WAYS OF IMPROVING THE MALE FROG TEST FOR PREGNANCY

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It is the purpose of this paper to present some observations on emission of spermatozoa by the leopard frog, *Rana pipiens*, which are pertinent to the procedures used in applying these animals to pregnancy diagnosis. These observations indicate methods of increasing the accuracy of the test by lowering the percentage of false negatives and by avoiding possible false positives.

The male leopard frog, *Rana pipiens*, was first reported as a test animal for pregnancy by Wiltberger and Miller (1948) and Robbins and Parker (1948). Pregnancy is indicated when mature living spermatozoa appear in the urine of the frog (designated as positive) following the subcutaneous injection of 4 cc. of first morning urine. Absence of spermatozoa (designated as negative) indicates no pregnancy. The validity of the male frog test for pregnancy has been verified by Bodine et al (1950), Maier (1949) and Brody (1949).

The results of further experiments in our laboratory indicate that factors such as the quantity of liquid injected, the salinity of the liquid, the season of the year, the temperature of the room in which they sit during the test and conditions of storage and handling are influential in their sensitivity.

We have induced discharge of spermatozoa in the laboratory by injections of dilute liquids and by rapid absorption of water following drying. Hundreds of frogs were injected with 7 cc. or more of various liquids such as distilled water, tap water and pond water and over 50 per cent discharged mature living spermatozoa with their urines. Accordingly experiments were devised to determine the extent of these factors in influencing discharge of spermatozoa and their possible effects upon the use of male frogs in clinical practice.

METHODS AND MATERIALS

Except where otherwise stated, the frogs, *Rana pipiens*, used in these experiments were delivered by express from commercial dealers and placed in aquaria containing about one inch of tap water. These aquaria were then placed in a refrigerator where the temperature was maintained at approximately 45° F. They were used in the experiments from two to sixty days after refrigeration began. All injections were subcutaneous into the lateral lymph sacs. Microscopic examinations for spermatozoa were made by removing, with a pipette, a drop of fluid from the jars in which the frogs urinated after injections. If a frog failed to urinate after 90 minutes, pressure was applied to the sides of the frog which usually emptied the urinary bladder.

THE POSSIBILITY OF FALSE POSITIVE DIAGNOSIS

Robbins and Parker (1949), while checking the source of false positives when using *Xenopus laevis*, have found that *Rana pipiens* as well as *Xenopus laevis* responds to some adrenergic substances by emitting spermatozoa. However, since the adrenergic substances to which *Rana pipiens* responded are not known to be present in human urines they discount them as a possible source of false positives.

The absence of false positives in the literature indicates that if they occur they are recognized as such because of abnormalities in the animal or faulty procedures. Thousands of urines of frogs have been examined in our laboratory and, except for the conditions to be mentioned below, the indications are that frogs kept in the laboratory under conditions conducive to longevity do not discharge spermatozoa unless gonadotropic substances are administered.

One of the conditions to be mentioned is the effect of injections of large quantities of liquids. An experiment was carried out to determine the minimum quantity of water necessary to stimulate spermatozoa emission.

Four to twelve cc. of *distilled* water were injected into 14 frogs of average size and their urines examined for spermatozoa. The results are found in Table I. A separation of the March and June data indicates that the danger of false positives due to large quantities of dilute solutions is greater in March than in June; in other words greater before the end of overwintering than after the breeding season.

TABLE I

THE RESULTS OF INJECTIONS OF VARIOUS AMOUNTS OF DISTILLED WATER

Amount of cc. in Distilled Water	4	5	6	7	8	9	10	11	12	Totals
IN MARCH										
Number emitting spermatozoa	0	3	4	2	3	5	2	6	6	31
Number not emitting spermatozoa	7	4	3	5	4	2	5	1	1	32
Death	0	0	0	0	0	0	0	0	0	00
IN JUNE										
Number emitting spermatozoa	0	0	0	2	1	0	2	4	4	13
Number not emitting spermatozoa	6	7	7	5	6	6	5	3	3	48
Death	1	0	0	0	0	1	0	0	0	2

In order to check the possibility of obtaining false positives from large amounts of urines, 10 cc. amounts of human male and non-pregnant female urines were injected into thirty frogs. Since it has been our experience that a large quantity of urine usually kills the frog, we also decided to check the effect of a non-toxic saline solution. Ten cc. amounts of cold-blooded Ringer's solution were injected into 30 frogs. Thirty frogs, received in the same shipment and stored under the same conditions as the other two groups, were injected with 10 cc. amounts of distilled water. The results are found in Table II.

TABLE II

THE RESULTS OF INJECTIONS OF 10 CC. OF NON-PREGNANT URINES, RINGER'S SOLUTION AND DISTILLED WATER

Number of Frogs	Injected with 10 cc. of:	Results		Death
		Positive	Negative	
30	Non-pregnant Urines	0	12	18
30	Ringer's Solution	1	29	0
30	Distilled Water	18	12	0

An analysis of these data suggest a remote possibility of obtaining a false positive even with a large quantity of saline solution. It does not eliminate the possibility of large quantities of dilute non-lethal urines inducing emission of spermatozoa. This was observed under controlled conditions in our laboratory. It resulted when an injection of 7 cc. of dilute urine from a non-pregnant woman followed one hour later by an injection of 10 cc. of the same urine. This case was not considered a false positive because the abnormally high amount of urine injected would not have been used clinically.

Another condition likely to result in a false positive diagnosis concerns the condition of the frogs. Our experiments indicate that abnormally dry frogs may emit spermatozoa following their introduction into water. In the winter and spring of 1950 some frogs were placed in jars without water. When they had lost

about 35 per cent of their previous weight, water was added and four hours later their urine was found to contain mature living spermatozoa. Control frogs were subjected throughout the experiment to the same environmental conditions except that they sat continuously in about one inch of water. These frogs did not emit mature living spermatozoa.

Frogs are usually received from commercial suppliers with a near normal water content. Hundreds of these have been examined by us upon their arrival, after they were placed in tap water at room temperature, and after refrigeration. None have ever discharged mature living spermatozoa. However, when very dry frogs are placed in tap water after arrival in early spring they have been seen to clasp and an examination of their urines revealed, in some cases, mature living spermatozoa. This indicates the necessity of care in preserving the water content at all times, of a conditioning period if received dry, and of checking all frogs before their use as test animals for pregnancy. The checking is quite simple and is practiced routinely in many clinical laboratories.

CONDITIONS INFLUENCING FALSE NEGATIVES

A false negative diagnosis may arise from a low sensitivity of the frog or an insufficient amount of gonadotopin, or a combination of both of these conditions. The lowest percentage of false negatives occurs from urines of women in the first trimester of pregnancy. It was shown by Miller and Wiltberger (1948) that there is a declining and fluctuating amount of chorionic gonadotropins after the first trimester of pregnancy. Bodine et al (1950) reported no negative responses

TABLE III

THE INFLUENCE OF CHORIONIC GONADOTROPIN ON EMISSION OF SPERMATOZOA AT VARIOUS TEMPERATURES

Temperature of	60	67	75	85	95
Number emitting spermatozoa after 90 min	17	19	16	11	11
Number emitting spermatozoa after 120 min	17	19	18	16	12
Number in which death occurred	0	0	0	0	4

from the first trimester of pregnancy. They also found the amount of chorionic gonadotropin was highest during the 2nd and lowest during the 6th month of pregnancy.

From these investigations it appears that maximum accuracy should be obtained by using urines from the first trimester and by using concentration procedures, which increase the amount of gonadotropins without injecting large quantities of solution. Wiltberger and Miller (1948) suggested the possibility of a seasonal variation of the frogs and Sampson (1950) reported a seasonal sensitivity to chorionic gonadotropin.

Some experiments were carried on by us which indicate that the sensitivity of the frog can be influenced by several factors under the control of the technician. The results of our investigations with liquids and chorionic gonadotropins in the summers of 1948 and 1949 indicate that the frogs did not emit spermatozoa as often when they were placed, after injections, in a room where the temperature was high (i. e., 90° F.). In order to determine whether the temperature at which the frogs were placed during the test was a factor in their sensitivity, we injected Ayerst brand of "Anterior Pituitary-Like" chorionic gonadotropin in amounts of 50 and 100 International Units, diluted to 2 cc. with distilled water, into each of 20 frogs over a range of five different temperatures. No significant difference was observed between those injected with 50 International Units and those injected with 100 International Units. The results are shown in Table III.

At temperatures below 60° F. considerable difficulty was encountered in getting the frogs to urinate within an hour's time. From these data it appears that the optimum temperature is around 67° F. This experiment was carried out in August, 1949, and at this time of the year, even with optimum temperature, some frogs remained negative after injections of a large amount of gonadotropins. The data also suggest that an increased length of time after injections is necessary before emission of spermatozoa by leopard frogs standing in temperatures above 75° F.

Another experiment indicated that an additional factor in their susceptibility to gonadotropins is the temperature at which the frogs have been stored. Frogs

TABLE IV

COMPARISON OF THE EFFECTS OF GONADOTROPINS ON EMISSION OF SPERMATOZOA BETWEEN FROGS STORED IN A REFRIGERATOR AND THOSE STORED AT ROOM TEMPERATURE

Number of Frogs	Kind of Storage	Number Emitting Spermatozoa
20	Refrigeration	13
20	Room Temperature	1

TABLE V

COMPARISON OF THE EFFECTS OF DISTILLED WATER ON EMISSION OF SPERMATOZOA BETWEEN FROGS STORED IN A REFRIGERATOR AND THOSE STORED AT ROOM TEMPERATURE

Number of Frogs	Kind of Storage	Number Emitting Spermatozoa
30	Refrigerated	16
30	Room Temperature	4

TABLE VI

COMPARISON OF THE EFFECTS OF DISTILLED WATER AND PREGNANCY URINE ON SPERMATOZOA EMISSION OF FROGS STORED IN DRY REFRIGERATION

Number of Frogs	Injected With	Number Emitting Spermatozoa
4	13 cc. of distilled water	3
5	4 cc. of pregnancy urine	0

from one shipment in May, 1950, were divided into two groups. One group was stored in a refrigerator (about 45° F.) and the other at room temperature (about 74° F.). Both groups were in one inch of water.

They were all injected with 1 cc. of Ringer's solution containing 5 International Units of Ayerst Brand of Chorionic Gonadotropin ("APL"). The Summary is found in Table IV.

It appears from this experiment that refrigerated frogs are much more sensitive to gonadotropins. It also appears that 5 International Units is close to the minimal dosage of gonadotropin necessary to cause emission of spermatozoa in May frogs.

In a similar experiment 12 cc. of distilled water was substituted for gonadotropins. The results are shown in Table V.

From these two experiments on the temperature of storage it appears that refrigerated frogs are better test subjects but consequently more susceptible to over-injections.

In a preliminary experiment 10 frogs were refrigerated (in an aquarium) without water and the effects of injections of distilled water and pregnancy urine on emission of spermatozoa were compared. The gonadotropic potency of the pregnancy urine used was tested on five frogs stored in a refrigerator in one inch of water and all five emitted spermatozoa. A preliminary test gave the results shown in Table VI.

Although the number of animals used in this experiment is small the results seem to indicate that dry frogs are not good test animals. They are not only less sensitive to gonadotropins but are more sensitive to distilled water.

CONCLUSIONS

From the foregoing experiments we suggest the following ways of improving the accuracy when using *Rana pipiens* as a test animal in clinical diagnosis of pregnancy.

1. Do not use more than 5 cc. of fluid if possible in a single injection. Quantities of distilled water and very dilute solutions have caused some emission of spermatozoa.
2. Always use a salinity as near to normal (Ringer's solution) as possible. Non-pregnancy urines and cold Ringer's solution showed little danger of false positives even when quantities of 10 cc. were injected.
3. Avoid large quantities of fluids in autumn, winter and early spring especially. The frogs are more sensitive to excessive quantities in these seasons than in summer.
4. Avoid excessive drying of test animals before using. Frogs that stood at room temperatures until they had lost about 35 per cent of their weight by drying were then placed in water for four hours, after which some emitted spermatozoa.
5. Store frogs in shallow water and in refrigeration if possible. Such frogs are more sensitive to gonadotropins than those stored at room temperatures.
6. Run tests at 60° F. to 75° F. if possible. The reactiveness of the frogs decreases at temperatures above and below this range.
7. By concentrating and extracting the hormones from the urine greater accuracy may be obtained without injecting large quantities into the frog.

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