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Improved method for vaginal plug detection in rats

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Introduction

Time mated rodents are needed in many research procedures for example when foetuses of a certain age are required, as well as in transgenic and knockout technology, where pseudopregnant females must be used as recipients for embryos. The vaginal plug is commonly used as evidence that mating has taken place. There are several studies on plug formation, evaluating the function and usefulness of plug detection in rodents, however, most have been performed in mice (*Erbeck & Kruckenberg* 1995).

In rodents, male accessory glands secrete constituents that form the copulatory plug in the female's vagina after mating (*Blandau* 1945, *Campean et al.* 1980). The function of vaginal plug has been studied by several groups. In an early paper, *Leuckart* (1847) suggested that plug prevents the outflow of sperm from the vagina. Later, it has been shown that the plug has an important role in transporting sperm through the cervix to the uterus (*Blandau* 1945, *Toner et al.* 1987, *Sofikitis et al.* 1990, *Cukierski et al.* 1991). The location of plug has effects on sperm count in uterus. A tight plug fills most of the vaginal-cervical junction and uterine sperm count is higher when there is a tight plug than with a loose plug (*Matthews & Adler* 1977, *Matthews & Adler* 1978). Plug fit has been shown to be important in transport of sperm to uterus, but the mechanism is not known (*Toner et al.* 1987). It has been suggested that copulatory plug reduces the high tonus of cervix and allows the entry of spermatozoa into the uterus (*Sofikitis et al.* 1990).

Although formation of the plug is important, it is not essential for pregnancy in mice (*Campean et al.* 1980). In rats, however, the plug appears to be an essential factor in determining whether pregnancy occurs after natural matings (*Cukierski et al.* 1991).

The copulatory plug is usually examined in the morning, since mating takes place nocturnally. The predictability of pregnancy by plug evaluation is 91-93% in mice (*Szabo et al.* 1969, *Champlin et al.* 1973), a figure which is obviously related to the fertility of the strain used. In rats (CRL:COBS CD (SD)bs), a vaginal plug was found only in 13 % of animals, but a pan plug (plug in the faeces pan) was seen in 71 % of pregnant rats (*Szabo et al.* 1969).

Copulatory plugs can be either vulvar or deep. In mice, there are differences between strains in the location of the plug. Deep plugs are not easily seen by vulvar inspection alone. The use of instruments such as a metal probe and a magnifying loupe improve detection of deep plugs in mice (*Erbeck et al.* 1989).

There are other possibilities to confirm mating in rodents as well. Together with vaginal plug, plugs dropped to the faeces pan, pan plugs, can be observed (*Szabo et al.* 1969). Vaginal lavage on the next morning after mating reveals sperm if the

mating has occurred. However, both methods have disadvantages. Pan plugs are difficult to observe if the animals are housed on the solid floor with bedding. Vaginal lavage sampling may induce pseudopregnancy, and detection of sperm is more time consuming than plug detection.

The disadvantage in rat vaginal plugs is that the plugs are often deep and difficult to detect by vulvar inspection. In this study, a human otoscope was used as a vaginoscope for detection of these deep plugs. Furthermore, the predictive value of vaginal plugs for pregnancy was estimated.

Materials and methods

Animals used were barrier-bred outbred Wistar (Bkl:WIST) rats (National Laboratory Animal Center, University of Kuopio, Kuopio, Finland). Altogether 132 primiparous female rats, 10-22 weeks old, were time mated to yield 14 to 17 day old foetuses. Males used for matings were experienced males tested for fertility. The same males were used during the whole study. At the beginning they were 24 and at the end 48 weeks old.

Females were kept in groups of three in solid bottom stainless steel cages (48.5 cm x 28.5 cm x 20 cm, length x width x height). Males were kept singly in similar cages. Cages, bedding and water bottles were changed twice a week. Aspen chips were used as bedding (Type HIM, Tapvei Oy, Kaavi, Finland), 1.6 l per cage. Pelleted rat food (R36, Lactamin, Stockholm, Sweden) and tap water in bottles were available *ad libitum*. Light and dark cycle of the animal room was 12 hours light and 12 hours dark, with lights switched on at 07.00 and off at 19.00 hours. The room temperature was 21 ± 1°C and air humidity 55 ± 5 %.

Vaginal cell smears were taken, stained and examined in the afternoon between 12 and 14 hours to choose rats in proestrous. Females were placed together with males in the males' cages, one female per single male. Each male rat was used for mating maximally every second day and only twice per week.

Mating was confirmed by the presence of the vaginal plug. Plugs were examined on the next morning after pairing, between 8.00 and 9.00 hours. For examination, an otoscope (Welch Allyn®, Skaneateles Falls, NY, USA) was used. The otoscope is originally meant for inspection of human external ear canal. The otoscope has an internal light and lens, through which the examination is done. The plastic head piece of the otoscope is removable and the outside diameter of the tip of the head piece is 4,5 mm. The structure of the otoscope is shown in Figure 1.

Figure 1. A human otoscope used in the study. Inspection is done through the lens seen on the left side of the scope. The removable, black plastic head is on the right side.

In examination of vaginal plugs, the rat was firmly held either in hand (Figure 2) or it was kept on the top of the cage hopper (Figure 3). The head of the otoscope was gently pushed a few mm into the vagina. Plugs were seen through the lens of otoscope under the scope's light (Figure 4).

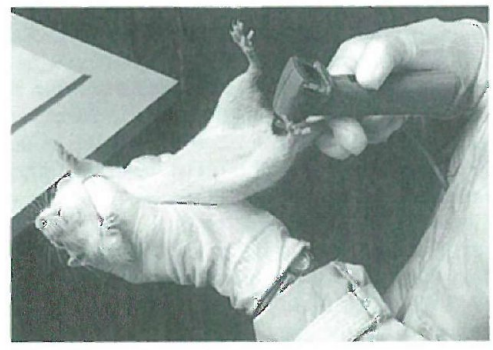

Figure 2. Examination of rat vaginal plug with the otoscope by holding the rat in the hand.

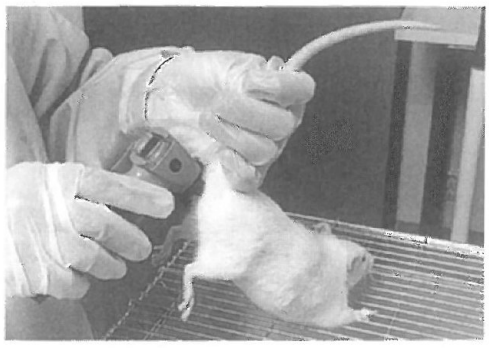

Figure 3. Plug detection with the otoscope when the rat is kept on the top of the cage hopper.

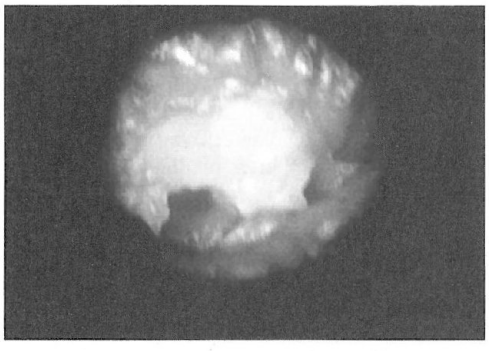

Figure 4. A deep vaginal plug seen through the lens of the otoscope.

After inspection, the females were returned to their original cages. The head piece of the otoscope was cleaned between examination of each female. Pregnancies were confirmed between days 14 to 17 of gestation, when the females were euthanized.

The number of pregnant animals was counted and females were grouped according to presence or absence of plugs to the following groups:

1. Females with plug and pregnant (positive).
2. Females with plug, but with no foetuses (false positive).
3. Females with no plug and with no foetuses (negative).
4. Females with no plug, but pregnant (false negative).

The sensitivity, specificity and positive and negative predictive values of vaginal plug method were calculated.

Results

All the plugs were deep inside the vagina and invisible without the use of the scope. The pregnancy rate of all females in proestrous was 69.7 %. Out of all 132 rats 72.7% animals had a vaginal plug, and 91.7% of these rats became pregnant. Table 1 shows the number of animals in positive, false positive, negative and false negative groups. The sensitivity of the vaginal plug method was 95.7 % and specificity 80.0 %. The positive predictive value of the method was 91.7 % and the negative predictive value 88.9 %.

Table 1. The number of females with or without vaginal plug, n=132.

Female group	n	%
Positive (Plug and pregnant)	88	66.7 %
False positive (Plug, no foetuses)	8	6.1 %
Negative (No plug, no foetuses)	32	24.2 %
False negative (No plug, but pregnant)	4	3.0 %
Total	132	100.0 %

Discussion

The visualisation of the vaginal plug in rat is rather difficult. In this study, detection of plugs was performed with a human otoscope. All the plugs were deep, thus the use of instrument was obligatory. The method is quick, easy and painless to the animals, it is also easily learned by the technicians.

The vaginal plug detection with the otoscope is an effective way for predicting the number of pregnancies. The positive predictive value of proestrous was 69.7 %. With plug detection the positive predictive value was 91.7 %. Thus, predictability of pregnancies increases by the detection of the vaginal plug. Both the sensitivity and specificity of vaginal plug detection method were good, which implies that the method is reliable.

The method was quick and no pseudopregnancies were detected, although the otoscope was pushed into the vagina. Compared to vaginal lavage, the use of the scope is more efficient since no microscoping is needed. The pan plugs were not observed because the rats lived in solid floor and pan plug detection would have been both uncertain and laborious. However, the good results obtained by plug detection indicate that there is really no need to examine pan plugs.

The number of vaginal plugs detected here is clearly larger than in the study by *Szabo et al.* (1969), where vaginal plugs were seen only in 13 % of pregnant females. When combined with a number of pan plugs (in 71 % of pregnant females) the total number of plugs seen was 84 % in pregnant cases. This comes close to the results of this experiment. The remaining difference may be due to the different rat strains used. Direct comparison of results is, however, difficult since in *Szabo's* study, the method for examining the vaginal plug is not stated, and the time when the plugs were observed was delayed (between 9 and 11 am). Furthermore, it is not clear if the vaginal smear examination for detecting sperm was done before or after the vaginal plug examination.

Nonetheless, there were a few false positives, *i.e.* non pregnant rats who did have a plug. In animal breeding, there is always a small percentage of pregnancy failures with some unknown reason. The size of the false positive group was equal to that in the study be *Szabo et al.* (1969) (vaginal and pan plug together, false positive in 10 % of females with plug). Furthermore, the pregnancy rate in rats with a plug is comparable to that seen in mice with a vaginal plug (*Champlin et al.* - 1973).

The false negative group did not have plugs, but turned out to be pregnant. Some of the plugs may have been small or unclear and therefore remained undetected. In mice, in rare cases the plug can fall out before morning, although the plugs usually stay firmly in place for 24 h (*Whittingham & Wood* 1983). These fallen plugs can sometimes be found on the floor of the cage or on the faeces pan. In the study by *Szabo et al.* (1969) the number of pan plugs was remarkable. However, the pan plugs were not observed in this study, although observation could have improved the plug detection rate. Detection of pan plugs would have increased work and it was not suitable for the cages used in the experiment. Moreover, the purpose was to develop a quick method, hence pan plug detection was omitted.

In conclusion, the method described appears to offer an improved and straightforward method for plug detection with high predictability of pregnancy in rats.

Summary

Time mating of rodents is essential in many experimental procedures. The presence of the vaginal plug is frequently used as an indicator to confirm that mating has occurred. In rats, vaginal plugs are often deep and therefore difficult to detect by vulvar inspection. This study was carried out to assess the applicability of a human otoscope for detection of deep plugs, and the predictive value of plugs for pregnancy. A total of 132 Wistar female rats in proestrous were mated and examined for vaginal plugs on the next morning with an otoscope. All the plugs were located deep inside the vagina and could not be seen without the scope. The pregnancy rate of all females in proestrous was 69.7 %. Of all females, 72.7% had a vaginal plug and the positive predictive value of plug detection was 91.7 %. The sensitivity of the vaginal plug method was 95.7 % and specificity 80.0%. In conclusion, this study shows that detection of plugs has value for predicting pregnancies in rats. The method devised is quick, straightforward and painless to the animals and it is easily taught to technicians.

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