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An Allometric Study on Relating the Growth of the Follicle to That of the Oocyte in Dairy Cows with Ovarian Cyst(s)

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Abstract: *Four hundred fifty-one oocytes were recovered from 115 Holstein cows with ovarian cyst(s) and 159 oocytes were recovered from 36 noncystic normal Holstein cows, respectively. The oocytes recovered from cystic cows were classified as healthy or degenerative by cumulus investment and by nucleus configuration, as well. The relative growth relating follicle diameter to the oocyte diameter in each classification was examined by using regression models. The best fitting models in each group were the hyperbolic regressions with R^2 values of 0.994 to 0.999. The growth rates of the oocyte calculated from the differentiated equations of the hyperbolic equations were found to be an asymptotic depression into zero less than 0.5 μm since the follicle grew more than 6.0 mm in diameter. The linear regressions for the growth of the follicle more than 6.01 mm in diameter in relation to that of the oocyte in each abovementioned group all indicated that the true slopes (β)=0 lay somewhere in the 95% confidence intervals of these regressions by the statistical analyses. These results corroborated that the oocytes did not grow but the follicles grew more than 6.0 mm in diameter in both normal and cystic cows. There was no difference between the growth patterns in cystic cows from which healthy and degenerative oocytes were recovered. This was regardless of the judgment by either cumulus investment or nucleus configuration.*

Key words: *Dairy cow, Ovarian cyst, Follicle, Oocyte, Oocyte growth rate.*

It was demonstrated that seven of 25 cows with cystic follicle(s) (28%) became pregnant by artificial insemination within 24 h after treatment with LH-RH analogue, and also that LH-RH analogue administration induced the ovulation not only of cystic follicles but also of smaller follicles as shown by postmortem examination performed 4 to 9 days after LH-RH analogue administration [1]. The latter finding is in agreement with those reported by Bierschwal *et al.* [2] and Kesler *et al.* [3, 4]. These results confirm the possibility of the existence of oocytes with a fertilization potential in the cystic follicle. Because of a lack of findings from the basic studies on the dynamics of the growth pattern between oocytes and follicles in the cows with cystic follicles, it is interesting to clarify if the growth pattern of their oocytes in cystic follicles is similar to the growth pattern relating the oocyte to the follicular size culminating in ovulation of normal follicles.

A number of researchers have made histological studies on the growth of the oocyte in relation to that of the follicle in monotremes, marsupials [5] and eutherian mammals including cows [6–10]. It is difficult to interpret these results without considering artificial shrinkage due to a complex process such as the fixation and embedding for microscopical preparations.

The purpose of the present study was to allometrically analyze the relation between the growth of follicles and that of their enclosed oocytes without the fixation for ordinary histological observations in both normal cows and cystic ones, and to establish whether the quantitative patterns relating oocytes to follicular growth in healthy oocytes differ from those of degenerative oocytes by classifying the oocytes recovered from cows with cystic follicle(s) into two types, healthy and degenerative.

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Materials and Methods

Animals and oocyte recovery

Ovaries were obtained within 30 min after slaughter from 36 noncystic, cycling Holstein cows and from 115 Holstein cows with ovarian cyst(s) from 25th December, 1986 to 18th July, 1996 at the Kanazawa Municipal Abattoir. As noncystic ovaries were harvested from cows of unknown reproductive history, identification of the stage of the estrous cycle was based on reported corpus luteum at various days during the estrous cycles [11, 12] and by known characteristics of the ovary during the cycles [13, 14]. The numbers of cycling cows at the early luteal, luteal and follicular stages during the cycle were 6, 23 and 7, respectively. The identification of the cystic cows was based both on the presence of a single or multiple formation of fluid-filled, anovulatory cystic follicular structures measuring 22.5 mm or more in diameter on at least one ovary and on the absence of both an ovulation point and a corpus luteum in both ovaries. The diameter of the cystic follicle was estimated by measuring the length at both the maximum diameter and the diameter at right angles to it. The ovaries obtained at the abattoir were maintained at approximately 35°C in a thermos flask containing physiological saline solution (0.154 M NaCl) and brought to the laboratory within 2 h, where they were transferred to a tall beaker filled with the saline solution in a water bath at 35°C. Using noncystic cows as controls, 159 oocytes from the noncystic cows, and 451 oocytes from the cystic cows were recovered under a stereo-microscope. The oocytes from the follicles larger than 1.5 mm in diameter, including Graafian and cystic follicles, were recovered from their follicles submerged into the saline solution in a watch glass by puncturing with a gauge needle or by cutting with ophtalmologic scissors. In order to prevent clotting of the follicular contents derived from the large follicles like ovarian cysts, the follicular fluid containing the oocyte in the watch glass was carefully replaced at least three times with saline solution. When the oocytes could not be recovered, the cystic follicles were flushed three to four times with saline solution, and the inner lining of the cystic follicle was lightly scraped to recover the oocyte, as well. The oocytes from the small follicles that were less than 1.5 mm in diameter were recovered after the follicles were dissected free of stromal tissues from the ovaries. All of the cystic ovaries from which the oocytes were recovered were serially sliced with a knife at approximately 5 mm, and every slice was macroscopically examined in order to confirm the absence of the luteal tissue.

Oocyte classification from cumulus investment

Each oocyte was classified as either healthy or degenerative, judged by the morphological appearance of its cumulus investment under a stereo-microscope. Oocytes were judged to be healthy if they were surrounded by complete and compact cumulus cells more than three layers thick. These healthy oocytes corresponded to the score of 1 of the investment in the criteria defined by Leibfried and First [13]. In addition to their score 2 to 4 [13], the oocytes surrounded closely by one layer of collapsed cumulus cells like the mantle layer of the earth were judged to be degenerative oocytes. Oocytes without cumulus cells, surrounded by the cumulus cells partially impaired and less than three layers thick were similar in all artifacts during the processing of oocytes and spontaneous products. They were considered to be degenerative.

After estimating the investment, the cumulus cells were removed completely from the oocyte by gently pipetting the cumulus-oocyte complexes into and out of a finely drawn glass pipette with an inner diameter similar to that of the nude oocyte in order to carefully avoid the rupture of the zona pellucida. The collapsed cumulus cells that had tightly adhered to the oocyte were removed from it by dissolving them enzymatically with the addition of 0.25% trypsin solution (1:250; Difco Laboratories, USA). The oocytes were then washed twice in the saline solution and their diameters were measured, including the thickness of the zona pellucida with an ocular micrometer calibrated with a slide micrometer under a light microscope at low magnification.

Oocyte classification from nucleus configuration

Each naked oocyte was placed on a slide and covered with a cover slip supported by four spots of vaseline paraffin mixture (9:1). The oocyte was then immersed in Carnoy's fixative for a minimum of three days before it was stained with 1% orcein in 45% acetic acid [15]. Immersion in Carnoy's fixative was performed within 6 h after the animals were sacrificed [16]. The morphological features of the nuclei of the oocytes were examined under a phase contrast microscope [17, 18]. Without relation to the stages of the chromosome configurations from germinal vesicle to metaphase I, the oocytes were classified as healthy or degenerative. *Healthy oocyte*: The oocytes which possessed an intact germinal vesicle were characterized by a round, well-defined nucleus and a smooth outline. The nucleus sometimes contained a nucleolus. The nuclear membranes, chromatins or chromosomes which also had an affinity for basic dyes were stained deep blue to bluish purple or purplish blue. *De-*

generative oocyte: In the remaining oocytes, the nuclear material that exhibited various degrees of pycnotic condensation was stained dark red or deep ruby red to pinkish red. The nucleus had an irregular crenated outline caused by partial collapse of the nuclear membrane. Lastly, the appearance of all the oocytes as well as the nucleus was too degenerate or damaged to be recognizable as at any particular stage.

Statistical analysis

The linear regression model used was $y=a+bx$, and the nonlinear regression models were $y=a+bx+cx^2$ for a quadratic curve regression and $y=ax/(x+b)$ for a hyperbolic curve regression, where y is the diameter of the oocyte, including the thickness of the zona pellucida in μm, and x is the follicle diameter in mm, respectively. These regressions were obtained by an application of the method of least squares analysis. The correlation coefficient (r) and the coefficient of determination (R^2) were determined between the oocyte diameter and the follicle diameter. The comparison of fit among regression models depended on the R^2 values adjusted for degrees of freedom. The oocyte growth rate in μm per follicle growth in mm was calculated from the differentiated equation of the regression with the highest coefficient of determination. The oocyte growth rates derived from the differentiated equations for the hyperbolic curve indicated that the oocyte was approximately stationary in size since the follicle reached 6.01 mm in diameter. The linear regression, the correlation coefficient and the coefficient of determination were also calculated for an increase in follicle diameter of more than 6.01 mm in relation to that of the oocyte. Student's *t*-test was employed in the analysis of significant differences of the true intercept (α) and the true slope (β) in the regression lines relating to a follicle diameter of more than 6.01 mm to oocyte diameter. The 95% confidence intervals for the true slopes (β) of these regressions were computed to determine whether $\beta=0$ lay in these intervals or not. The *t*-test was also used to determine whether or not there were significant differences between individual regressions in the intercepts (a) and the slopes (b), and if the intercepts and the slopes in two regression lines relating follicle diameter more than 6.01 mm to oocyte diameter were different [19]. The results were expressed as means ± SEM.

Results

Table 1 shows the linear and nonlinear regression equations for the growth of the oocyte and that of the follicle in each group of noncystic normal cows and cystic cows. The best coefficients of determination (R^2) values resulting from the three regression analyses were obtained from the hyperbolic curves in each group (0.994 to 0.999). The R^2 values in the quadratic regressions were 0.195 to 0.507 lower than those in the hyperbolic regressions. The lowest R^2 values were seen in those of the linear regressions (0.160 to 0.392). The hyperbolic curve regressions obtained for (A) noncystic normal cows, (B) cystic cows, cystic cows from which (B-hc) healthy and (B-dc) degenerative oocytes were recovered, judged by cumulus investment, and cystic cows from which (B-hn) healthy and (B-dn) degenerative oocytes were recovered, judged by nucleus configuration, were as follows: (A) $y=160.84x/(x+0.165)$, $R^2=0.998$ (n=159); (B) $y=160.40x/(x+0.132)$, $R^2=0.998$ (n=451); (B-hc) $y=161.03x/(x+0.151)$, $R^2=0.999$ (n=311); (B-dc) $y=159.59x/(x+0.052)$, $R^2=0.994$ (n=140); (B-hn) $y=160.71x/(x+0.139)$, $R^2=0.999$ (n=305); and (B-dn) $y=159.92x/(x+0.100)$, $R^2=0.996$ (n=145), respectively (Fig. 1). As shown by the dotted curves in Fig. 2, the growth rates of the oocyte calculated from the differentiated equations for the hyperbolic curves resulted in a marked decline in the growth of the follicle of 130.2 ± 28.12 μm, 56.1 ± 9.8 μm, 16.4 ± 2.5 μm and 2.1 ± 0.3 μm (per 1 mm in the follicle growth) when the follicle reached 0.2 mm, 0.5 mm, 1.0 mm and 3.0 mm in diameter, respectively. But the growth rates of the oocyte were found to be asymptotic depressions into zero less than 0.5 ± 0.1 μm since the follicle grew more than 6.0 mm in diameter.

The linear regressions for the growth of the follicle in diameter of more than 6.01 mm in relation to that of the oocyte in each abovementioned group were as follows: (A) $y=156.05+0.194x$, $r=0.207$ (n=64); (B) $y=158.43+0.053x$, $r=0.077$ (n=224); (B-hc) $y=158.26+0.081x$, $r=0.145$ (n=102); (B-dc) $y=158.06+0.053x$, $r=0.066$ (n=122); (B-hn) $y=159.40+0.023x$, $r=0.037$ (n=108); and (B-dn) $y=156.59+0.115x$, $r=0.148$ (n=116), respectively (Fig. 2). Tests for the statistical significance of these six linear regressions resulted in highly significant intercepts (a) ($P<0.0001$) with regression slopes (b) ranging from 0.023 to 0.194 ($P=0.100$ to $P=0.707$), and the results indicated that the true slopes (β)=0 lay somewhere in the 95% range for confidence intervals for the true slopes of these regressions (Fig. 2).

If the true intercept and true slope in two regression lines of (A) noncystic normal cows and (B) cystic cows were designated α_A and β_A for noncystic normal cows and α_B and β_B for cystic cows, respectively, the hypothesis of the intercepts (Ho: $\alpha_A=\alpha_B$) could not be rejected

Table 1. The linear (1) and nonlinear {quadratic curve (2) and hyperbolic curve (3)} regressions, the correlation coefficients (r), and the coefficients of determination (R^2) for the growth of the oocytes and that of the follicles

Classification†	Regression equation	r	R^2
A	(1) $y=141.4+1.257x$	0.536	0.287
	(2) $y=130.5+5.368x-0.202x^2$	0.712	0.507
	(3) $y=160.8x/(x+0.165)$	0.999	0.998
B	(1) $y=143.8+0.651x$	0.582	0.338
	(2) $y=140.7+1.836x-0.040x^2$	0.641	0.411
	(3) $y=160.4x/(x+0.132)$	0.999	0.998
B-hc	(1) $y=142.6+0.779x$	0.551	0.304
	(2) $y=138.8+2.617x-0.068X^2$	0.626	0.392
	(3) $y=161.0x/(x+0.151)$	0.999	0.999
B-dc	(1) $y=152.3+0.276x$	0.400	0.160
	(2) $y=149.8+0.688x-0.012x^2$	0.441	0.195
	(3) $y=159.6x/(x+0.052)$	0.997	0.994
B-hn	(1) $y=142.2+0.789x$	0.565	0.319
	(2) $y=138.2+2.665x-0.069x^2$	0.646	0.418
	(3) $y=160.7x/(x+0.139)$	0.999	0.999
B-dn	(1) $y=152.0+0.285x$	0.443	0.196
	(2) $y=150.6+0.582x-0.009x^2$	0.463	0.215
	(3) $y=159.9x/(x+0.100)$	0.998	0.996

†The data derived from (A) noncystic normal cows; (B) cystic cows, cystic cows from which (B-hc) healthy and (B-dc) degenerative oocytes were recovered, judged by cumulus investment, and cystic cows from which (B-hn) healthy and (B-dn) degenerative oocytes were recovered, judged by nucleus configuration.

(t_{A-B}=|−0.19|, df=284, P=0.10). Similarly, the hypothesis of the slopes (Ho: $\beta_A=\beta_B$) also could not be rejected (t_{A-B}=0.62, df=284, P=0.10). If the true intercept and slope in two regression lines for cystic cows from which (B-hc) healthy and (B-dc) degenerative oocytes were recovered, judged by cumulus investment were designated α_{Bhc} and β_{Bhc} for cystic cows from which healthy oocytes were recovered and α_{Bdc} and β_{Bdc} for cystic cows from which degenerative oocytes were recovered, respectively, the hypothesis of the intercepts (Ho: $\alpha_{Bhc}=\alpha_{Bdc}$) could not be rejected ($t_{Bhc-Bdc}$=0.01, df=220, P=0.10). Similarly, the hypothesis of the slopes (Ho: $\beta_{Bhc}=\beta_{Bdc}$) also could not be rejected ($t_{Bhc-Bdc}$=0.30, df=220, P=0.10). If the true intercept and slope for cystic cows from which (B-hn) healthy and (B-dn) degenerative oocytes were recovered, judged by nucleus configuration were designated α_{Bhn}, β_{Bhn} and α_{Bdn}, β_{Bdn}, respectively, the hypothesis of the intercepts (Ho: $\alpha_{Bhn}=\alpha_{Bdn}$) could not be rejected ($t_{Bhn-Bdn}$=0.17, df=173, P=0.10). Similarly, the hypothesis of the slopes (Ho: $\beta_{Bhn}=\beta_{Bdn}$) also could not be rejected ($t_{Bhn-Bdn}$=|−0.98|, df=173, P=0.10). These results during the growth of follicles of more than 6.0 mm in diameter confirmed that the oocytes of both (A) noncystic normal cows and (B) cystic cows did not grow, and that there was no difference between the growth patterns of cystic cows from which healthy and degenerative oocytes were recovered, regardless of the judgment by either cumulus investment or nucleus configuration.

Discussion

Oocyte and follicular growth are interrelated. As in other mammals [5, 6, 8, 9, 20], the growth of the oocyte in relation to its follicle in the bovine ovary conforms to a biphasic pattern [7, 10]. During the first phase, the oocyte and its surrounding follicle grow coordinately. At the end of the first phase, the oocyte reaches its maximum size and ceases growth. Then, during the second phase, the oocyte remains stationary in size while the follicle continues marked growth. These two phases of growth have been expressed by two linear regressions for oocyte diameter and follicle size, but in these previous studies the fitting levels in the two regression lines were lower than those in the present study.

The present study shows that the two phases of

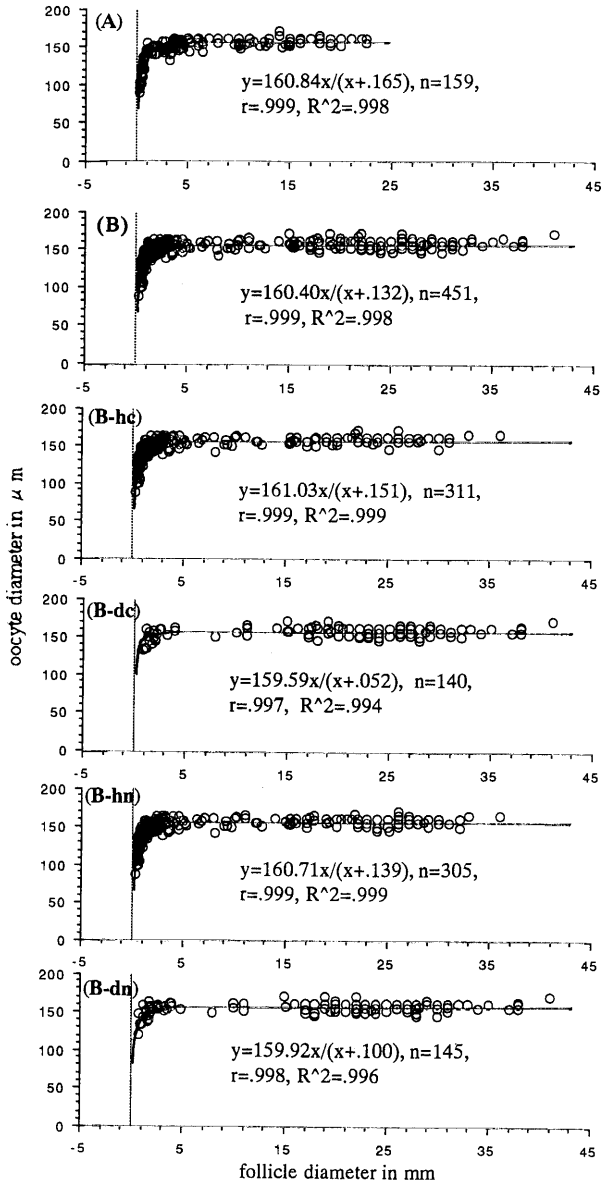

Fig. 1. The lines represent the hyperbolic regression curves for oocyte diameter and follicle diameter in (A) noncystic normal cows, (B) cystic cows, cystic cows from which (B-hc) healthy and (B-dc) degenerative oocytes were recovered, judged by cumulus investment, and cystic cows from which (B-hn) healthy and (B-dn) degenerative oocytes were recovered, judged by nucleus configuration.

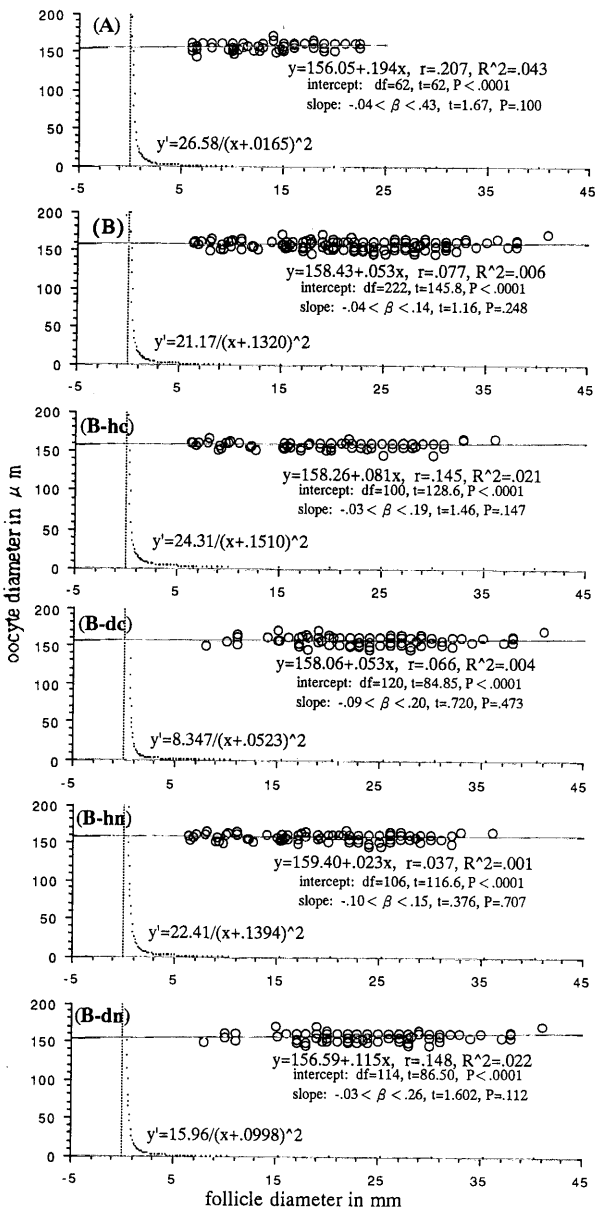

Fig. 2. The dotted nonlinear curves represent the changes in the oocyte growth rate in μm per follicle diameter in mm, whereas the solid linear lines represent the regressions between the oocyte in diameter and a follicle diameter greater than 6.01 mm.

growth relating the oocyte to follicular size fit only one hyperbolic curve regression with the best R^2 values of 0.994 to 0.999 in both normal cows and cows with cystic follicle(s) (Fig. 1). The growth rates of the oocyte calculated from the differentiated equations of these hyperbolic curves resulted in an asymptotic decline with the follicle growth (Fig. 2). The growth rates of the oocyte seem to reach nearly zero (strictly 0.53 μm/mm) at a follicular diameter of 6.0 mm, and thereafter the oocyte has almost ceased growing. As shown in Fig. 2, the slopes of linear regressions for the growth of the follicle in diameter to more than 6.0 mm corresponding to the second phase mentioned above were 0.023 to

0.194 μm/mm and seemed to be nearly zero. These are much lower than those of the previous study (0.0077 μm/μm=7.7 μm/mm) [10]. The pattern of the first phase of growth was reported as a straight-line in the previous studies [5, 6, 8, 10], which is quite different from those expressed as the hyperbolic curve regressions in the present study. This is because growth rates of the oocyte during the first phase did not remain constant but resulted in an asymptotic decline with the follicle growth. The regression lines during the first phase must not be linear. This implies that these hyperbolic regressions are the true pattern of growth during the first phase.

It was conceivable that the point at which the two regression lines intersect gives the approximate time when the oocyte almost completes its growth in relation to follicular size [8, 10, 21]. The oocyte in the present study completed its growth at a follicular diameter of 6.0 mm, which is considerably more than the values of 400 to 450 μm reported for the Japanese Black cattle in the previous study [10]. This disparity in size may have been due to artificial shrinkage when processing the oocyte for microscopical study.

When the time that elapsed between slaughtering (Time 0) and recovery of the oocytes was not more than 8.5 h, good maturation rates have been obtained in cattle [16]. In the present study fixation to observe oocyte nucleus configuration was performed within 6 h after the animals were slaughtered.

The increase in follicle size seems to be partially dependent upon the increased water content in follicular fluid as indicated by increased ion concentrations and decreased protein and albumin concentrations [22]. Why the changes in the fluid content in cystic follicles are much larger than preovulatory follicles is not clear. But the firdings obtained in the present study indicate that the content of the cystic follicular fluid should be favorable to the enclosed oocytes. The corresponding oocyte ceased growing without shrinkage or swelling when the cystic follicle was more than 22.5 mm in diameter and continued to grow.

Had it been possible to make clear the increase in the diameter of the nucleus, results more useful than those provided by the present study might have been obtained. The nucleus of the bovine oocyte, in contrast to that in mice, rats or hamsters, can rarely be observed under a microscope without extracting the yolk deposit in the ooplasm by immersion in Carnoy's fixative. It is difficult to assess the true size of the nucleus in the bovine oocyte because of the pressure on the oocyte when it is between the slide glass and the cover slip during Carnoy's fixation.

Increased knowledge of allometric changes in the growth of oocytes in noncystic and cystic ovaries in relation to that of follicles will lead to an understanding of the complexity of events surrounding the development of cystic follicles.

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