Ovarian Tissue Culture for Fertility Preservation in Male Transgender Patients After Hormonal Treatment

Alessandra Leal Bottini
Programa de Pós-Graduação em Ginecologia e Obstetrícia, Universidade Federal do Rio Grande do Sul
Vânia Marisia Fortes dos Reis
Programa de Pós-Graduação em Ginecologia e Obstetrícia, Universidade Federal do Rio Grande do Sul
Edison Capp (edcapp@gmail.com)
Programa de Pós-Graduação em Ginecologia e Obstetrícia, Universidade Federal do Rio Grande do Sul
https://orcid.org/0000-0002-1039-7940
Ilma Simoni Brum da Silva
Programa de Pós-Graduação em Ginecologia e Obstetrícia, Universidade Federal do Rio Grande do Sul
https://orcid.org/0000-0002-7428-825X
Lúcia Maria Lúcia Maria Kliemann
Programa de Pós-Graduação em Ginecologia e Obstetrícia, Universidade Federal do Rio Grande do Sul
Helena von Eye Corleta
Programa de Pós-Graduação em Ginecologia e Obstetrícia, Universidade Federal do Rio Grande do Sul

Research Article

Keywords: Ovary, Ovarian reserve, Fertility preservation, Tissue culture techniques, Sex reassignment surgery.

Posted Date: April 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-374230/v1

Abstract

The aim of this study was to evaluate the reproductive and histological characteristics of fresh cultured ovarian tissue from transgender male patients.

Methods: *In vitro* experimental study. Samples were collected during sex reassignment surgery for male transgender patients. Ovarian cortex was cut into fragments of 2, 3 and 4 mm and placed in a 96-well plate suitable for cultivation at days zero, 2, 4, 6 and 8, when the histology was analyzed.

Results: Stromal hyperplasia was observed in all samples. Presence of stromal hyperplasia was not associated with obtaining primordial or primary follicles. Peripheral reduction in cells number was also a recurrent finding. Primordial and primary follicles were identified with a heterogeneous pattern between fragments from the same patient and between different patients, and follicles in more advanced stages of development (secondary and antral) were not found. There was an association between the diameter of the ovarian fragments and the identification of primary follicles (p=0.036). The number of days in culture was associated with histological signs of tissue suffering in the fragments (p=0.002). The total number of follicles identified in the 2 mm diameter samples was significantly lower than in the 4 mm diameter samples (p=0.031).

Conclusion: Even after prolonged exposure to testosterone, ovaries presented primordial and primary follicles, maintaining viability over the days exposed to the culture. Follicles at more advanced stages of development were not identified. These findings suggest that in female to male transgender patients reproductive potential may be preserved to the time of reassignment surgery.

Introduction

The ovaries have a defined number of primordial follicles at birth, which constitute the ovarian reserve. The activation of these follicles is responsible for the irreversible decline in reproductive function over the years [1], the rate of this reduction being variable among women [2].

One of the major causes of potentially avoidable ovarian reserve reduction is the use of gonadotoxic drugs used in the treatment of neoplasms. These drugs can trigger exacerbated follicular activation/inhibition leading to accelerated and premature depletion of the follicular reserve. Thus, preventing ovarian dysfunction induced by chemotherapy would be important to preserve the possibilities of natural or medically assisted conception after treatments [3].

The methods currently used to preserve the fertility of patients undergoing cytotoxic and hormonal treatments are the cryopreservation of embryos, oocytes and ovarian tissue. However, the available techniques have an indication limited to the patient's age and clinical status [4, 5].

The development of culture conditions for immature germ cells is one of the biggest challenges in reproductive medicine, with the objective of obtaining competent oocytes. Complete *in vitro* growth of

primordial follicles with subsequent *in vitro* fertilization, followed by live embryo transfer, has been achieved, so far, only in mice [6].

In view of the complex regulatory system of follicular development, the challenge remains to create increasingly elaborate and complex culture systems in order to promote an environment similar to the ovary *in vivo* and thus, through cryopreserved ovarian tissue fragments, to support follicular growth. The impossibility of access to human ovarian tissue, free of diseases, of young women - due to ethical aspects - results in the difficulty of establishing an efficient *in vitro* ovarian culture.

The sex reassignment surgery for male transgenders, which includes the excision of the ovaries, allows the gonads to be used for study purposes. Testosterone therapy used for long periods by these patients leads to reversible amenorrhea, with preserved ovarian follicles, without depleting or affecting the development of primordial follicles [7].

Therefore, due to the limited amount of human tissue available for clinical use and research, as well as reduced numbers of follicles in samples obtained from older patients or with ovarian disease, the ovaries of male transgender patients are an opportunity to study this noble organ in research. The aim of this study was to evaluate the reproductive and histological characteristics of fresh an cultured ovarian tissue from transgender male patients.

Methods

Study Design

An experimental *in vitro* study was performed. The experiments were carried out in the Endocrine and Tumoral Molecular Biology Laboratories, installed in the Department of Physiology at UFRGS and in Experimental Pathology, at the Experimental Research Center of Hospital de Clínicas de Porto Alegre (HCPA). This project was supported by Fundo de Incentivo à Pesquisa e Eventos - HCPA (FIPE-HCPA #2018-0462).

Sample of ovarian tissue

The samples were collected from the ovarian cortex of patients from the Gender Identity Program (Programa de Identidade de Gênero - PROTIG), Hospital de Clínicas de Porto Alegre (HCPA), who underwent sexual reassignment surgery, with indication independent of this study. The criteria for inclusion in the study were patients who underwent pan-hysterectomy, aged between 20 and 45 years old and who did not present ovarian neoplasia. The sample size was determined by convenience.

After obtaining fragments of the ovarian cortex, the specimens were sent for anatomopathological analysis. The samples were identified and transported refrigerated to the laboratory in a medium composed of Hank's salt solution (Gibco BRL Grand Island, N.Y, USA) and 1% kanamycin. In a laminar flow hood, excess blood was removed, the samples were cut into fragments of 2, 3 and 4 mm of diameter with a disposable biopsy punch (Kolplast, Brazil). After cutting, the samples were plated.

In vitro ovarian fragments culture

The ovarian cortex fragments were placed in a 96-well plate. Each well was filled with 200µL of DMEM medium (Gibco BRL Grand Island, NY, USA) supplemented with 1% antibiotic (streptomycin), 25 mIU/mL recombinant FSH (GONAL-f®) and 5% fetal bovine serum (SBF - Gibco BRL Grand Island, NY, USA). The tissue fragments were grown at 37°C in a humidified incubator, with constant injection of 5% CO_2 for 2, 4, 6 and 8 days. The culture medium was changed every 48 hours and the fragments were observed everyday under an inverted microscope. After 2, 4, 6 or 8 days of culture, the fragments were fixed in 10% buffered formaldehyde.

Slide preparation and histological analysis

The formaldehyde fixed samples were sent to HCPA Experimental Pathology Unit where they were embedded in paraffin and cut into 5 µm series sections for histological analysis. The slides were stained with hematoxylin/eosin.

To limit the effect of heterogeneous follicular distribution within the samples of the ovarian cortex submitted to culture, random sections were performed per piece of ovary, with the purpose of covering the entire fragment. The slides were evaluated under an optical microscope by an experienced pathologist.

A primordial follicle was defined as the presence of an oocyte surrounded by a layer of spindle-shaped granulosa cells and a primary follicle is an oocyte surrounded by cuboidal granulosa cells. Secondary follicles are characterized by an oocyte that is completely surrounded by a pellucid zone and the presence of at least two layers of granulosa cells. Antral follicles are defined by the presence of an antral cavity [8].

Statistical analysis

The data were entered twice, revised and analyzed using the SPSS program, version 18.0 [SPSS Inc. Launched in 2009. PASW Statistics for Windows, version 18.0. Chicago: SPSS Inc.]. Qualitative variables were described as absolute (n) and relative (n%) frequencies. Fisher's exact test was applied and Yates's correction for continuity was used when indicated. Quantitative variables were expressed as medians, as distributed by the Shapiro Wilk normality test; so, the Kruskal-Wallis test and Dunn-Bonferroni *post hoc* test were applied. For all analyzes, the significance level was set at 5%.

Ethical aspects and biosafety

An informed consent form (ICF) was applied to authorize the use of ovarian fragments in the present study. Patients were informed about the research and invited to participate by signing the ICF, knowing that they could withdraw at any time.

After the experiments, the residues were packed in white bags, closed, sealed and identified with a biological residue label with all the required information and delivered to the competent collection service

of the institution. Phenol residues were treated as chemical waste and collected by the collection service based at the Chemistry Institute of UFRGS.

Results

Characterization of patients

The ovarian fragments studied were obtained at the time of sexual reassignment surgery (pan-hysterectomy). The age of patients ranged from 25 to 34 years old, all of whom had used testosterone before surgery. The onset age of using the hormone, the duration and time between suspension and surgery are shown in table 1. In the macroscopic analysis, 3 of the 4 ovaries had cystic follicles.

Histological analysis of ovarian tissue culture

The histology of the ovarian fragments was analyzed at zero, 2, 4, 6 and 8 days of culture. Stromal hyperplasia was observed in all samples, regardless of the culture day (Figure 1). Peripheral reduction in cells number was also a recurrent finding, related to the advancing days of culture. Primordial and primary follicles were identified with a heterogeneous distribution pattern between fragments from the same patient and between different patients, and follicles in more advanced stages of development (secondary and antral) were not found. In all the ovarian cortex fragments analyzed, the total number of primordial and primary follicles identified was 267 and 224, respectively.

The total number of follicles found per patient according to the culture day is shown in table 2. There was no association between the culture day and the number of follicles found.

There was an association between the diameter of the ovarian fragments and the identification of primary follicles. Fragments with a diameter of 3 mm or more showed significantly more follicles ($p=0.036$), which was not found in relation to primordial follicles (Table 3). The presence of stromal hyperplasia was not associated with obtaining primordial or primary follicles ($p=0.042$), detailed in table 4.

The number of days in culture was not associated with the identification of primordial and primary follicles (Table 5). However, it was associated with histological signs of tissue suffering in the fragments ($p=0.002$) (Table 6). The total number of follicles identified in the 2 mm diameter samples was significantly less than in the 4 mm diameter samples ($p=0.031$, Kruskal-Wallis test, Dunn-Bonferroni's *post hoc*, normality was tested by the Shapiro-Wilk test). However, when relating the 3 mm diameter samples to the 2 mm or 4 mm samples, no difference was found (Figure 2).

Discussion

The establishment of human ovarian tissue culture techniques *in vitro* would enable a better understanding of the mechanisms involved in follicular growth and, consequently, the expansion of

ovarian preservation methods. Ethical issues minimize access to human ovarian tissue for research, with consequent difficulty in standardizing and developing protocols for culturing this tissue. Transgender male patients, at the time of the sex reassignment surgery, which includes the excision of the ovaries, make it possible to obtain young human ovarian tissue without pathologies.

All patients undergoing this surgery undergo hormonal therapy with testosterone, as part of the treatment of adaptation to the identified gender. In this study, even after prolonged exposure to testosterone, the ovaries had viable primordial and primary follicles, maintaining viability over the days exposed to the culture. Broecke *et al.* (2001) proposed that the ovarian cortex of these patients could be used experimentally to obtain primary follicles, and from these, in xenograft models (mice), to reach more advanced stages of growth. However, obtaining antral follicles was not possible [7].

The heterogeneous distribution of follicles in the human ovarian cortex found in this sample was also reported by Schmidt *et al.* (2003), analyzing the cortex of three human ovaries. The primordial follicles showed a density variation of more than two orders of magnitude in random pieces of cortical tissue from the same ovary and the developmental stage of the follicles had a heterogeneous distribution [9]. Another important finding to be highlighted is that the cut size of the 4 mm fragment of the ovarian cortex allows the identification of a larger number of follicles than smaller cuts, as well as easier manipulation during plate cultivation, without showing a difference in relation to tissue suffering. It was also possible to identify peripheral hypocellularity, found with the advance of days in culture, which is characteristic of tissue culture, in which cells expand around the fragment and start to adhere to the material in the culture plate.

Stromal hyperplasia, a recurrent observation in the evaluation of fragments, has been described in studies that evaluated the ovarian tissue of patients with a history of hormone therapy with testosterone [10-13]. These authors relate this finding to the androgenic microenvironment, resulting from previous exposure to testosterone, and this association is due to the fact that this process is frequently observed in the ovaries of patients with polycystic ovary syndrome (PCOS), with the androgenic microenvironment being one of the foundations pathophysiology of this disease.

The presence of cystic follicles at macroscopy identified in this study is described in the literature in patients with PCOS. A study that evaluated the ovarian histology of 12 male transsexual patients described enlarged and multifollicular ovaries, a consequence of the direct or indirect effect of androgen on the proliferation and growth of stromal ovarian cells. The follicular morphological changes found were attributed to the increased stimuli of growth factors exerted by androgens [14].

Comparative studies, such as that by Pache *et al.* (1991), which included 29 ovaries obtained from 17 transgender patients in amenorrhea after androgenic hormone therapy and 14 control ovaries, showed greater ovarian volume, collagen thickening of the cortex and stromal hyperplasia accompanied by clusters of stromal cells in the ovaries of transgender patients [15]. Another comparative study included ovaries of 19 transgender patients and 12 control patients and found, in the ovaries of transgender

patients, an increase in volume, multiple cystic follicles (89.5%), diffuse ovarian stromal hyperplasia (84.2%), collagenization of the external cortex (68.4%) and luteinization of stromal cells (26.3%) [16].

Obtaining follicles in more advanced stages of development was not possible, nor was there any development of follicles in vitro, probably due to inhibition by testosterone, despite what was previously shown by Van Den Broecke, 2001 [7]. It has been shown that sex reassignment surgery is a unique opportunity to obtain human ovaries for research, with proven follicular and tissue viability in up to 8 days of culture. Further studies aiming at developing cortex culture protocols capable of supporting follicular survival, growth and germ cell maturation in humans are essential.

Declarations

Funding: This research was supported by Fundo de Incentivo à Pesquisa (FIPE), Hospital de Clínicas de Porto Alegre, Brazil. Project number 2018-0462.

Conflict of Interest Statement: None of the authors has any conflicts of interest related to this study, whether financial or of any other nature. None of the authors has any relevant financial or nonfinancial relationships to disclose.

Ethics approval: This project was evaluated and approved by the ethics research committee of the Hospital de Clínicas de Porto Alegre under the number 2018-0462.

Consent to participate: An informed consent form (ICF) was applied to authorize the use of ovarian fragments in the present study. Patients were informed about the research and invited to participate by signing the ICF, knowing that they could withdraw at any time.

Consent for publication: All authors read and approved the final manuscript as submitted.

Availability of data: The data that support the findings of this study are available from the corresponding author, EC, upon reasonable request.

Code availability: Not applicable

Authors' contributions: Helena von Eye Corleta and Alessandra Leal Bottini conceptualized/designed the study. Alessandra Leal Bottini and Vânia Marisia Santos Fortes dos Reis worked on the experiments. Lúcia Maria Kliemann, Helena von Eye Corleta and Alessandra Leal Bottini performed the analysis of the slides. Helena von Eye Corleta, Alessandra Leal Bottini, Vânia Marisia Santos Fortes dos Reis, Lúcia Maria Kliemann, Edison Capp, Ilma Simoni Brum da Silva carried out the analyses, drafted the initial manuscript, critically reviewed and revised the manuscript. All authors read and approved the final manuscript as submitted.

Acknowledgments

This research was supported by Fundo de Incentivo à Pesquisa (FIPE), Hospital de Clínicas de Porto Alegre, Brazil. Project number 2018-0462. Edison Capp is scholarship recipient from CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil.

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Tables

Table 1 - Patient characteristics regarding age and use of transsexual hormone therapy.

Characterization of patients				
	Patient 1	Patient 2	Patient 3	Patient 4
Age (years)	34	25	27	32
Age beginning treatment (years)	31	22	24	29
Duration of use (months)	27	30	22	33
Pre-surgical hormone suspension (months)	1	2	12	3

Table 2 - Total number of follicles per patient according to the culture day (samples in triplicates).

	Number of follicles				
	Patient 1	Patient 2	Patient 3	Patient 4	Total
Day 0	18	4	14	36	72
Day 2	0	19	4	45	68
Day 4	40	8	13	24	85
Day 6	12	4	13	74	103
Day 8	13		5	37	55
Total	83	35	49	216	383

Table 3 - Association between tissue fragment diameter and the presence of primordial and primary follicles in fragments up to 2 and 3 mm or more.

	Tissue diameter		P-value *
	Up to 2 mm	3 mm or more	
Primordial follicle			
No	7 (46.7%)	13 (39.4%)	0.755*
Yes	8 (53.3%)	20 (60.6%)	
Primary follicle			
No	9 (60%)	8 (24.2%)	0.036#
Yes	6 (40%)	25 (75.8%)	

*Fisher exact test. #Yates's correction for continuity. Statistical significance accepted when p-value ≤ 0.05.

Table 4 - Association between primordial and primary follicles and stromal hyperplasia.

	Stromal hyperplasia		P-value *
	Não	Sim	
Primordial follicle			
No	12 (41.4%)	5 (31.2%)	0.541*
Yes	17 (58.6%)	11 (68.8%)	
Primary follicle			
No	6 (20.7%)	8 (50%)	0.090#
Yes	23 (79.3)	8 (50%)	

*Fisher exact test. #Yates's correction for continuity. Statistical significance accepted when p-value ≤ 0.05.

Table 5 - Association between the presence of primordial and primary follicles and the number of days in culture (≤ 4 days or ≥ 6 days).

	Number of days of culture		P-value *
	≤ 4 days	≥ 6 days	
Primordial follicle			
No	13 (44.8%)	7 (36.8%)	0.803#
Yes	16 (55.2%)	12 (63.2%)	
Primary follicle			
No	8 (27.6%)	9 (47.4%)	0.274#
Yes	21 (72.4%)	10 (52.6%)	

#Yates's correction for continuity. Statistical significance accepted when p-value ≤ 0.05.

Table 6 - Association between culture time (≤ 4 days and ≥ 6 days) and histological signs of tissue suffering.

	Number of days of culture		P-value *
	Up to 4 days	6 days or more	
Tissue suffering			
No	20 (74.1%)	5 (26.3%)	0.002*
Yes	7 (25.9%)	14 (73.7%)	

*Fisher exact test. Statistical significance accepted when p-value ≤ 0.05.

Figures

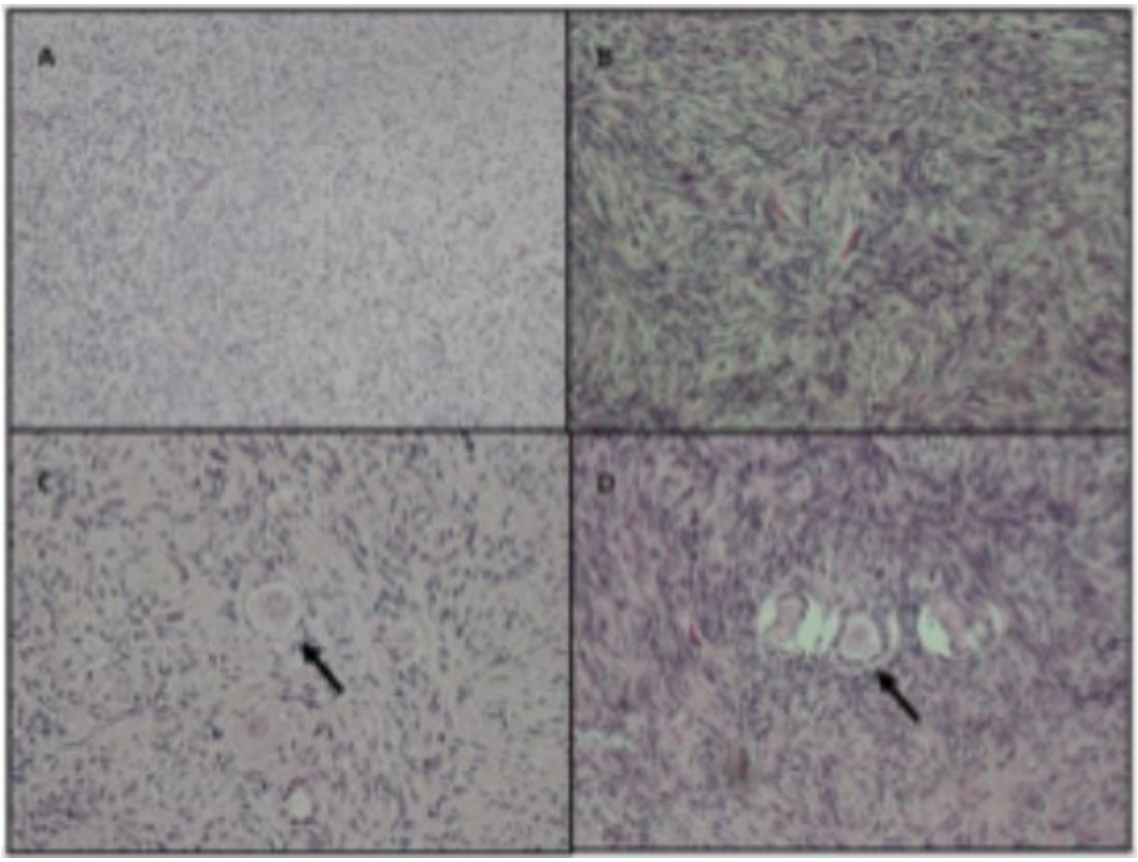

Figure 1

Histological sections of ovaries stained with hematoxylin and eosin (H&E) showing (A) stromal hyperplasia (4x); (B) stromal hyperplasia with the magnification of the objective lens (40x); (C) primordial follicle and (D) primary follicle (40x).

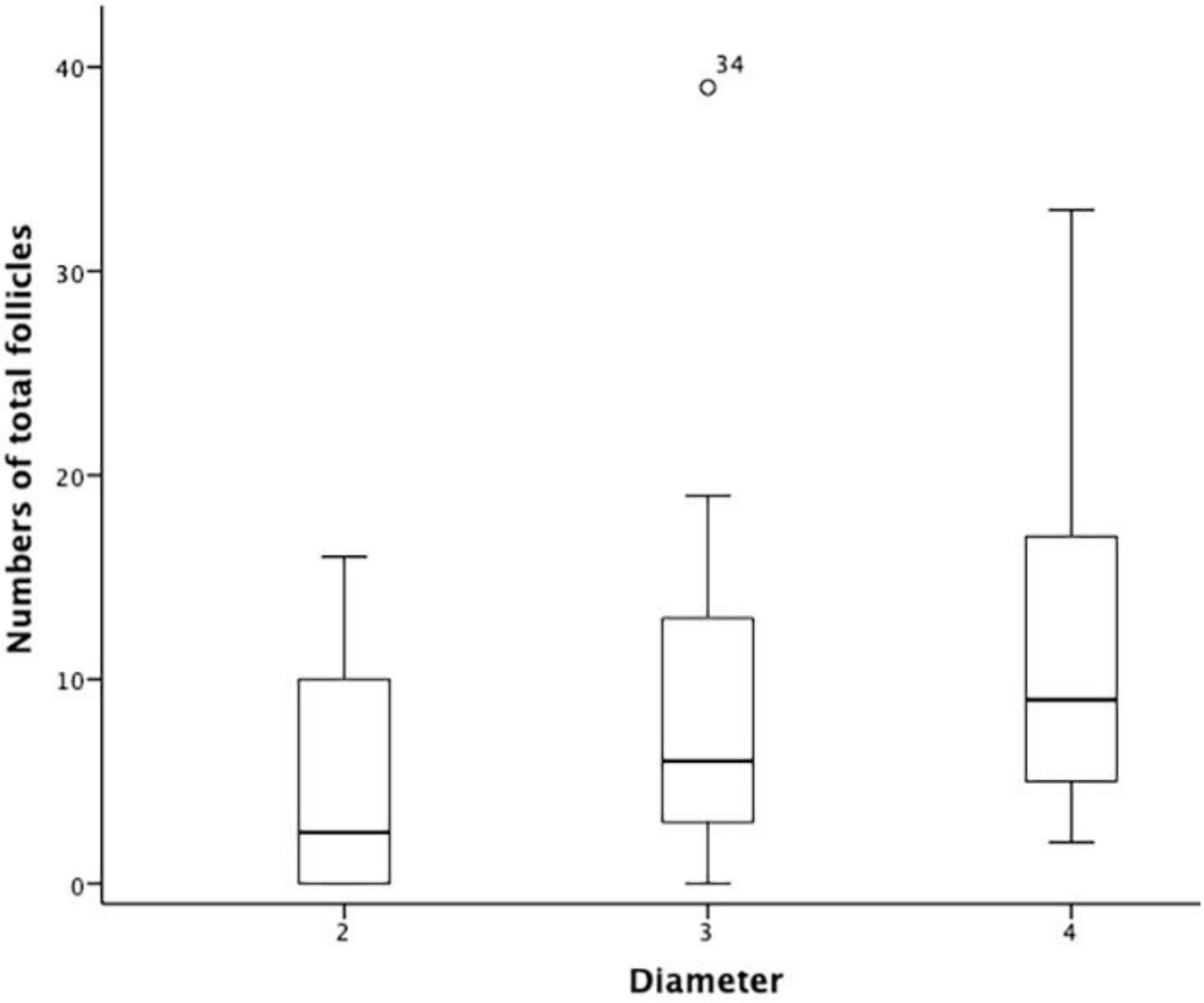

Figure 2

Total number of follicles and 2, 3 and 4 mm in diameter. *2mm different from 4 mm.