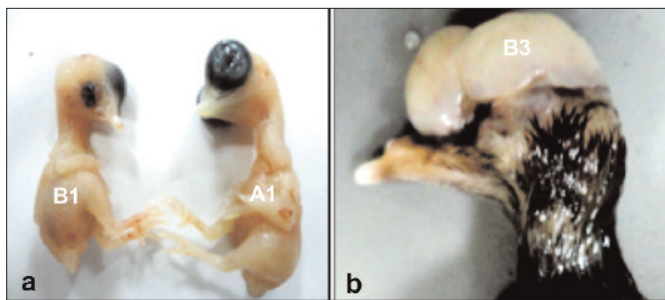


Figure 1: (a) Comparison regarding the eye size of glucose exposed B1 and control A1 chick embryos at day 10 of incubation (p-value 0.698). (b) Animal from subgroup B3 with anophthalmia.

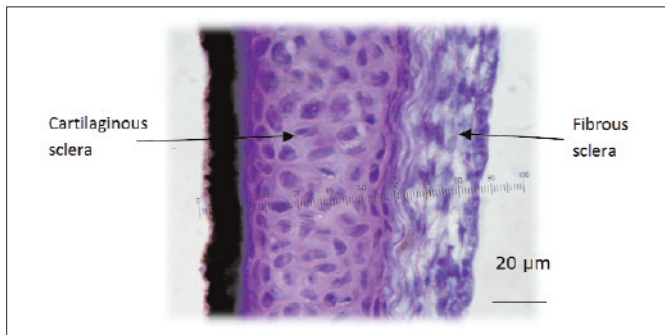


Figure 2: Haematoxylin and eosin stained section showing fibrous and cartilaginous parts of sclera in day 10 control embryos.

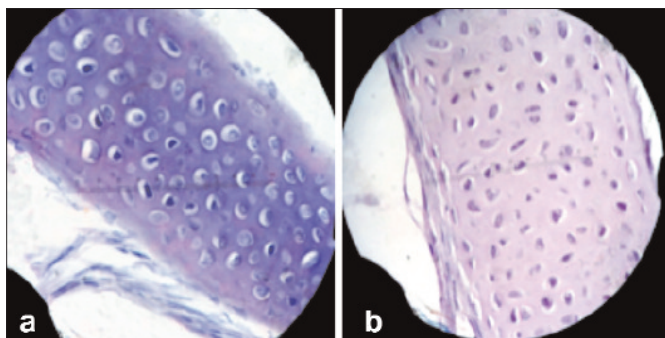


Figure 3: (a) Comparison of cartilaginous sclera regarding the number of chondrocytes. (a) Showing cartilaginous sclera in day 10 control embryos, (b) cartilaginous sclera of glucose exposed day 10 embryos with less number of chondrocytes per unit area. Scale bar=20µm. Hematoxylin and eosin stained sections.

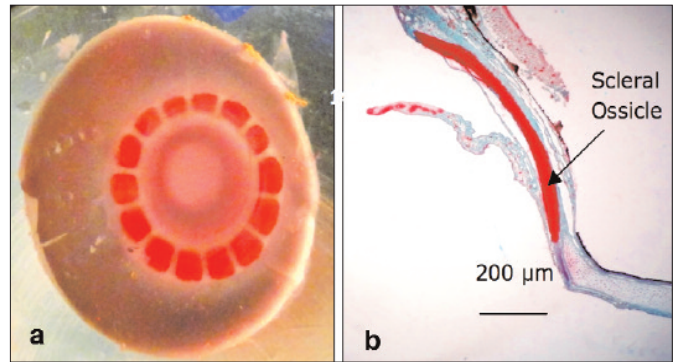


Figure 4: From control embryos day 12 of incubation, (a) Alizarin Red stained whole anterior half of eye seen under stereomicroscope, showing 14 ossicles in the ossicular ring of sclera, (b) histological section showing scleral ossicle after staining with Alizarin Red and counter stained with light green. Scale bar=200µm.

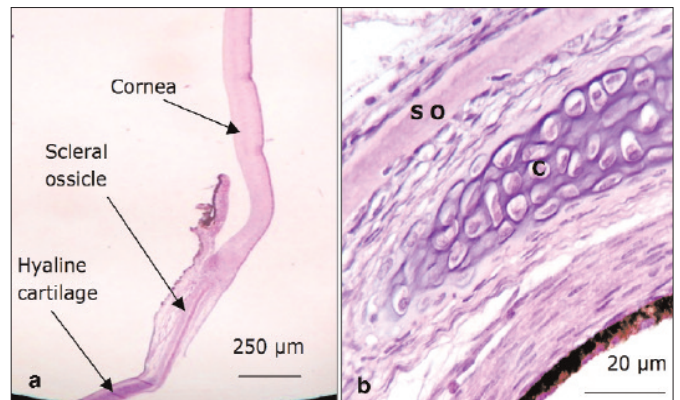


Figure 5: From control embryos at day 15 of incubation; (a) showing cornea, scleral ossicle and hyaline cartilage of sclera. Hematoxylin-eosin staining. Scale bar=250µm, (b) High magnification photomicrograph showing scleral ossicle (SO) and scleral hyaline cartilage(C). Hematoxylin-eosin staining. Scale bar=20µm.

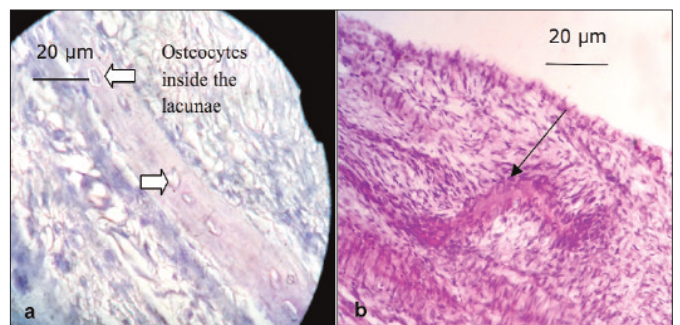


Figure 6: Comparison of histology of scleral ossicle in day 15 embryos; (a) photomicrograph of control embryo showing osteocytes in scleral ossicle (arrows), (b) scleral ossicle from glucose exposed embryos with no osteocytes. Hematoxylin-eosin staining. Scale bar=20µm.

DISCUSSION

Glucose is essential for growth and metabolism but an excess amount is detrimental to developing embryo. A positive reciprocity is present between hyperglycemia during embryogenesis and congenital anomalies as evidenced by clinical,⁴ and experimental data.^{5,6}

The current study was planned to investigate the outcome of exogenous glucose on the development of sclera in the chick embryos keeping in mind the increasing prevalence of diabetes, particularly among the women in their reproductive years.¹⁵ It is essential to have a better understanding of its influence on the health of developing embryos. The administration of glucose provides an opportunity to access the direct effects of this individual metabolite rather than using animal models of streptozocin induced diabetes.

In the present study, the results obtained after the application of the statistical tests and interpreted with the literature available from the previous studies indicate that glucose caused some histomorphological differences in the sclera of developing chick embryos between control and experimental groups. The gross eye defects were seen in 6.75% of experimental embryos. These were microphthalmia and anophthalmia. These findings are consistent with previous studies done after prenatal exposure of hamsters to glucose leading to microphthalmia in the developing fetuses.¹⁶ The role of neural crest cell in development of eye is established and abnormal migration, distribution and differentiation of this cell population have been documented for disrupted eye development in clinical as well as animal based research.¹⁷ The findings of our study could be due to effect of glucose on this vulnerable cell population as there is lack of antioxidant enzymes in these cells during early development and excess glucose results in oxidative stress.

No significant difference was observed in the thickness of cartilaginous sclera between control and glucose exposed subgroups. However, the number of chondrocytes per unit area of cartilaginous sclera was significantly lowered in the glucose exposed subgroups B1 and B3. This confirms an earlier report of decreased cartilage formation in diabetic rats due to decreased proliferation of cartilage precursor cells. Several anatomical changes have been associated with oxidative injury to cartilage with a thinning of the proteoglycan and collagen layers and loss of matrix proteins and chondrocytes.¹⁸

The TGF- β dependant expression of *Pitx2* is required for NCC to adapt scleral fate as these transcription factors are present in POM. Scleral agenesis has been observed in *Pitx2* mutant mice.¹⁰ Altered expression of TGF- β by glucose might be a causative factor for decreased number of chondrocytes in our research as glucose causes an increased level of TGF- β ,¹⁹ and

disrupted anterior segment development by over expression of TGF- β has been reported in mice.²⁰

Regarding the ossicular sclera, the number of ossicles remained same in both control and experimental subgroups in our study, but histological picture showed improper formation of ossicular plate with no osteocytes visible. This is in accordance with previous research works in animal models that have demonstrated hyperglycemia to be associated with bone loss, decreased osteoblast activity and osteoblast death. The same experimental model showed decreased rate of mineral opposition and bone formation in the mandible as examined by the histomorphometric analysis.²¹

Scleral ossicles are membranous bones. Previous works done showed deranged development of craniofacial bones which are also membranous bones that receive contribution from the NCC, the altered development of scleral ossicles in this study might be due to effect of glucose on this important cell population.

CONCLUSION

Administered glucose resulted in alterations in the normal histology of chick sclera. The reason may be the effect of glucose on neural crest cells involved in the morphogenesis of sclera.

REFERENCES

1. Yajnik CS. Nutrient mediated teratogenesis and fuel-mediated teratogenesis: Two pathways of intrauterine programming of diabetes. *Int J Gynaecol Obstet* 2009; **104** Suppl 1:S27-31.
2. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabet Care* 2011; **34**: 82-89.
3. Frank LA, Sutton-McDowall ML, Brown HM, Russell DL, Gilchrist RB, Thompson JG. Hyperglycemic conditions perturb mouse oocyte *in vitro* developmental competence via beta O-linked glycosylation of heat shock protein 90. *Hum Reprod* 2014; **29**:1292-303.
4. Schelbach, Cheryl J, Robker, Rebecca L, Bennett, Brenton D, *et al.* Altered pregnancy outcomes in mice following treatment with the hyperglycaemia mimetic, glucosamine, during the periconception period. *Reprod Fertil Dev* 2013; **25**: 405-16.
5. Wang XY, Li S, Wang G, Ma ZL, Chuaj M, Cao L, *et al.* High glucose environment inhibits cranial neural crest survival by activating excessive autophagy in the chick embryo. *Sci Rep* 2015; **5**:18321.
6. Barisic I, Odak L, Loane M. Prevalence, prenatal diagnosis and clinical features of oculo-auriculo-vertebral spectrum: A registry-based study in Europe. *Eur J Hum Genet* 2014; **22**: 1026-33.
7. Creuzet S, Vincet C, Couly G. Neural crest derivatives in ocular and periorbital structures. *Int J Dev Biol* 2005; **49**:161-71.
8. Grocott T, Johnson S, Bailey AP, Streit A. Neural crest organizes the eye via TGF- β and canonical Wnt signaling pathway. *Nat Commun* 2011; **2**:265.
9. Evans AL, Gage PG. Expression of the homeobox gene *Pitx2* in neural crest is required for optic stalk and ocular anterior segment development. *Hum Mol Genet* 2005; **14**:3347-59.

10. Zhang G, Boyle DL, Zhang Y, Rogers AR, Conrad GW. Development and mineralization of embryonic avian scleral ossicles. *Molecular Vision* 2012; **18**:348-61.
11. Sadler TW. Medical embryology, 11th ed. Philadelphia: *Lippincott Williams & Wilkins* 2010; 276.
12. Thompson H, Griffiths JS, Jeffery G, McGonnella IM. The retinal pigment epithelium of the eye regulates the development of scleral cartilage. *Dev Biol* 2010; **347**:40-52.
13. Murillo OR, Moreno JAR, Garzon JFR, Morenilla MTP, Chiquero JAM, Nacle FA, *et al.* Ultrasound analysis of the eye of chick embryos exposed to low-frequency magnetic fields. *Eur J Anat* 2002; **6**:71-4.
14. Tona K, Onagbeasan O, Bruggeman V, Mertens K, Decuypere E. Effect of turning duration during incubation on embryo growth, utilization of albumen and stress regulation. *Poult Sci* 2005; **84**:315-20.
15. Guariguata L, Linnenkamp U, Beagley J, Whiting DR, Cho NH. Global estimates of the prevalence of hyperglycaemia in pregnancy. *Diabetes Res Clin Pract* 2014; **103**:176-85.
16. Gale TF. Effects of *in vivo* exposure of pregnant hamsters to glucose.1. Abnormalities in LVG strain fetuses following intermittent multiple treatments with two isomers. *Teratology* 1991; **44**:193-202.
17. Chen BY, Chang HH, Chen ST, Tsao ZJ, Yeh SM, Wu CY, *et al.* Congenital eye malformations associated with extensive periocular neural crest apoptosis after influenza B virus infection during early embryogenesis. *Mol Vis* 2009; **15**: 2821-8.
18. Kayal RA, Alblowi J, McKenzie E. Diabetes causes the accelerated loss of cartilage during fracture repair which is reversed by insulin treatment. *Bone* 2009; **44**:357-63.
19. Smoak IW. Hyperglycemia induced TGF-beta and fibronectin expression in embryonic mouse heart. *Dev Dyn* 2004; **231**: 179-89.
20. Robertson JV, Siwakoti A, West-Mays JA. Altered expression of transforming growth factor beta-1 and matrix metallo-proteinase-9 results in elevated intraocular pressure in mice. *Mol Vis* 2013; **19**:684-95.
21. von Wilmowsky C, Stockmann P, Harsch I, Amann K, Metzler P, Lutz R, *et al.* Diabetes mellitus negatively affects peri-implant bone formation in the diabetic domestic pig. *J Clin Periodontol* 2011; **38**:771-9.

