ORIGINAL RESEARCH

Tissue Dimensions Affect Follicle Survival and the Development of *in Vitro* Human Ovarian Culture

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Abstract

Objective: To determine the relationship between ovarian cortical tissue dimensions and the growth and survival of human primordial follicles *in vitro*.

Design: Human study.

Setting: Research laboratory of university hospital.

Patient(s): Women undergoing benign gynaecological procedures.

Intervention(s): Fresh ovarian cortical tissues were removed and cut into 32 smaller pieces of various sizes/shapes and 22 were cultured in serum-free medium for 7 days.

Main outcome measure(s): Follicle viability, density and growth potential assessed by histology.

Result(s): Strong correlations were observed between the density-weighted shape descriptor and the reciprocals of viability and growth ratio.

Conclusion(s): The correlation between follicular surface area and viability/development suggests the use of the smallest practical size as the most efficient way to maximise follicular viability and growth potential. Our mathematical model may also provide an analytical technique for future research relating to *in vitro* tissue culture.

Keywords: primordial follicle, tissue dimension*, in vitro* culture, ovarian cortical tissue

Introduction

The risk of premature ovarian failure following chemo- or radiotherapy is an increasingly recognised complication of cancer treatment. With improving long-term survival after treatment, the new challenge facing cancer survivors, particularly those of prepubertal age is that of preserving their future fertility. Cryopreservation of ovarian tissue followed by auto-transplantation is a potential option and has resulted in pregnancies and live births in humans.[1,2] However, complications associated with immunosuppressants in heterologus transplantation and the ethical and safety issues of potentially reintroducing malignant cells restrict the clinical potential of transplantation.[3,4] Advances in the tissue/follicle culture systems for growing and maturing the abundant population of primordial follicles to maturity *in vitro* have been gathering pace in recent years.[5] Using such culture systems, offspring have been produced in mice models from oocytes developed *in vitro* from primordial follicles.[6,7] Feasibility of this approach in human has been demonstrated by the development of antral follicles from primordial/primary stages using a two-step culture system.[8]

The protracted growth span from primordial to Graafian follicle stage *in vivo*[9] suggests that an extended culture period for primordial follicles growth would be needed *in vitro* for developmental competence. Thus primordial follicle survival in current *in vitro* culture system poses a major challenge for success in human model.[10]

The success of *in vitro* growth (IVG) and *in vitro* maturation (IVM) systems are influenced by the nature of the tissue and the culture environment.[10] A variety of factors including base media, culture supplements, growth factors and duration of exposure have been examined.[10] However, little is known about the optimal ovarian tissue dimension that supports primordial follicle growth *in vitro*.

Earlier studies using *in vitro* culture of whole ovaries[11] was unsuccessful because of its inability to maintain the cell survival in the inner medulla region due to anoxia, depletion of nutrients and accumulation of metabolites leading to necrosis. Attempts at culturing isolated primordial follicles *in vitro* have also been unsuccessful.[12] Follicles rapidly lose their three dimensional structure[13,14] and degenerate after 24 hour. Improved culture success in the presence of stromal cells suggests that the lack of effective communication from the extra-follicular cortical cells contributes to the demise of isolated primordial follicles *in vitro*.[15]

To overcome these problems, the use of ovarian cortical fragments or slices has been adopted. This technique avoids the damage caused by the follicle harvesting procedure and minimises necrosis through increasing the surface area of the tissue for gaseous and nutrient/waste exchange. More importantly it provides a complex support system resembling the *in vivo* environment for primordial follicles allowing them to remain in "communication" with the surrounding stromal cells.[10] This approach allows follicles to remain viable and morphologically normal for up to three weeks of culture.[16,17]

The evidence highlights the importance of structural support in early follicular survival and growth. Both slices and cubes of human cortical tissue have been used in previous culture systems,[8,17–20] however no systematic study has been performed to examine the relationships between different tissue dimension markers and the follicle viability and development.

This study aims to examine the relationship between ovarian cortical tissue dimensions and follicle viability *in vitro*, with a view to maximise the reproductive potential of ovarian tissue.

Materials and Methods
Patients
Ovarian cortical tissues were collected from two women aged 35 and 39 years undergoing bilateral oophorectomies for benign gynaecological procedures at the Edinburgh Royal Infirmary. The study was approved by the local ethics committee and informed written consent was obtained from each patient.

Ovarian tissue culture
Ovarian tissue collected during the surgical procedure was transported immediately to the laboratory on gauze soaked in normal saline. The ovarian cortical tissue was striped to 1–2 mm in thickness and further cut into smaller pieces of various sizes (ranging from approximately 1 to 25 mm²) and shapes (cube and rectangular). Note that these designed dimensions

only serve as a guide for cutting tissue samples, the actual measured tissue dimensions were used in the data analysis.

Five pieces of fresh tissue sample from each patient were fixed immediately for histological analysis, whilst the remainder were cultured in pre-warmed 24 well culture dishes (Nunclon, Nunc, Denmark) with inserts (0.4 um Millicell Culture Plate Inserts, Millipore, France) for 7 days. For each tissue sample placed on the insert, 200 ul of culture medium [McCoys 5A modified medium supplemented with Human Serum Albumin (5 mg/ml), Penicillin G/Streptomycin Sulphate (0.1 mg/ml), CGMP (1.1 mg/ml), Fresh Ascorbic Acid (50 ug/ml), L-Glutamine (3 mM), Transferrin (2.5 ug/ml), Selenium (4 ng/ml), Insulin (10 ug/ml) and FSH (0.5I U/ml), all Sigma Chemicals Poole] were added both inside and outside of the insert within each well. Culture plate was then placed in a humidified incubator at 37 °C with 5% CO_2 in air. 200 ul of culture medium within the inserts was removed and replaced with fresh medium every second day.

Fixation and histological analysis

Fresh and cultured tissues were fixed in Bouin's solution for 12 hours before being dehydrated in ethanol (70%, 90% and 100%). Tissue was then submersed in cedar wood oil (BDH Laboratory Supplies, Poole UK) for 24 hours before placed in toluene (Fisher Scientific UK Ltd, Loughborough UK) for 30 minutes. After removing the oil, tissue was further embedded in paraffin wax for 4 hours at 60 °C before serial sections of 6 μm thick were cut and stained with haematoxylin and eosin.

Follicle and tissue dimensions (surface area, volume, density) measurements

Zoom Browser EX (Cannon) software was used to capture images of follicle and section of cortical tissue. ImageJ software (http://rsbweb.nih.gov/ij/) with its integrated measuring tool was used to perform accurate calculation of slice area and circumference after calibration with a stage micrometer.

Morphological analysis of follicles was carried out on every 10th section to avoid double counting and only follicles with the presence of oocyte nucleolus were counted and photographed.

The final count was adjusted using a correction factor. Assuming every ith of k slices ($i \leq k$) is selected for examination and the shape of nucleus is a ball with an average diameter of d and the thickness of a slice is t (6 um), for the nucleus to present in the sample slice, the centre of the nucleus should either be inside the slice or within half of its diameter distance from the edge of the slice. If the centre of a nucleus is inside the k slices, the probability P that it presents in the sampled slice is:

$$P = (d + t)/(k * t)$$

Therefore, the calibrated count is:

$$N_c = N_o/P$$

N_o is the observed count.

For surface area, volume and follicle density calculations, we first used the polygon selections tool in ImageJ to mark the outline of the tissue area, from which the area a and the circumference c is automatically calculated.

For a tissue cutting into n slices of thickness t, its volume is:

$$V = t \sum_{i=1}^{n} a_i$$

And its surface area is:

$$A = t \sum_{i=1}^{n} c_i + a_1 + a_n$$

Here a_1 and a_n are the area of the first and last slices in the series.

Consequently, we calculate the follicle density of a tissue as:

$$FD = N_c/V$$

Morphological criteria for follicle viability and development

Viability of the follicle was classified and grouped using a previously described method:[8] Viable follicle is defined as intact oocyte (regular shape and even cytoplasm) or misshapen oocyte still surrounded by granulosa cells with $\geq 10\%$ pyknotic granulosa cells; Nonviable follicle is described as oocyte dissociated

from granulosa cells or fragmented oocyte with ≥10% pyknotic granulosa cells.

Developmental stages of viable follicles were classified and grouped based on granulosa cell morphology surrounding the follicle: 1) Resting follicles (Primordial and Transitional stage): oocyte surrounded by a partial or complete layer of flattened granulosa cells; 2) Growing follicles (Primary and Secondary stage): oocyte surrounded by one or more complete layer of cuboidal granulosa cells.

Descriptors representing tissue dimensions

The size of a tissue sample is represented by its volume. For shape description, we used a dimension-free descriptor for 3D shapes known as sphericity:[22]

$$S = \frac{P^{\frac{1}{3}}(6V)^{\frac{2}{3}}}{A} \qquad (1)$$

Here V and A are the volume and surface area of a 3D shape. Effectively S is the ratio of the surface area of a sphere to the surface area of a 3D shape with the same volume of the sphere. S has a value range of $0 < S \leq 1$ and has the maximum value of 1 for any sphere (the most compact 3D shape).

Interestingly, description of a shape does not restrict it to the measurement of a single unit. Sphericity can also be defined for a collection of 3D shapes. When a single 3D shape is cut into multiple new shapes, the overall volume remains the same where the sum of surface area of these new shapes will be greater than the surface area of the original shape. Consequently, the sphericity value defined for these new shapes as a whole is smaller than the sphericity of the original shape.

It should be noted that there are also other dimension-free shape descriptors with characteristics similar to sphericity, for example:

$$S_2 = \frac{6\pi^{\frac{1}{2}}V}{A^{\frac{3}{2}}}$$

We have also tested this descriptor and the result is in fact slightly more statistically significant than the result using sphericity. Since the functional form of sphericity is more desirable for our specific modelling

and analysis, we will only present in detail the results based on sphericity.

Statistical analysis

Correlation analysis and linear regression (Minitab 15) were used to explore the relationships between various follicle dimension factors and the viability and growth ratio. Normality test and t-test of two samples assuming uneven variance were also used in the process.

Results

Original data

A total of 1980 follicles from 32 pieces (22 cultured pieces) of human ovarian cortical tissue were analysed in this study. The proportion of viable follicles in the uncultured tissues was 97% (SEM = 0.63). The proportion of growing follicles (primary and secondary stage) is significantly increased in all cultured tissue after seven days (79.31%) compared with uncultured fresh tissue (18.75%) ($P < 0.001$, see Table 1).

Figure 1 demonstrates the typical histological images of follicles in both uncultured and cultured tissue.

Original data of the measured volume (V), surface area (A), follicle counts [total (Fc), live (Flc) and growing (Fgc)] and the calculated shape factor—sphericity (S) for each tissue are shown in Table 2. Here the total follicles refers to both live and dead follicles in a particular tissue sample; Live follicles refers to the number of live follicles (both undeveloped: primordial and transitional as well as developed: primary and secondary follicles); Growing follicle count refers to the number of live follicles at primary and secondary stage in a particular tissue sample.

Table 1. The proportion (mean + SEM) of viable follicles at different stages of development (primordial to secondary) in both pre and post-cultured tissue are presented in this table.

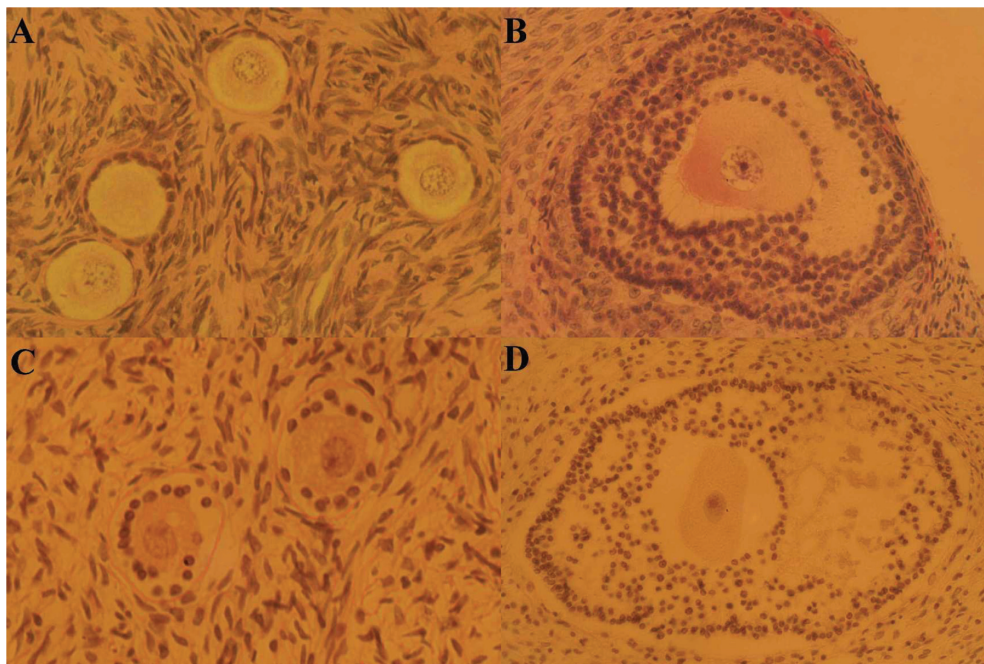|	Resting follicles (%) (primordial and transitional stage)	Growing follicles (%) (primary and secondary stage)
Uncultured tissues	81.25 + 1.87	18.75 + 1.87
Culture tissues	20.69 + 2.57	79.31 + 2.57

Figure 1. Follicles photographed under 400× magnification using a light microscope. Uncultured fresh ovarian tissue with primordial follicles (**A**) and antral follicle (**B**). Primordial follicles (**C**) and antral follicle (**D**) after 7 days of culture.

Data transformation and the result of the initial analysis

Data transformation

Below are the definitions of some derived variables used in our analysis:

Follicle density (***FD***) = ***Flc***/***V***; Follicle viability ratio (***FV***) = ***Flc***/***Fc***; Follicle growth ratio (***GR***) = ***Fgc***/***Fc***; Surface area per follicle (***AF***) = ***A***/***Fc***.

As both ***FV*** and ***GR*** are percentage data, they need to be transformed to improve normality. Conventionally *arcsine* transformation is used for this type of data. However, in our case we have found *reciprocal* transformation yields better results. The two variables created by the transformation are $FV^{-1} = 1/FV$ and $GR^{-1} = 1/GR$.

No significant correlation between *V*, *S* and *FV*⁻¹, *GR*⁻¹

After data transformation, correlation analysis was applied and no significant correlation was detected between dimension descriptors (***V*** and ***S***) and FV^{-1} or GR^{-1}.

Analysis post density calibration

A density-weighted shape descriptor

One factor that has not been considered in our analysis is the well known large variations in follicle density (***FD***) among tissue samples which potentially may have significant impact on the relationships between tissue size/shape and the follicle viability/growth ratio. To address this issue, we used the follicle density as a weight and designed a new descriptor ***S'*** as:

$$S' = S * FD^{d}$$

Here ***d*** is a real number that allows us to adjust the intensity of the weight factor. Currently we simply use ***d*** = 1. From this definition, we may make the following derivation:

$$AF = \frac{A}{Fc} = \frac{1}{S'}\left(\frac{\pi}{V}\right)^{\frac{1}{3}} = \frac{1}{S * FD}\left(\frac{\pi}{V}\right)^{\frac{1}{3}}$$

which demonstrates the relation between the surface area per follicle (*AF*) and other indicators.

Strong correlation between *S'* and *FV*⁻¹/*GR*⁻¹

Strong correlations were detected between ***S'*** and FV^{-1}, and between ***S'*** and GR^{-1}, with a correlation coefficient of 0.672 and 0.806 respectively (see Fig. 2), both are significant at α = 0.001 for *d.f.* = 20.

Table 2. Tissue dimensions and follicle counts (adjusted and rounded).

Tissue	Patient	Volume (mm³)	Surface area (mm²)	Sphericity	Total follicle count	Live follicle count	Growing follicle count
1	0	24.057	47.786	0.8434	73	39	36
2	0	6.3767	23.675	0.7024	17	14	11
3	0	18.838	39.254	0.8722	151	109	84
4	0	16.492	46.038	0.6806	117	89	64
5	0	9.8707	22.514	0.9884	142	100	56
6	0	8.059	16.951	1.00	117	47	36
7	1	16.3547	37.882	0.8225	53	39	36
8	1	20.507	43.444	0.8340	67	56	42
9	1	21.839	48.871	0.7731	47	39	28
10	1	22.942	48.949	0.7977	31	28	17
11	1	13.232	36.861	0.7340	45	36	25
12	1	14.74	38.226	0.7605	8	8	8
13	1	17.096	45.519	0.7051	22	20	20
14	1	17.104	42.338	0.7583	89	78	59
15	1	12.125	37.207	0.7763	39	28	17
16	1	12.923	42.834	0.6217	50	39	33
17	1	17.414	52.15	0.6230	17	14	11
18	1	16.251	45.369	0.6839	70	59	53
19	1	19.477	40.363	0.8673	28	20	17
20	1	11.862	33.976	0.7403	20	14	11
21	1	10.274	31.298	0.7302	87	56	45
22	1	34.027	70.544	0.7198	20	20	17

Regression models for FV^{-1}/GR^{-1} using S' as the independent variable

Two regression models were built using S' as the independent variable to describe the relationship between S' and GR^{-1}, and between S' and FV^{-1}.

$$\text{Model 1: } GR^{-1} = a + b * S'$$
$$\text{Model 2: } FV^{-1} = c + d * S'$$

Prior to the regression analysis, normality tests on the patient-specific subsets of GR^{-1} and FV^{-1} were carried out and normality assumptions were not rejected at $\alpha = 0.05$. Subsequently t-test for equal mean was performed and for both GR^{-1} and FV^{-1} the hypothesis was not rejected at significance level of 0.05.

The regression equation for model 1 ($P < 1\text{E-}12$ for intercept and $<6.1\text{E-}06$ for variable, $R^2 = 0.6489$) is:

$$GR^{-1} = 1.3707 + 0.1015 * S'$$

And the equation for model 2 ($P < 4.6\text{E-}13$ for intercept and $<6.2\text{E-}4$ for variable, $R^2 = 0.4516$) is:

$$FV^{-1} = 1.1467 + 0.0543 * S'$$

Interpretation of the regression model

The significance of the regression results allows us to perform further inference on the model. Here model 1 is used as an example.

$$GR^{-1} = a + b * S' \Rightarrow$$
$$GR = \frac{1}{a + b * S * FD} = \frac{1}{a + b * \left(\frac{\pi}{V}\right)^{\frac{1}{3}} * \left(\frac{A}{Fc}\right)^{-1}}$$
$$= \frac{1}{a + b * \left(\frac{\pi}{V}\right)^{\frac{1}{3}} * AF^{-1}} \tag{2}$$

Two observations may be made from equation 2. First, for the same follicle density (**FD**), the growth ratio (**GR**) increases when S decreases (ie, the geometric shape is less compact, less like a sphere). Second, for the same tissue volume (**V**), if the surface area per follicle (**AF**) increases, the growth ratio (**GR**) increases as well.

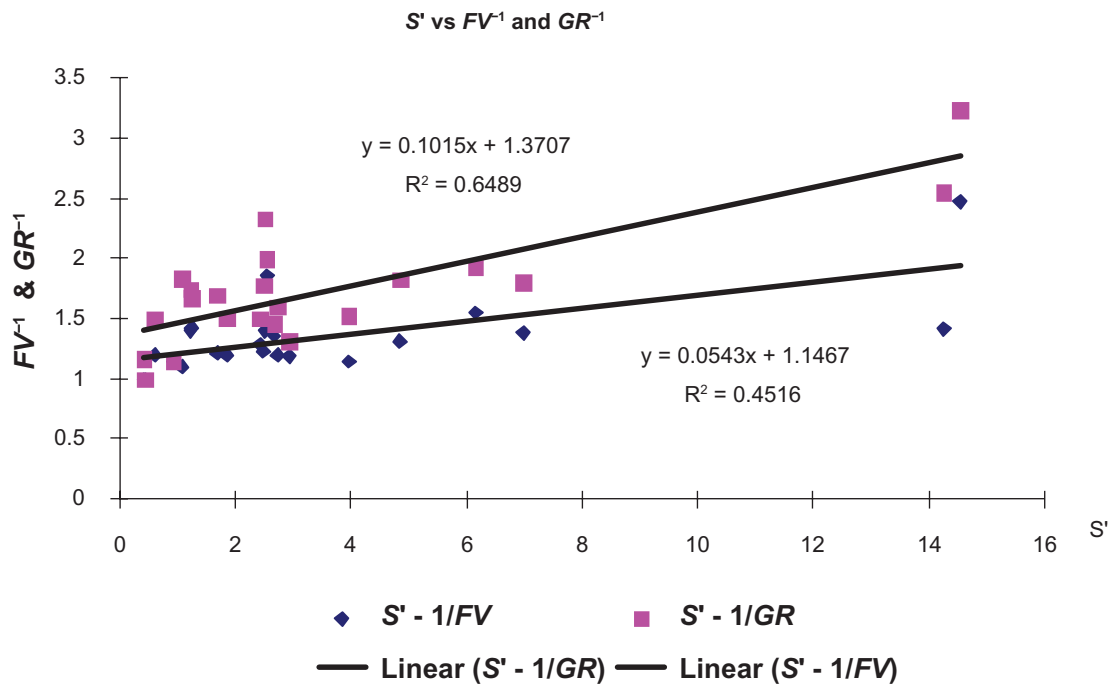These observations also apply to the follicle viability (Model 2).

Figure 2. Strong correlation between density-weighted tissue shape factor (S') and the reciprocals of viability and growth ratio.

We may thus conclude, the key to increase the viability and growth ratio is to reduce the shape factor (S) of the tissues. Furthermore, the reduction of S can be further interpreted as to increase the surface area per follicle. From the definition of S, the simplest solution to reduce S is to cut a tissue into the smallest possible size. Note that this conclusion has been drawn solely on the basis of the experimental conditions and outputs of this study. Other factors potentially restricting the applicability of this solution are beyond the scope of our experiment.

Discussion

A balanced biochemical and physical component of the ovarian tissue is central to its optimal development in the *in vitro* culture. The complex structural requirement and its adaptation during growth hold an important key in controlling this balance.[22] This forms the theoretical basis of the current multistep ovarian tissue culture system for primordial follicles *in vitro*.

This is the first study to systematically examine the role of tissue dimension in the survival and development of primordial follicle *in vitro*, in a quest to find supportive evidence for the current culture model.

After using the density-weighted shape descriptor we developed to address the large follicle density variation between tissues, a strong correlation is found between both follicle viability and growth ratio and surface area per follicle. Our result agrees with findings from a previous study using tissues of two different sizes and shapes (1 mm³ cubes verses 1–2 by 5–8 mm slices) and found improved follicle viability and growth ratio in the smaller cubes compared to the larger slices.[19]

In our cultured tissue, a strong positive association between the surface areas per follicle and the follicle viability suggest an increased surface area for gaseous and nutrient/waste exchange per follicle unit is a likely explanation for improved survival in primordial follicles. This implies that the nutritional demands for the follicular unit are different to the surrounding stromal cells and the ovarian follicle is more sensitive to impoverished culture conditions than previously believed. In addition, atretogenic/apoptosis signals (eg, TGFβ, IL-6, androgens, reactive oxygen species, bax, Fas antigens, p53, TNF and caspases)[23] originating from neighbouring cells may be weakened with the increase of distance and therefore contribute to the improved follicular survival.

The positive correlation between the surface area per follicle and follicle growth ratio concurs with the frequent *in vivo* observations of growing follicle migrating from compact cortical region to the less follicle dense cortical/medullary border.[24,25] It suggests that the benefit of the medullary environment for developing follicles is not restricted to its ability to meet demands of a raising metabolic requirement and growing gonadotrophic dependence by its increased blood supply. Moreover, it is likely that the widened space between follicles in the medullary layer plays an important role in the initiation of primordial follicles by reducing the inhibitory signals reaching the index follicle. An example of such inhibitory factor is the Anti Mullerian Hormone[26] produced by granulose cells of surrounding developing follicles.[27,28]

These findings all serves as further evidence to support the current multistep culture strategy,[8,10,29] isolating growing follicles out of compacted cortical tissue after gaining growth potential from the initial period of tissue culture, and thus to ensure adequate nutrient and oxygen supply meeting the change in demand.[18]

Our proposed mathematical model derived from the data enables us to have a clearer understanding of the relationships between primordial follicle viability/development and tissue dimensions. The density weighted shape descriptors (and its variations) may also be a useful analytical tool for future research concerning tissues with uneven cellular density, especially when the sample size is relatively small.

As this study did not examine tissue volume of less than 1 mm^3, we are unable to comment if the current model could be extrapolate to tissues with a lesser volume. Previous studies of culturing mechanical or enzymatic isolated primordial follicles[12–14] indicates the use of very small dimensions in cell culture results in poor follicular viability, which suggests there is a lower threshold to the tissue dimension beyond which the viability will deteriorate. Further study is required to locate this threshold.

In conclusion, based on our results we suggest tissue dimensions within the cortical strips play an important role in maintaining survival and the initiation of primordial follicles growth of human *in vitro* culture system. For culturing primordial follicles *in vitro*, we recommend for any given size, ovarian biopsy tissue should be cut up into the smallest practical size (0.5 to 1 mm) to achieve maximum overall follicular viability and growth potential.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

Investigations on Human Subjects

This study was approved by the local ethics committee.

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