AUTORADIOGRAPHIC AND CYTOLOGICAL STUDY OF BLASTODERMAL CELLS IN TURKEY EGGS SUBJECTED TO EXTENDED PRE-INCUBATION STORAGE[1, 2]

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The length of the interval between oviposition and start of incubation is the most important single factor that affects the subsequent developmental history of the turkey gastrula: prolonged storage progressively reduces the early embryo's capacity to re-initiate and maintain ontogenesis (Kosin and Mun, 1965). Cytological evidence, based on the observations of chicken blastoderms (Arora and Kosin, 1967), suggests that cells of the early avian gastrula continue to exhibit some mitotic activity at temperatures even as low as 7.2° C. However, the same study has shown that the mitotic cycle of these cells is incomplete—it does not proceed beyond metaphase. The objective of the present study, reported below, was to extend similar cytological observations, augmented by the use of tritiated thymidine, to cells of turkey gastrulae, both during pre-incubation storage and the first hours of post-storage incubation.

Materials and Methods

Two genetically distinct lines of Broad Breasted Bronze turkeys were used in the study, both originating in 1954 from a single heterogeneous population. Of the two, Line 1 was a random-bred population while the other, Line 3, was single-trait-selected for high egg production. Although Line 3 turkeys, at the time the data were collected, were characterized by a substantially higher rate of egg production than Line 1, with respect to body size and hatchability, both lines were equal. The eggs were gathered daily, at approximately hourly intervals, between 9:00 AM and 4:30 PM from single-sire pens. All eggs were identified according to the hens that laid them. Shortly after 5:00 PM the eggs were transferred from the pens to a holding room maintained at 13° C. and 80% relative humidity. During storage, the eggs were kept with the large (blunt) end up.

Series 1. After overnight cooling, Line 1 eggs were allotted randomly to two groups: control and experimental. In the control fertile eggs, the germ discs were removed and fixed for cytological examination. The fertile eggs in the experimental group, after 3 days of storage, were injected with tritiated thymidine, sealed, and stored for an additional 6 days. The injection procedure was as follows: the shell was cleaned with 70% ethanol, and a small window (1 cm.^{2}) was cut in the large end of the egg. (Because the egg had been kept throughout in the vertical position, the blastoderm was usually found to be located just under the air-cell.)

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The cut piece of the shell and its membrane were then reflected and the solution containing the isotope was injected, directly under the blastoderm, with a 1.0-ml. micrometer syringe, equipped with a 12-mm. 27-gauge needle, the tip of which was slightly turned up to facilitate the delivery of the solution under the blastoderm. The total volume injected per blastoderm was 0.05 ml. containing 1.6 μc. of T-H^3, with specific activity of 6.64 μc./μM. The solution containing the isotope was held at 13.0° C. Following the injection, the cut piece of the shell and its membrane were reflected back into place, the opening was sealed with paraffin and plastic tape, and the eggs returned to storage at 13.0° C.

After the storage period, the treated eggs were removed and blastoderms fixed for 4 hours in a 19:1 mixture of absolute ethanol and acetic acid at −6° to −8° C., according to the method described by Wolman and Behar (1951). This was followed by dehydration in absolute ethanol at room temperature overnight. The blastoderms then were cleared in xylene, embedded in paraffin and cut serially at 7 μ. For comparative purposes, adjacent sections from different regions of the experimental and control blastoderms were mounted on the same slide. After staining, which involved the Feulgen-Fast Green reaction, the slides intended for the preparation of autoradiographs were held in water at 40–45° C. before the diluted emulsion was applied to the slides. The emulsion (NTB—Kodak) was diluted 1:2 with distilled water at 40–45° C., and the slides were dipped twice, individually, and then allowed to stand on end in a rack until the emulsion dried completely (*cf.* Messier and Leblond, 1957). The slides were placed in plastic boxes containing Drierite, sealed with black tape and stored in the refrigerator (4–5° C.) for 20–28 days. Care was taken that the slides were in a horizontal position in the box, with the emulsion side down. Following this exposure, the slides were developed for 3 minutes in Kodak D-19 at 18° C., rinsed in tap water, and fixed for 6 minutes in Kodak Acid Fixer and then washed in running tap water for 10–15 minutes. Finally, the slides were dehydrated in ethanol, cleared in xylene and mounted in Permount.

The determination of the frequency of mitotic, necrotic, and labeled nuclei was based on counts within at least 30 random unit areas from various sections of each blastoderm. The unit area measured approximately 0.48 × 0.07 mm. The values for these three parameters were expressed per 100 nuclei examined. Morphologigally, some necrotic nuclei appeared pycnotic, while others became much enlarged. They were all characterized by loss of structural detail and deep staining following the Feulgen reaction. All nuclei showing a grain count above the level that could be ascribed to background fogging were considered to be labeled. To be counted as "mitotic," the nuclei could be in any stage of the mitotic cycle.

Series 2. Line 3 eggs were allotted randomly to storage treatments of 1, 7, 14, or 21 days' duration at 13.0° C. and 80% relative humidity. Following storage each egg received the injection of a saline solution containing T-H^3. The injection procedure followed the general plan described for the experimental eggs in Series 1, except for volume of the solution and concentration of T-H^3: 0.07 ml. of the solution containing 7.77 μc. of the isotope at the specific activity of 9.2 μc./μ M. After the injection, the eggs were incubated for 10 hours. At the end of this period the blastoderms were removed, fixed and sectioned serially at 5 μ. Adjacent sections from different regions of each blastoderm were mounted on the same slide.

TABLE I

Frequency (%) of mitotic and necrotic cells in the blastoderms of oviposital and stored eggs. Series 1

Category	Mitotic cells	Necrotic cells
Oviposital (non-stored)		
$\bar{x}$	5.2	1.9
Range	(1.1–9.3)	(0.4–3.9)
Stored		
$\bar{x}$	5.6	2.9
Range	(1.7–9.5)	(1.1–5.4)

Thus two slides, I and II, were prepared from each blastoderm. All slides were subjected to the Feulgen-Fast Green staining reaction. Prior to the preparation of autoradiographs, the I and II slides were treated as follows:

I: These were stained and mounted permanently for histo-cytological studies. They were not exposed to the emulsion.

II: After the usual steps involving xylene, a descending series of ethanol and, finally water, the slides were treated for 5 minutes with 5% TCA at 4° C. The slides were then stained and allowed to dry at room temperature. A few hours before the application of the emulsion, the slides were dipped in a 1% collodion solution for a few seconds (prepared in a 1:1 mixture of ether and absolute ethanol) and allowed to dry in the air (*cf.* Gross *et al.*, 1951, and Kopriwa and Leblond, 1962). From then on the procedures for the preparation of autoradiographs and subsequent cytological analysis were the same as outlined earlier in Series 1.

RESULTS

The data concerning the frequency of mitotic and necrotic nuclei in the oviposital (*i.e.* non-stored) and Line 1 blastoderms are summarized in Table I. The fre-

TABLE II

Frequency (%) of labeled nuclei in blastodermal cells of stored eggs, exposed to tritiated thymidine. Series 1

Blastoderm no.	Frequency of labeled nuclei
1	2.5
2	1.1
3	2.2
4	1.6
5	1.3
6	0.0
7	0.9
8	1.8
9	1.0
10	2.1
11	1.7
12	0.0
$\bar{x}$	1.6

TABLE III

Frequency (%) of T-H³ labeled nuclei in the blastoderms of eggs subjected to different pre-incubation storage periods, followed by 10 hours of incubation. Series 2

Storage length (days)			
1	7	14	21
3.14[a]	3.21	0.91	0.24
2.64	4.10	0.56	0.00
1.02	0.92	2.98	2.10
3.19	3.12	1.21	0.00
4.21	0.98	3.46	1.20
3.21	2.98	2.98	1.12
1.92	0.94	0.29	0.42
2.62	4.10	4.21	2.81
4.12	0.92	0.82	0.00
5.21	....	1.10	0.00
4.00	....	....	....
$\bar{x}$ 3.21	2.30	1.85	0.69[b]
			1.15[c]
Range 1.02–5.21	0.92–4.21	0.29–4.21	0.00–2.81

[a] Each value represents the frequency of T-H³-labeled nuclei in a single blastoderm.
[b] Based on total number of blastoderms involved.
[c] Based on the number of embryos showing labeled nuclei.

quency value in each category was based on 12 blastoderms. The data show that there was an increase in the frequency of these nuclei in the blastoderms stored for extended period, indicating that mitotic activity continued during storage at 13° C. Although the differences were not large, their consistency was unmistakable. The majority of the mitotic figures was associated with the epiblast. By contrast, the necrotic cells were largely found in the hypoblast and in the zone of junction.

The autoradiographic studies demonstrated the incorporation of tritiated thymidine into some of the nuclei of the stored blastoderms. The frequency of the nuclei labeled with T-H³ in the stored blastoderms is summarized in Table II.

The incorporated T-H³ was observed in interphase and early prophase, sometimes in metaphase, but never in anaphase. In general, the frequency of T-H³-labeled nuclei was low.

Table III shows the frequency of nuclei labeled with T-H³ in the blastoderms incubated for 10 hours following different pre-incubation storage treatments of Line 3 eggs (Series 2). It will be seen that the frequency of nuclei decreased markedly when the length of storage was more than 7 days. Among the labeled cells, most were in interphase or in early prophase, although some were in metaphase. Not all the mitotic figures observed in sections were labeled. Moreover, there was extreme individual variation among blastoderms with respect to the incidence of labeled nuclei, particularly among the blastoderms of eggs stored for 14 and 21 days. In the latter group, 4 blastoderms of the 10 examined were devoid of labeled nuclei. In these blastoderms, and in a few from other treatments, the evidence of radioactivity was either exclusively or largely localized around the yolk granules. Such blastoderms lacked the sub-germinal cavity.

TABLE IV

Frequency (%) of mitotic, fragmented, and necrotic nuclei in the blastoderms of eggs subjected to different pre-incubation storage periods, followed by 10 hours of incubation. Series 2

Storage length (days)	Type of nuclei		
	Mitotic	Fragmented	Necrotic
1	5.26	1.81	3.58
7	4.54	2.68	3.94
14	2.83	5.74	4.90
21	2.63	5.15	8.22

Data bearing on the further cytological examination of the slides which were not exposed to the emulsion (Slide I) are presented in Table IV. The frequency value in each classification was based on 10–12 blastoderms. One sees from it that the frequency of fragmented nuclei increased as the storage was extened to 14 days or beyond, whereas the opposite was true for mitotic figures, indicating an inverse relationship between these two parameters. The fragmented nuclei were characterized by the presence of both large and small, usually round, masses of nuclear material which stained deeply with nuclear dyes. These "micro-nuclei" were found either enclosed within the intact nuclear membrane or within the cellular plasm in the cells in which the nuclear membrane could no longer be detected.

In addition, the blastoderms from eggs first stored for extended periods and then incubated for 10 hours revealed the presence of various types of nuclear irregularities. Among these the most frequent were: tripolar mitosis, amintosis and chromosomal aberrations such as double chromatid bridges.

DISCUSSION

An earlier report from this laboratory (Arora and Kosin, 1967) has shown that the "physiological zero" for chicken blastoderms (when the term is used to designate the absence of activity recognizable at the intracellular level with the aid of a light microscope) is below 13° C., the temperature recommended for holding hatching eggs before incubation. Some mitotic activity continued even when eggs were kept at 7.2° C., for as long as 21 days. In the present study, based on turkey blastoderms, the experimental design called for a single pre-incubation temperature at 13° C. Keeping this point in mind, the parallelism of results obtained in the comparable phases of the two studies is striking. In both, the mitotic activity in aging blastoderms was blocked at metaphase: no cells were observed to proceed with mitosis beyond that stage during the experimental holding period. Consequently, progressive lengthening of storage was accompanied by an accumulation of "blocked" cells.

The existence of mitotic activity in blastodermal cells at 13° C. was corroborated by the observation, in the present study, that the DNA synthesis continued within such cells. Evidence for the incorporation of tritiated thymidine in the nuclei was unequivocal, although the frequency of cells involved in the process was low. The presence of mitotic figures devoid of the isotope among the cells of blastoderms

which received T-H^3 early in the storage period suggests an explanation for two possible conditions which are not necessarily mutually exclusive: one is that the cells in mitosis were present *before* the initiation of "quiescent" (storage) period and that they remained in that stage; the other is the availability of the isotope to some of the cells at least was reduced by physical means. For example, the frequently observed absence of the sub-germinal cavity in the early gastrulae of eggs exposed to 37.5° C., following an extended period of temperature-induced quiescence, indicates that the liquefaction of the yolk under such blastoderms does not proceed at a normal rate. It is possible that the solution containing the isotope, when injected under the blastoderm of this type, was prevented from reaching the physiologically active cells of the epiblast. This would, of course, introduce an error in the subsequent cytological analysis of the physiological state of the affected blastoderm.

The aging process in the avian blastoderm is characterized by a steep rise in the frequency of necrotic nuclei. No direct evidence is available on the possible relationship between cells blocked at metaphase and those classified as "dead." However, the close and consistent association between their respective frequencies strongly indicates that necrotic cells may be a by-product of blockage. As suggested earlier (Arora and Kosin, 1966a, 1966b, 1967), this chain of events on the cellular level may be responsible for the reduced viability, during subsequent incubation, of chicken and turkey blastoderms previously subjected to extended storage. Support for this comes from the present study: following pre-incubation storage, the turkey blastoderm upon being exposed to 37.5° C., optimal for normal embryogenesis, responds by a decreased level of T-H^3 labeling of the nuclei and an increased frequency of fragmented nuclei and of mitotic irregularities.

Summary

An autoradiographic and cytological study involved blastoderms of Broad Breasted Bronze turkey eggs subjected to storage up to 21 days at 13° C. and 80% relative humidity. In one phase of the study, the blastoderms were treated with tritiated thymidine during storage; in the other phase, the blastoderms were first stored, then treated with T-H^3 and, finally, incubated for 10 hours at 37.5° C.

Results indicate that:

1. The frequency of mitotic and necrotic cells in the blastoderms increased during pre-incubation storage.

2. Nuclei, labeled with T-H^3, were found present in the blastoderms exposed to the isotope, both during and after storage.

3. The "aged" blastoderms, when incubated for 10 hours, showed a high incidence of necrotic nuclei, of nuclear fragmentation, of mitotic irregularity, and a decreased frequency of labeling with T-H^3.

The conclusions reached are that:

1. The turkey blastoderm is physiologically active during extended storage at 13° C.: it exhibits evidence of some DNA synthesis and of undergoing limited mitosis.

2. The accumulation of cells blocked at metaphase during storage may be a major factor responsible for the subsequent moribundity of such blastoderms.

LITERATURE CITED

ARORA, K. L., AND I. L. KOSIN, 1966a. Changes in the gross morphological appearance of chicken and turkey blastoderms during pre-incubation storage. *Poultry Sci.*, **45**: 819–825.

ARORA, K. L., AND I. L. KOSIN, 1966b. Developmental responses of early turkey and chicken embryos to pre-incubation holding of eggs: Inter- and intra-species differences. *Poultry Sci.*, **45**: 958–970.

ARORA, K. L., AND I. L. KOSIN, 1967. Changes in the gross morphology and mitotic pattern of chicken blastoderms subjected to three levels of pre-incubation holding temperatures. *Physiol. Zöol.*, (in press).

GROSS, J., R. BOGOROCH, N. J. NADLER AND C. P. LEBLOND, 1951. The theory and methods of the radioautographic localization of radioelements in tissues. *Amer. J. Roentgenol.*, **65**: 420–458.

KOPRIWA, B. M., AND C. P. LEBLOND, 1962. Improvements in the coating technique of radioautography. *J. Histochem. Cytochem.*, **10**: 269–284.

KOSIN, I. L., AND A. M. MUN, 1965. Some factors affecting the biological quality of turkey eggs. *Poultry Sci.*, **44**: 31–39.

MESSIER, B., AND C. P. LEBLOND, 1957. Preparation of coated radioautographs by dipping sections in fluid emulsion. *Proc. Soc. Exp. Biol. Med.*, **96**: 7–10.

WOLMAN, M., AND A. BEHAR, 1951. Methods of fixation for enzyme-cytochemistry and cytology. *Exp. Cell Res.*, **3**: 619–621.