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Difference in Chromosomal Pattern and Relative Expression of Development and Sex Related Genes in Parthenogenetic Vis-A-Vis Fertilized Turkey Embryos

Tomar NS[1], Goel A[1], Mehra M[1], Majumdar S[1], Kharche SD[2], Bag S[3], Malakar D[4] and Bhanja SK[1]*

[1]*Central Avian Research Institute, Izatnagar-243122, U.P, India*
[2]*Central Institute for Research on Goats, Makhdoom, Farah-281122, U.P, India*
[3]*Indian Veterinary Research Institute, Izatnagar-243122, U.P, India*
[4]*Animal Biotechnology Center, National Dairy Research Institute, Karnal, India*

Abstract

Turkey hens show spontaneous parthenogenesis (embryo development without any male contribution) which is influenced by genetic and environmental factors. Chromosome pattern and differential expression of genes associated with parthenogenetic development in turkey eggs were investigated in the present study. The metaphase spread obtained from parthenogenetic embryos was classified as haploid, diploid, polyploidy or aneuploidy based on the proportion of 'n' number of chromosomes. With the advancement of embryonic age, per cent of haploid cell (38.73 to 20.44) or other ploidy decreased while those of diploid cell increased (21.10 to 42.06) and the transition of ploidy continued till 48 h of embryo dveleopment. Early developmental stages presented higher ratios of W chromosomes in comparison to Z chromosomes while ZW combination was absent. Freshly laid parthenogenic eggs had higher *Sox2* gene expression, but 24hrs old embryo had higher *Sox3, GATA-4* or *PouV* genes expression. Expression of male specific genes (*DMRT* and *AMH*) was higher in 12 h or older parthenogenetic embryos, but the female specific genes, *ASW* and *P450* were expressed more in freshly laid parthenogenetic eggs. It is concluded that transition of ploidy from haploid to diploid or poly-ploidy continued till 48 h or beyond parthenogenetic development. Significantly higher expression of *Sox3* or *GATA-4* gene in parthenogenetic embryos could potentially be used as marker gene for indication of parthenogenesis in turkey.

Keywords: Blastomere; Chromosomal pattern; Gene expression; Parthenogenesis; Turkey birds

Introduction

Parthenogenetic development in birds was reported in domestic fowl *Gallus domesticus,* domestic pigeon *Columba livia* [1] but found to be most common in turkey (*Meleagris gallopavo)* with almost 20% incidence in general [2-5]. Parthenogenetic birds (invariably males in turkey) are derived entirely from the maternal genome; the equivalent of self-fertilization will be achieved when parthenogenetic males will be bred to their mothers. Use of parthenogenetic males as sire could substantially reduce the number of embryonic lethal present in the genotype which is a major concern for early chick mortality in turkey.

Embryonic development is often delayed in parthenogens and hatching occurs 2 to 3 days later in comparison to normal embryos derived from inseminated hens [2]. In our earlier study also there was mostly unorganized development after 36 h of incubation of parthenogenetic eggs [6]. It is reported that parthenogenetic development began in haploid cells and subsequently became diploid embryos [7-9]. Few haploid cells have also been identified in parthenogens between five to nine days of development [10] and even small proportion contributes to adult parthenotes life [7]. There is hardly any information available on the transition pattern of chromosomes (haploid to diploid or aneuploid) in turkey parthenotes. As not all the partheniogenetic development leads to embryo formation and subsequent hatching [6], the exact mechanism involved in this process needs to be explored for increasing the incidence of parthenogenesis.

There are few transcription factors like *Sox2* (the earliest molecular markers for the neural plate) or *Sox3* (important for normal oocyte development and male testis differentiation and gametogenesis) plays an important role in early embryo formation [11,12]. Parthenogenesis is a very useful method of derivation of embryonic stem cells (ESCs), which may be an important source of histocompatible cells and tissues for cell therapy. The maintenance of pluripotency and ability to self-renew has been shown to be governed by the transcription factors PouV (homologus of mammalion oct-4) and Nanog [13,14]. These genes are expressed in early embryos or in germ cells just before gastrulation. *GATA-4* is required to initiate formation of the genital ridge in both XX and XY fetuses [15]. *DMRT1* and *AMH* genes are found to be consistently higher in males [16,17]. Whereas, W-linked novel genes, *ASW* and *P450* express only in ZW female gonads at the onset of morphological differentiation [18]. The correlation of pluripotent and sex specific genes expression with ploidy status of parthenotes will help in understanding the physiology of the early development that can be used to manipulate the parthenogenesis in turkey birds. Identifying few marker genes can also help to differentiate parthenogenetic development from that of fertilized one. The present study investigates the difference in chromosomal pattern of parthenogenetic and fertilized turkey embryo and correlated the ploidy

***Corresponding author:** Bhanja SK, Central Avian Research Institute, Izatnagar-243122, India, Tel: 09359105979; E-mail: subratcari@gmail.com

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of the parthenogenetic embryo with that of expression pattern of early development related and sex specific genes.

Materials and Methods

Birds, parthenogenetic eggs and isolation of blastomere

Eighty virgin turkey hens of 30 week old from a single hatch were maintained on deep liter system for collection of parthenogenetic eggs and another batch of thirty females and five males were reared for fertile egg collection (by pen mating). Eggs were daily collected, fumigated and set in a force draft incubator at 37.5°C temperature and 60% relative humidity. The germinal disc with diameter of 6 mm or more were classified as having undergone parthenogenetic development as described by Olsen and Buss [19]. The blastomere/embryo was collected from freshly laid eggs (just before incubation, 0 hr) or incubated eggs (12, 24, and 48 h) as per the method described in Tomar et al. [6]. The blastomere was separated from the vitelline membrane by slipping the hair loop under the disc. The isolated blastomere was transferred to an eppendorf tube for total RNA isolation using standard commercial kit (Trizol method, Life Technologies Ltd., USA).

Macro-chromosome analysis

Before collection of blastomeres for chromosomal analysis, cut was made at the broad end of the eggs and 0.05 to 0.1 ml of colchicine (0.05%) was injected around the blastomere to arrest the cells in metaphase stage. Following injection the cut end was sealed with paraffin and the eggs were incubated for 45-60 minutes before harvesting the blstodermal cells. The procedure for collection of blastomere was same as discussed earlier.

For standard karyotyping, the peripheral blood mononuclear cells (PBMCs) of adult male and female were isolated and processed for chromosomal analysis using the method of Tomar et al. [20] and was used as reference for identification of diploid ZZ and ZW, respectively. The blastomeres were transferred to a 15 ml tube containing 4-5 ml of 0.56% of hypotonic KCl and kept at 37°C for 10 min, thereafter cells were fixed with 3:1 methanol: acetic acid (chilled and freshly prepared) and slides were prepared by putting few drops of cell solution on to it from a height of about 3 feet. A minimum of six slides were prepared for each period of parthenogenetic embryo and three slides for PBMCs of male and female turkey. The slides were then air dried and kept overnight in the incubator (37.5°C). Next day the slides were stained with 4% Giemsa solution in PBS for 25-30 minutes and then rinsed gently under running tap water and dried. The slides were observed under trinocular compound microscope at 100X oil immersion and photographed for karyotyping and chromosomal analysis using Motic Image Plus 2.0 software. Each slide was screened at ten positions to identify the sets of macro-chromosomes (First five numbers including the sex specific Z and W). The chromosomes in the proportion of 'n' number of chromosome were termed as haploid and '2n' as diploid. The parthenogenetic embryo whose sex was not identified clearly and the cells which contain more than two sets of haploid genome (polyploidy) and the cells lacking chromosome in the proportion of 'n' number of chromosome (aneuploidy) were classified as other ploidy.

Total RNA isolation and first strand cDNA synthesis by RT-PCR

Total RNA was extracted from the blastomere by TRIZOL method for quantitative RT-PCR of development related genes (*Sox2*, *Sox3*, *GATA-4* and *PouV*) and sex specific genes (*DMRT*, *AMH*, *ASW* and *P450*). The cDNA synthesis was performed by using Revert Aid™ first strand cDNA synthesis kit (MBI, Fermentas) and was used as template for amplification of above mentioned genes by quantitative real time PCR.

Standardization of primers and quantitative PCR

The primers for this study were designed using DNA Star Lasergene software (DNASTAR, Inc., Madison, WI, USA) and there specificity was checked by NCBI blast program. The specificity of primers was checked by NCBI blast program. PCR products of different genes were sequenced and the sequences were submitted to NCBI, USA or EMBL, UK for confirmation and accession numbers were obtained for those genes (Table 1). The amplification of selected genes were carried out in IQ5 cycler (Bio-Rad, USA) in 25µL volume containing 1X QuantiTect SYBR Green PCR master mix (SYBR Green 1 dye, Hot Start Taq DNA polymerase and dNTPs in optimized buffer components; QIAGEN GmBH, Germany), a 0.2µM concentration of each gene-specific primer and 1µL of cDNA template. PCR cycling conditions included initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30s; annealing for 30s and extension at 72°C for 45s. For each gene of interest, negative and positive controls were also included. Relative gene expression was calculated as ΔCt (cycle threshold) of the experimental sample by normalizing Ct value of target gene using a housekeeping gene (28S rRNA). The relative abundance of gene was determined using the formula Relative quantity=$2^{-\Delta\Delta Ct}$ [21].

Statistical analysis

The ploidy data and mRNA expression levels (relative expression) of developmental and sex specific genes were analyzed by one way ANOVA using SPSS software package Version 16.0 (2007). Difference in mean values was considered as significant at the level of 95% ($P<0.05$) and 99% ($P<0.01$).

Results

Chromosomal pattern of PBMC of male and female turkey was

Gene[1]	Sequence (5' → 3')	Annealing Temp. (°C)	Product size (bp)	Accession Number
Sox 2	F-gcagagaaaagggaaaaagga	48.0	170	HG313861
	R-tttcctagggaggggtatgaa			
Sox 3	F-tgttcgcttccgagtcttaaa	48.0	231	JQ280473
	R-cctttccgtaggaacaaaacc			
Gata-4	F-tgagaaaagagggcattcaga	47.0	262	JN639851
	R-gcaggatgaattgaagatcca			
PouV	F-gttgtccgggtctggttct	54.0	189	HE608817
	R-gtggaaaggtggcatgtagac			
DMRT	F-gtggcagatgaagggaatggag	57.0	187	AF123456
	R-gctgggcggcgaaaacac			
AMH	F-accgtctacgcagccaacaact	57.0	182	HE646741
	R-gccgacacgctgatgatgag			
ASW	F-gcctgggcttgaccgatggat	60.0	201	JN942586
	R-ggtggctaggctgacgggcaac			
P-450	F-attgcagacgaccccacagtg	59.0	239	J04047
	R-atgcgtccaatgttgagaataatg			
28s	F-caggtgcagatcttggtggtagta	58.0	274	JN942581
	R-gctcccgctggcttctcc			

[1]Sox-2=SRY (sex determine region Y)-box 2, Sox-3=SRY (sex determine region Y)-box 3, GATA-4 =GATA-4 Transcription factor, POU-V=POUV POU domain class 5 transcription factor, DMRT: Doublesex/Mab-3 Related Transcription Factor, AMH: Anti-Mullerian Hormone, ASW: Avian Sex-specific W-linked, P450: Cytochrome P450.

Table 1: Oligonucleotide sequence of pluripotent and sex related genes of turkey.

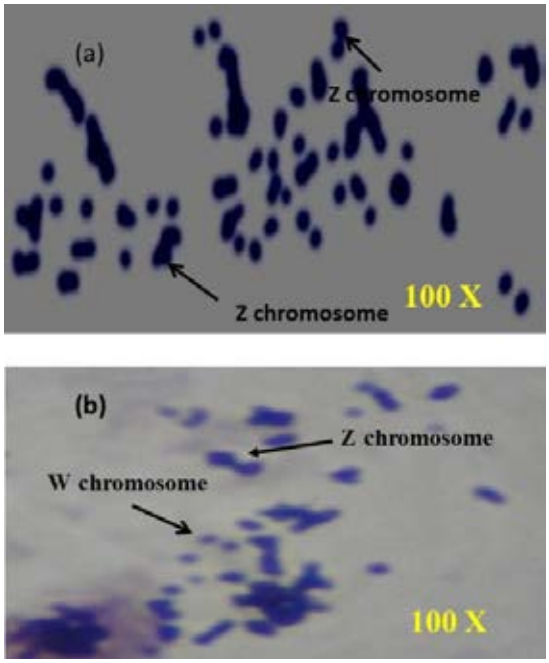

Figure 1: Diploid chromosome pattern of PBMC cells of adult male (a) and adult female (b) turkey birds seen at 100x magnification.

taken as reference (Figure 1a and 1b) for ploidy determination. The photographs showing ploidy of parthenogenetic egg at different hours of incubation have been presented in Figures 2 and 3. There was no significant difference in relative length, size and morphology of chromosomes in parthenogenetic and PBMC cultured cells. At initial stage of development (0 hr), percentage of haploid cells (38.73%) was higher than the diploid cells (21.10%). The metaphase spread obtained from parthenogenetic embryo whose sex was not identified clearly and having more than two sets of chromosome (polyploidy) and those not in the proportion of 'n' number of (aneuploidy) were classified as other ploidy (Figure 4). With advancement of age, percentage of haploid cells decreased from 38.73 to 20.44% while that of diploid cells increased from 21.10 to 42.06%. The proportion of other ploidy also decreased with embryonic age (Table 2). The proportion of W chromosome containing cells was higher at lay but decreased with the advancement of age, while reverse trend was observed in Z chromosome. No ZW combination was observed in parthenogenetic embryos at any point of time during the whole experiment.

Differential expression of pluripotent genes

In freshly laid parthenogenetic eggs, *Sox2* expression was significantly (P<0.05) higher but decreased in 12 h or older embryos in an age dependent manner. An upward trend but not significantly different *Sox3* expression was seen in parthenogenetic embryos till 24 h of age compared to normally fertilized embryos (Figure 5). Differential expression of *GATA-4* revealed that parthenogenetic embryos except that of 48 h old had higher expression than the normal fertilized embryos. In parthenogenetically derived eggs expression of *PouV* was higher than fertilized embryo but in 24 h old embryos the expression increased to almost 40 fold *(P<0.05)* (Figure 5).

Expression of sex specific genes

Relative expression level of male sex specific gene (*DMRT*) was compared between normal fertilized and parthenogenetic turkey embryos at different stages of development (Figure 6). The expression was very low in freshly laid parthenogenetic eggs (before incubation), but significantly increased (P<0.01) in 24 h old embryo. The expression of anti-mullerian hormone (*AMH*) in parthenogenetic eggs was also lower in freshly laid parthenogenetic eggs as compared to normal fertilized eggs. The expression increased in 12 and 24 h old pathenogenetic embryos. The expression of *ASW* (avian sex specific w linked) a candidate ovary determining gene, was higher in freshly laid parthenogenetic eggs. However, the expression level decreased in 12 and 48 h old parthenogenetic embryos. The expression of *P450* gene (female specific) was found to be lower in freshly laid parthenogenetic

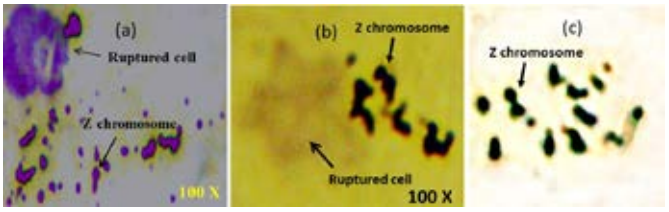

Figure 2: Haploid chromosome pattern at the time of lay (a), 12 h (b) and 24 h (c) of incubation in parthenogenetically developed turkey eggs/embryo.

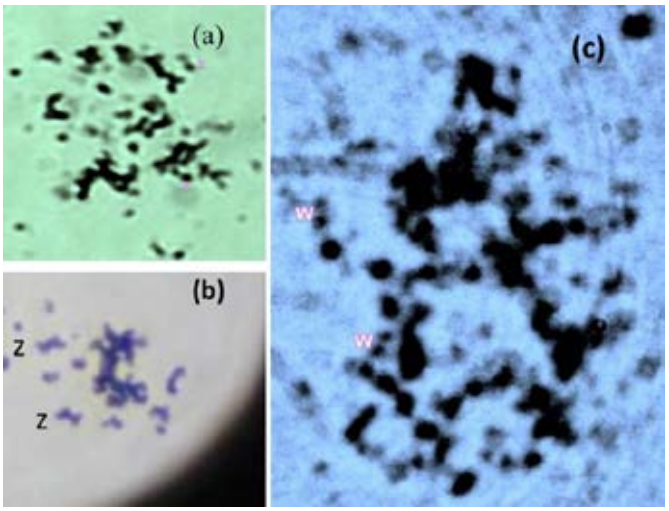

Figure 3: Metaphase spread of parthenogenetic turkey blastomere cells showing diploid-ZZ at 12 h (a), 24 h (b) and diploid-WW at 48 h (c) of embryo development.

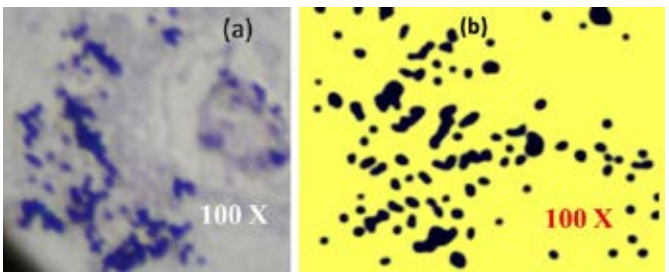

Figure 4: Metaphase spread of blastomere cells of parthenogenetic turkey embryo showing aneuploidy at 12 h (a) of age and polyploidy at 24 h (b) of development.

	Haploid			Diploid			Other ploidy
Hours of development	**Z**	**W**	**Total**	**ZZ**	**WW**	**Total**	
0 hr	20.83	17.90	38.73[c]	10.00	11.10	21.10[a]	40.16
12 hrs	18.33	18.33	36.66[c]	13.88	14.16	28.05[a]	35.27
24 hrs	19.38	9.187	28.57[b]	29.11	12.33	41.44[b]	29.98
48 hrs	12.27	8.16	20.44[a]	30.51	11.55	42.06[b]	37.48
Pooled SEM	3.53	3.44	1.92	4.34	3.34	2.77	1.64
Probability	NS	NS	P<0.01	NS	NS	P<0.01	NS

a,b superscripts in a column refers to significant at P<0.05, NS-represents non-significant at P>0.05.

Table 2: Ploidy percentage of parthenogenetic blastomeres/embryos at different stages of development.

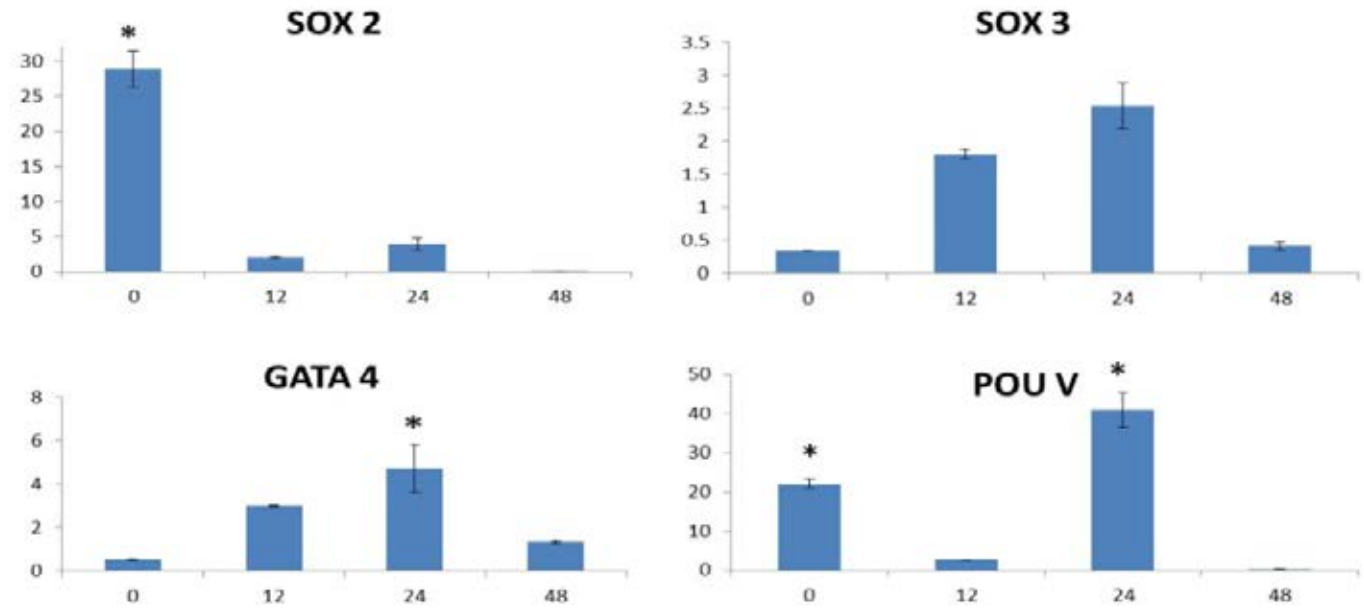

Figure 5: Differential expression of development related genes (*Sox2*, *Sox3*, *GATA-4* and *PouV*) at 0-48 h of embryonic development. * and ** denotes significance at 5% and 1% , respectively.

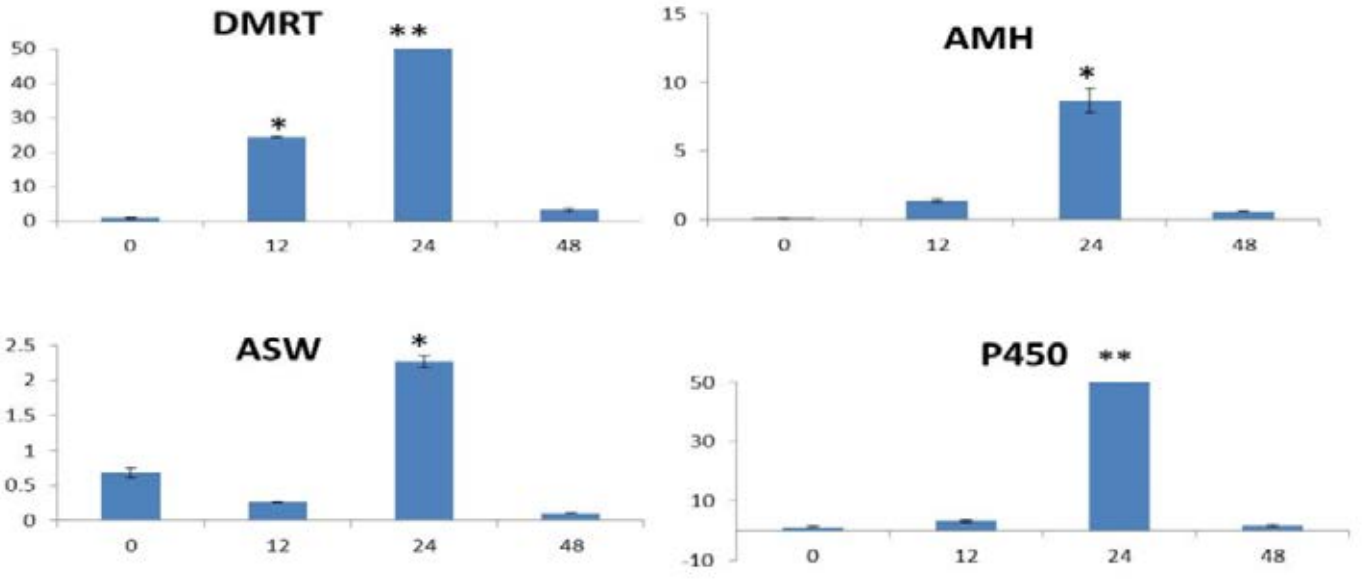

Figure 6: Differential expression of male (*DMRT* and *AMH*) and female specific (*ASW* and *P450*) gene during 0-48 h of embryo development. * and ** denotes significance at 5% and 1% , respectively.

eggs but the expression increased significantly *(P<0.01)* in 24 h old partenogenetic embryos (Figure 6).

Discussion

Higher proportion of haploid and other ploidy (polyploid, aneuploid and unidentified) at the time of laying in the present study indicates more number of chromosomal abnormalities at initial stage of development. Lower rate of survival of usually 20% turkey parthenotes [2] could be the result of these chromosomal aberrations. We found, significant decrease in the number of haploid cells in 48 h old parthenogenetic embryos than the freshly laid parthenogenetic egg (38.73 to 20.44%) and simultaneously increase in the proportion of diploid cell (21.10 to 42.06%). Increase in the ratio of diploid cells might be due to restoration of diploidy from haploid cells. This finding was supported by the views of Darcey and Buss [8], Harada and Buss [22] and Olsen [3] who suggested that parthenogenetic development in the turkey begins in a haploid oocyte, and at subsequent developmental stages, the diploid chromosome number is established. Hatched parthenogenetic poults reported so far have invariably been males, and more than 87% of their cells have been found to be diploid [23]. We did not find any ZW combination in our metaphase spreads during the study from freshly laid eggs to 48 h of incubation. We also observed decreasing trend in the ratio of haploid W and diploid WW containing cells, while reverse trend was observed for Z and ZZ combination containing cells. We did not observed any haploid cells in normal fertilized embryos while ratio of haploid cells was significantly *(P<0.01)* higher in parthenogenetic embryos. The presence of haploid cells and diploid cells in all parthenogenetic embryos at later stage of development provide strong evidence that parthenogenetic development initiates in the haploid ovum which subsequently became diploid.

Expression of *Sox2* gene used to be higher before the differentiation of embryonic stem cells in to specialized cells. During early development, when the embryo has three layers of cells (ectoderm, mesoderm, and endoderm), a region of the ectoderm called the neural plate becomes specified to generate the entire nervous system. One of the earliest molecular markers for the neural plate is the transcription factor *Sox2*, which is critical for cells to acquire neural progenitor character [11]. In the present study, at early stages of development (up to 36 h of incubation) the expression of *Sox2* was higher in parthenogenetic eggs, which revealed that organogenesis might have not started in those embryos, so less differentiating cells were present in the blastomere. This also correlates with the result on ploidy restoration, which continued till 48hrs of parthenogenetic embryo development.

Increased *Sox3* expression was seen in parthenogenetic embryos till they attained 24 h of age. *Sox3*, an X-linked member of the family, is considered to be one of the earliest neural markers in vertebrates, playing a role in specifying neuronal fate [24]. Studies performed on *Sox3* null mice [12] showed that this gene is important for normal oocyte development and male testis differentiation and gametogenesis. Early expression of both male and female specific genes in parthenogenetically derived turkey embryos also correlated with the higher expression of *Sox3* gene.

Chicken *PouV* (cPOU-V) and *Nanog* (cNanog), homologus of mammalion *oct-4* and *Nanog* genes are expressed in early embryos and thereafter, in germ cells before gastrulation [13]. In the present study, we found that the expression level of *PouV* was higher in parthenogenetic embryos, suggesting that parthenogenetic embryos had less differentiating cells as compared to normal fertilized embryos. This result also correlates with macroscopic analysis [6] where, development in parthenogenetic embryo was slower as compared to normal fertilized embryos. Over expression of *PouV* decreased the expression of *Nanog* but increased the expression of *GATA-4* gene [13]. However in the present study we did not find such positive correlation between *PouV* and *GATA-4* gene, rather the expression of *GATA-4* gene was higher irrespective of *PouV* expression.

The *DMRT*1 mRNA was expressed in both sexes beyond 5th day of incubation in chickens, but the amount of *DMRT1* mRNA in male gonads was about two times as much as in female gonads [25]. In the present study, expression of both *DMRT* and *AMH* were higher in 24 h old parthenogenetic embryos, which is quite earlier than the expected gonadal differentiation. Ploidy study also revealed that Z specific haploid and diploid cells were more in parthenogenetic eggs, which might have associated with higher expression of male specific genes. It is also reported that in female-to-male sex reversed chicken, *DMRT1* was expressed higher in parthenogenetic gonads than in normal female gonads [17]. It is assumed that in normal fertilized egg there is probability of 50:50 either ZZ or ZW chromosome combination, whereas, in case of parthenogenetic eggs at early stages of development before ploidy restoration, probability of Z or ZZ and W or WW is equal. Due to presence of higher proportion of W chromosome at early stages of development in parthenogenetic eggs might have resulted in higher expression of the female specific genes.

The expression of *P450* gene was lower in freshly laid parthenogenetic eggs but increased in 24 h old partenogenetic embryos. Two terminal enzymes necessary for estrogen synthesis of P-450 aromatase and 17bHSD, are expressed only in ZW female gonads [18]. In our chromosomal analysis also we did not encounter any ZW chromosome in parthenogenetic embryos, so the expression was low in freshly laid eggs. However, increase in *P450* gene expression in 24 h partheneogenetic embryo could be attributed to WW diploidy.

In conclusion, we found that transition of ploidy from haploid to diploid or poly-ploidy continued till 48 h or beyond parthenogenetic development. Most of the surviving parthenogenetic embryos beyond 48 h of incubations were happens to be in ZZ combination (male) thus providing an opportunity to turkey grower for customized production. Significantly higher expression of *Sox3* or *GATA-4* gene in parthenogenetic embryos could potentially be used as marker gene for parthenogenesis in turkey.

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