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DETERMINATION OF FERTILE AND INFERTILE DAYS OF THE MENSTRUAL CYCLE OF WOMEN BY USING THE SALIVA CRYSTALLIZATION TEST (“LADY TEST” - “GANOP TEST”)

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Introduction

In health institutions, there are numerous possibilities for determination of fertile and infertile days of the menstrual cycle of women. All those methods are ovulation based (Pschyrembel W. 1977). We shall enumerate only some of the methods : measurement of basal temperature, vaginal cytology, cervical changes (external uterine orifice dilatation, the quantity of mucus, distensible quality, crystallization, cell contents), endometrial biopsy, determination of hormones in blood and urine, sonography (development of the follicle and endometrial changes). None of the methods is absolutely reliable in confirming the ovulation. They are based on changes in levels of ovarian hormones, estrogen and progesterone, occurring in the course of the menstrual cycle.

The only direct and absolutely positive methods indicating the existence of ovulation are the pregnancy incepted in the observed cycle or finding of the ovum in genital organs.

All those methods require every day visits to a specialist - gyneacologist in a health institution. The only way a woman can determine the time of ovulation without consulting the doctor is by measuring basal body temperature. Rising of temperature by about 0.5 to 0.6 C^0 results from progesterone effects on thermoregulatory center in hypothalamus. Such a rise in basal temperature occurs in case of ovulation with appearance of corpus luteum and progesterone generation (Mladenovic D. 1973). However, the precise day of ovulation cannot be determined in advance by the measurement of basal temperature. The rise in basal temperature occurs only 1-2 days after ovulation so that, as a result, the existence or non-existence of ovulation may be determined only afterwards by using temperature tables. (Speroff L.,Glass R.H., Kase N.G. 1976).

Determination of fertile days in clinical practice is most frequently done by FERN-TEST - the test of the cervical secretion crystallization. Papanicolaou (Papanicolaou G.N., 1942) was the first who observed that the vaginal mucus during ovulation, smeared and dried on glass slide, manifests crystallization in the fern -like form. This phenomenon is most evident in the periovulatory period, 3-4 days before the occurrence of ovulation.

Crystallization results from biophysical and biochemical changes in cervical mucus under the influence of ovary hormones (Abarbanel H.R.,1946).

Secretion activity of the cervical epithelium during the menstrual cycle is effected by ovary hormones. The cervix is under the influence of estrogen in the course of the whole menstrual cycle. This influence is the strongest 3 - 4 days before the ovulation when the secretion of estrogen reaches the highest level in the course of the cycle and has a strong effect on cervical epithelium. As a result of different levels and ratios of estrogen and progesterone, cyclical variations appear in the quantity of cervical mucus and certain inorganic salts (Hagenfeldt K.,1972).

Mac Donald and Roland (Mac Donald R.R.,1969; Roland N.,1958) consider sodium the main component of electrolytes of cervical mucus and along with calcium ions brings about the phenomenon of crystallization, known as FERN mucus reaction. According to Toyoshima (Toyoshima K.,1956), sodium chloride accounts for 90% of all inorganic salts in cervical mucus related to ramification.

Cervical mucus crystallization - FERN TEST - the test of branching, is tested every day in the periovulatory period of the cycle in the following way: First, the external uterus orifice is wiped by gauze and then a stick covered with cotton wool is inserted into the cervical canal up to internal cervical orifice. Cervical mucus is taken by a circular movement and then smeared on a glass slide. After drying for 10-15 minutes at the room temperature, the plates are examined under a microscope.

On the 10th and 11th days , as a result of weak estrogen activity, there is only partial crystallization of NaCl with occasional tiny fern branches - like “frost on the window”. Such findings are registered as mildly evident positive FERN test and are marked by (+). On 12th and 13th days , as a result of increased estrogen activity, the crystallization appears in the form of tender fern branches that occupy only a portion of the microscope field . Such a positive FERN test is marked by (++). On 14th and 15th days, at the time of maximum estrogen activity , crystallization appears in the form of rough and thick fern or palm leaves that occupy the whole microscope field. Such findings are marked by (+++).

Due to changes in the uterine cervix, the examination of the uterine cervix secretion is sometimes made difficult. Excessive secretion of the uterine cervix glands or the abundant presence of leukocytes and other cells, can alter the crystallization of the secretion , which is called “dysmucorrhea”. In certain cases, examination is made difficult or impossible due to bleeding of the cervix in contact with the stick.

Determination of the ovulation by means of the FERN test requires every day visit to a gynaecologist.

Crystallization of the saliva

The ovulation related phenomenon of saliva crystallization in a fern-like pattern was first observed in 1957 by Andreoli and Della Porta from University in Torino . J.M.Biel Casals (Casals J.M.B., 1968) was the first to investigate this phenomenon more extensively. He examined 493 saliva samples and concluded that the intensity of crystallization is directly dependent on closeness of ovulation. The method used for determination of crystallization is simple: a drop of saliva is placed on a glass slide and left to dry at the room temperature. When completely dry, the sample is examined using small magnification.

Salivation (Gayton C.A., 1981)

Main salivary glands are: parotid, submandibular and sublingual glands; there is also a lot of small buccal glands. Daily salivation ranges from 1000 to 1500 ml. Saliva consists of two kinds of secretion: (1) serosus, containing ptyalin, enzyme (alpha-amylases) for starch digestion , and (2) mucous secretion which protects and moists the oral cavity. The saliva pH is between 6.0 and 7.4 . Salivary glands are compound glands consisting of acinuses covered by secretory glandular cells and system of small ducts conducting saliva into the oral cavity.

The secretion of saliva takes place in two stages; at the first stage, acinuses and, at the second, ducts are involved. The acinuses secrete the so called primary secretion containing saliva enzymes in ionized solution which, as regard ions, does not considerably differ from plasma. However, the ion composition of the primary secretion is significantly modified in the ducts by two important active transportations.

In the first place, sodium ions are actively reabsorbed from excretory ducts and potassium ions are actively secreted in exchange. Accordingly, the concentration of sodium as well as chloride ions decreases while the concentration of potassium increases. Secondly, the bicarbonate ions are secreted into excretory ducts by way of enzyme carboanhydrasis that is in epithelial cells of excretory ducts. In exchange for bicarbonates, additional quantities of chloride ions are passively reabsorbed from excretory ducts . As a result of processes of active transportation , in conditions of rest, the concentration of sodium and chloride ions in saliva is about 15 mEq/lit each , being approximately 1/7 to 1/10 of their concentration of plasma. On the other hand, the concentration of potassium ions is about 30 mEq/lit being approximately 7 times higher than in plasma. The concentration of bicarbonate ions is about 50-90 mEq/lit , being 2 to 4 times higher than in plasma.

When saliva is been secreted more intensively, the concentration of ions in saliva considerably changes because the primary secrete in acinuses is secreted 20 times faster. That increase in fastness causes such an acceleration of the flow of the secrete through excretory ducts so that changes in its composition that occur during the passage take place to a considerably lesser extent than they otherwise do. Accordingly, when saliva is secreted more intensively, the concentration of NaCl is higher than usual and amounts to 1/2 to 2/3 of the one in plasma, while the concentration of potassium is lower than usual - only 4 times higher than in plasma.

Due to the fact that a concentration of potassium ions in saliva is high, any abnormal state accompanied by long-term hypersalivation can cause a serious loss of potassium ions and severe potassium defecit.

Mechanisms for regulation of saliva secretion

- Influence of local incitement
- The food mechanically irritates the surface of glandular cells and causes saliva secretion. The presence of smooth objects in oral cavity (e.g. pebble) results in substantial hypersalivation while rough objects cause weaker salivation and maybe even stop it.

- Chemical irritation, specially the taste of sour, results in abundant hypersalivation, often as high as 5 ml per minute or 8 to 20 times more than the basal saliva secretion.
- Nervous regulation of saliva secretion
- Submandibular and subligual glands are controlled by neural impulses from upper salivary nucleuses and parotid gland by impulses from lower salivary nucleuses. Those nucleuses are situated close to the border of medulla oblongata and pons and they are stimulated by receptors for taste and touch of the tongue and other areas of oral cavity.
- Salivation can be stimulated or inhibited by impulses coming to salivation centers from higher centers of the central nervous system. For example, salivation intensifies when someone smells or eats the food he likes. The center for appetite in the brain regulating this is located very close to parasymphatic nucleuses in frontal hypothalamus and activity of that area is to a large extent a reaction to impulses coming from the center for taste and smell, located in almond nucleuses in cortex.

The role of autonomous nervous system - parasymphatic stimulation

Stimulation of parasymphatic nearly always intensifies gland secretion. Stimulation of symphaticus, by itself, has a slight effect on secretion. However, weak parasymphatic stimulation often results in reduced secretion due to vasoconstriction and poorer blood supply.

Hormonal regulation of salivation

Certain hormones, above all aldosteron, estrogen and progesterone, play an important role in salivation and composition of saliva .

Excessive secretion of aldosteron brings about increased reabsorption of Na and Cl while the secretion of K increases; thus, the concentration of NaCl in saliva is reduced and the concentration of K is increased.

Stimulation by estrogens intensifies the secretion of saliva with the larger quantity of water, Na and Cl. Increased concentration of NaCl in saliva leads to its crystallization. Progesterone causes decrease in quantity of saliva, its water share and concentration of Na and Cl.

Ultrasound of ovaries and uterus

(Prelevic G.M., 1992)

Ultrasound of ovary and uterus are irreplaceable methods for evaluation of ovulation in clinical practice. This is a reliable method for the establishment of the size and morphology of ovaries and uterus, dynamics of the follicle development as well as changes in endometrium quantitative and qualitative terms both in spontaneous and stimulated cycles.

Transabdominal and transvaginal methods may be used.

Ovary follicles 20-25 mm in size with the appearance of " double contour" and the echo of cumulus oophorus are considered to be the sign of the forthcoming ovulation. The ultrasonographic sign of the ovulation is considered to be the disappearance of the ovary follicle with the appearance of a new sign on endometrium, the so-called ovulation ring.

However, this method is not simple in the least and requires the most modern ultrasound devices, great experience and expertise of doctors as well as daily clinic attendance of the woman in periovulatory period.

Clinical investigation covered by this paper is a prospective study carried out at the Clinic of Gynaecology and Obstretics "Narodni Front" in Belgrade, Sterility Department, under the professional guidance of Assistant Professor dr Ratomir Ganovic, the head of the department, and associates.

The preliminary name of the test, "LADY TEST", borrowed from foreign literature, was later replaced by "GANOP TEST" after the name of its authors and the Clinic.

Purpose of the project

The following objectives have been specified in the course of investigation:

1. Evaluation of the utility and clinical value of GANOP test of saliva in determination of fertile and infertile days in menstrual cycle.
2. Determination of approximate time of ovulation in patients under observation by using FERN test - analysis of cervical mucus.
3. Ultrasonographic monitoring of the dynamics of the follicle growth and development as well as changes in thickness and structure of endometrium throughout the menstrual cycle confirming at the same time the occurrence of ovulation.
4. Compare results of the FERN test and GANOP test with the results of ultrasonographic folliculometry.
5. Compare the results obtained with the ones of other researchers.

A special objective was to determine validity, sensitivity, specificity and diagnostic doctrine of GANOP test as a saliva crystallization test i.e. a test for determination of fertile and infertile days in the menstrual cycle.

Material and methods

The investigation covered 71 woman of generative age. Out of 71 patients, those with non stimulated cycle accounted for 81.69% (58), while in 11.27% (8) patients the cycle was stimulated by Clomifen Citrate and, in 7.04%cases (5), the stimulation was carried out by Clomifen citrate and Human menopausal gonadotropin in combination.

The tests were conducted from the 8th to 20th day of the cycle. A total number of the examinations performed was 213. Every patient was examined at 3 different points of time and each time the following was carried out:

- ultrasonographic folliculometry and sonoendometry (apparatus ALOKA SSD620, transvaginal probe 5mhz)
- Test of crystallization of cervical mucus - FERN test (microscope ZEISS)
- Test of crystallization of saliva - GANOP test (minimicroscope MAYBE BABY with enlargement 52 times, product of "OPTIX", Belgrade).

All three examinations were carried out quite separately and the results for every patient were entered into forms specially devised for this research.

For evaluation purposes, results of ultrasonographic folliculometry were recorded in terms of the size of follicle in millimeters or "ovulatio"; the results of FERN and GANOP tests were marked by (0), (+), (++),(+++), depending on the quantity of "fern" in the dried sample of cervical mucus or saliva.

During the research, microscopic shots were taken of FERN and GANOP tests samples. Characteristic photographs are attached to this paper.

The obtained results were statistically processed by chi-square test, analysis of variance and analysis of correlation (Pearson and Spearman correlation)

Results

The study covered 71 patients.

The average age was 32.6 $\pm$ 5.5 years in the range from 21 to 45 years, as showed in table 1.

Table 1. Age of patients.

Age	No of patients	%
21-24	9	12.7
25-29	32	45.1
30-34	22	31.0
35-39	5	7.0
40-45	3	4.2
Total	71	100.0

x years= 32.6 $\pm$ 5.5

In all patients the ultrasonographic folliculometry was performed as a reference method. Along with ultrasonographic folliculometry, FERN and GANOP test were

performed, all three in morning hours; that morning, the patients didn't eat, smoke or drink.

The results of ultrasonographic folliculometry and FERN test are shown in table 2.

Table 2. Results of ultrasonographic folliculometry and FERN test.

Ultrasound			Fern test							
Size of foll.(mm)	Total				+		++		+++	
	N	%	N	%	N	%	N	%	N	%
Up to 10	56	26.3	41	60.3	6	13.3	5	9.1	4	8.9
11-17	67	31.4	15	22.1	24	53.3	18	32.7	10	22.2
18-25	69	32.4	9	13.2	13	28.9	22	40.0	25	55.6
Ovulatio	21	9.9	3	4.4	2	4.5	10	18.2	6	13.3
TOTAL	213	100	68	100	45	100	55	100	45	100

X^2=61.058; p<0.001
Phi=0.512; C=0.423; p<0.001
Pearson's correlation r=0.316; p<0.01
Spearman's correlation ro=0.317; p<0.01

The size of follicle under 10 mm was sonographicaly registered in 56 (26.3%) examinations, 11-17 mm in 67 (31.4%) examinations, 18-25 mm in 69 (32.4%) examinations and in 21 examination (9.9%) the ovulation was registered.

The higher percentage of negative FERN test results was found in small size follicles while, in larger size follicles, there was a higher percentage of positive results. Those differences are statistically significant (Chi-square = 61.058; P<0.001).

A significant and high correlation was established between the size of follicle and FERN test results (Phi=0.512; C=0.423; P<0.01).

The results of ultrasonographic folliculometry and GANOP test are shown in table 3.

Table 3. Results of ultrasonographic folliculometry and GANOP test.

Ultrasound			Ganop test							
Size of foll.(mm)	Total				+		++		+++	
	N	%	N	%	N	%	N	%	N	%

Up to 10	56	26.3	36	67.9	9	17.3	7	12.5	4	7.7
11-17	67	31.4	9	17.0	27	51.9	18	32.1	13	25.0
18-25	69	32.4	6	11.3	13	25.0	23	41.1	27	51.9
Ovulatio	21	9.9	2	3.8	3	5.8	8	14.3	8	15.4
TOTAL	213	100	53	100	52	100	56	100	52	100

X^2=63.539; p<0.001
Phi=0.541; C=0.476; p<0.001
Pearson's correlation r=0.370; p<0.01
Spearman's correlation ro=0.371; p<0.01

The higher percentage of negative GANOP TEST results has been found in small size follicles while, in larger size follicles, there has been a higher percentage of positive ones (Chi-square = 63.539 ; P < 0.001). There is a highly significant correlation between the size of the follicle and the results of GANOP TEST (Phi=0.541; C=0.476 ; P< 0.01).

Comparative analyses of FERN and GANOP tests in relation to sonographic folliculometry are shown in tables 4,5,6 and 7.

Table 4. Comparative values of Fern and Ganop tests at the size of follicles up to 10 mm (n=56)

Results	θ		+		++		+++		Total	
	N	%	N	%	N	%	N	%	N	%
Fern test	41	73.2	6	10.7	5	8.9	4	7.2	56	100
Ganop test	36	64.3	9	16.1	7	12.5	4	7.1	56	100
X^2=1.039; DF=1; p>0.05										

Comparative values of Fern and Ganop tests at the size of follicles up to 10 mm (n=56) are shown in the table 4. At this size of the follicle, there was no statistically significant difference in the percentage of positive results (Chi square= 1.039; P > 0.05). The percentage of positive results in FERN test and GANOP test was 26.8% (15) and 35.7% (20) respectively.

Table 5. Comparative values of Fern and Ganop tests at the size of follicles from 11 to 17 mm (n=67)

Results	θ		+		++		+++		Total	
	N	%	N	%	N	%	N	%	N	%
Fern test	15	22.4	24	35.8	18	26.9	10	14.9	67	100
Ganop test	9	13.4	27	40.3	18	26.9	13	19.4	67	100
X^2=1.827; DF=1; p>0.05										

Comparative values of Fern and Ganop tests at the size of follicles from 11 to 17 mm are shown in the table 5. The percentage of positive results was 77.6% (52) in FERN test and 86.6% (58) in GANOP test. There was no statistically significant difference in the number of positive results between these two test (Chi square=1.827; P>0.05).

Table 6. Comparative values of Fern and Ganop tests at the size of follicles from 18 to 25 mm (n=69)

Results	θ		+		++		+++		Total	
	N	%	N	%	N	%	N	%	N	%
Fern test	9	13.0	13	18.9	22	31.9	25	36.2	69	100
Ganop test	6	8.7	13	18.9	23	33.3	27	39.1	69	100
X^2=0.673; DF=1; p>0.05										

Comparative values of Fern and Ganop tests at the size of follicles from 18 to 25 mm are shown in table 6. At this size of the follicle, FERN test was positive in 87% (60) cases and GANOP test in 91.3% (63) cases. The differences are not statistically significant (Chi square =0.673; P>0.05).

The percentages of positive results of both tests are shown in graph 1.

Graph 1. Percentages of FERN and GANOP tests positive results according to the size of the follicle

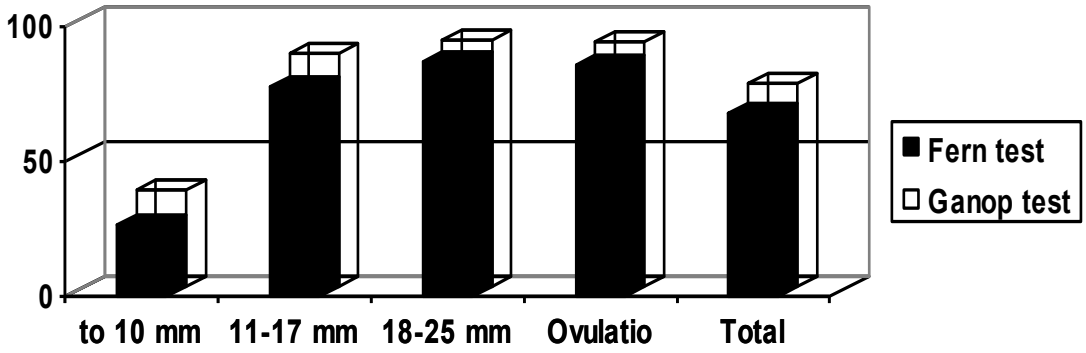


Table 7. Comparative values of Fern and Ganop tests in patients with sonographically proved ovulation (n=21)

Results	θ		+		++		+++		Total	
	N	%	N	%	N	%	N	%	N	%
Fern test	3	14.3	2	9.5	10	47.6	6	28.6	21	100
Ganop test	2	9.5	3	14.3	8	30.1	8	30.1	21	100
X^2=0.227; DF=1; p>0.05										

Comparative values of FERN and GANOP tests in patients with sonographically proved ovulation are shown in table 7. There were 21 patients with sonographically proved ovulation and, out of these, FERN test was positive in 85.7% (18) cases while GANOP test was positive in 90.5% (19) cases. Those differences are not statistically significant (Chi square=0.227; P>0.05).

The overall review of FERN and GANOP tests results is shown in table 8.

Table 8. Comparative values of Fern and Ganop tests (n=213)

Results	θ		+		++		+++		Total	
	N	%	N	%	N	%	N	%	N	%
Fern test	68	31.9	45	21.1	55	25.8	45	21.2	213	100
Ganop test	53	24.9	52	24.4	56	26.3	52	24.4	213	100
X^2=2.879; DF=3; p>0.05 (θ:+) X^2=2.597; DF=1; p>0.05										

Out of 213 examinations, the results of FERN test were negative in 68 (31.9%) cases and positive in 145 (68.1%) cases. As for GANOP test, the results were negative in 53 (24.9%) and positive in 160 (75.1%) cases.

As for different modalities of positive FERN test, the results were marked by (+) in 21.1% cases, by (++) in 25.8% and by (+++) in 21.2% cases. Positive GANOP test results were marked by (+) in 24.4% cases, by (++) in 26.3% and by (+++) in 24.4% cases.

There was no statistically significant difference between both tests either for modalities of positive results (Chi square =2.879; P>0.05) or for the ratio of positive and negative results (Chi square=2.597; P>0.05).

Analysis of variance of FERN and GANOP test results, according to the size of follicles, are shown in tables 9 and 10.

Table 9. Relation between the FERN test results and mean value of the foll. size

Fern test	N	Size of follicle (mm)			
		x	SD	SE	min-max
θ	68	11.9	6.7	0.81	5.0 - 22.8
+	45	14.8	5.9	0.88	7.5 - 23.2
++	55	15.6	7.4	1.00	8.4 - 24.0
+++	45	18.3	6.0	0.89	10.0 - 24.5
Analysis of variance		F=7.504; p<0.01 LSD-test (0: +,++,+++) (+: +++) p<0.05			

The relation between the FERN test results and the size of the follicle was investigated by analysis of variance, as shown in table 9. With regard to FERN test results, statistically significant differences in the size of follicle (F=7.504; P<0.01) have been established. Accordingly, mean values of the size of follicles were significantly higher in positive FERN test results than in negative ones. Highly positive (+++) results of FERN test had significantly higher mean values of the size of follicles in relation to mildly positive (+) results, P<0.05.

The relation between the GANOP test results and mean value of the size of follicles was investigated by analysis of variance as shown in table 10.

By the analysis of variance, statistically significant differences (F=7.926; P<0.01) between GANOP test results and the size of the follicle were established.

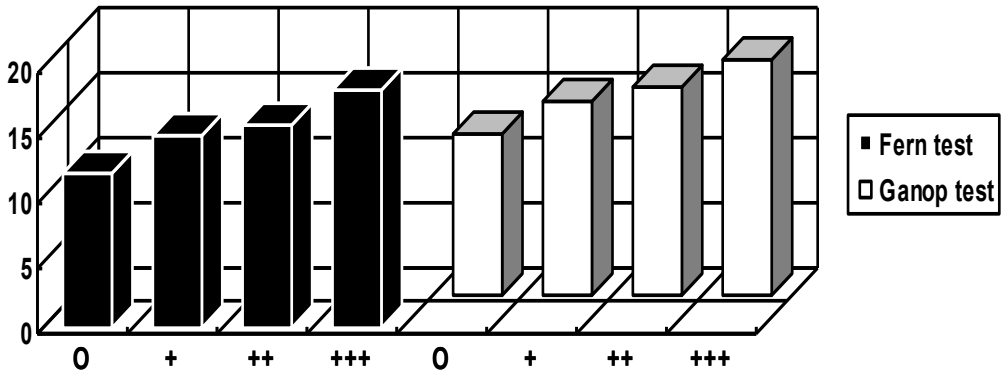
Table 10. Relation between the GANOP test results and mean value of the size of the follicle

Ganop test	N	Size of follicle (mm)			
		x	SD	SE	min-max
θ	53	12.4	6.3	0.9	5.6 - 22.5
+	52	14.9	8.1	1.12	7.0 - 23.4
++	56	16.0	6.9	0.92	7.6 - 24.2
+++	52	18.1	6.2	0.86	1.0 - 24.8
Analysis of variance		F=7.926; p<0.01 LSD-test (0: +,++,+++) (+: +++) p<0.05			

Mean values of the size of follicle were significantly higher in positive results compared to negative results. Highly positive (+++) results of GANOP test had significantly higher mean values of the size of follicles in relation to mildly positive (+) results, P<0.05.

Relation between FERN and GANOP test results and the size of follicle is shown in graph 2.

Graph 2. Relation between FERN and GANOP test results and the size of the follicle



By the analysis of all these parameters, it was established that, out of 213 examinations, FERN test had 15 false positive and 12 false negative results amounting to a total of 7.98% (27) false results.

Out of 213 examinations, GANOP test had 20 false positive and 8 false negative results - a total of 13.15% (28) false results.

In 23 cases, false results of FERN and GANOP tests coincided.

Discussion and conclusions

The possibility of determining fertile and infertile days of the menstrual cycle by using the saliva crystallization test (GANOP TEST) is viable, its validity has been proved and its sensitivity is high. The special validity of this test is its certainty of reaction, specially in the preovulatory period and the period of ovulation.

GANOP test shows a higher tendency towards positivism 4-5 days before the ovulation and 2-3 days after the ovulation.

FERN test of cervical mucus is undoubtedly more precise while GANOP test of the saliva is more sensitive.

Comparable indicators of GANOP test and FERN test in relation to sonographic changes in follicles or their increase indicate that GANOP test is as valid and sensitive as FERN test and correlative values of both tests with regard to sonographic folliculometry are high.

The advantage of GANOP test is its simplicity and possibility for wide application. “MAYBE BABY” - a mini microscope , with instructions, may be easily used by any woman .

A special value of GANOP test is the establishment of fertile and infertile days in the course of the menstrual cycle , as a method of family planning and reduction of unwanted effects to health , and economization in the field of contraception and unwanted pregnancy.

We are of the opinion that GANOP test, owing to its simplicity and validity, deserves a systematic application in determining fertile and infertile days in the course of the menstrual cycle.

A more extensive study is needed in order to judge real values and scope of application of this test. In so far as the real value of the test is established and confirmed in course of time, brilliant clinical future may be predicted for it.