Morphometric analysis of ovarian follicles of Indian flying fox *Pteropus giganteus giganteus* (Brunnich)

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Abstract: The present study was undertaken to know the development of ovarian follicles in Indian flying fox *Pteropus giganteus giganteus*. Ovaries were collected and fixed for histological examination. Histomorphological details of graffian follicles with their respective oocyte and nucleus were described. Analysis of correlation coefficient and regression equations between diameters of graffian follicles of developmental stages, respective oocyte and their nucleus were calculated. Result of the study revealed that growth of graffian follicle was not consistent. Oocyte diameter was not directly proportional to the graffian follicular diameter of developing stages. Non linear increase of nucleus diameter was noted when compared with diameter of graffian follicle. Significant ($P < 0.01$) positive correlation has been observed between oocyte and nucleus of antral follicle. Non significant increase in cytoplasmic volume of oocyte has been observed during successive development of oocyte except bilaminar secondary follicle to multilaminar preantral follicle.

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1. Introduction

Mammalian ovary is the vital organ because of its important role in synthesis of hormones, growth factors and oogenesis (Johnson, 2003). Mammalian ovary comprises of thousands to millions of graffian follicle in different maturity stages. Ovary supports the maturation and development of graffian follicle as well as ovulation under the influence of hormones FSH and LH (Cortvrindt and Smitz, 2001). Researchers had classified graffian follicles as morphologically normal follicle in different species (van Wezel and Rodgers, 1996; Fair et al., 1997; Lundy et al., 1999; Rodgers and Irving-Rodgers, 2010). Morphometric analysis of graffian follicle and oocyte is the important criteria to understand the successive developmental process of oogenesis and pattern of growth of graffian follicle and oocyte in mammals. Therefore, aim of present study is to understand the process of oogenesis in *Pteropus giganteus giganteus* by the morphometric analysis of graffian follicle and oocyte at each stage of development. Statistically significant changes were determined in diameter, surface area and volume of graffian follicle, oocyte and nucleus. Correlation coefficient in between diameters of graffian follicle, oocyte and nucleus was calculated at each stage of graffian follicular development. Regression equation between diameter of graffian follicle and oocyte was also calculated to observe linearity during growth.

2. Material and Methods

2.1 Collection of Specimens

Pteropus giganteus giganteus (Brunnich) is an Indian megachiropteran bat of pteropidae family commonly known as Indian flying fox. A colony of 50 to 500 individuals had been observed on the large tree of *Ficus bengalensis* near the water reservoir at Padmapur village. All the specimens used during entire study period were obtained from natural populations from feeding site at Padmapur village near Armori. [Longitude 20^{0}. 22' North and latitude 79^{O}.48' East (Dist-Chandrapur, Mahrashtra)]. The specimens were collected from December 2008 up to December 2010 in such a way that entire reproductive cycle was represented. Feeding sites were identified by the examination of guano of the *Pteropus giganteus giganteus*. Five specimens were collected during each reproductive stage. Complete reproductive cycle of *Pteropus giganteus giganteus* had been studied and anoestrous, oestrous, early pregnancy, mid pregnancy, parturation and lactation stages were confirmed by the histological examination of uterus and ovaries, morphological examination of mammary glands and breeding behavior at roosting site.

2.2 Procedure for morphometric analysis

The ovaries were fixed in alcoholic Bouins fixative and sectioned serially at a thickness of 5µm. Sections were stained with hematoxylin-eosin staining technique (Humason, 1979). Microphotographs of histological sections of ovaries were captured using Labomade DG-3 compound microscope camera at 400X magnification. Tagged image file format was used to calculate the diameter of the graffian follicle, oocyte and nucleus. The oocyte and nucleus were assumed to be spherical. Oocyte and nucleus diameter was measured (*a*) and the diameter at right angles to this (*b*) (Wiliams, 1977). The diameter (D) was calculated using the following equation:

Diameter (D) (mm) = $\sqrt{ab}$.

The mean diameter (D) for each oocyte and nucleus was calculated. This was then converted to an oocyte and nucleus volume and surface area using the following equations:

Volume of graffian follicle (mm^3) $= \frac{4}{3}\pi\left\{\frac{D}{2}\right\}^3$

Surface area of graffian follicle (mm^2) = $4\pi r^2$

The volume of cytoplasm in the oocyte was calculated by using fallowing equation:

Volume of Cytoplasm (mm^3) = Volume of oocyte - Volume of nucleus

2.3 Statistical analysis

A statistical analysis of these data was performed to determine the significant changes in graffian follicle and its respective oocyte and nucleus diameter, surface area and volume during successive developmental stages. Mean, Standard error, Standard deviation, Variance and ANOVA with post hoc Tucky's HSD test, correlation and regression were calculated by using Statistical Package for Social Sciences (SPSS 10.0).

3. Results

3.1 Ovarian Follicular Morphology and Development

Comparision between the diameter of graffian follicle**,** oocyte and oocyte nucleus; as well as surface area and volume of oocyte, surface area and volume of nucleus and volume of cytoplasm in *Pteropus giganteus giganteus* has been depicted in the table 1 and 2, respectively.

In the present investigation, there was no significant difference found in diameter of type1 and type 2 follicles. However in later follicular development, significant increase in diameter at ($P < 0.01$) was observed in between type 3, type 4 and type 5 follicles.

During the development of oocyte in type1 to type 2 follicles and type4 to type 5 follicles there was no significant increase in the diameter of oocyte. However significant increase was evident during the development of oocyte from type 2 to type 3 and type 3 to type 4 follicles.

Oocyte showed non-significant increase in volume and surface area during development of type 1 to type 2 follicle and type 2 to type 3 follicles. While it was significantly increased during the development of type 3 to type 4 follicle.

During the successive stage wise development of oocyte, a significant change in the diameter of oocyte nucleus has been observed. However non significant increase in volume and surface area of oocyte nucleus during development of type 1 to type 2 follicle and type 2 to type 3. In the later developmental stages such as type 3 follicle to type 4 and type 4 follicle to type 5 follicle, significant increase in volume and surface area of oocyte nucleus has been noted.

Non-significant increase in the volume of cytoplasm was observed during development from type1 to type 2, type 2 to type 3 and type 4 to type 5 follicles. However significant increase in the cytoplasmic volume has been evident during development from type 3 follicle to type 4 follicle.

3.2 Analysis of Correlation and regression between diameters of graffian follicle, oocyte and nucleus:

Correlation matrix of the follicle diameter, oocyte diameter and nucleus diameter of primordial, primary, secondary, preantral and small antral follicle are presented in the table 3, 4, 5, 6 and 7 respectively. Significant correlation coefficient between follicular diameter and oocyte diameter of primordial, primary, secondary, preantral and antral follicles was found to be $r = 0.921$, $r = 0.812$, $r = 0.644$, $r = 0.48$ and $r = 0.612$, respectively. However non-significant correlation coefficient was observed between diameter of primordial, primary, secondary, preantral and antral follicles and respective oocytes and their nucleus. Regression equation for follicular diameter and oocyte diameter $Y= 0.770x + 4.971$, $Y= 0.447x + 25.84$, $Y= 0.272x + 72.47$, $Y= 0.325x + 68.18$ and $Y= 0.205x + 6.535$ were calculated for primordial, primary, secondary, preantral and antral follicles, respectively (figs. 1 to 5).

4 Discussion

4.1 Graffian follicle, Oocyte and Nucleus diameter

The follicles have been classified as proposed by Lundy et al. (1999). There was no statistical difference between the oocyte diameters of the primordial and primary follicles. This suggests that the main component responsible for follicular growth at this stage was the granulosa cells. From primary follicle to multilaminar preantral follicle stage, the changes in the follicular diameters were characterized by significant increase in the granulosa cells as well as diameter of oocyte and nucleus. The preantral follicle was characterized by changes in granulosa cells. The follicle becomes activated and flattened granulosa cells appeared cuboidal in shape (Erickson, 1986). Development of preantral follicle into antral follicle showed the significant increase in nucleus diameter while there was no significant increase in oocyte diameter during this stage. Herting et al. (1976) in *Aotus trivirgatus* (owl monkey); Nagle et al. (1980) in *Callitrichix jacchus*; Oeke (1995) in *Cebus paella* (capuchin monkey) and Lundy et al. (1999) in *Ovis aries* (sheep) have observed the diameter of the antral follicles varies from 0.3 mm to 12 mm. In women, the preovulatory follicle can reach up to 20 mm (Baker and Wai, 1976). Diameter of an antral follicle in *Pteropus giganteus giganteus* was ranging from 877.22 to 1384.8 µm which may increase in preovulatory antral follicle. Observations in present study showed that antrum formation occurred when the follicle reached 429±17.05 µm in diameter.

Table 1: Comparision of mean graffian follicle, oocyte and nucleus diameter in *Pteropus giganteus giganteus*.

Sr.No.	Follicle Type	Mean Graffian Follicular Diameter ± S.E in µm	Mean Oocyte Diameter ± S.E in µm	Mean diameter of oocyte nucleus ± S.E in µm	Number of follicles Measured
1	Primordial Follicle	59.59±1.45[a]	50.86±1.21[a]	29.26±0.47[a]	19
2	Unilaminar Primary Follicle	126.94±6.06[a]	82.60±3.33[a]	38.96±1.56[b]	12
3	Bilaminar Secondary Follicle	242.22±8.91[b]	138.5±3.77[b]	50.20±1.13[c]	12
4	Multilaminar Preantral Follicle	429.93±17.05[c]	208.22±11.56[c]	67.51±1.48[d]	16
5	Antral Follicle	1090.94±46.71[d]	217.69±15.68[c]	89.94±1..93[e]	10

Within column Mean ± S.E. with the same superscripts are not significantly different at ($P < 0.01$).

Table 2: Area and volume (Mean± S.E.) of oocyte and nucleus with respect to follicular type in *Pteropus giganteus giganteus*.

Group	Area of oocyte (mm^2)	Area of nucleus (mm^2)	Volume of oocyte (mm^3)	Volume of nucleus (mm^3)	Volume of cytoplasm (mm^3)	Number of follicles Measured
Primordial Follicle	8.21±0.39[a]	2.70±0.08[a]	71.08±5.26[a]	13.30±0.62[a]	57.77±5.33[a]	19
Unilaminar Primary Follicle	21.82±1.83[a]	4.85±0.38[a]	311.68±40.76[a]	32.62±3.84[ab]	279.06±39.00[a]	12
Bilaminar Secondary Follicle	60.75±3.32[a]	7.96±0.36[b]	1425.39±117.11[a]	67.35±4.72[bc]	1358.03±115.58[a]	12
Multilaminar Preantral Follicle	142.51±14.13[b]	14.42±0.63[c]	5352.6±714.07[b]	164.63±10.97[d]	5187.98±710.32[bc]	16
Antral Follicle	155.83±20.11[b]	25.52±1.11[d]	6123.5±1084.74[b]	385.80±25.44[e]	5737.69±1064.70[c]	10

Within column Mean ± S.E. with the same superscripts are not significantly different at ($P < 0.01$).

Table. 3: Correlation matrix of the Follicle diameter, Oocyte diameter and Nucleus diameter of primordial follicle. [The values (*r*) ranged above 0.456 and 0.575 are significant at $P < 0.05$(2-tailed) and $P < 0.01$(2-tailed), respectively].

Correlations for all pairs of data series (method=pearson)			
r	Follicular diameter	Oocyte diameter	Nucleus diameter
Follicular diameter	1		
Oocyte diameter	0.921	1	
Nucleus diameter	0.127	-0.044	1

Table. 4: Correlation matrix of the Follicle diameter, Oocyte diameter and Nucleus diameter of primary follicle. [The values (*r*) ranged above 0.576 and 0.708 are significant at P < 0.05(2-tailed) and P < 0.01(2-tailed), respectively].

Correlations for all pairs of data series (method=pearson)			
r	Follicular diameter	Oocyte diameter	Nucleus diameter
Follicular diameter	1		
Oocyte diameter	0.812	1	
Nucleus diameter	0.18	0.445	1

Table. 5: Correlation matrix of the Follicle diameter, Oocyte diameter and Nucleus diameter of secondary follicle. [The values (*r*) ranged above 0.576 and 0.708 are significant at P < 0.05(2-tailed) and P < 0.01(2-tailed), respectively].

Correlations for all pairs of data series (method=pearson)			
r	Follicular diameter	Oocyte diameter	Nucleus diameter
Follicular diameter	1		
Oocyte diameter	0.644	1	
Nucleus diameter	0.32	0.313	1

Table. 6: Correlation matrix of the Follicle diameter, Oocyte diameter and Nucleus diameter of preantral follicle. [The values (*r*) ranged above 0.48 and 0.623 are significant at P < 0.05(2-tailed) and P < 0.01 (2-tailed), respectively].

Correlations for all pairs of data series (method=pearson)			
r	Follicular diameter	Oocyte diameter	Nucleus diameter
Follicular diameter	1		
Oocyte diameter	0.48	1	
Nucleus diameter	0.064	0.247	1

Table. 7: Correlation matrix of the Follicle diameter, Oocyte diameter and Nucleus diameter of antral follicle. [The values (*r*) ranged above 0.632 and 0.765 are significant at P < 0.05(2-tailed) and P < 0.01(2-tailed), respectively].

Correlations for all pairs of data series (method=pearson)			
r	Follicular diameter	Oocyte diameter	Nucleus diameter
Follicular diameter	1		
Oocyte diameter	0.612	1	
Nucleus diameter	0.505	0.773	1

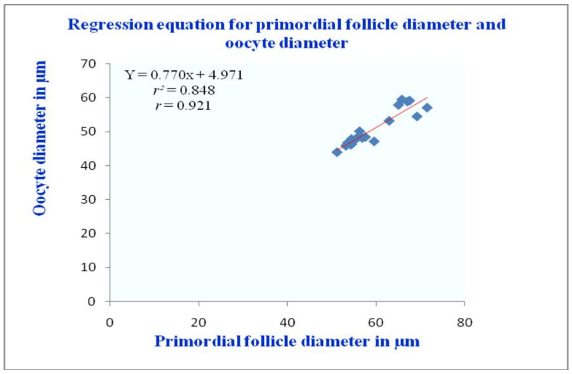

Fig.1: Linear regression equation showing relationship between primordial follicle and oocyte diameter in *Pteropus giganteus giganteus*.

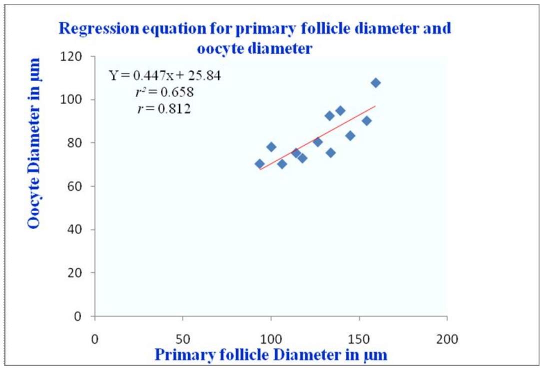

Fig.2: Linear regression equation showing relationship between primary follicle and oocyte diameter in *Pteropus giganteus giganteus*.

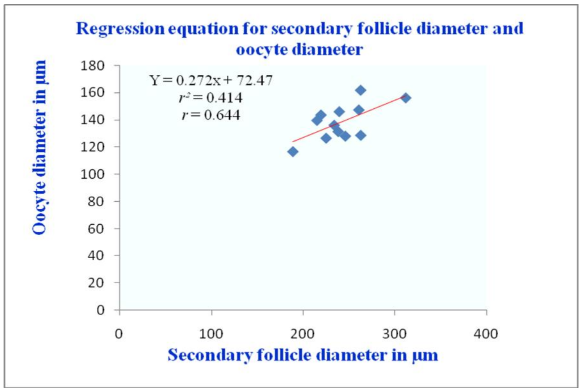

Fig.3: Linear regression equation showing relationship between secondary follicle and oocyte diameter in *Pteropus giganteus giganteus* Oncentrati.

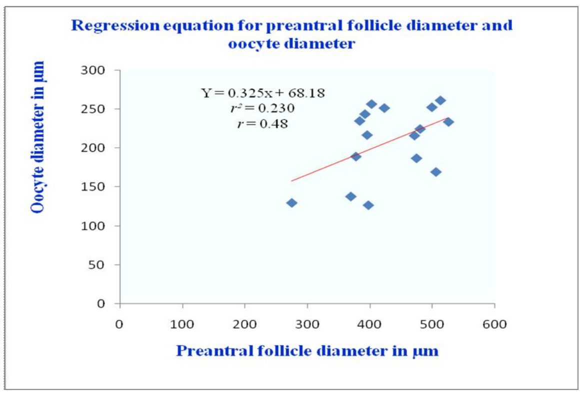

Fig.4: Linear regression equation showing relationship between preantral follicle and oocyte diameter in *Pteropus giganteus giganteus*.

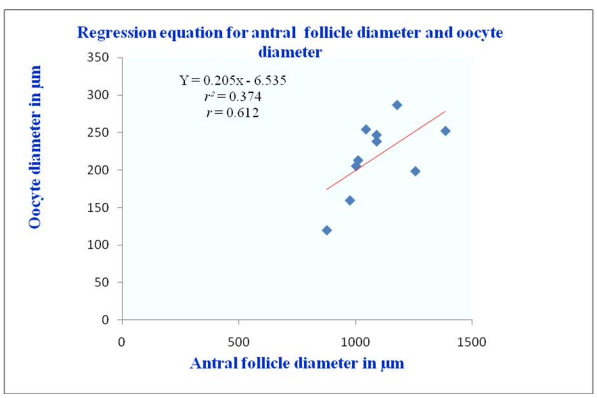

Fig.5: Linear regression equation showing relationship between antral follicle and oocyte diameter in *Pteropus giganteus giganteus*.

The mean antral follicle diameter and oocyte diameter was approximately 1090±46.71 µm and 217.69±15.68 µm respectively.

Before antrum formation, follicular diameter and oocyte diameter were positively and linearly correlated. Oocyte reaches more than 90% of its growth at the stage of antrum formation. After antrum formation non significant increase in oocyte diameter contrasting with a significant follicle development has been observed. The zona pellucida was absent in primordial and primary follicles, however it appeared at the bilaminar secondary stage and reached almost 7-8 µm at the pre-ovulatory stage. In the present study, it was clearly evident that the structure of ovarian follicles in *Pteropus giganteus giganteus* has similarity to that in other mammalian species.

Considering the results obtained in the present study, it may be concluded that during the preantral stage, there were two phases of follicular growth. The first phase was characterized by transformation of flattened to cuboidal granulosa cells and increase in the oocyte size. However second phase of graffian follicular growth was characterized by the increase in the oocyte size and proliferation of granulosa cells which mainly contribute for increase in follicular size such a biphasic pattern of follicle and oocyte growth has been noted in *Pteropus giganteus giganteus*. These results are in agreement with the findings of Gougeon and Chainy (1987), Hulshof et al. (1994), Codón et al. (2001) and Reynaud et al. (2009). The statistical differences recorded in the follicular diameter could be used as a parameter for the classification of the preantral follicles.

Antral follicles were more susceptible to follicular atresia due to nuclear pyknosis and nuclear karyorrhexis. During the final differentiation stage of the granulosa and theca cells, few antral follicles get degenerated, and this is in agreement with results recorded by Lussier et al. (1987).

4.2 Correlation and regression between diameters of graffian follicle, oocyte and nucleus in *Pteropus giganteus giganteus*

4.2.1 Oocyte diameter versus follicular stage

The growth rate of the follicle during each stage of maturation was not consistent. The difference in follicle diameter between stages became progressively greater with each stage. ANOVA results suggested significant increase in diameters ($P \leq 0.01$) during each stage of maturation except primordial to primary follicles. This indicates that the follicle growth from one stage to the next was progressive, but not linear. (van den Hurk and Zhao, 2005).

4.2.2 Oocyte diameter versus follicle diameter

The relationship of oocyte diameter to follicular diameter has been previously reported for many mammalian species at the early stages of follicular growth (Knigge and Leathem, 1956; Gougeon and Chainy, 1987; Hoage and Cameron, 1976 and Morbeck et al., 1992). Regression equations for primordial, primary, secondary, multilaminar preantral and antral follicle to their respective oocyte diameter were reported to be Y= 0.770x + 4.971(r=0.921), Y= 0.447x + 25.84 (r=0. 0.812), Y= 0.272x + 72.47 (r=0.644), Y= 0.325x + 68.18(r=0.48) and Y= 0.205x + 6.535 (r=0.612), respectively. The change in oocyte diameter was not directly proportional to the follicular diameter.

4.2.3 Nucleus diameter versus follicle diameter

Significant increase in diameter of oocyte nucleus has been noticed during each stage of maturation of graffian follicle. Correlation coefficient (r) in between primordial, primary, secondary, multilaminar and antral graffian follicles and their respective nucleus diameter was found to be 0.127, 0.18, 0.32, 0.064 and 0.050 respectively. This indicates non linear growth of nucleus diameter when compared with diameter of graffian follicle.

4.2.4 Nucleus diameter versus oocyte diameter

Correlation coefficient (r) in between oocyte of primordial, primary, secondary, multilaminar and antral graffian follicles and their respective nucleus diameter was found to be 0.004, 0.445, 0.313, 0.247 and 0.773 respectively. Significant positive correlation coefficient ($P \leq 0.01$) was only recorded in between oocyte and its nucleus diameter of antral follicle.

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