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Effect Of Oocyte Aging On Dna Methylation Pattern In Buffaloes.

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Abstract:

DNA methylation is an important epigenetic mechanism that plays a vital role during gamete and early embryo development. The present study aimed to investigate expression levels of the 5-Methylcytosin in the fresh and aged oocytes of buffalo. Buffalo cumulus-oocyte complexes (COCs) were subjected to culture in *in-vitro* maturation media for 24h (fresh) and 30h (aged). The methylation pattern of buffalo oocytes were studied by labeling with anti-5-Methylcytosin and the ratio of fluorescence intensity was assessed. There was a significant difference ($P < 0.05$) was observed at the fluorescence intensity of fresh and aged oocytes (20.08 ± 5.04 vs 45.77 ± 4.34) and 5-Methylcytosin was differently localized in the buffalo oocytes. Further studies are required to understand the mechanism of differently expressing DNMTs in the early developmental periods.

Keyword*: - *Aged oocytes, methylation pattern, 5-Methylcytosin, in vitro maturation, buffalo.

INTRODUCTION:

DNA methylation is one of the most important epigenetic marks involved in the allele-specific silencing of imprinted genes. During gametogenesis, the genome-wide profiles of DNA methylation patterns of parental genomes undergo drastic changes, with a new methylation pattern established through de novo methylation. Failures in the process of epigenetic reprogramming could lead to the loss of imprinting for many but not all imprinted genes, as noted by Reik and Walter (2001)[1]. Lopes et al. (2009)[2] investigated the relationships between reproductive and epigenetic outcomes associated with increasing maternal age in C57BL/6 mice. They found that although morphological abnormalities and delayed development were related to the age of mice (43–47 weeks old), the monoallelic expression of imprinted genes H19 and Snrpn was normal in the blastocysts of aged female mice where the DNA methylation patterns of the differentially methylated regions (DMRs) of Snrpn, Kcnq1ot1, U2af1-rs1 (Zrsr1), Peg1, Igf2r, H19 were not altered. They also observed that there were no significant differences in genome-wide DNA methylation patterns in embryos and placentas from aged female mice. Lienget al. (2011)[3] observed that postovulatory oocyte aging caused a decline in reproductive outcomes but did not lead to defects in DNA methylation imprinting acquisition in the oocytes

from viable offspring. In contrast, Yue et al. (2012)[4] found that the changes in genome-wide DNA methylation in Oocytes and preimplantation embryos of 35- to 40-week-old mice were associated with decreased expression of DNMTs. The pregnancy rate of older Kunming mice (35–40 weeks old) was lower than that of younger mice, and the stillbirth and fetal malformation rates were higher in the older group compared with the younger group, which might be associated with abnormal DNA methylation in oocytes. To provide basic information regarding epigenetic changes associated with oocyte aging and gain a better understanding of the relationship between oocyte DNA methylation and reproductive competence during female aging, further research has been conducted on fresh and aged buffalo oocytes to examine the methylation pattern.

MATERIALS AND METHODS:

The culture media, chemicals, growth factors, and hormones used in this study were obtained from Sigma Chemicals Company (St. Louis, Missouri, USA), while the plastic wares used were purchased from Nunc in Denmark. Fetal sheep serum was obtained from GIBCO, Invitrogen in the USA.

Buffalo ovaries were acquired from a slaughterhouse and transported to the Department of Animal Biotechnology's Assisted Reproductive Technology Laboratory, Centralized Embryo Biotechnology Unit at TANUVAS in Chennai. The ovaries were transported in 0.9% normal saline containing penicillin (100 IU/ml) and streptomycin (50 mg/ml) at 30-35ºC in a thermos flask within 2 hours of slaughter. In each experiment, only oocytes obtained from buffalo ovaries collected on a single day were used for in vitro maturation. The purpose of the study was to examine the methylation patterns of fresh and aged buffalo oocytes. Buffalo cumulus-oocyte complex (COCs) were cultured in in vitro maturation media for 24 hours (fresh) and 30 hours (aged). The methylation pattern of buffalo oocytes was analyzed by labeling them with anti-5-Methylcytosine, and the ratio of fluorescence intensity was evaluated. The ovaries were trimmed of any adherent tissues and ligaments, and washed thoroughly with running tap water, followed by five washes in normal saline supplemented with penicillin and streptomycin, and one wash in 70% ethanol.

Oocyte retrieval was performed using the slicing method, which involved holding the ovaries with forceps and making cross-hatched incisions on the entire ovarian surface using a sterile scalpel blade (No. 11). The ovarian tissues were flushed with oocyte collection medium in a 60 mm Petri dish to remove the cumulus-oocyte complexes (COCs). Subsequently, oocytes were screened under a stereo zoom microscope (Nikon, Japan) and graded based on the investment of cumulus cells and homogeneity of ooplasm, following the method described by Nandi et al. (1998).[5]

Table -1 Number Of Matured Oocytes Considered For Methylation Study

	Fresh oocytes	Aged oocytes
1	13	13
2	20	20
3	13	12
TOTAL	**43**	**45**

Grade A: Oocytes with many unexpanded complete layers (greater than 3 layers) of cumulus cells and with homogenous evenly granular ooplasm.

Grade B: Oocytes with thin or incomplete layers (2-3 layers) of cumulus cells and with homogenous evenly granular ooplasm.

Grade C: Oocytes with either partially denuded or with 1-2 layers of cumulus cells and with irregular and dark ooplasm.

Grade D: Oocytes with no cumulus cells and irregular and dark ooplasm.

Grade E: COCs with highly expanded or scattered cumulus cells and with irregular and dark ooplasm.

Only COCs of grades A, B, and C were used for in vitro maturation. The A, B, and C grade oocytes were collected and subjected to four washings in TCM 199 + 10 %FBS and a final wash in IVM medium, consisting

of TCM-199 supplemented with 10 % FBS, 1 µg/ml of Folltropin (FSH), 0.02 IU/ml of Luteinizing Hormone (LH), 1 µg/ml of estradiol and10 ng/ml of epidermal growth factor (EGF). The COCs were transferred to 50 µl droplets of maturation medium in a 35 mm Petri dish and covered with sterile mineral oil to avoid medium evaporation. COCs were cultured for 24 h at 38.5˚C in a humidified atmosphere of 5 % CO2 in the air. The maturation rate was assessed based on the degree of cumulus expansion, evaluated and classified as described by Ravindranatha et al. (2002).[6]

Table –Ii Matured Buffalo Oocytes Cultured In Tcm-199 Medium (Mean ± Se)

Experiment	No. of replicates	No. of oocytes		Maturation rate (Mean ± SE)
		Cultured	Cumulus expanded	
I	3	37	26	**84.46± 0.98**
II	3	44	40	**85.21± 0.89**
III	3	38	25	**86.98± 0.15**
Total	19	119	91	

The data provided shows the results of an experiment where matured buffalo oocytes were cultured, and the maturation rates were measured. The experiment was performed three times, with three replicates each time (total of 9 replicates). The mean number of oocytes cultured was 119, and the mean number of cumulus-expanded oocytes was 91.

The maturation rate was calculated as the percentage of cumulus-expanded oocytes out of the total number of oocytes cultured. The mean maturation rates for each experiment were 84.46% ± 0.98%, 85.21% ± 0.89%, and 86.98% ± 0.15%, respectively.

The overall mean maturation rate across all experiments was 85.88% ± 0.56%. This suggests that the culture conditions used are effective in supporting the maturation of buffalo oocytes. However, there was some variation in the maturation rates between the experiments, with experiment III showing the highest maturation rate.

Overall, the results indicate that the culture conditions used are suitable for the maturation of buffalo oocytes, with an average maturation rate of 85.88% and some variation between experiments. Further studies may be needed to investigate the factors contributing to the variation in maturation rates between experiments.

Table –Iii Morphological Assessment Of Matured Oocytes

	deg2	deg1	deg 0
1	14	12	11
2	25	15	4
3	13	12	13
TOTAL	**52**	**39**	**28**

Degree 2: The cumulus cells were uniformly spread, and there were no more clustered cells present (complete cumulus cell expansion).

Degree 1: The cumulus cells were slightly expanded, and clustered cells were still visible (moderate cumulus cell expansion).

Degree 0: There was no visible morphological change compared to fresh COCs (minimal or no expansion).

Oocytes that reached the 2 and 1-degree of cumulus expansion were considered mature. The matured Oocytes were collected from the culture drops, washed in a solution containing 10mM phosphate-buffered saline (PBS) and 1% polyvinylpyrrolidone (PVP), and then fixed with a 4% paraformaldehyde solution in PBS-PVP for 15 minutes. The fixed embryos were washed three times with PBS-PVP, permeabilized using 0.25% Triton X-100 in PBS for 30 minutes, and washed three times with a wash buffer containing 0.1% Tween-20 and 10 mg/ml fraction V BSA. The embryos were then exposed to RNase A, which was diluted in PBS-PVP, and incubated at 37°C for 1 hour. After three washes with the wash buffer, the embryos were incubated with 3M

HCl/0.1% PVP at 37°C for 30 minutes, and then neutralized by incubation with 100M Tris-HCl (pH 8.5) containing 1% PVP for 10 minutes. Finally, the embryos were washed three times with the wash buffer.

To prevent non-specific binding, embryos were treated with a solution of PBS containing 5 mg/ml BSA for 1 hour. Then, the embryos were transferred to a solution of 1 µg/ml anti-5-methylcytosine (an affinity-purified mouse monoclonal antibody) that was diluted in PBS containing 0.05% Tween 20 and 0.01% BSA. A negative control was used by treating the embryos with 2% BSA instead of the primary antibody. After 1 hour, the embryos were washed three times in a wash buffer and transferred to a solution of 1 µg/ml fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG. After three washes, the nuclei were labeled with propidium iodide (PI) and the embryos were observed using a Zeiss confocal scanning microscope. Images were captured using a 40× objective and FITC, blue, and rhodamine filters, with constant exposure times for all embryos in each replicate.

The intensity of immunofluorescence was evaluated by using ImageJ software (version 1.60_41, NIH, Washington DC, USA). In images that showed two colors (green for 5-methylcytosine and red for nuclei), individual nuclei were identified by labeling with PI and then outlined using the free-hand tool in ImageJ. The mean gray intensity of the green and red images were measured independently. The ratio of intensity for green and red (which represents 5-methylcytosine/DNA) was computed for each nucleus. The data for each of the studied nuclei in that oocyte were summed to get the average level of methylation for that oocyte. Red fluorescence represented the entire DNA, while green fluorescence denoted methylated DNA.

MEAN VALUES OF FLUORESENCE OF FRESH OOCYTE -1

S.I.No	NUCLEUS	CYTOPLASM
1	24.302	0.741
2	24.517	3.088
3	19.833	3.384
4	19.495	3.262
5	24.126	3.333
6	27.084	4.158
Average	23.2261667	2.99433333

The Average Difference of Nuclear and cytoplasmic Fluoresence intensity is 20.23

MEAN VALUES OF FLUORESENCE OF AGED OOCYTE -1

S.I.No	NUCLEUS	CYTOPLASM
1	55.108	2.354
2	53.122	2.67
3	43.171	2.58
4	71.595	2.755
5	39.53	2.707
6	42.876	2.071
Average	50.9003333	2.52283333

The Average Difference of Nuclear and cytoplasmic Fluoresence intensity is 48.37

MEAN VALUES OF FLUORESENCE OF FRESH OOCYTE -2

S.I.No	NUCLEUS	CYTOPLASM
1	38.421	2.253
2	29.464	2.275
3	34.87	2.339
4	42.201	2.616
5	22.668	3.154
6	23.485	2.357
Average	31.8515	2.499

The Average Difference of Nuclear and cytoplasmic Fluoresence intensity is 29.35

MEAN VALUES OF FLUORESENCE OF AGED OOCYTE -2

S.I.No	NUCLEUS	CYTOPLASM
1	40.533	2.253
2	41.554	2.275
3	38.387	2.339
4	46.201	2.616
5	32.668	3.154
6	39.485	2.357
Average	39.8046667	2.499

The Average Difference of Nuclear and cytoplasmic Fluoresence intensity is 37.30

MEAN VALUES OF FLUORESENCE OF FRESH OOCYTE -3

S.I.No	NUCLEUS	CYTOPLASM
1	15.173	2.172
2	16.337	2.336
3	15.242	4.296
4	16.646	3.872
5	14.464	3.737
6	11.384	3.621
Average	14.8743333	3.339

The Average Difference of Nuclear and cytoplasmic Fluoresence intensity is 10.66

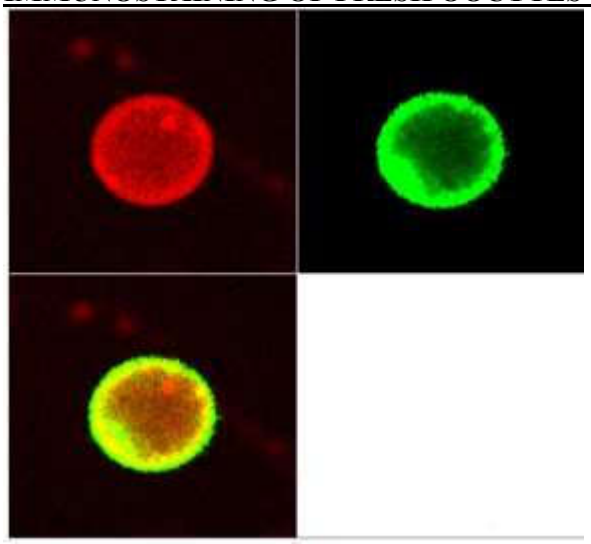
MEAN VALUES OF FLUORESENCE OF AGED OOCYTE -3

S.I.No	NUCLEUS	CYTOPLASM
1	72.56	3.26
2	56.96	2.93
3	49.23	3.45
4	47.56	4.23
5	62.89	3.33
6	41.47	3.56
Average	55.1116667	3.46

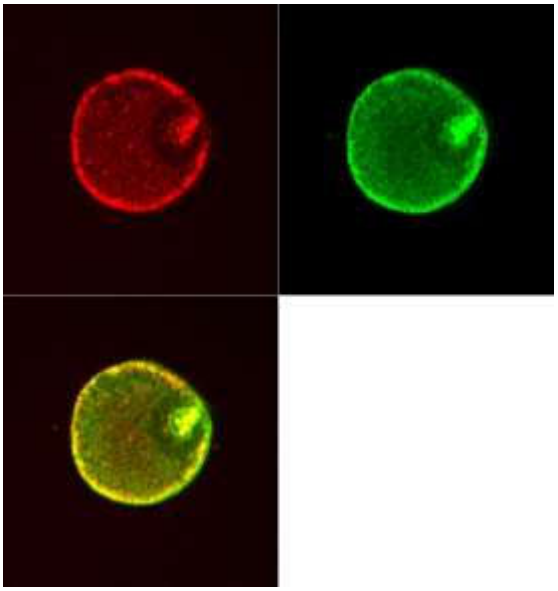
The Average Difference of Nuclear and cytoplasmic Fluoresence intensity is 51.65

(Fig-1) Differential Interference Contrast Image Of A Bovine Oocytes Stained With Anti 5methylcytosine (Green) And Propidium Iodide (Red)

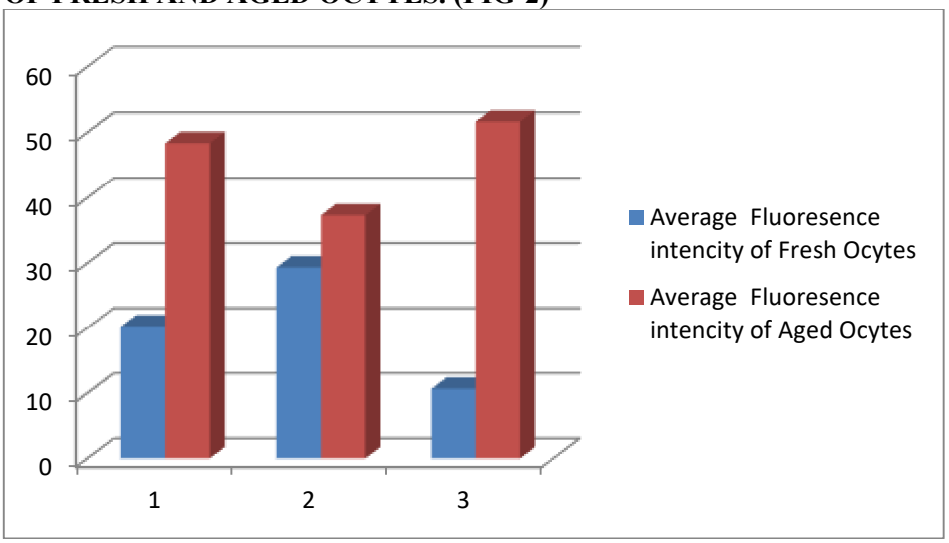
IMMUNOSTAINING OF FRESH OOCYTES UNDER CONFOCAL MICROSOPY



IMMUNOSTAINING OF AGED OOCYTES UNDER CONFOCAL MICROSOPY



GRAPHICAL REPRASENTATION OF AVERAGE DIFFENCE OF FLUORESENCE INTENCITY OF FRESH AND AGED OCYTES. (FIG-2)



RESULTS AND DISCUSSION:

The statistical analysis of the data followed the Snedecor and Cochran (1994)[7] method. The mean percentage value with standard error was calculated for oocytes of different ages (mean % ±S.E). The maturation rate data were analyzed using the student T-test and significant differences were indicated at 1 percent ($P < 0.01$). Table 5 presents the average differences in fluorescence intensity between fresh and aged oocytes. The fluorescence intensity of aged oocytes (45.77± 4.34) was significantly higher ($P < 0.05$) compared to fresh oocytes (20.08 ± 5.04).

The number, quality, and recovery rate of COCs harvested from buffalo ovaries by the slicing method are presented in (Table-IV). The buffalo COCs graded based on their cellular investment and homogeneity of ooplasm are presented in plate 1. The percentage of A, B, C, and D-grade oocytes retrieved in all three experimental trials in the present study was 49.58, 36.36, 12.39, and 1.65 respectively. The COCs yield per ovary (Mean± SE) for A, B, C, and D grades by slicing method were1.01±0.02, 1.10± 0.02, 1.50± 0.03, and 0.49± 0.02, respectively with an average total yield of 4.38± 0.08 COCs per ovary. The in vitro maturation rate (%) of buffalo COCs cultured in TCM 199 medium is presented in (Table-II). In all three experiments (n=3 each) carried out with TCM 199 medium, only A, B, and C-grade oocytes were used for in vitro maturation. The maturation rate was assessed based on the cumulus expansion (Plate 2). Out of 37,44 and 38 oocytes cultured in experiments I, II, and III, 26, 40 and 25 oocytes showed cumulus expansion with mean maturation rates of 84.46± 0.98, 85.21± 0.89, and 86.98± 0.15 percent, respectively.

Table –Iv Number, Quality And Recovery Rate (Per Cent) Of Oocytes Harvested From Buffalo Ovaries By Slicing Method**.**

No. of ovaries used	Grades	A	B	C	D	Total
21	No. of o ocytes	**60**	**44**	**15**	**2**	**121**
	Percentage	49.58	36.36	12.39	1.65	100
	Number per ovary (Mean± SE)	1.01±0.02	1.10± 0.02	1.50± 0.03	0.49± 0.02	4.38± 0.08

(* In three experiments (n=3 each)**)**

The study found that 5-methylcytosine had differences in expression between fresh and aged oocytes, with a significant difference in fluorescence intensity observed between the two (20.08 ± 5.04 vs 45.77 ± 4.34). Additionally, the DNMT1 protein was mainly found in the cytoplasm and had a gradual decrease and increase from fresh to aged oocytes. DNMT3A, on the other hand, was mostly located in the nucleus and had fluctuations in expression from fresh to aged oocytes. DNMT3B was more highly expressed in the nucleus and had similar expression patterns to DNMT3A. DNA methylation is an epigenetic mechanism that regulates the expression of genes important for oogenesis and early embryonic development. During oogenesis, the

levels of global DNA methylation progressively increase in growing GV oocytes from birth to puberty, and they reach their highest levels in MII oocytes in mice(Saitou et al. 2012)[8]. After fertilization, the levels of global DNA methylation gradually decrease from 1-cell to 16-cell morula stage embryos, and the genes that are imprinted by both parents also become demethylated during the late stages of embryogenesis(Yang et al. 2007)[9]. In blastocysts, the levels of global DNA methylation are highest because de novo methylation processes are activated, and maintenance methylation establishments are maintained, resulting in the establishment of new genomic imprints. In the current study, we have shown that the levels of DNMT1 and DNMT3B proteins increased significantly from fresh to aged oocytes, while the levels of DNMT3A remained unchanged in both types of oocytes (as depicted in Fig-I). This prominent increase in DNMT1 or DNMT3B expression is consistent with the rise in global DNA methylation levels from fresh to MII oocytes. These proteins are most likely involved in the establishment of maintenance and de novo methylation processes in both GV and MII oocytes. Ratnam et al. (2002)[10] discovered that Dnmt1mRNA is present in postnatal oocytes collected at various time points (days 1, 5, 10, 15, 21, and 35) in mice (Ratnam et al. 2002). In bovines, DNMT1 has been identified in all GV oocyte stages (from GV0 to GV3) and in MII oocytes (Lodde et al. 2009). Like in mice and bovines, DNMT1 is expressed in human GV and MII oocytes (Huntriss et al. 2004)[11]. Although DNMT1 is predominantly found in the nucleus of GV oocytes, it is highly localized in the cytoplasm of MII oocytes in humans (Petrussa et al. 2014)[12].

In our study, we found that both DNMT3A and DNMT3B proteins are mainly located in the nucleus and have weak cytoplasmic localization in oocytes. There are noticeable differences in the intracellular intensity of these proteins in buffalo oocytes among different studies, which may be attributed to the use of different techniques and/or oocyte strains. In bovine oocytes, our study suggests that 5-methylcytosine plays a role in establishing genomic imprinting and maintenance methylation during early development.

CONCLUSION:

In conclusion, this study provides important insights into the role of DNA methylation in oocyte aging and early embryo development. Our findings demonstrate significant differences in the expression levels and sub-cellular localizations of 5-Methylcytosin proteins between fresh and aged buffalo oocytes, highlighting the importance of proper epigenetic reprogramming during gametogenesis. These results suggest that the fluctuations in the expression of DNMT proteins in fresh and aged oocytes could contribute to the establishment of DNA methylation patterns and genomic imprinting. Further studies are needed to fully understand the molecular mechanisms underlying the observed differences in DNMT expression and DNA methylation patterns during early development. Nonetheless, this study provides valuable information for improving reproductive outcomes in both animal and human assisted reproductive technologies.

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COMPETING INTERESTS

‘The authors declares that there is no conflict of interest’

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