Journal of Animal and Veterinary Advances 11 (4): 531-538, 2012
ISSN: 1680-5593
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A Novel Oocyte Chromatin Configuration Classification Method: Based on the Degree of Aggregation and Spatial Distribution of Germinal Vesicle Chromatin

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Abstract: There is a lack of quantitive and objective method study the chromatin configuration of mammalian oocytes. This study attempts to solve this problem. The Germinal Vesicle (GV) chromatin was stained with Hoechst33342 and photos were taken under the fluorescence microscope. The photos are then processed and the Integral Optical Density (IOD) of fluorescent areas and areas of germinal vesicle are measured. The researchers regard the IODs of fluorescent areas as Aggregation Degree (AD) and the ratio of fluorescent areas to the areas of GV as Spatial Distribution (SD). According to the traditional classification method of chromatin configuration in mouse, pig and goat, the researchers divided the photos of chromatin into several groups and calculated the AD, SD and IOD of chromatin configuration in these three species. The conclusions are as follows: quantitative fluorescence analysis technology established in this study can be applied in studying the chromatin configuration of mammalian oocytes. All the oocytes with poor capability to resume meiosis and support embryo development have a common feature that the value of AD is the smallest and the value of SD is the biggest in their own species. SD is a key parameter of chromatin configuration in relation to transcription. Transcription continued when SD>0.4 and ceased when SD<0.3. The value of AD increased and the value of SD decreased when the capabilities of the oocytes to resume meiosis and to support embryo development were enhanced. The value of IOD does not vary in healthy oocytes during the follicular growth stage.

Key words: Configuration, pig, goat, species, transcription, traditional, classification

INTRODUCTION

Chromatin configuration is the appearance of global heterochromatin and partly condensed chromosomes. It is a reflection of the movement and localization of telomeres and centromeres, the course of chromatins being transformed into chromosomes and changes the metabolic state according to a recent research (Gasser, 2002). The chromatin configuration has tight relationship with epigenetic modifications and global repression of gene expression in the oocytes. The most famous chromatin configurations in mammalian oocytes are the SN (where the nucleolus is surrounded by chromatin) and NSN (where the nucleolus is not surrounded by chromatin) (Debey *et al*., 1993). The mouse oocytes with SN configuration have bigger size, better capability and potency in restoring meiosis, maturation and embryo development (Wickramasinghe *et al*., 1991; Zuccotti *et al*., 1998). The configurations which look like NSN and SN have also been found in other species such as human (Miyara *et al*., 2003), pig (Sun *et al*., 2004), bovine (Liu *et al*., 2006), sheep (Russo *et al*., 2007), canine (Lee *et al*., 2008), rabbit (Wang *et al*., 2009) and ferret (Sun *et al*., 2009). It seems that this configuration is a popular indicator of oocyte's quality. But there are many difficulties in classifying all the animals' chromatin configurations with only these two configurations. What method shall the researchers use in chromatin configuration research?

In the past few years, scientists have tried to study chromatin configuration by observing the patterns of chromatin stained with dyes (De La Fuente 2006; Tan *et al*., 2009). Hoechst33342, DAPI and CMA_3 are commonly used dyes and they bind to the AT double bond or GC triple bond of DNA (Wang *et al*., 2009; Liu *et al*., 2006). With a fluorescence microscope, the configurations of stained chromatins can be observed and divided into groups according to the patterns. But such classification method mostly depends on the subjective judgment of researchers. Different researcher may have different judgment on the same pattern. For example, Lee and his colleagues divided the canine GV chromatin configurations into five groups called GV-I to GV-V (Lee *et al*., 2008) but Reynaud and his colleagues

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classified the canine GV chromatin into three groups (Reynaud *et al*., 2009). Bovine oocyte chromatin configuration was divided into five categories by Liu *et al*. (2006) but Lodde divided it into four categories (Lodde *et al*., 2008). Hinrichs *et al*. (2005) divided the horse GV chromatin configuration into two groups which were named CC and FN (Hinrichs *et al*., 1993) but Gable classified horse chromatin configuration into nine types (Gable and Woods, 2001). Although, many researchers prefer the CC and FN classification (Love *et al*., 2002; Pedersen *et al*., 2004), the researcher reclassified the horse chromatin configuration into five kinds which were fibrillar, intermediate, loosely condensed, tightly condensed and fluorescent nucleus (Hinrichs *et al*., 2005). These remind us that a much more objective method is greatly needed for the classification. Further more such morphological classification is not favorable for the comparison among different species. For example, the SN configuration is popular in many species but it's not found in the goat oocyte (Sui *et al*., 2005). Each species has its own configuration that is distinct from others and this makes it difficult to compare chromatin configuration among different species. The meaning of the same chromatin configuration pattern is also different from species to species. For example, the SN oocytes have better maturation capabilities in mouse and pig but the configuration before ovulation is not SN in bovine oocyte (Liu *et al*., 2006). Although, the mouse SN oocytes appear transcriptionally repressed (Liu and Aoki, 2002), the rabbit SN oocytes are transcriptionally active (Wang *et al*., 2009). The researchers thus think the method used before is not appropriate. A method suitable for the research of oocyte GV chromatin configuration is needed.

Here, the researchers developed a new method to classify chromatin configurations. It can be used to compare chromatin configuration among different species. Using this method, the researchers established a correlation between chromatin configuration and the quality of oocytes.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

Oocyte collection: Immature oocytes of three kinds of animals have been used in the experiments. The female mice of 6-8 weeks old were each injected with 10 IU PMSG first and killed 48 h later to collect the ovaries. The follicles were punctured with a needle in M2 medium and the COCs were collected. The pigs were slaughtered in a local slaughterhouse and the ovaries were transported to the lab within 3 h. The follicles were pumped with syringe and the COCs were picked into a dish. Wash the COCs in D-PBS 3-4 times before use. The goats were slaughtered in the slaughterhouse too. The ovaries were transported to the lab in the physiological saline within 3 h. The follicles were cut with knife inside a dish filled with D-PBS and COCs were collected.

Fluorescent staining: The cumulus cells were removed before fluorescent staining. M_2 was used as the operating medium for mouse and D-PBS was used for pig and goat. COCs were denuded of cumulus cells by pipetting in operated medium with a proper glass tube. The oocytes were incubated in the operating medium containing 10 μg mL^{-1} Hoechst33342 at 37°C under 5% CO_2 in humidified air for 10 min. The stained oocytes were washed 6-8 times in drops of operating medium (1 min in each drop). Oocytes were transferred 10 at a time to a glass slide and covered with cover slip.

Image acquisition and processing: Hoechst33342 fluorescence was obtained by excitation at 220-360 nm using a mercury lamp (50 W) with neutral filters. Leica DFC420C CCD was used to acquire the images of chromatin. Adjust photographic system to confirm the gray scale of photos to be linear. Then, fix a suitable exposure parameter of the software, avoid super saturation and take photos of the oocytes one by one (Fig. 1a).

Image-pro plus was used to process the images of oocytes. Open an image and convert the image to gray scale 8 and invert the image. Then, snap picture of image with measurement overlays and convert the picture to gray scale 8 again. Calibrate the optical density in intensity calibration window. Chose Std. Optical Density to define a standard optical density response curve. Click the option button to calibrate the incident level by clicking the image button and select pixel in the image for incident value. After processing the image, the researchers come to the count/size menu.

Click options button and confirm that the fill holes option is not checked. Then, select AREA and IOD in the measurement menu. Use the automatic dark objects option to count the region of fluorescence (Fig. 1b). View the statistics and export data to excel. After measuring the whole area of GV (Fig. 1c), the researchers obtained one oocyte's data.

Data analysis: According to the current classification in mouse, pig and goat, the researchers divide the data into groups. Each group corresponds with one chromatin configuration. Data were analyzed by ANOVA in SPSS 19.

Different groups were compared using one-way analysis of variance after being transformed via LSD. The $p<0.05$ was considered significant.

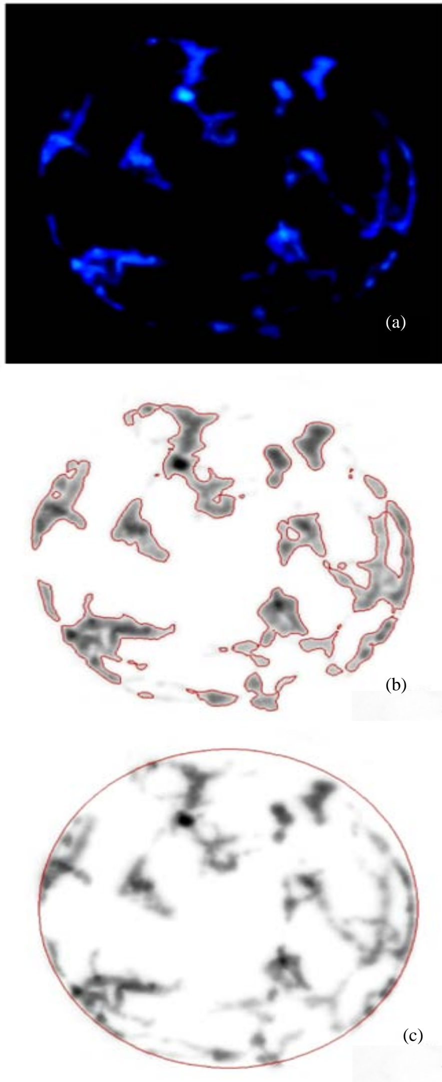

Fig. 1: Image processing. Image a, b and c are the same goat oocyte chromatin. Image a shows the chromatin stained by Hoechst33342. Image b is the process result of image a, Region of Interest (ROI) is the fluorescence chromatin. Image c is the process result of image a too, ROI is the germinal vesicle

RESULTS AND DISCUSSION

Quantitative image analysis: The researchers regard Average Optical Density (AOD) as the Aggregation Degree (AD) of chromatin. AOD could be calculated from IOD/area.

The researchers regard fluorescent area/GV area as the Spatial Distribution (SD) of chromatin. The method of calculate AD and SD describe in Eq. 1:

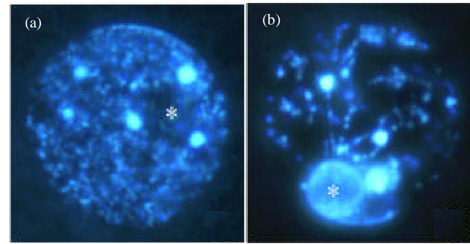

Fig. 2: Chromatin configuration of mouse oocyte. Image a is NSN configuration. Image b is SN configuration. Star shows the nucleolus

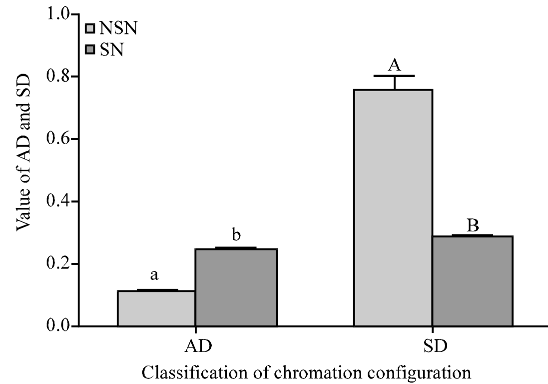

Fig. 3: Value of AD and SD in mouse chromatin configuration. Different minuscule or majuscule on top of the columns indicates that the difference is significant. The columns and bars represent mean±SEM

$$\text{AD} = \frac{\text{Integrated optical density}}{\text{Fluorescent area}} \qquad \text{SD} = \frac{\text{Fluorescent area}}{\text{Germinal versical area}} \tag{1}$$

These two parameters reflect the aggregation and spatial distribution of oocyte chromatin. The researchers established the relationship between these two parameters and the traditional chromatin configurations.

Mouse chromatin configuration: There are two chromatin configurations in the mouse oocytes according to previous research namely, SN (where the nucleolus is surrounded by chromatin) and NSN (where the nucleolus is not surrounded by chromatin). The researchers assorted the images into NSN and SN groups first (Fig. 2) and then processed the SN and NSN images separately. Data analysis shows that the aggregation degree of the NSN configuration is significantly looser than the SN configuration (0.1085 ± 0.0087 vs. 0.2463 ± 0.0074, $p<0.05$) and the spatial distribution of NSN configuration is more widespread than SN configuration (0.7630 ± 0.0445 vs. 0.2871 ± 0.0085, $p<0.05$) (Fig. 3).

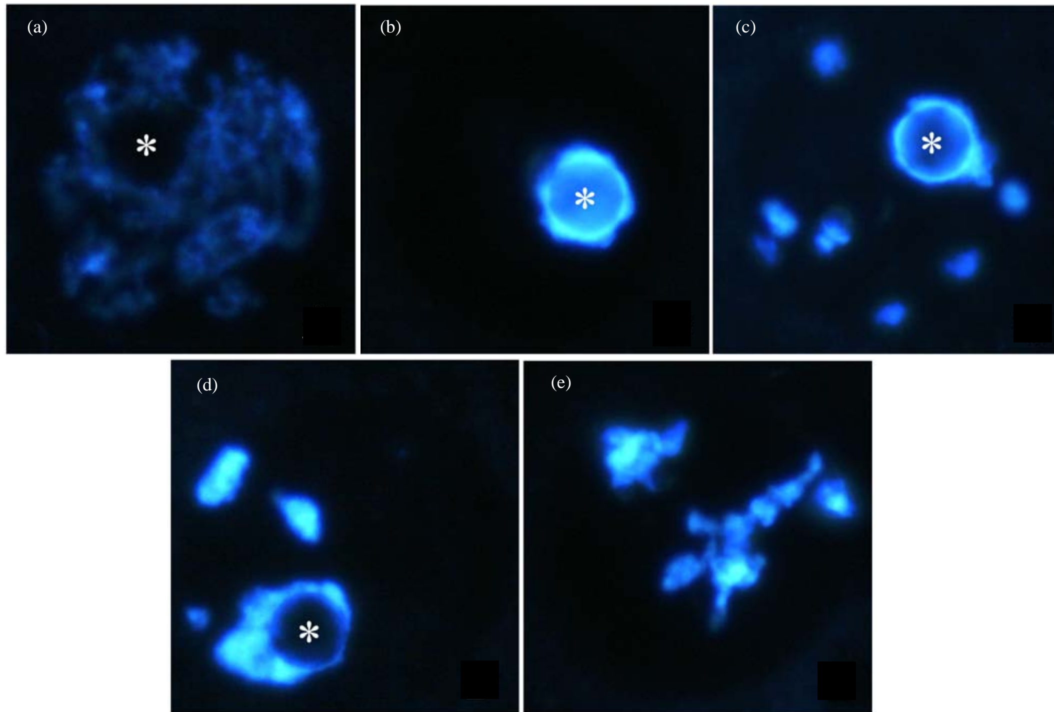

Fig. 4: Chromatin configuration of pig oocytes. Star shows the nucleolus. Image a shows the GV0 chromatin configuration, the diffuse filamentous chromatin is distributed in the nuclear area. Image b shows the GV1 chromatin configuration, nucleolus was surrounded by condensed chromatin. Image c shows the GV2 chromatin configuration, the condensed chromatin not only surrounded the nucleolus but also formed a few clumps near the envelope. Image d shows the GV3 chromatin configuration. Chromatin was further condensed into clumps or strands distributed throughout the nucleoplasm. Image e shows the GV4 chromatin configuration. Condensed chromatin clumps or strands were still there but the nuclear membrane was less distinct and the nucleolus disappeared completely

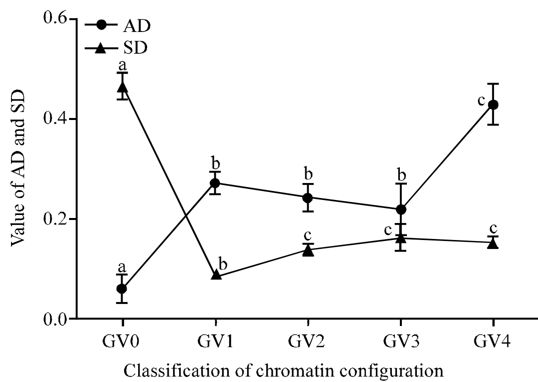

Fig. 5: Value of AD and SD in pig chromatin configuration. Different letter with the same color indicate that the difference is significant ($p<0.05$)

Pig chromatin configuration: There are five chromatin configurations in the pig oocytes a ccording to previous studies which have been termed GV0-GV4. The researchers divided the fluorescent images of pig chromatins into the same five groups (Fig. 4) and calculated the data. As shown in the study, the aggregation degrees of GV1, GV2 and GV3 configurations have no difference ($p>0.05$) and they are more compact than GV0 but obviously looser than GV4 ($p<0.05$). The spatial distribution of GV0 is the most widespread chromatin configuration and the spatial distribution of GV1 is the most restricted (Fig. 5).

Goat chromatin configuration: There are six chromatin configurations in the goat oocytes according to previous studies (Fig. 6). The aggregation degrees of GV1, GV2n and GV3n is looser than other configurations ($p<0.05$). The spatial distribution of GV1 is the most widespread ($p<0.05$). GV2n is also more widespread than other chromatin configurations except GV1 ($p<0.05$). The remaining chromatin configurations have similar spatial distribution patterns except that the GV3c is a little restricted (Fig. 7).

Rule of IOD in different chromatin configuration: There is no difference of IOD between the two chromatin

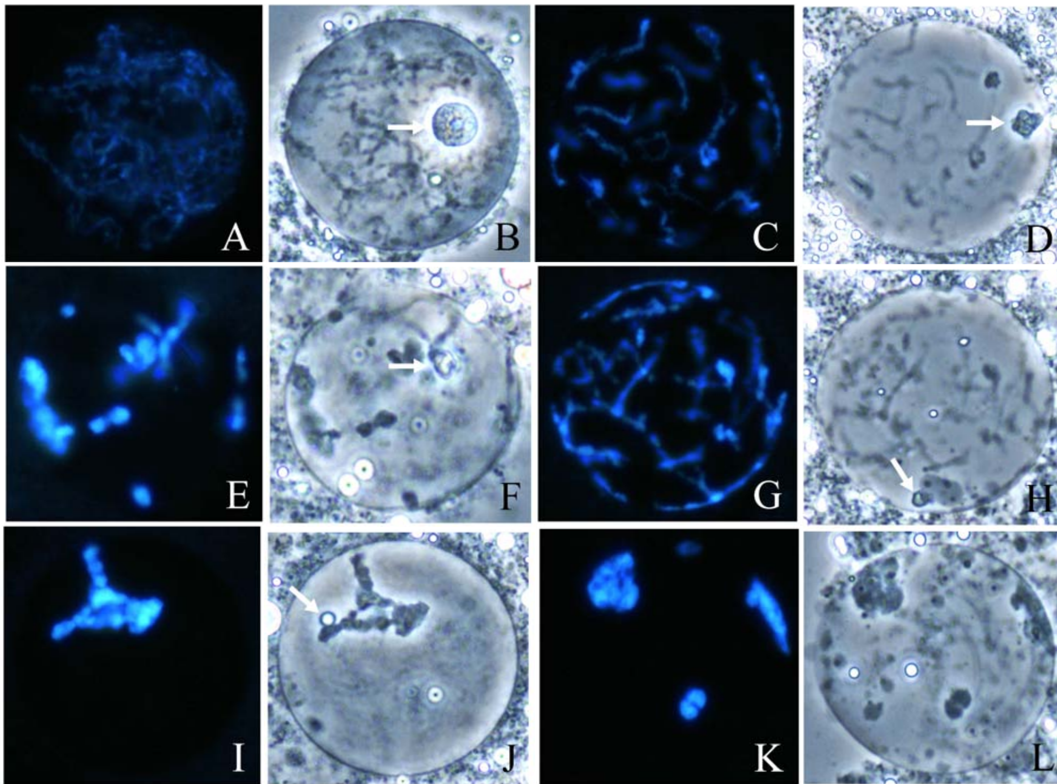

Fig. 6: Chromatin configuration of goat oocytes. Arrow shows the nucleolus. Images with the same letter are the same oocytes observed with fluorescence and phase contrast microscopy. Image A and B show the GV1 chromatin configuration with the diffuse filamentous chromatin over the nuclear area and a large nucleolus. Image C and D show the GV2n chromatin configuration with the condensed net-like chromatin and a medium-sized nucleolus. Image E and F show the GV2c chromatin configuration with the condensed clumped chromatin and a medium-sized nucleolus. Image G and H show the GV3n chromatin configuration with the condensed net-like chromatin and a small nucleolus. Image I and J show the GV3c chromatin configuration with the condensed clumped chromatin and small nucleolus. Image K and L show the GV4 chromatin configuration with condensed clumped chromatin but no nucleolus there

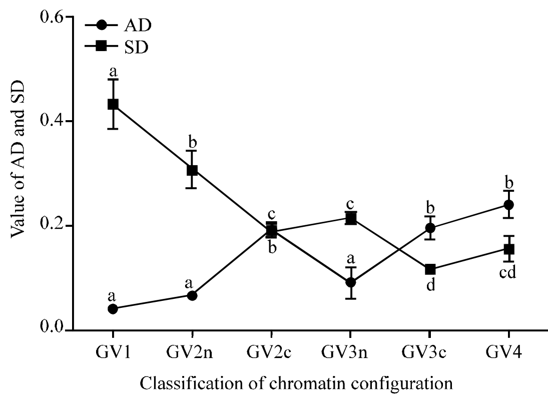

Fig. 7: Value of AD and SD in goat chromatin configuration. Different letter with the same color indicate that the difference is significant ($p<0.05$)

configurations of mouse (NSN, 7899.98±347.29 vs. SN, 7839±234.29) ($p<0.05$). But the IOD was different in pig and goat. GV4 has the highest IOD (8161.82±1213.27) in pig and the highest IOD in goat were GV2c and GV4 (5930±632.34 and 5658.77±1187.76) in goat. The rest of the chromatin configurations of pig and goat have an equal IOD (Fig. 8).

A quantitative method of chromatin configuration is greatly needed. The traditional method is quite subjective and different studies may yield different results in the same species. One example is the conflicting research results in canine (Reynaud *et al*., 2009; Lee *et al*., 2008) and bovine (Liu *et al*., 2006; Lodde *et al*., 2008). Although, the data may look perfect in their own studies, it is difficult to compare the data from different laboratories. In this study the researchers describe a quantitative and much more objective method for classification of

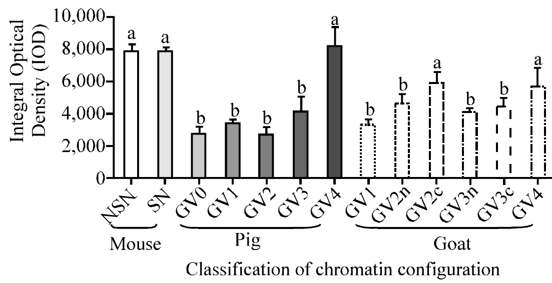

Fig. 8: Values of IOD in different chromatin configurations. Different letter with the same color on top of the columns indicate that the difference is significant. The columns and bars represent mean±SEM

chromatin configurations. The researchers compared the chromatin configuration with this new method in several animals by the parameters of AD and SD according to the ability of oocytes. There is a common rule in the chromatin configuration of NSN in mouse, GV0 in pig and GV1 in goat: the AD value is the smallest while the SD value is the biggest. Transcription in such oocytes of mouse (Miyara *et al*., 2003), pig (Bjerregaard *et al*., 2004) and goat (Sui *et al*., 2005) is the most active. The AD and SD data directly support the conclusion. Widespread and looser chromatin favors transcription. The conclusions also provide some clues to the research in human (Miyara *et al*., 2003), rabbit (Wang *et al*., 2009) and cattle (Lodde *et al*., 2008) in which studies focus on the transcription of different chromatin configuration.

The oocytes with NSN, GV0 and GV1 chromatin configurations have also been considered as having lower capability to resume meiosis, mature and support embryo development in mouse (Zuccotti *et al*., 2002), pig (Wu *et al*., 2006) and goat (Ma *et al*., 2003; Han *et al*., 2006). In the studies, the AD and SD values in these chromatin configurations have the most difference from the values of mature oocytes. The immense difference indicates such oocyte is far away from maturation than the oocytes with other chromatin configurations. Transcription activity determined by AD and SD also indicates that there are lots of proteins need to be synthesized during oocyte maturation and embryo development. That may be another reason that such oocytes do not have enough ability to resume meiosis, mature and support embryo development.

The values of SD decreased ($p<0.05$) when the chromatin configurations SN, GV1 and GV3n were compared to NSN, GV0 and GV1 in mouse, pig and goat, respectively. The values of AD are increased in such comparison although, the difference was not significant in goat. The transcriptional activity should be weaker due to the increase of AD and decrease of SD. The studies of chromatin configuration in mouse (Miyara *et al*., 2003), pig (Bjerregaard *et al*., 2004) and goat (Sui *et al*., 2005) support the hypothesis. Moreover, the chromatin configurations with intermediate AD and SD values exhibited a moderate activation of transcription. Such transformations in AD and SD reduce the difference of GV oocytes and MII oocytes. Such oocytes should have more ability to resume meiosis, mature and support embryo development. The studies of chromatin configuration in mouse (Zuccotti *et al*., 2002), pig (Wu *et al*., 2006) and goat (Han *et al*., 2006) support the hypothesis.

There are some common characteristics of SD in different species. The value of SD is never <0.4 in the chromatin configuration with high transcriptional activity and never >0.3 in the chromatin configuration with no transcriptional activity. This indicates that the chromatin needs to compact to a certain degree to cease the transcriptional activity. These results proved the hypothesis that large-scale chromatin structure regulates the spatio-temporal expression of gene during development and differentiation (De La Fuente, 2006).

CONCLUSION

Previous studies on chromatin configuration lack quantification due to the limit of the methods. The method provides a new approach to address this problem. There are no differences among different chromatin configurations except the GV4 chromatin configuration in pig and the GV2c and GV4 chromatin configurations in goat.

These special IODs indicate that the oocytes with these chromatin configurations are different from others. The oocytes with GV4 chromatin configuration in pig may represent stages toward atresia or transient events prior to GVBD according to previous research (Sun *et al*., 2004). There are similar results in the research of goat oocytes with GV2c and GV4 chromatin configurations (Sui *et al*., 2005). These results may explain the results about the special IOD.

ACKNOWLEDGEMENTS

The researchers are grateful to Prof. Li BaoJie at Bio-X Center of Shanghai Jiao Tong University and Dr. Liu YiFei at Fuyang Teachers College for comments on this manuscript. This research was supported by grants from the National Natural Science Foundation of China (Nos: 31071310 and 30871415) and the Key Project of the Natural Science Research Program of Anhui Higher Education Institutions of China (No. KJ2009A030).

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