Influence of Heat Stress on Development of Chick Embryo (*in ovo)*

تأثير الإحتباس الحراري على نمو أجنة الدواجن (داخل البيض)

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كلية التقنية العليا \الخوير مسقط / سلطنة عمان

Abstract

The present study was conducted to determine the adverse effects of high incubation temperature on growth, development and genome stability of broiler chick embryo *in ovo*). One hundred twenty broiler eggs from Cobb Company, USA were weighted and divided into two groups. The first group was incubated at 37^{o}C ± 0.5^{o}C, and the second group was incubated at 41^{o}C ± 0.5^{o}C from 0 to 18th day. Starting on day 4th and every other day; three eggs from each group were examined following performed measurements as weight of eggs post incubation, embryo, yolk, and egg shell for measuring growth index. Blood smear was also prepared for counting heterophiles, and lymphocytes to determine H/L ratio. Micronucleus formation and presence of binucleated red blood cells were investigated as genome stability parameters, in 2000 cells. Significant reduction (P<0.01) in growth indices was observed in embryos grown at 41^{o}C compared to those grown at 37^{o}C ± 0.5^{o}C. Reduction in H/L ratio was statistically significant (p≤0.01) in embryos of 2nd group comparing to 1st group embryos. Blood of embryo from heat stress group group (2) showed Red blood cells with micronuclei and binucleated cells while no such phenomenon could be seen in embryos from control group group (1). These results suggested that heat stress is influencing cell division at telophase and induces chromosomal damage. 88% of chicks from group (1) were hatched on day 21st; only 18% of chicks from group (2) were hatched lately on day 23rd, while the others were found dead. These results indicate that heat stress not only adversely affects growth and development of embryo stem cells but also induces genome instability which intern resulted in poultry production losses.

المستخلص

تهدف هذه الدراسة الى الكشف عن التأثيرات السلبية لدرجات الحرارة العالية على النمو والمادة الوراثية لأجنة الدواجن . قسمت 120 بيضة دجاج لحم ملقح (من شركة كوب) الى مجموعتين : المجموعة الأولى 60بيضة حضنت تحت درجة 37م ± 0.5 والمجموعة الثانية 60 بيضة حضنت تحت درجة 41م ± 0.5 بدأً من اليوم الرابع ولغاية اليوم 18 فحص البيض من النواحي التالية: وزن البيضة ، نمو الجنين ، المح ، القشرة لقياس دالة النمو . كما حضّرت شرائح مجهرية من دم الجنين خلال تلك الفترة وتم إجراء الفحوص التالية على الدم: نسبة خلايا الدم الهيتروفيلية / اللمفاوية L/H ، كذلك أجري التحري عن ظهور النواة المجهرية ونسبة الخلايا المتعددة النواة في كريات الدم الحمراء . ظهر إختزالاً معنوياً (بإحتمالية أ ≥ 0.01) وإنخفاضاً في نمو الأجنة وأوزانها ونسب خلايا L/H في الدم نتيجة حضن البيض تحت درجة41م مقارنة بنمو الأجنة المحضونة تحت 37م . ظهر عدداً ملحوظاً ومعنوي الإحتمالية من كريات الدم الحمراء في الأجنّة النامية تحت 41م يحوي على أنوية مجهرية وخلايا ثنائية النواة . بينما لم تلاحظ هاتين الظاهرتين في دم الأجنة المنماة تحت 37م . كما لوحظ تأخير في فقس البيض لمدة يومين في البيض المنمى تحت 41م مقارنة بالبيض النامي تحت درجة 37م.

إن ظهور الخلايا الثنائية النواة يعد دليلا على حدوث خللاً في عملية الإنقسام الخلوي ومرحلة تكوين الغشاء الخلوي . تشير هذه النتائج الى إن الإرتفاع في درجات الحرارة تؤثر سلباً ليس فقط على نمو الأجنّة وأوزانها ومواعيد فقسِها ، بل على مجينها الوراثي وإنقسامات خلاياها .

Introduction

It has been well documented that when incubation conditions were optimal, chick embryos are developed normally and hatched in approximately 21 days, but turning, temperature; vital gas exchange, humidity and other factors have been shown to affect embryo growth [1]. Temperature has been suggested to be the most important factor controlling embryo growth and development [2]. Abnormal incubation temperatures, however, have been shown to affect organ development of chick embryo and accelerate hatching time after egg incubation of the embryo for 14 days at 39^{o}C [3]. However, thermal manipulation in late-term chick embryos have immediate and longer term effects on myoblast proliferation and skeletal muscle hypertrophy [4].

Moreover, there are different physiological effects have been detected the influence of changing in incubation temperature on the development of chick embryo, such as myocardial glycogen [5], stimulation of brain development [6], weight of embryo, weight of yolk sac, weights of different organs [3], mortality and hatchability [7]. Heat shock protein is also used as a biomarker for detection of thermally stressed chick embryo [8]. Furthermore, blood leukocytes could also be used for detecting the influences of environmental stress on chick embryo development. Percentages of heterophiles and lymphocytes, as immune blood cells, were affected by heat stress [9].

Micronucleus formation in chick embryo cells provides a simple and rapid indirect measurement of the induction of structural or numerical chromosome aberrations that are resulted from exposure to environmental stress on the development of embryo. On other hand, failure of cytokinesis to occur in tissue cells will lead to reproduction of binucleated or multinucleated cells. It was reported that heat stress induced micronuclei and inhibits cytokinesis in mouse bone marrow, *in vivo*, and in human blood lymphocytes, *in vitro* [10]. Cytokinesis is a process leading to separation of the cytoplasm living eukaryotic cells and its contents after mitosis. These process in animal cells begins during anaphase as a cleavage furrow, an indentation of the membrane. Actin filaments constrict to deepen the furrow until the cytoplasm is separated between the two daughter cells. On the other hand, myocin II generates force for the division of eukaryotic cells through its interaction with actin filaments. The cell cycle is completed after cytokinesis [6].

The present study is aimed to detect the influence of heat stress on development of broilers embryos by applying; growth index, heterophile/ lymphocyte ratio, binucleation and micronucleus formation parameters in red blood cells of chick embryo incubated at 37.0 ± 0.5^{o}C and 41.0 ± 0.5^{o}C for eighteen days to detect the effects of heat stress on the development and genome stability of the chick embryo.

Materials and Methods

*Eggs source

One hundred twenty fertile broiler eggs were purchased from Cobb Company.USA through Muscat Poultry Company in Oman. Average egg weight at moment of

incubation was 68.5gm (S.D=5.7). These have a percentage of fertility ranging 80-85% (Company information).

Egg Incubation

Egg shell is wiped with iodine-alcohol solution (2%/10% v/v) for cleaning, then weighted and coded. These eggs were divided into two groups; Group (1) was incubated at 37 ± 0.5^{o}C and group (2) was incubated at 41 ± 0.5^{o}C. The incubator was manufactured by a team in the Department of Applied Science, and Department of Engineering, The Higher College of Technology and was quite efficient in growing and hatching of chicks under electronically controlled optimal conditions, such as temperature, humidity and air circulation. Incubation humidity was adjusted to be 65-75%. On day 4th of incubation, the eggs from both groups were candled and were 82% fertile.

***Embryo growth measurements**

Starting day 4th of incubation and every other day, 3 eggs from each group were examined for embryo growth by measuring the weight of the egg post incubation, opening the egg and blood was withdrawn for smear preparation and then they were poured into a Petri-dish for separation of the embryo from the yolk sac. The embryo was weighted as well as the yolk and egg shell.

***Blood cell analysis**

Blood smear was prepared from each embryo. The obtaining blood smears were stained by applying May-Gruenwald-Giemsa procedure. Differential cell counts were performed based on at least 1000 cells per sample. The criteria of Lucas and Jamroz [11] were used to distinguish lymphocytes from other erythropoitic, granulo-cytopoietic, thrombocytopoietic and heterophilic cells as well as undifferentiated blast cells. Smears were coded and examined independently by two observers to avoid intra-individual errors. Heterophiles and lymphocytes were counted in 1000 white blood cells for counting the heterophil/ lymphocyte ratios.

Micronucleus and binucleated cell analysis

Two thousands mature red blood cells were scored for presence of micronucleus and cells with binuclei in smear from each embryo during the developmental period, following [12] method except that we used conventional microscopic system instead of semiautomatic image analysis system.

***Statistical Analysis**

Data were submitted to one-way analysis of variance (temperature) and subsequently expressed by polynomical functions to justify the differences between the treatment means. Growth index data were evaluated by Fishers Test 5%. All statistical analyses were performed using General Lenear Model (GLM) procedure of SAS (Statistical Analysis System) [13].

Results and Discussion

The present study is focusing on growth period starting on 4th day of incubation till the day of transferring the eggs from the incubator to hatcher at the end of day 18th of incubation as a test period.

Effects of heat stress on broiler embryos growth are summarized in Table (1). It shows a gradual increase in embryo weight as a function of incubation days at 37^{o}C.

Significant (P<0.01) reduction and fluctuation in embryo weight were seen in embryos incubated at41.0 ± 0.5^{o}C for entire period. Clearly growth index (weight of embryo/ total egg weight post incubation) presented the response of embryo to increase of incubation temperature Figure (1). Stable increase in growth index was seen in embryos grown at 37^{o}C group (1), while those grown at 41^{o}C group (2) showed disturbed growth indices. The weights of fresh broiler eggs for first group (1) and second group (2) were 69.0-74.6 gm and 66.6 to 78.4 gm respectively Table (1). With respect to egg weight loss, there was a significant reduction in weight (P<0.05) as a function of incubation from day 4th to 18th comparing to the weight before incubation. That reduction was ranging from (1-10) % with no difference between the two groups.

Table (1): Effects of heat stress on growth of broiler chick embryos measured by weight:

Incubation day	Weight of egg before incubation(gm)	Weight of egg after incubation (gm) at the day of test	Weight of embryo (gm)	Weight of yolk(gm)	Egg shell weight
	A: Incubation Temperature 37.0±0.5 C^{o}				
4	69.8	69	0.1	61.5	7.4
6	69	67	0.4	58.6	8
8	70.4	67.2	1.2	56.3	9.7
10	74.6	71	1.6	57.3	12.1
12	68	63.4	8.4	48.5	6.5
14	69.6	64.2	11.4	33.5	19.3
16	68.3	62.1	17.3	37	7.8
18	69.2	64.4	20.1	30.1	14.2
	B: Incubation Temperature 41.0±0.5 C^{o}				
4	78.4	77.7	0.2	67.8	9.7
6	67.5	65.7	1.9	55.6	8.2
8	70.9	68.5	1.4	60.4	6.7
10	73.1	71.2	0.5	43.1	27.6
12	66.6	61.3	0.7	44.1	16.5
14	70.3	65.3	12.6	44.4	8.3
16	65.9	60	13.1	44.4	2.5
18	71.9	66.1	0.3	45.5	20.3

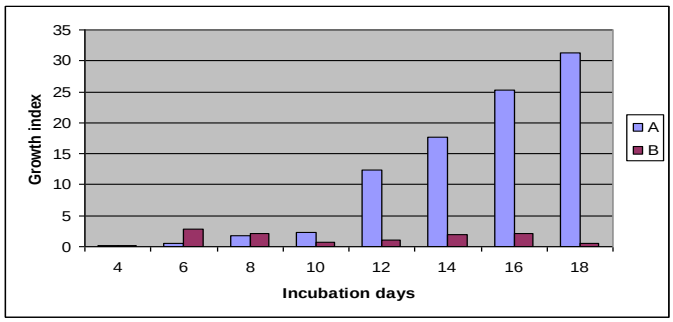


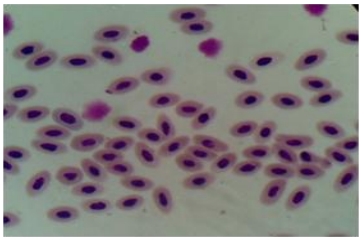
Fig (1): (A) Represents data for embryos grown at incubation temperature 37.0±0.5 C^{o} group (1). (B) Represents data for embryos grown at incubation temperature 41.0±0.5 C^{o} group (2).

Similar effects of incubation on weight loss were reported in broiler eggs by [14] suggesting that this loss was resulted from water loss from the eggs. However, we have extended our observation on egg weight loss by including longer incubation days (till hatching day 21 at 37^{o}C). The rate of weight loss reached to 15%.

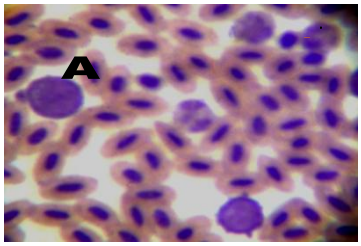
It is generally agreed that each egg is built with a complete capacity to produce a perfect new organism. In the sequence of events following embryo development, protein and energy are first obtained from the albumine, and derived from the yolk only after 14 days of incubation. Minerals are also primarily obtained from the yolk and from the shell only after the chorioallantoic circulation is established. Bone mineralization process requires phosphorus derived from the yolk phosvitin. The phosvitin reacts with calcium from the shell. An intense metabolism involving carbonic acid and enzymes is in effect of mineral removal from the egg [15]. Together with the results presented in Table (1), these nutritional growth requirements of embryo described why this study established the ratio of embryo weight to total egg components (shell, albumin, and yolk) weight after incubation and represented the index of growth. These results showed that this index is highly affected by heat stress.

It is interesting to note that hatchability of groups (1, 2) chicks reached to 88% and 18%, respectively. The unhitched chicks from group (1) were survived but weak to be hatched which is being expected in this breed (Company information's). Chicks from group (2), however, were hatched on day 23 of incubation, while the unhatched eggs had dead embryos. These results indicated that heat stress causes embryo death at last stages of growth as well as slowing down the maturation to some whom have heat tolerance.

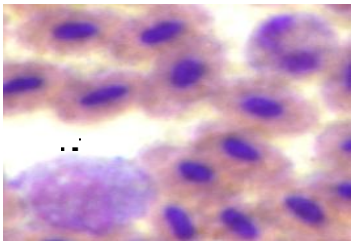
Blood heterophil and lymphocytes were counted from blood smear which were prepared from embryos of both groups after incubation for 18 days Figure (2). Constant increase in the heterophil/lymphocyte (H/L) ratio as a function of incubation time was observed, it was ranging from 0.4% at day four till reach 0.7% at day 18 of embryo grown at 37.0 ± 0.5^{o}C Figure (3). H/L ratio for embryos grown at 41.0 ± 0.5^{o}C; however, were four times more than the first groups and declined gradually as a function of incubation period till reach to 0.96%.



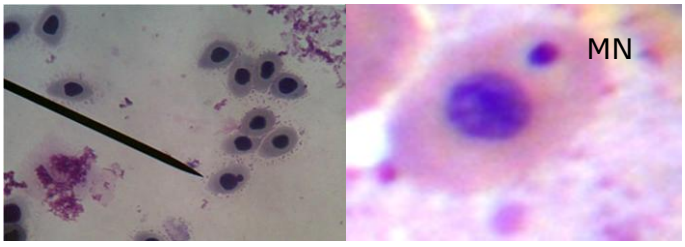
(a) Normal Red Blood Cell



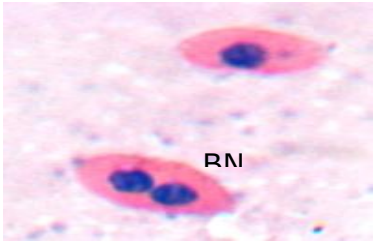
(b) Blood film from chick embryo grown at 37C^{o}



© Blood film from chick embryo has heterophil (H)



(d) Immature blood cells with micronuclei (MN) from chick embryo grown at 41 ^{o}C.



(e) binucleated red blood cell from chick embryo grown at 41°C.

Fig (2): illustrated chick embryo blood cells: RBC, heterophil, lymphocyte, immature blood cells with micronuclei and binucleated red blood cells.

Results presented in Figure (3) suggested that heterophiles are generally outnumbered lymphocytes in chick embryo, their numbers are raised during mildly or moderately stressful conditions of heat and consequently the H/L ratio can be used to detect the presence of physiological stress. Similar results were observed in other avian species by Maxwell and Robertson (1998). Moreover, heat stress effects on H/L ratio from broiler embryos grown at 41.0 ±0.5^{o}C showed a biphasic curve. It seems that this phenomenon is unique to Aves [9].

Results presented in Figure (3) showed that the periods between days 10th, 12th and 18th were heat sensitive or critical periods in chick embryo development. At this period the growth index were significantly (P≤0.01) lower than those in the first week or week after. The cause of presence of such phenomenon is unknown. However, exposure to environmental stressors can result in biochemical, physiological and histological (tissue) alteration in chick embryo [9, 16]. The presence of these alterations may not be seen unless exposing the embryo for entire period rather than at random choosing period. These results indicated that this system is quite sensitive model to be used for detecting the effects of heat stress on avian immune system. It also can facilitate the study of nutrition supplementation that could reduce heat stress effects on broilers.

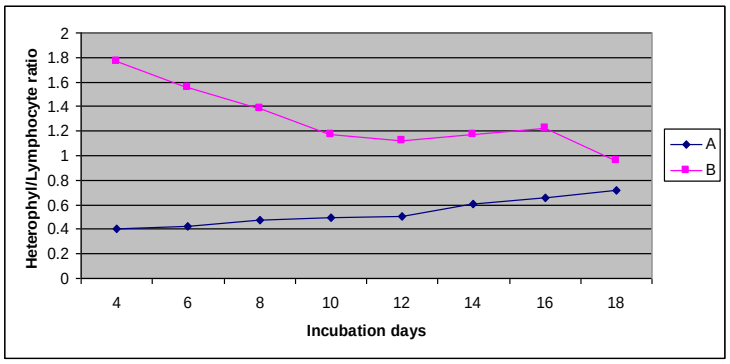


Fig (3): Effects of heat stress on heterophil/lymphocyte ratios
(A) Represents H/L ratio for embryo grown at 37.0±0.5 Cº.
(B) Represents H/L ratio for embryo grown at 41.0±0.5 Cº.

Micronucleus analysis was performed on 2000 red blood cells from each blood embryo grown on 37.0 ±0.5^{o}C and 41.0 ± 0.5^{o}C for 18 days. A total of 32,000 RBCs were analyzed for the presence of micronuclei. Neither micronucleus nor binucleated RBCs could be detected in blood obtained from embryo grown at optimum temperature 37.0 ± 0.5 ^{o}C. A significant number of RBCs with micronucleus and/or binuclei (Fig.1) were seen in blood from embryos grown at 41.0^{o}±0.5C^{o}, starting on day 8th of the incubation Table (2). There was no correlation (r=0.332) between presence of cells with micronucleus or binucleated cells and duration of incubation, although those abnormalities were started present at day 8th till the day 18th except on day 12th when no such phenomenon could be detected. The percentages of those abnormalities, however, were independent from embryo stage of maturity.

Table (2): Percentages of red blood cells with micronucleus and binuclei from chick embryo grown for 18 days at 41.0°±0.5C°.*

Incubation days	% of RBCs with Micronucleus	% of Bionucleated RBCs
4	0.00	0.00
6	0.00	0.00
8	0.00	0.25
10	0.28	1.94
12	0.00	0.00
14	0.14	0.00
16	0.29	1.26
18	0.15	0.31

There were not nither micronuclei nor could binucleated cells in blood cells from embryos grown at 37.0o±0.5Co be seen.

Micronucleus (MN) assay was developed as a short-term screening test. In this test, chromosomal aberrations are detected indirectly via chromatin less from the nucleus leading to MN in the cytoplasm of the cell. MN are defined as a small round-DNA containing cytoplasmic bodies formed during cell division by loss of both acentric chromatin. It can be induced by different environmental stressors (for review see 17). It was indicated that, for genotoxicity assay, the erythrocytes from chick embryo blood are relevant target cells and independent from their stage of maturity (polychromatic as well as normochromatic definite erythrocytes) for environmental stressors [12].

Induction of micronucleus by increasing incubation temperature was seen in mouse bone marrow cells [10] as well as in human blood lymphocytes. Data presented in Table (2) showed that long duration of heat stress leads to formation of MN in chick embryo erythrocytes. The mechanism(s) of such induction is unknown. However, it could be due to heat disturbance of the mitotic apparatus [10], and/or impairment of DNA repair enzyme function and process [18] as a result of increase in incubation temperature leading to micronucleus formation. Moreover, results presented in this study indicated that mononucleated chick embryo RBCs have developed micronucleus Figure (2) Table (2).

The results of the present study may indicate that heat stress is an aneugenic agent, and one possible mechanism of MN formation in mononucleus RBC is that MN is formed in immature RBC before maturation process has taken place. This may be an interesting additional parameter in Micronucleus-assay in chick embryo system.

Different mechanisms have been proposed to amount for the origin of binucleated cells: *i*) binucleated cells can be arised by nuclear mitosis without cytoplasmic division [19]. *ii*) Failure in the formation of the actin-myosin contractile ring could cause cells to be defective in cytokinesis. And these actin-myosin molecules involved in the later stages of cytokinesis may be responsible for incomplete cytokinesis during the binucleation process [20]. *iii*) Cell binucleation might be due to direct breakage of the actin filaments [21]. In chick embryo, failing of cytokinesis will lead to reproduction of binucleated or multinucleated cells. However, these processes are cytosolic Ca^{++} -dependent [22], and such dependency is different from one cell type to others and it is also heat sensitive process. Finally, *iv*) it was reported that heat stress

leads to production of heat shock protein that can act as an inhibitor of actins polymerization [23].

Result from present study concluded that the formation of binucleated RBCs in chick embryo growing continuously under heat stress is an indicator of growth hypertrophy and as a result of mitosis without cytokinesis

These results also concluded that heat stress not only adversely affects the growth and development of chick embryo (*in ovo*), but also induces genome instability. The three parameters that we applied together in this assay; growth index, heterophil/ lymphocyte ratio, micronucleus/binucleated cells presence were highly sensitive to detect heat stress effects on stem cells during differentiation.

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