Fractal organization of feline oocyte cytoplasm

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**Dedicated to Prof. Benoît Mandelbrot in honour of his 80th Birthday*

The present study aimed at verifying whether immature cat oocytes with morphologic irregular cytoplasm display self-similar features which can be analytically described by fractal analysis. Original images of oocytes collected by ovariectomy were acquired at a final magnification of 400 X with a CCD video camera connected to an optic microscope. After greyscale thresholding segmentation of cytoplasm, image profiles were submitted to fractal analysis using FANAL++, a program which provided an analytical standard procedure for determining the fractal dimension (FD). The presentation of the oocyte influenced the magnitude of the fractal dimension with the highest FD of 1.91 measured on grey-dark cytoplasm characterized by a highly connected network of lipid droplets and intracellular membranes. Fractal analysis provides an effective quantitative descriptor of the real cytoplasm morphology, which can influence the acquirement of *in vitro* developmental competence, without introducing any bias or shape approximation and thus contributes to an objective and reliable classification of feline oocytes.

Key words: cytoplasm structure, *cumulus oophorus*, FANAL ++ software, feline oocyte, fractal analysis, fractal dimension, ovariectomy, greyscale thresholding segmentation.

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In recent years, spontaneous and experimental animals of various species have been produced by *in vitro* oocyte maturation (IVM) and *in vitro* fertilisation (IVF) techniques. In this context, among the factors affecting the acquirement of *in vitro* developmental competence, oocyte cytoplasm morphology at the age of collection appears to be of great importance in some animal species. On the basis of the microscopic appearance of their cytoplasm, oocytes are usually subjectively categorised in several subgroups, each displaying greater or lesser developmental capability (Dell'Aquila *et al.,* 1997; Hewitt and England, 1998; Luvoni ad Oliva, 1993; Pope et al, 1997; Nagashima *et al.*, 1996; Blondine and Sirard, 1995). Morphologic structural information relevant for diagnosis or useful for assessing oocyte developmental capability is mostly acquired by means of a subjective visual inspection. This invariably leads to results which are difficult to reproduce, a problem which generally occurs whenever one is dealing with a complex system of cells and tissues. Even using conventional microscopic examination, *ex vivo* cat oocytes reveal a very complex cytoplasm appearance, due to a great amount of lipid droplets distributed within an intracellular framework of highly connected membranes and organelles of irregular morphology (Jewgenow and Stolte, 1996; Nagashima *et al.*, 1996; Pope *et al.*, 1997). In this context, it is likely that almost all conventional morphometric tools and computer-assisted image analysis will provide rather ambiguous and poorly comparable data on morphologic dimensional properties because these methods are inadequate to quantitatively describe irregular cell components that cannot be assessed with a unique Euclidean scale of measure chosen *a priori* (Losa & Nonnenmacher, 1996; Losa, 2002). In contrast, the fractal geometry discovered by Mandelbrot (1983) may offer an appropriate way to quantitatively unravel contour length, surface area and other dimensional parameters of almost all irregular and

morphologically complex biological tissues (G.A. Losa, 2002). The present study aims to verify (1) whether feline oocytes could be recognised as self-similar fractal elements, (2) to evaluate the fractal dimension (FD) on a series of distinct cytoplasm features revealed in oocytes with or without the *cumulus oophorus* (COC), which has been shown to affect the oocyte developmental potential (Pope *et al.*, 1997), and finally to provide a reproducible method for the morphologic characterisation and the objective classification of immature feline oocyte cytoplasm.

Materials and Methods

Ovary collection and Oocyte recovery

Ovaries from two healthy female cats, 8 months and 6 years old respectively, obtained by ovariectomy were stored at room temperature in phosphate-buffered saline (PBS) supplemented with 100 IU/mL^{-1} penicillin-G potassium salt and 100 g/mL^{1} sulphated streptomycin (Sigma Chemical Co. St. Louis MO, USA) for 30-120 min. Eight (8) oocytes from each animal were collected by repeatedly puncturing the ovaries with a 22-gauge needle. Oocytes with an intact *corona radiata*, attached *cumulus oophorus* (COC) and medium to dark pigmented cytoplasm were pooled and washed twice with PBS containing antibiotics and 0.1% (w/v) polyvinyl alcohol (Sigma Chemical co St. Louis MO, USA).

Assessment of oocyte cytoplasm morphology

Selected oocytes with intact COC and successively denuded of COC using finely-drawn glass capillary pipette (Figure 1: A-A_1) were submitted to fractal analysis. Original images of each oocyte were acquired using a 40x objective lens and a CCD video camera connected to an optic microscope. Three different picture profiles of the oocyte cytoplasm were segmented using a computer assisted image analysis system (Sistema MONO, Immagini e Computer, Milano, Italy). A binary image was obtained by first grey thresholding the area occupied by the grey-dark cytoplasm (Figure 1: B-B_4); thereafter, two different outlines were extracted from this binary image by applying a Roberts filter: one pertained to the internal texture of the grey-dark cytoplasm area as well as to the scattered grey-dark particles within the cytoplasm, while the other referred to the external profile of the grey-dark cytoplasm only (Figure 1: C-C_1 and Figure 1: D-D_1).

Fractal analysis and Fractal Dimension Evaluation

The fractal analysis of segmented cytoplasm profiles was performed by means of three different methods, two of them yielding the mass fractal dimension and one the fractal surface dimension. The FANAL++, a program run on a workstation equipped with Linux S.U.S.E.8.2, enabled us to identify the true fractal domain within the bi-asymptotic curve achieved by the box counting method (Dollinger *et al.*, 1998). The fractal domain corresponded to the middle part of the curve, precisely defined by upper [ε_2] and lower [ε_1] limits which appears as a straight line on a double log-log plot (Figure 2). From the slope of this straight line it was possible to estimate the corrected fractal dimension FD. Both scaling limits [ε_1,ε_2] were estimated by an automatic procedure based on a least square fit algorithm which defines the widest interval [logε_1, logε_2] within which the standard deviation of the estimated slope does not exceed a given limit, corresponding to a 95% confidence interval. The FANAL++ program has been tested on exact self-similar mathematical constructs and found to produce results close to the exact theoretical fractal dimensions (Nonnenmacher *et al,* 1994; Dollinger *et al,* 1998). All images were also analysed using BENOIT 1.3 (TruSoft Int'l Inc., 204 37th Ave. N # 133, St. Petersburg, FL 33704), an alternative box counting method which, however, did not include the principle of the fractal interval within the bi-asymptotic curve and therefore was not equipped for FD calculation based on *fractal window* recognition (Losa & Nonnenmacher, 1996). The external contour profile of oocyte cytoplasm was lastly also analysed using the Image-pro plus 4.1 program (Media Cybernetics, Springfield, USA), which performed a modification of the hand and divider method (yardstick method) introduced for the first time by Richardson (1967). In the present study the FANAL++ program was adopted as the reference method, to which FD values were compared to those obtained with the other methods.

Statistics

Data were found to be normally distributed and

Table 1. Fractal Dimension of cytoplasm profiles segmented from oocytes with and without COC.

Oocytes with intact COC			Oocytes without COC		
Image profiles	**FANAL**	**BENOT 1.3**	**IMAGE-PRO**	**FANAL**	**BENOIT 1.3**	**IMAGE-PRO**
Figure 1: D-D1 	1,27±0,08	1,19±0,05	1,25±0,09	1,27±0,06	1,19±0,04	1,23±0,05
Figure 1: C-C1 	1,54±0,08**	1,44±0,09	n.m.	1,55±0,11**	1,43±0,08	n.m.
Figure 1: B-B1 	1,91±0,02*	1,80±0,02	n.m.	1,91±0,03*	1,78±0,01	n.m.

*Results are means ± one SD of 48 examined images. Figure 1:B-B1, *FD values significant different at p<0,001; Figure1: C-C1,**FD values significant different at p<0,05; Figure1: D-D1, statistically not different. n.m. = not measurable with Image-pro plus 4.1. Images of Figure 1 are adequately explained in the Material and Methods section.*

were analysed for parametric statistics (ANOVA and T-test) with the Microsoft Excel program.

Results

All the examined profiles of feline immature oocyte cytoplasm displayed a self-similar pattern with fractal properties defined by characteristic FD values which were assessed by three different approaches (table 1). For each set of oocyte images, either with intact COC or without COC as depicted in Figure 1: B-B1, C-C1, D-D1, FD mean values appeared comparable between the two categories, i.e. with or without COC, when estimated using the same fractal analysis program and between methods within either the intact COC group or the denuded COC group (Table 1). However, by measuring the more complex profiles of both oocyte categories, i.e. when the entire cytoplasm of oocytes with or without COC was analysed by means of FANAL ++ and Benoit 1.3 [Figure 1: B-B1], FD absolute mean values were found to be extremely elevated for FANAL ++ and statistically different from Benoit 1.3 at *p< 0.001*, namely FD 1.91±0.02 vs.1.80±0.02 and 1.91±0.03 vs. 1.78±0.01 respectively (table 1). Mean FD values of the images reported in Figure 1: C-C1 were also found to be statistically different, even at *p<0.05* for the two fractal analysis methods. Unexpectedly, the mean FD values of external profiles of oocytes with COC and without COC (Figure 1:D-D1) obtained by Image-pro plus 4.1, were found to be consistently lower, due to their smoother appearance, but statistically not different from those obtained with FANAL ++ and Benoit 1.3 programs.

Discussion

We have developed a new morphometric strategy that uses grey level thresholding segmentation and fractal methodology to 1) verify the theoretical assumption that the oocyte cytoplasm morphology possesses a self-similar fractal behaviour, 2) quantitatively describe segmented features of immature feline oocytes by fractal analysis and assess the respective fractal dimension [FD], and 3) provide a critical comparison of data obtained by three different methods. In this context, the FANAL++ program was considered the method of reference because it enabled us to calculate the fractal interval, also called *scaling window,* in which real fractal behaviour of a biological structure exists, i.e. where the data can be represented adequately by a straight line (Dollinger *et al.*, 1995). Specific fractal dimensions obtained with the FANAL++ program showed that immature feline oocyte cytoplasms shared statistically scale-invariant proper-

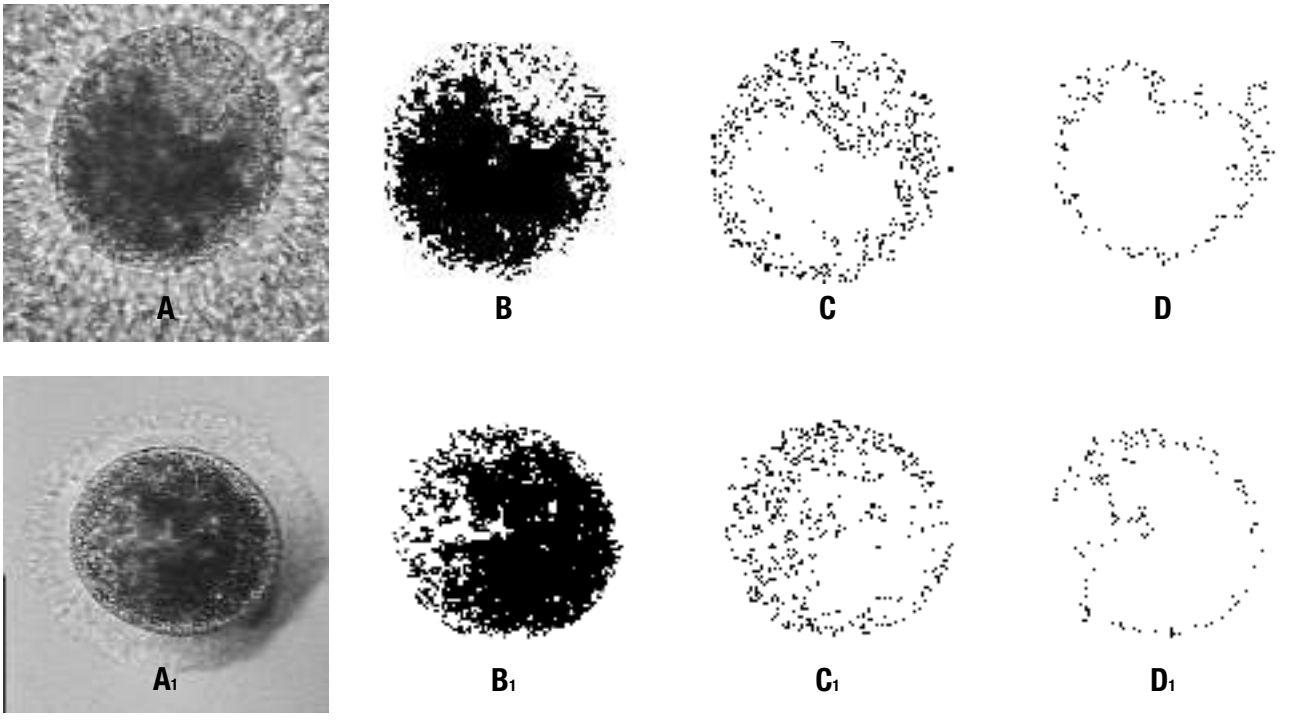

Figure 1. (A-A1) Oocytes with intact COC and successively denuded of COC using finely-drawn glass capillary pipette. A binary image was obtained by grey tresholding the area occupied by the grey-dark oocyte cytoplasm (B-B1); two different outlines were extracted from this binary image by applying a Roberts filter: one pertained to the internal texture of the grey-dark cytoplasm area as well as to the scattered grey-dark particles within the cytoplasm (C-C1), while the other referred to the external profile only of the grey-dark cytoplasm (D-D1). Original images of each oocyte were acquired using a 40x objective lens.

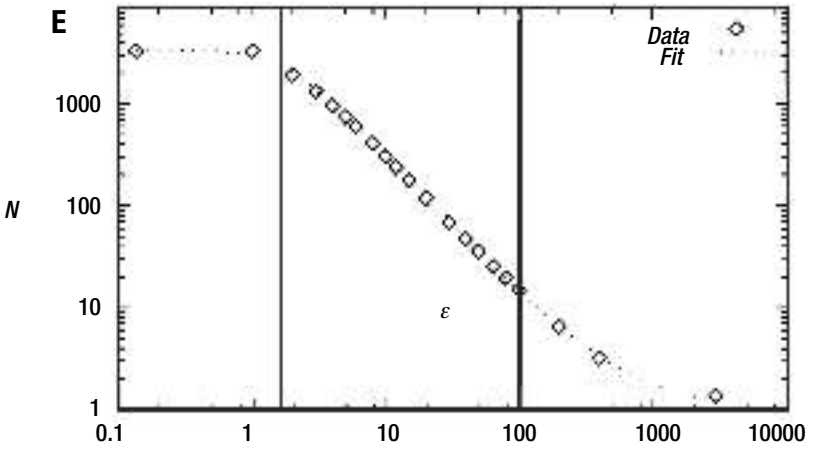

Figure 1 E. 1 FANAL ++ calculation of the fractal dimension. The fractal region is defined by upper and lower limits (vertical lines) on the straight middle part of the bi-asymptotic curve. The scale is in pixels (1pixel = 0,63 μm).

ties within a scaling domain [ε_2 / ε_1] covering a factor of two orders of magnitude, which is a conventional requisite for defining fractal structures (Nonnenmacher, 1994; Nonnenmacher et. al, 1994). For all the distinct cytoplasm profiles examined, the data obtained with the other methods appeared different from those obtained with FANAL++. The reason is that Benoit 1.3 and Image pro plus 4.1 programs, unlike FANAL ++, both lack the theoretical foundation focusing on scaling behaviour, which prevents the identification of the appropriate scaling region that contains fractal-like features of cells and tissues, namely self-similarity and scale invariance for a defined region, being taken into account (Losa & Nonnenmacher, 1996). Two other important observations deserve to be mentioned here: first, the analysis of different cytoplasm profiles revealed increasing FD values in

relation to the increasing complexity of their features, which actually occurred by passing from the simplest external outline (Figure 1: D-D_1) to the most complex grey-dark profile of the oocyte cytoplasm (Figure 1:B-B_1). Second, the data obtained with FANAL ++ concerning cytoplasm features of increasing complexity showed a higher statistical significance with respect to the data obtained with the other two methods (Table 1). Therefore, high FD values underlined the reliability of FANAL ++ for measuring highly complex and irregular cell structures, such as grey dark cytoplasm profiles of feline oocytes, without introducing any morphologic approximation or simplification, which is unavoidable when Euclidean morphometric methods are used. Cytoplasm profiles of feline oocytes are curve lengths (Mandelbrot, 1967) related to the surfaces from which they were obtained by binary segmentation of microscopic views from ex vivo preparations, rather than from histological sectioning. Their relative FD findings, however, could not be critically compared, because of the absence of experimental results for feline oocytes and, therefore, should be qualified in the light of those FDs obtained in other biological systems. Actually, the fractal dimensions reported in this study were compatible with fractal dimensions pertaining to most cell organelles and cell tissues, either in normal physiologic (Paumgartner *et al.*, 1981), pathologic or tumour conditions (Losa *et al.*, 1996; Losa *et al.*,1992; Einstein *et al.*, 1998; Landini *et al.*, 2000; Nielsen *et al.*, 2000; Dioguardi *et al.*,1999). In general, membranes and cytoplasm organelles in a metabolic active state, such as feline oocytes, breast cancer cells triggered by estrogens (Losa *et al.*, 1998) and several other neoplastic tissues (Bianciardi *et al.*, 2002; De Vico *et al.*, 2002), with the exclusion of leukemic cells (Losa *et al.*, 1992), shared FDs higher than those found in subcellular components of differentiated or quiescent elements, in non neoplastic tissues or in cells prone to apoptosis (Castelli *et al.*, 2001). Taken together, our findings further confirmed previous results; namely, that fractal structures observed in human and animals are self-similar only within a limited range of scale lengths to be experimentally defined. On the bases of these premises the fractal analysis performed with the FANAL ++ program has enabled us to unravel the morphologic richness and the structural irregularity of immature oocyte cytoplasm. It has also provided quantitative information useful for an albeit partial description of the real morphology and for an objective method allowing a reliable oocyte classification. Furthermore, the observation that oocytes with or without COC collected ex vivo after ovariectomy displayed close FD values could be heuristically relevant. The methodology presented here might be of particular value in the case of domestic cats, whose oocytes are currently used as models for non-domestic Felidae to prevent the extinction of endangered species through the selection of oocytes for further IVM/IVF (Luvoni *et al.*, 1993).

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