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Morphological changes induced by colchicine in the chick optic cup in early stages of development. A stereological study

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Summary. In this study chick embryo optic cups at HH stage 13 of development were analyzed under normal conditions and after inoculation with colchicine for 1, 2, 4, and 8 h. Several changes were seen after these periods of treatment: 1) modifications of the structure, with thicker regions in the cup and a general decrease in the total volume according to the duration of exposure to the drug (about 4 times less than normal, 5,035 x 10^3 μm^3 vs 1,334 x 10^3 μm^3 after 8 h of treatment); 2) enlargement of the ventricular cavity and its closure, due to failure of approximation of retinal and pigmentary layers; 3) failure of lens development, with delay and impairment of pit formation and deformation of all structures; lens volume was less than normal (about 4 times less, 2,148 x 10^3 μm^3 vs 658 x 10^3 μm^3. after 8 h of treatment); 4) a general segregation of the cells making up the structure, principally in the more active proliferating zones. The local alterations found are described.

Key words: Optic cup development, Stereology, Colchicine

Introduction

In the chick embryo a lateral outpocket of the diencephalon, named primary optic vesicle, appears at about 28 h of incubation. From Hamburger and Hamilton's stage HH10 (Hamburger and Hamilton, 1951) onward, the optic cup comes into contact with the ectodermal layer in a well-delimited region which will become the lens (McKeehan, 1951). As development progresses, both the primary vesicle and the lens rudiment invaginate and then close at the ends of the originally curved structure, a process accompanied by cell proliferation and kinetic migrations of the nuclei (Sauer, 1935a,b, 1936, 1937). Several hypotheses have attempted to explain how both the optic cup and lens attain their final shape: cytoskeletal action (microtubules and/or microfilaments) (Wilson, 1970; Messier, 1978; Linville and Shepard, 1972; Morriss-Kay and Tuckett, 1985), extracellular matrix component actions (Bernfield and Wessells, 1970); and modifications in cell cycle timing (Smith and Schoenwolf, 1988) have all been invoked. Given these antecedents, it is clear that the products that may affect the nucleus' normal movements during the cell cycle as well as the synthesis of the intra- and extracellular matrix proteins will disturb the normal constitution of the structures. Analysis of the changes in the early stages of the development after exposure to this kind of drug serves to characterize the alterations produced as well as the different types of reactions.

To characterize the type and intensity of modifications caused by antimitotic drugs, we experimentally analyzed the action of colchicine in the chick embryo at HH13 of development, after exposure to the drug for periods ranging from 1 to 8 h.

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Materials and methods

Fertile White Leghorn chicken eggs were incubated at 38.5 °C in a forced air incubator with automatic turning, until the embryos reached HH13 (approximately 48h of incubation).

A window was opened in the shell, and under a dissection microscope, 0.1 ml 2.5 x 10^{-5} M colchicine in saline solution (10 µg/ml) was injected near the head (anteroinferior region in the vicinity of the preamniotic cephalic fold) in 20 eggs, while in another 20, saline alone was injected as a control. All eggs were resealed and returned to the incubator. The concentration of colchicine used has been previously shown to be the most effective in causing constant alterations (Schoenwolf and Powers, 1987).

After incubation for 1, 2, 4 or 8 h, embryos were removed and placed in 2% glutaraldehyde-formaldehyde (v/v) in 0.1M sodium cacodylate buffer (pH 7.4, 340 mOsmol) at 4 °C for 12 h, dehydrated in acetone, embedded in Spurr's resin and cut into 4 µm frontal and transverse serial sections. The sections were stained with

0.5% toluidine blue solution in 0.5% sodium tetraborate.

Sections of the optic vesicle were observed under light microscope, and areas were measured in images recorded with a television camera and stored in the computer's memory, using a semiautomatic image analysis system (IMCO10-Kontron). The areas were used to calculate optic cup volume with Cavalieri's method, based on the equation:

$$V_t=(d+t)\sum A$$

in which d is the distance between sections measured, t is mean section thickness, and A is the area of each section used. In our material, d was 12 μm, and t was 4 μm.

Results

A number of factors that affect the development of the chick optic cup can vary depending on individual variations; some factors, on the other hand, are not

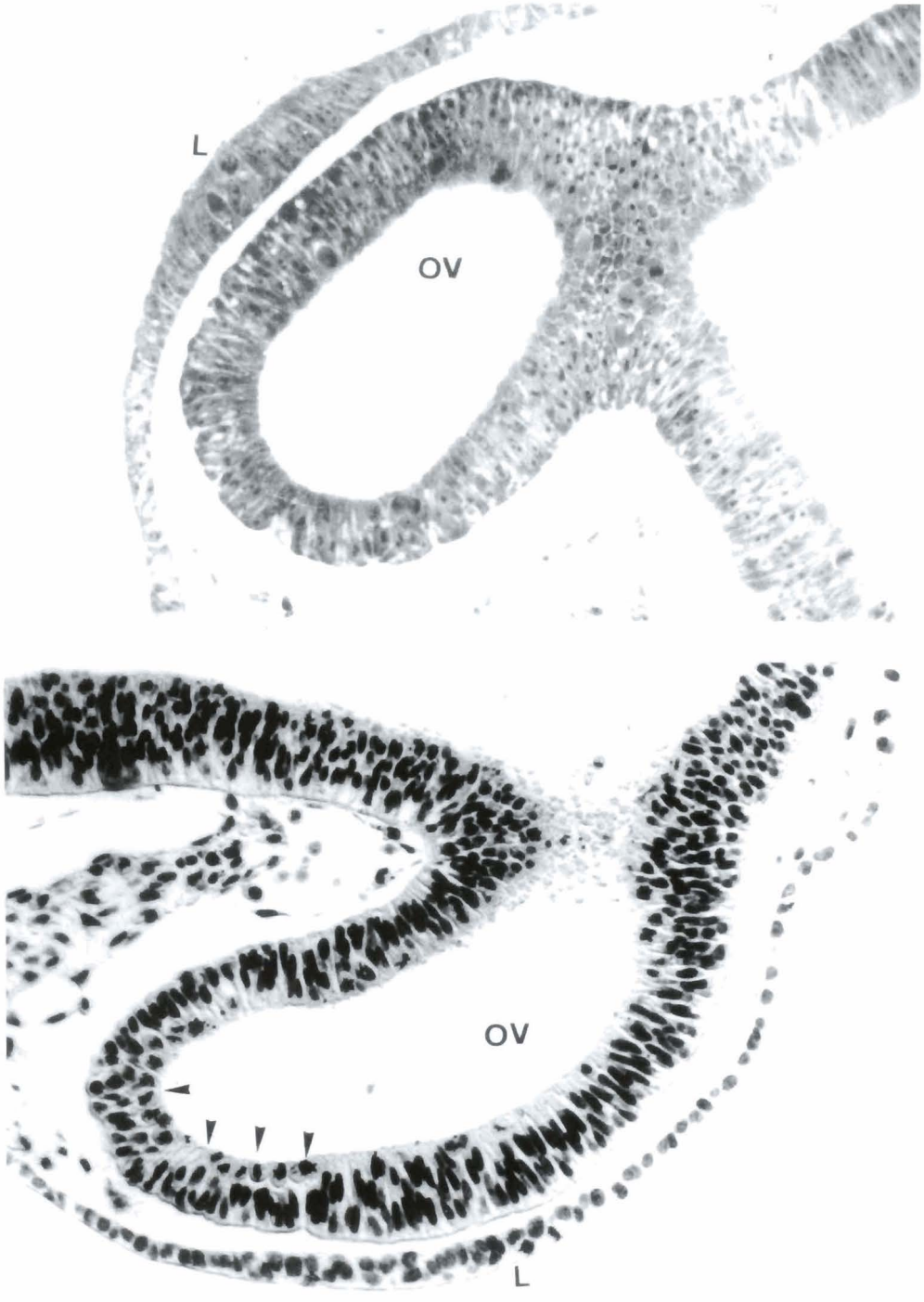

Fig. 1. a. Primary optic vesicle, consisting of a dilated ventricular cavity (OV), in an HH stage 13 chick embryo. The ectoderm is slightly thickened at the level of the lens rudiment (L). x 350. **b.** An analogous HH13 chick embryo after treatment with colchicine for 1h. Although no change in optic vesicle formation is noted when compared to control embryos, some star-shaped C-metaphase figures are present (arrowheads). L=lens; OV=optic ventricle. x 420

amenable to analysis because they arise from variables that cannot be controlled during incubation. In the present study, we discarded all embryos showing such variations, and considered only those of normal appearance after 48 h of incubation. Therefore, the control results described below refer only to normal embryos presenting an acceptable range of variation, as described in previous studies (Abadía-Fenoll et al., 1985, 1987; Calvente et al., 1988; Abadía-Molina et al., 1989).

Morphological description

HH13 + 1 h embryos

Controls (untreated) (Fig. 1a). Most embryos had developed to HH13, at which time the optic vesicle showed a dilated ventricular cavity, and had not yet invaginated. The prospective retinal layer was composed of pseudostratified epithelium with three rows of nuclei. Cells in different phases of mitosis were aligned along the ventricular margin.

The lens primordium appeared as an initial placode, therefore no quantitative determinations were made.

Colchicine treatment for 1 h (Fig. 1b). After treatment with colchicine for 1h, no effects on the general structure of the eye primordium were observed when compared to the control embryos. Cytological observations included a slight increase in the number of metaphases in both the optic vesicle and the lens placode. The superficial features of the ventricular margin in the retina, and the external margin in the lens, were unchanged with respect

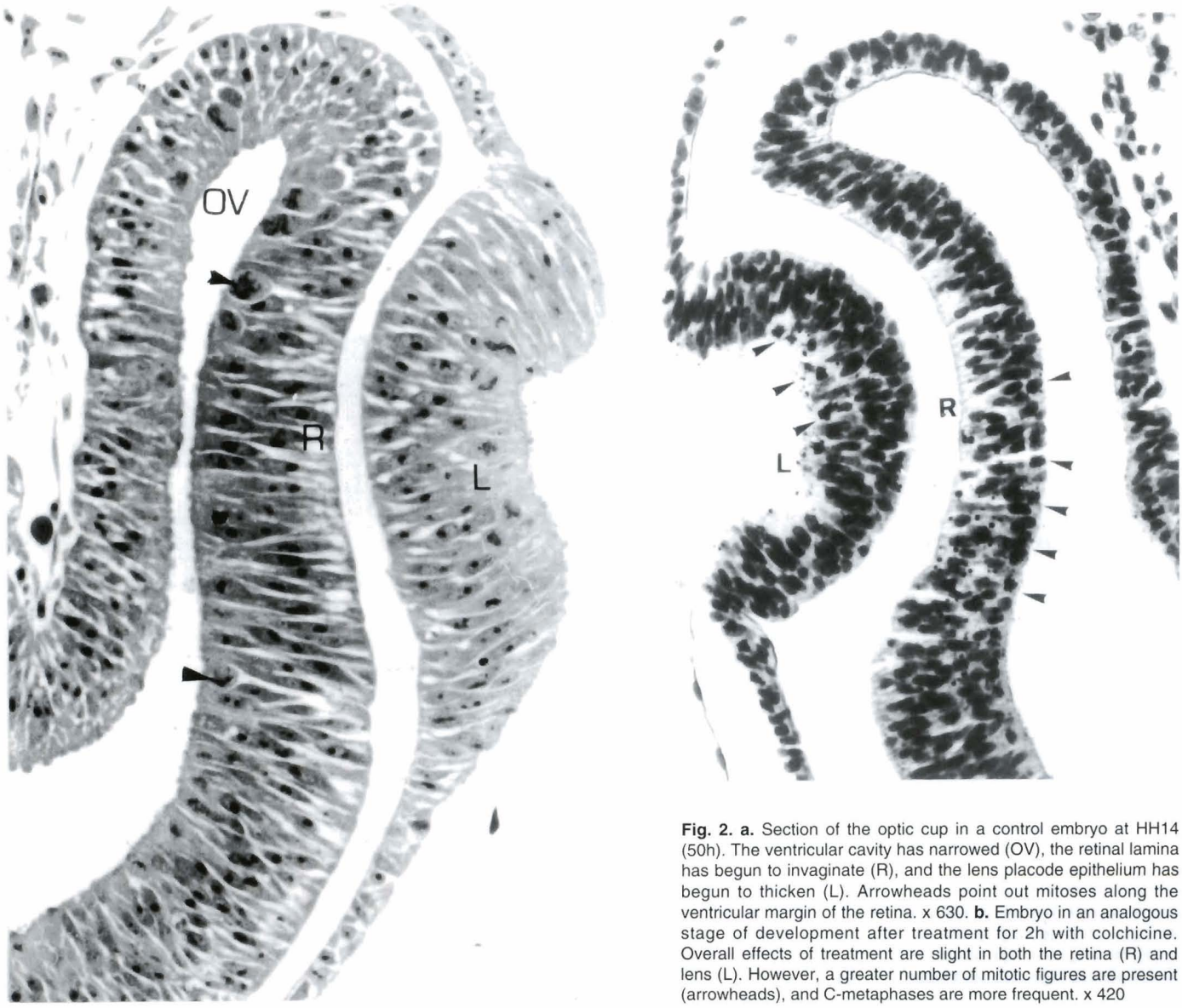

Fig. 2. a. Section of the optic cup in a control embryo at HH14 (50h). The ventricular cavity has narrowed (OV), the retinal lamina has begun to invaginate (R), and the lens placode epithelium has begun to thicken (L). Arrowheads point out mitoses along the ventricular margin of the retina. x 630. **b.** Embryo in an analogous stage of development after treatment for 2h with colchicine. Overall effects of treatment are slight in both the retina (R) and lens (L). However, a greater number of mitotic figures are present (arrowheads), and C-metaphases are more frequent. x 420

to controls.

HH13 + 2 h embryos

Controls (untreated) (Fig. 2a). Some embryos were apparently still in HH13, although most had progressed to HH14. At this stage, the optic vesicle and the lens placode began to invaginate, the latter forming the so-called lens pit. The future neural retina was formed of three rows of nuclei; as in the ectodermal margin of the lens pit, the ventricular surface appeared velvety. Numerous mitotic figures were seen in the optic cup and the lens pit.

Colchicine treatment for 2 h (Fig. 2b). Treatment with colchicine for 2 h delayed development, and caused deformations in the optic cup: the ventricular cavity was excessively dilated when compared to control embryos, with cells protruding into the ventral region of the retinal layer.

The lens was abnormally invaginated, with detached cells within the cavity of the lens pit.

HH13 + 4 h embryos

Controls (untreated) (Fig. 3a). After 4 h of incubation with saline solution, most embryos had attained HH15. Invagination of the optic cup had progressed, and was most evident in the dorsal zone of the optic stalk. The retinal layer consisted of four rows of nuclei.

Invagination of the lens had also progressed; the margin of the lens cavity appeared velvety.

Cells in different phases of mitosis were seen in both the retinal layer and the lens rudiment.

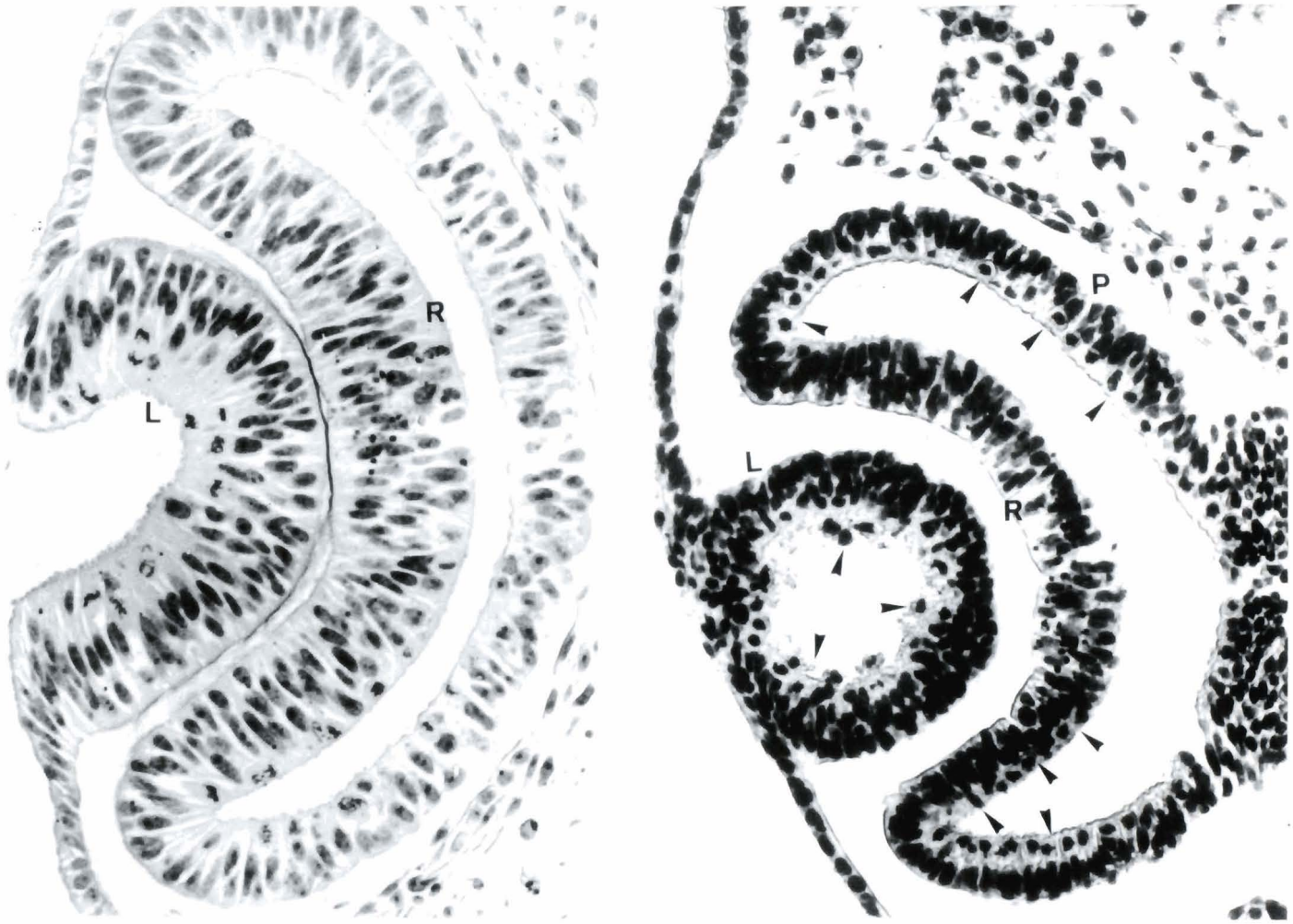

Fig. 3. a. Section through the optic cup and lens in an HH15 chick embryo (52h), showing normal development in an untreated embryo. L=lens; R=retina. x 500. **b.** Embryo in an analogous stage of development after treatment with colchicine for 4h. Development in all three layers (pigmentary, P; retina, R; lens, L) is less advanced than in untreated embryos. Although this section was taken more laterally than the one shown in Figure 3.a, the ventricular and lens cavities appear more dilated than normally at this stage of development. Arrowheads point out the increase in metaphase figures. x 420

Colchicine treatment for 4 h (Fig. 3b). Incubation for 4 h with colchicine produced evident alterations, with a marked reduction in size of the optic cup. The superficial regions of the cup were abnormally thickened due to the accumulation of mitotic cells.

HH13 + 8 h embryos

Controls (untreated) (Fig. 4a). Most control embryos had reached HH16, in which little change occurs in the structure of the eye primordium with respect to the preceding stage. Invagination of the optic cup had progressed, the ventricular cavity becoming clearly narrowed dorsally, and remaining relatively open ventrally. The ventricular margin of the retina was mostly smooth in dorsal regions, whereas ventral regions were still velvety in appearance. The retinal layer was composed of four rows of nuclei.

The lens pit began to close in its central region, remaining open ventrally at this stage. Closure was completed within a few hours after HH13 began, to give rise to a vesicle. The velvety appearance of the margin of the lens was less marked than in previous stages.

Mitotic figures were less numerous than in previous stages, both in the optic cup and the lens pit.

Colchicine treatment for 8 h (Fig. 4b). Exposure to colchicine had marked effects, manifested as a further reduction in optic cup volume with respect to the previous stage, together with morphological abnormalities, and general structural disorganization.

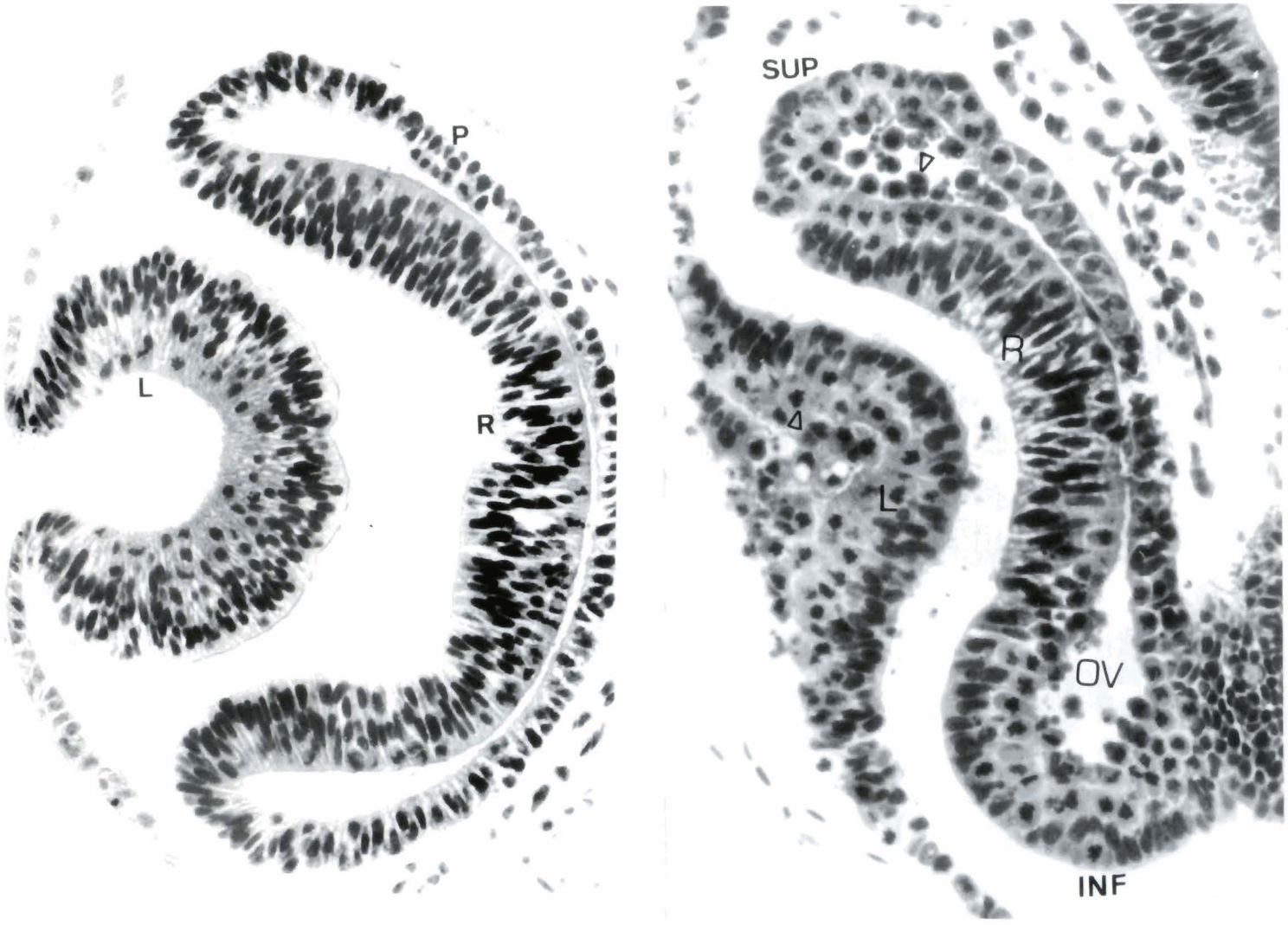

Fig. 4. a. Normal structure of the developing optic cup and lens in an HH16 chick embryo. L=lens; R=retina; P=pigmentary epithelium. x 450. **b.** Chick embryo at an analogous stage of development after treatment with colchicine for 8h. The lens structure is deformed (L), incurving of the cup is weaker, and many mitotic figures (open arrows) appear in the upper (SUP) and lower zones (INF) of the structure. x 600

In the retina, C-metaphases were abundant, and some interphase nuclei were noted along the vitreous margin. In peripheral regions, most of the sloughed-off cells in the ventricular cavity were in C-metaphase; some interphase cells were also noted. In lateral regions of the optic cup, signs of invagination were present. However, in the central region, disorganization led to decreased curvature with abundant cell fragments, giving an overall picture of obvious structural modifications.

In the lens, many C-metaphase cells and cell fragments had become detached and were seen loose in the lens cavity.

Estimation of retinal and lens volumes at different periods

To evaluate the extent of the developmental modifications caused by colchicine, retinal and lens volumes were measured at each stage of treatment (Tables 1 and 2). As noted above, no attempt was made to measure lens volume in embryos treated for 1h.

Table 1. Mean retinal volume (± standard deviation) in chick embryos treated with colchicine, and untreated controls, after different exposure times. All values are the mean of 5 independent determinations.

	RETINAL VOLUME (x1000μm³)			
	1h	2h	4h	8h
COLCHICINE	1683±112	1818±104	1834±108	1334±95
CONTROL	1636±109	1972±102	3520±207	5035±372

Table 2. Mean lens volume (± standard deviation) in chick embryos treated with colchicine, and untreated controls, after different exposure times. All values are the mean of 5 independent determinations.

	LENS RUDIMENT VOLUME (x1000μm³)			
	1h	2h	4h	8h
COLCHICINE	...	391±21	624±30	658±58
CONTROL	...	532±96	1543±86	2146±306

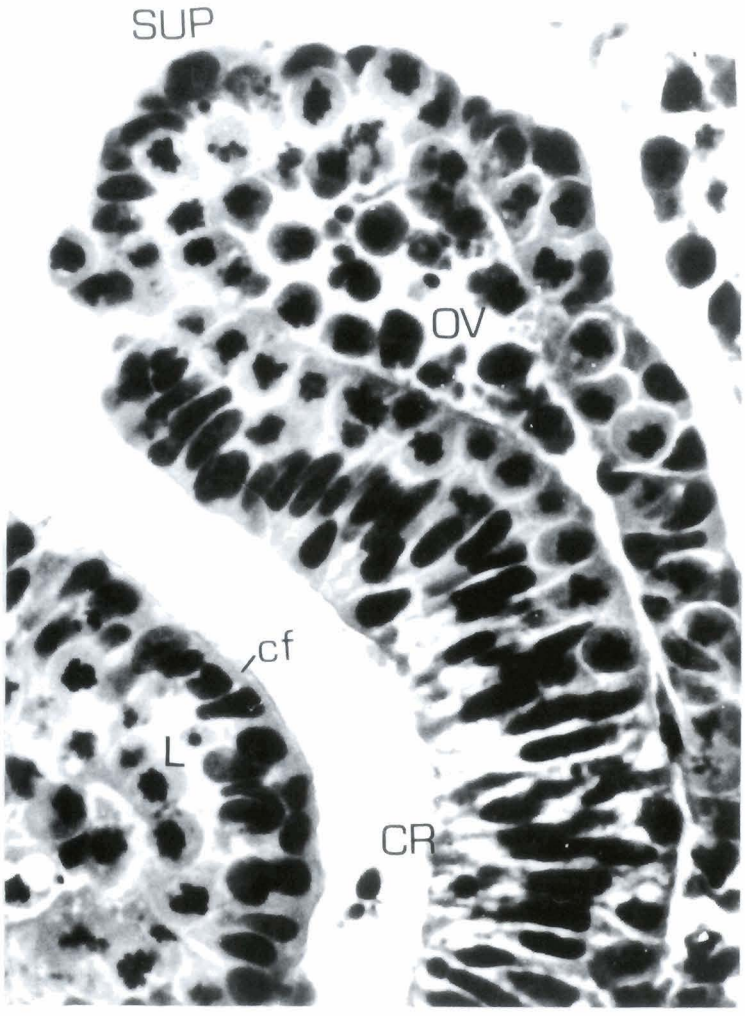

Fig. 5. Detail of the upper and central zones of Figure 4b, showing the different behaviour of cells in the central zone of the retina (CR) and the upper zone (SUP). Some mitotic figures appear detached and free in the ventricular cavity (OV), In the lens, cells of the presumptive lens fibres appear retracted (LF), and the area contains abundant mitotic figures. x 1,200

Discussion

We have described the decreases in the volume of the organs in the chick embryo eye in terms of the increasingly severe effects of colchicine treatment after longer exposures. The modifications we observed included cessation of cell production due to blockage of metaphase, and increased cell necrosis, situations that have also been reported in similar studies. Effects found by other authors included a decrease in intercellular spaces (García-Porrero and Ojeda, 1981) and a decrease in cell height, resulting from the absence of normal elongation (Schoenwolf and Powers, 1987). However, these modifications would not be expected to have a significant effect on the overall reduction in organ volume, because the cells undergo separation processes and become rounded, increasing in diameter upon being arrested in division.

On comparing control chick embryos with those treated with colchicine, we noted that the drug not only reduced organ volume, but also caused structural disintegration, especially in proliferating regions. This suggests that colchicine may also impede the normal process of cell adhesion. The volumetric alterations in the lens paralleled those seen in the retinal layer.

Despite the modifications described above, some of the main events that characterize normal eye formation (O'Rahilly and Meyer, 1959; Schook, 1980; Abadía-Fenoll et al., 1985; Calvente et al., 1988; Nordquist and McLoon, 1991) were still observable during these stages of development in colchicine-treated embryos. The retina and lens continued to thicken, especially in embryos subjected to brief (1 or 2h) treatment, and invagination took place. These findings raise the question of the exact nature of the forces that determine shape and invagination in the nervous system, particularly in the lens placode and optic vesicle. The deformations and variations in volume found in the present study affect the conformation of the developing eye, whereas folding and invagination appear to depend, in part, on factors originating in the superficial cells and their environment, as well as the influence of increases in occupied volume, which depend on proliferative phenomena.

By examining the regions of curvature together with those of cell divisions, we found that in the lens primordium, dividing cells were located on the limiting surface facing the lens cavity, in the areas where C-metaphase cells were found in treated embryos. This outer region, or surface (Fig. 5), is the future site of optic pit closure. However, in the optic cup, mitotic division was concentrated on the opposite limiting surface; i.e., the ventricular or inner margin (Fig. 5). Closure of the lens pit and optic vesicle and therefore difficult to explain exclusively on the basis of pressure due to the increased cell population, and the influence of phenomena taking place between the two ends of the structures, as well as that of the surrounding mesenchyme, are likely to be involved. Although the physical process of approach and fusion of the two ends of the curve is similar in the lens and retinal layer, the significance of the surfaces that limit this curvature of the lens and retinal layer differ in the two structures.

The findings of the present study suggest several conclusions regarding the triggering effects of colchicine on morphogenesis. Cell proliferation phenomena at the beginning of morphogenesis offer a plausible explanation for normal development, which prepares structures for subsequent tissue differentiation. Although the antimitotic action of colchicine affects growth during these initial moments, phenomena such as the closure of the optic cup and lens pit are not completely disrupted at these early stages. Therefore, other factors from the mesenchyme must influence closure and subsequent differentiation. In this connection, a further observation of note was the lack of effect on or delay in lens thickening due to cell division, despite the anomalies caused in the retina. Deformation, accumulation of mitoses and modifications in the pattern of development did not equally affect all parts of the developing eye, suggesting that the optic vesicle, as early as HH12 onward, is a structure formed by cells of different constitution, with different competences (Abadía-Fenoll et al., 1985, 1987). As a result of the different origins and fates of the cells in the developing eye, the abnormalities caused by colchicine are differentially manifested not only as a function of the duration of treatment, but also depending on the moment when the drug exerts its effect, the «window» apparently being between HH stages 11 and 22.

Stereological analysis provides a reliable approach to the collection of observations such as those presented in the present article, and avoids spurious findings and equivocal interpretations in the absence of an analysis of three-dimensional organization.

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