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Comparison of the Efficacy of Different Hydrocortisone-Induced Cataract Models in Developing Chick Embryos

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Authors' contributions

This work was carried out in collaboration between all authors. Author Reşat Duman designed the study. Authors Reşat Duman and TE performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors Reşat Duman, AV, TK and AB managed the analyses of the study. Authors Reşat Duman and Rahmi Duman managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate and compare the efficacy of different hydrocortisone-induced cataract models in chick embryos.
Material and methods: On the 15th day of the study, hydrocortisone succinate sodium (HC) (0.5 µmol/egg or 0.25 µmol/egg) was administered directly into the air sac (AS) or into the chorioallantoic membrane (CAM) in 4 HC-groups. In two control groups, phosphate-buffered saline (PBS) was administered in the same manner. On the 17th day of the study (48 hours after the injection), the lenses were removed and classified into five stages according to lens opacification

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under a stereoscopic microscope. In addition, reduced glutathione levels (GSH) of the lenses were measured by High-performance liquid chromatography (HPLC) method.
Results: The mean scores of lens opacity and cataract formation in HC-groups were significantly higher than those of control groups (p = 0.00). In addition, the mean reduced glutathione levels were significantly lower in HC-groups compared to control groups (199-245 ps vs. 291-294, p = 0.00). The mean scores for lens opacity, cataract formation, and the mean GSH levels did not significantly differ among 4 HC-groups (p > 0.05).
Conclusion: Both doses of HC (0.25 or 0.50 µmol/egg) and both injection methods (into the AS or CAM) may be used efficiently for cataract formation in chick embryo steroid-induced cataract models.

Keywords: Cataract; eye; hydrocortisone; chick.

1. INTRODUCTION

Despite the developing technology, cataract still remains the leading cause of visual impairment worldwide [1]. Although the major risk factors for cataract are aging, trauma, diabetes mellitus, uveitis, ultraviolet light (UV) exposure, smoking and the long-term treatment with steroids [2], the exact etiopathogenetic pathways still need to be clarified. Animal models of cataract formation are essentially important for both studying the etiopathogenesis of cataract and also testing potential preventative therapeutics. Previously different animal models (Mouse, Rat, Zebra Fish, Chick Embryo, Dog, Guinea pig, Calf, Rabbit, Bovine) have been used for studying different type cataracts including age-related cataract, diabetic cataract, UV-induced cataract, steroid-induced cataract, oxygen-induced cataract, and secondary cataract [3].

Cataract formation, especially in the form of posterior subcapsular lens opacities, is a widely known complication of corticosteroid therapy with an incidence of 22-58% [4]. Although the exact molecular events causing steroid-induced cataract still need to be clarified, there have been many theories about etiopathogenesis of SIC such as metabolic changes, osmotic failure, gene transcription events, and oxidative stress in lens epithelial cells [5,6]. Transient steroid-induced cataract model in developing chick embryo was firstly reported in 1983 by Nishigori et al [7]. Although several ongoing studies have used steroid-induced cataract models in chick embryos, there is still lack of a standard model and method of drug administration. Thus in the present study, we aimed to evaluate and compare the different steroid-induced cataract models-using different drug dosages and methods of drug administration to find out the most practical, efficient and usable steroid-induced cataract model in developing chick embryos.

2. MATERIALS AND METHODS

2.1. Animals and Treatments

60 fertilized Specific Pathogen-Free (SPF) eggs were enrolled in the study. All experiments were conducted in accordance with the animal research protocol of Afyon Kocatepe University Ethics Committee (Approval number: AKUHADYEK-203-17). Hydrocortisone succinate sodium (HC) were purchased from Sigma-Aldrich Chemical. The study was conducted in the research laboratories of the departments of Anatomy and Biochemistry and Clinical Biochemistry at Afyon Kocatepe University. All eggs were placed in the incubator and monitored in the incubator at 37.5°C and 68% relative humidity. Then, eggs were randomly divided into 6 groups each having 10 SPF fertilized eggs as 4 groups injected with HC, and 2 control groups injected with phosphate-buffered saline (PBS). On the 15th day of incubation, SPF eggs were removed from the incubator. The surfaces of the egg shells were sterilized with 70% ethyl alcohol, and then holes were created in the egg shells with the help of a tiny electric drill. After creating holes in the egg shells, HC/PBS was injected with insulin injectors either into the air sac (AS, inner shell membrane intact) or into the chorioallantoic membrane (CAM, inner shell membrane removed), (Fig. 1). After injection, the puncture was sealed with sterile cellophane tape, and the eggs were further incubated for 48 hr in the same incubator. Study groups were summarized in Table 1.

2.2 Evaluation of Opacity of Removed Lenses

On the 17th day of the study (48 hours after the injection), the lenses were removed from chick embryos under the dissection microscope with corneal limbus incision. The states of the

lenses were determined under a stereoscopic microscope and their photographs were taken.

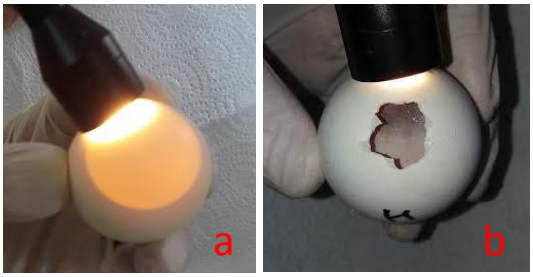

Fig. 1. The photograph of 15-day old egg showing the site of injection of air sac (a), and The hole in the egg shell to reach the chorioallantoic membrane (b)

A previously described staging system was used to score the lenses on a 5-grade scale: 1: clear lens (no lens opacity); 2: lens with a faint opaque ring between the cortical region and the nuclear region; 3: lens with a distinct opaque ring between these regions; 4: lens with a pinhole-sized clear area in an opaque nucleus; 5: lens with an opaque nucleus (Fig. 2), [5].

2.3 Measurement of GSH Levels in the Removed Lenses

The removed lenses were immediately frozen and stored at -80ºC until the measurement of the GSH level. Two lenses of each chick embryo served as one sample. The sample was taken out of the deep freezer and sonicated in ice with a Dr. Hielscher (Germany) sonicator with 0.1 M pH: 7.4 phosphate buffer. The homogenates are then centrifuged at 10,000 g for 15 min. Reduced glutathione levels were determined Glutation assay kit (Chromsystems Diagnostics; Munich / Germany) with High-performance liquid chromatography (HPLC) method using HPLC fluorescence detector (Ex: 385 Em: 515 nm) supplied by Thermo Scientific Ultimate 3000 HPLC device.

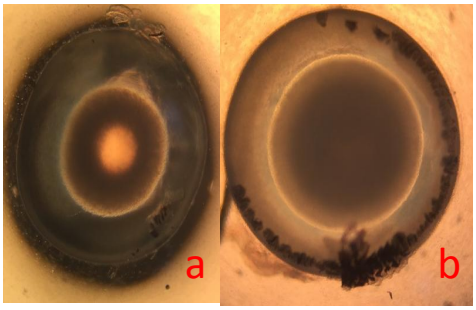

Fig. 2. The stage 4 lens with a pinhole-sized clear area in an opaque nucleus (a), and a stage 5 lens with an opaque nucleus (b)

2.4 Statistical Analysis

The statistical analysis was performed with SPSS software version 18.0 (SPSS Inc, Chicago, IL). Continuous variables were presented as a mean ± standard deviation and categorical variables as frequencies and percentages. Differences between groups were determined using one-way analysis of variance test. The level of statistical significance was set at $P < 0.05$.

Table 1. Characteristics of 6 separate groups enrolled in the study (HC: Hydrocortisone succinate sodium; as Air sac; CAM: chorioallantoic membrane; PBS: phosphate-buffered saline)

Group	Agent	Dosage	Method of administration	Volume	Delivery frequency	Effect duration
1 HC/AS 0.50	HC	0.50 µmol	AS	0.1 ml	1 time	15- 17. day
2 HC/AS 0.25	HC	0.25 µmol	AS	0.1 ml	1 time	15- 17. day
3 HC/CAM 0.50	HC	0.50 µmol	CAM	0.1 ml	1 time	15- 17. day
4 HC/CAM 0.25	HC	0.25 µmol	CAM	0.1 ml	1 time	15- 17. day
5 PBS/AS	PBS		AS	0.1 ml	1 time	15- 17. day
6 PBS/CAM	PBS		CAM	0.1 ml	1 time	15- 17. day

3. RESULTS

In control groups all the lenses (100%) were clear (stage 1), whereas in HC-groups the lens opacity scores more than stage 3 was seen in 70% (HC/AS 0.50), 60% (HC/AS 0.25), 80% (HC/CAM 0.50) and 70% (HC/CAM 0.25), respectively. In addition, the mean scores of lens opacity were significantly higher in HC-groups compared to control groups (3.7-4.3 vs. 1, $p < 0.001$).

The mean scores of lens opacity did not significantly differ between 4 different HC-groups ($p > 0.05$). Mean scores in each group were given in Table 2. The mean GSH levels were significantly lower in HC-groups compared to control groups (199-245µmol/L in HC-groups vs. 291-294 µmol/L in control groups, $p = 0.00$ (Table 2). The mean GSH levels of 4 HC-groups were not significantly different ($p > 0.05$). Mean scores within each group were given in Table 2.

4. DISCUSSION

The present study aimed to evaluate and to compare the different approaches of steroid-induced in vivo cataract models in the developing chick embryos. In recent years the chick embryo cataract model has gained attention as an easy and useful method to study cataract formation and effectiveness of anti-cataract drugs with antioxidant activity [2,7]. As it's clearly known that steroids cause cataract formation, HC-induced cataract models have been one of the best-established cataract models in the literature. Previously, several studies on HC-induced cataracts in chick embryos used different approaches (injection into the AS or CAM) and drug dosages (0.025 µmol/egg, 0.25 µmol/egg, or 0.50 µmol/egg HC) and there is still lack of a standard method.

Previously Lee JW et al. evaluated cataract development after HC injection in chick embryos at various ages (from day 9 to day 17). They reported no cataract-inducing effect on day 9 or day 11; whereas stage 4, 5 lenses were observed on day 13 (63%) and on day 15 (90%). And they showed that HC administration on day 15 and evaluations of the lenses on day 17 gave the highest incidence and best reproducibility [8]. Thus we performed the present study between day 15 and 17 to yield best results.

Nishigori H et al. reported that cataractogenic activities of steroids varied depending on the structure and dose of the drug [7]. They found that 0.025 µmol/egg HC had no cataract-inducing effect (0%), whereas HC doses of 0.25 µmol/egg had a significant cataract-inducing effect (88.8%). Similarly, most of the previous studies used HC with a dose of 0.25 µmol/egg and reported close incidences of stage 4, 5 cataract formation ranging between 80 and 94% [9-15]. The dose of 0.5 µmol/egg HC was used in only one study by Ishikawa et al., and as an interesting result the percentage of stage 4-5 cataract formation was reported to be very low (20%) [2]. There is no previous study comparing HC doses of 0.25 µmol/egg and 0.50 µmol/egg. In the present study, we found that two different doses of HC (0.5 µmol/egg and 0.25 µmol/egg) had similar stage 4- 5 cataractogenic effects. In

Table 2. Opacity scores of removed lenses and mean reduced glutathione levels in study groups. (HC: Hydrocortisone succinate sodium; AS: Air sac; CAM: chorioallantoic membrane; PBS: phosphate-buffered saline)

Group	Opacity scores of removed lenses						GSH levels (µMOL/L)
	1	2	3	4	5	Mean (min-max)	(mean ± SD)
1 HC/AS 0.50	0	1	2	3	4	4 (2-5)	209,1 ± 69
2 HC/AS 0.25	0	2	2	3	3	3,7 (2-5)	245,1 ± 48
3 HC/CAM 0.50	0	0	2	3	5	4.3 (3-5)	199,4 ± 41
4 HC/CAM 0.25	0	1	2	3	4	4 (2-5)	241,4 ± 44
5 PBS/AS	10	0	0	0	0	1	293,9 ± 64
6 PBS/CAM	10	0	0	0	0	1	290,9 ± 60

contrast with the study findings by Ishikawa et al. we found a high cataractogenic effect with HC dose of 0.5 µmol/egg. In addition, although it was statistically not significant, the higher cataractogenic effect was observed with a dose of 0.50 µmol/egg compared to 0.25 µmol/egg. Methods used in previous studies were summarized in Table 3.

In previous studies with chick embryo cataract models, HC injection was administered mostly into the AS or rarely into the CAM (Table 3). In the present study, we compared these approaches to clarify if injection into the CAM increased the cataractogenic effect or not, and we found that the two methods had similar cataractogenic effects. This finding supports that the internal shell membrane in the developing chick embryo absorbs HC. As an injection into the AS is an easier and less invasive method with less risk of complications including infection, and injection into the CAM do not significantly increase the cataractogenic effect, it may be efficiently preferred for cataract models.

It's known that oxidative stresses contribute to cataract formation by denaturating lens proteins (including enzymes, crystallins, and other chaperones) [15]. It has been shown that glutathione plays an important role in maintaining lens transparency. Several previous studies showed decreased reduced glutathione levels in cataractous lenses supporting the protective role of glutathione from oxidative attacks [16-20]. Nishigori H et al. found that the lenticular reduced glutathione levels decreased with the appearance of lens opacification in developing chick embryos after HC administration [7]. Similarly, Lee et al. and Kosano et al. reported lower reduced glutathione levels in cataractous lenses compared to controls [12,18]. Inconsistency with their findings, we also showed that reduced glutathione levels significantly decreased with the cataract formation. However different from previous studies, we used HPLC method to determine the levels of reduced glutathione. And our results support that HPLC method may be used as a specific and sensitive method for measuring reduced glutathione levels in chick embryo cataract models. And our results support that HPLC method may be used as a specific and sensitive method for measuring reduced glutathione levels in chick embryo cataract models.

Table 3. Methods of previous studies using HC-induced cataract model. (HC: Hydrocortisone succinate sodium; AS: Air sac; CAM: chorioallantoic membrane)

Author	Agent	Dosage	Method of administration	Stage 4/5 lens (%)
NISHIGORI H ET AL. (7)	HYDROCORTISONE ACETATE	100 µG/0.2 ML	CAM	92
NISHIGORI H ET AL. (8)	HC	0.025 µMOL/EGG	AS	0
		0.25 µMOL/EGG		88.8
LEE JW ET AL. (9)	HC	0.25 µMOL/EGG	AS	94
SETOGAWA T ET AL. (10)	HC	0.25 µMOL/EGG	AS	92
WATANABE H ET AL. (11)	HC	0.25 µMOL/EGG	AS	92
HAMAMICHI S ET AL. (6)	HC	0.25 µMOL/EGG	AS	80
ISHIKAWA S ET AL. (2)	HC	0.50 µMOL/EGG	AS	20
KOSANO H ET AL. (15)	HC	0.25 µMOL/EGG	AS	94
THE PRESENT STUDY	HC	0.50 µMOL/EGG	AS	70
		0.25 µMOL/EGG	AS	60
		0.50 µMOL/EGG	CAM	80
		0.25 µMOL/EGG	CAM	70

5. CONCLUSION

In conclusion, both doses of 0.25 µmol/egg or 0.50 µmol/egg HC may be used efficiently for cataract formation in chick embryo steroid-induced cataract models. Administration into the CAM does not seem to increase the cataractogenic effect. Although both methods had similar efficiency, injection into the AS seems more reasonable because of being easier and less invasive procedure. Furthermore, HPLC method is an efficient and reliable method for evaluating reduced glutathione levels in chick embryo HC-induced cataract models. Furthermore, HPLC method is an efficient and reliable method for evaluating reduced glutathione levels in chick embryo HC-induced cataract models.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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