Can Wheat Germination Test Be Used to Predict Pregnancy in Ewes?

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RESEARCH ARTICLE

Abstract
Several invasive diagnosis methods to predict pregnancy in humans and animals were used in the past. Since Antiquity, grains germination test was used to predict more safely the pregnancy in women in order to avoid the possibility of miscarriage. The abscisic acid is a component of pregnant females’ urine that prevents germination of grains. Considering this principle, 20 samples of urine were collected from 10 pregnant ewes and 10 from non-pregnant animals. After urinalysis, 15 ml urine sample was added to 20 grains of wheat in a Petri dish, followed by a dilution of 1:4 with distilled water. Only water has been added in the control sample. There were performed duplicates for each animal. For 10 days, all plates were kept in a constant environmental temperature (22°C) and humidity (42%). Statistical analysis revealed that after 5 and 10 days, wheat grains belonging to pregnant ewes were less germinated, and values regarding shoot length were lower than those from the control group. Even if the number of samples is small, it can be concluded that wheat germination test can be used to predict pregnancy in ewes.

Keywords: sheep; Punyakoti test; wheat germination; pregnancy.

INTRODUCTION
Seed germination test, used for pregnancy diagnosis, is an example of ancient knowledge that could be found in the ancient Egyptian history of biology and medicine. A description of this test was written in Berlin Papyrus number 3038 (Grossi et al., 2019). At the beginning, this test, also called Punyakoti (Grossi et al., 2019), was used not just to test pregnancy in women, but also for social, legal, and political grounds. For sure these reasons, were certainly of supreme importance for such a highly organized Pharaonic Egypt society because wedding contracts with previously married women emphasize that interest (Ghalioungui et al., 1963) As soon as the results of this test were found to have benefits in humans, physicians started to test this method for animals, after Venna and Narendranath (1993) adopted suitable modifications to be used for pregnancy diagnosis in cattle. Nowadays, good results are obtained in pregnant cows (Narayana Swamy et al., 2010; Dilrukshi and Perera, 2012; Rine et al., 2014; Juodžentytė and Žilaitis, 2016; Skálová et al., 2017; Lázničková et al., 2020), buffaloes (Hussain et al., 2016; Rahman and Saha, 2020), ewes (Islam et al., 2014), goats (Islam et al., 2014), sows (Kumar et al., 2017), and alpacas (Kubátová and Fedorova, 2016). Throughout the time, the interest for a precocious pregnancy diagnosis in animals has increased in terms of herd management. There is a study (Nirmala et al., 2008) that claims that urinary reproductive hormones (especially estrogen and progesterone) do not affect the seed germination or shoot length, but Lázničková et al. (2020) believe that there is weak correlation between these hormones and the two
parameters mentioned. However, excluding hormonal assays or the use of ultrasonography and, the Punyakoti test can be used to predict pregnancy as early as 28 days, in 68% of the cases, and between 35-45 days of pregnancy, 100% respectively, in cows (Rao Krishna and Veena, 2009; Skálová et al., 2017).

The principal of Punyakoti method is represented by the abscisic acid (ABA) action against different types of seeds. ABA interferes with the differentiation of stem cells in the primary root meristem, which means that the subsequent development of tissues and organs is affected (Chen et al., 2020). This acid is a phytohormone that regulates abiotic stresses, such as temperature variations, water intake, high salinity, heavy metals and ultraviolet radiation quantity (He et al., 2018; Chen et al., 2020), and is generally considered as a plant growth inhibitor, but there are numbers of experimental data that show that ABA also supports root and shoot growth (Humplík et al., 2017; Bechtold and Field, 2018). ABA was first identified in the early 1960s in abscising cotton fruit and leaves of sycamore trees (Finkelstein, 2013), later in fungi (Takino et al., 2018), marine sponges (Zocchi et al., 2017) and in humans (Magnone et al., 2009), but since then, it has been used for biomedical applications (Bassaganya-Riera et al., 2010).

It seems that ABA is a physiological element of pregnant female urine, and according to Rine et al. (2014) the inhibitory effect of pregnant cow's urine on seeds starts by the end of the first month of pregnancy. The present study is the first one in Romania and the aim was to evaluate the result of Punyakoti test in a half breed sheep herd.

**MATERIALS AND METHODS**

The study was carried out in accordance with the current legislation of the European Union (Directive 2010/63/EU of the European Parliament and Council of 22 September 2010 on animal protection used for scientific purposes (Lázničková et al., 2020). Animals were familiarized gradually to the sampling procedure and there were not brutally handled in order to prevent stress.

**Animals**

The study involved 20 half breed ewes, between 2 and 5 years of age, divided into two groups (pregnant P n=10, and nonpregnant NP n=10) based on clinical and paraclinical investigations; P group contained 5 nulliparous and 5 multiparous ewes. The animals belong to a 1300 herd from Novaci city, Gorj county, in the south-west part of Romania.

During the study period (October-November 2019) the animals were housed separately in free-stall barns for pregnant, and non-pregnant ewes, and for rams. During this time all the sheep were kept on a permanent straw bedding, there were fed with dried hay and corn grains (600-700 g), twice per day, and the water intake was provided through fresh water troughs, *ad libidum*.

**Experimental design**

In this farm, all females were naturally breeded between August and September (short day seasonal breeders) with a male to female ratio of 1:20. One month after breeding, the ewes were ultrasonographically examined by transrectal way (Tringa Linear VET®, Esaote, The Netherlands) after a technique already described by Karen et al. (2004). At the same time, the ewes’ body condition score (BCS) (from 1 to 5, where 1 is thin and 5 is obese) was evaluated in the same manner as Russel described in 1991, for the first time. Thus, the spinous and transverse processes and loin muscles were palpated.

A second ultrasound exam was repeated one month later to exclude the embryonic mortality. Based on this procedure, the two groups of animals were created. After the last exam, in the following morning, urine samples have been collected (Figure 1) from each female using a urinary catheter, either spontaneous urination.

![Figure 1. Urine samples and urinalysis strips.](image)

Right after collection, urinalysis has been performed for each sample using dipsticks (Urinalysis Reagent Strips®,...
Laboquick, Turkey) indicating glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrite and leukocytes levels.

The germination test was performed by diluting 15 ml from each urine sample with distilled water at a ratio of 1:4. For each animal, four Petri dishes with twenty wheats (Triticum aestivum) seeds each have been used (Illico®, Syngenta, Romania). On three Petri dishes (U, U1, and U2), diluted urine (15 ml) was added and, on the other one, it was added distilled water (15 ml) – control group (C), as it was already described by Veena and Narendranath (1993) for sorghum seeds germination test in cows.

Materials used in this study were properly cleaned and sterilized and the wheat seeds were uniformly spread over a single layer into Petri dishes. In this study 1600 wheat seeds were used: 20 seeds for each Petri plate (4) for each ewe (20).

All Petri dishes were covered with their caps and were kept at a constant temperature (22°C) and humidity (42%) away from sunlight for the next 10 days. Five and ten days later, all seeds were evaluated, both the germinated and the ungerminated ones, in terms of bud length and color of the ungerminated seeds. The sprout length was measured in centimeters using a digital caliper. Two different examinations were performed; the first one after five days and the last one after ten days. Within these examinations, Petri dishes were observed and the germinated seeds were counted (out of all twenty seeds) in order to determine the percent of germinated seeds, using a formula explained slightly below. Accordingly, after the first five days, the ewes were considered pregnant if most of the seeds moisturized with diluted urine were not germinated and have turned blackish.

The percent of germinated seeds (PGS) was calculated using the following formula:

\[ \text{PGS} = \frac{\text{Total no. of germinated seeds}}{\text{Total no. of seeds/dish}} \times 100 \]

All data analysis, including the statistical one, were processed in Microsoft Excel 2016.

RESULTS AND DISCUSSIONS

The present study investigated the result of wheat germination test, and if there are some differences in urine parameters of pregnant and non-pregnant ewes.

The study shows that none of the animals were excluded based on disease criteria, all of them being healthy, with mean BCS for all females between 2.5 and 3.

In the same time, none of the ewes were affected, stressed or injured during investigations.

The percent of germination

Regarding the results of the germination test, it can be seen in the Figure 2 that more than 84% of wheat seeds belonging to the control groups germinated, in comparison with the seeds used in non-pregnant and pregnant ewes' urine, where less than 43%, respectively less than 15% of seeds germinated. In the same figure, it is showed that there are small differences between duplicates of non-pregnant and pregnant groups. From all 1600 wheat seeds used in this study, only 674 of them germinated.

![Figure 2. The results for wheat germination test in pregnant and non-pregnant ewes](image-url)
In the same time, standard deviation for seeds germination (Table 1) shows a small difference between test replicates of pregnant and non-pregnant females.

**Table 1. Standard deviation for seeds germination in all groups**

<table>
<thead>
<tr>
<th></th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Pregnant</td>
<td>8.32%</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>9.66%</td>
</tr>
</tbody>
</table>

By comparison to our study, Islam et al. (2014) obtained similar results, as following: a germination rate of 89.20 ± 0.95% for the control group, 75.16 ± 3.51% for non-pregnant females, and 15.55 ± 0.69% in pregnant sheep.

Given that there is just one paper in sheep on this subject, it can be said that the results of wheat seed germination test in ewes are similar to those in 45 days pregnant cows (Rine et al. 2014), buffaloes (Rahman and Saha, 2020) goats (Islam et al., 2014), pigs (Kumar et al., 2017) and alpacas (Kubátová and Fedorova, 2016).

The percentages of wheat germination test in pregnant and non-pregnant ewes are presented in Figure 3, respectively in Figure 4.

![Figure 3](image1.png)

**Figure 3.** The percentages of all Petri dishes belonging to each pregnant ewe

![Figure 4](image2.png)

**Figure 4.** The percentages of all Petri dishes belonging to each non-pregnant ewe

In Figure 5, the shoot length of wheat seeds by the 5th day of germination can be observed. Comparing to Figure 5 (b), Figure 5 (a) and 5 (c) show a higher percentage of germinated seeds and, at the same time, the shoot length was longer than the shoot length of pregnant ewes.
Studying the data analysis of shoot length after 5 days of germination (Figure 6), the pregnant sheep group has showed a very slow rate of shoot growth, with a mean of 0.30 cm.

The situation remains constant as well after the second control (10 days) in this group, as can be seen in Figures 7 and 8, where the mean shoot length was 0.57 cm.

By comparison, in the non-pregnant group, after 5 and 10 days of germination, the average shoot length was higher (1.22 cm, respectively 2.91 cm) than that for the pregnant group (Figure 9 and 10). Furthermore, the values for the non-pregnant group are very close to the control group values, where the average value at 5 days was 1.70 cm. The situation is different after 10 days because the mean shoot length is much higher in the control group compared to the non-pregnant group (6.01 cm, respectively 2.91 cm).

Islam et al. (2014) obtained similar values in their study after 5 days (0.68 cm and 2.31 cm in pregnant and non-pregnant ewes, respectively and 4.79 cm in control group). In cows, Juodžentyte and Žilaitis (2016) obtained...
answers in accordance with current results.

**Figure 7.** The shoot length of pregnant sheep group after 10 days of germination

![Figure 7](image)

**Figure 8.** (a) Shoot length for non-pregnant ewes after 10 days of germination; (b) Shoot length for control group after 10 days; (c) Shoot length for pregnant ewes after 10 days of germination

![Figure 8](image)

**Figure 9.** The shoot length of non-pregnant sheep group after 5 days of germination

![Figure 9](image)
It is known that through urine, some hormones like estradiol and progesterone are excreted and because of that, it was speculated that these can influence the germination of seeds and the shoot length. However, Nirmala et al. (2008) tested this hypothesis as null.

Urinalysis results

Regarding to urinalysis results, it can be seen in Table 2 that none of the animals showed ketone bodies or glucose. The pH level varied between 6 and 7, and the specific gravity between 1.005 and 1.020, both in pregnant and non-pregnant females.

In a work published by Athanasiou et al. (2018), it was observed that the urine dipstick pH values were situated between 6 and 8.5 in all categories of sheep. In the same paper, the authors obtained larger range values (5.98-8.73) using pH-meters.

Table 2. The urinalysis results obtained with dipstick pH kit for pregnant (1-10) and non-pregnant ewes (11-20) after some seconds, depending by each parameter.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Leukocytes at 120 s (Leu/μl)</th>
<th>Nitrite at 60 s (mg/dl)</th>
<th>Urobilinogen at 60 s (mg/dl)</th>
<th>Protein at 60 s (mg/dl)</th>
<th>pH at 60 s</th>
<th>Blood at 60 s (Ery/μl)</th>
<th>Specific gravity at 45 s</th>
<th>Ketone at 40 s (mg/dl)</th>
<th>Bilirubin at 30 s (mg/dl)</th>
<th>Glucose at 30 s (mg/dl)</th>
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</table>

‡ s – seconds
‡ neg. – negative value

Taking all of these into account, it can be speculated that, regarding herbivores, the dipstick measurements are suitable for pH evaluation with high applicability in field conditions, as Defontis et al. (2013) had already showed in calves.

Also, regarding urinalysis, in the Table 2 it can be seen that 5 out of 20 animals presented traces of blood in the urine samples. This fact can be due to bladder catheterization.
CONCLUSIONS

Using the Wheat Germination Test (also known as Punyakoti test), our study confirms that the physiological state of the ewes (pregnant or nonpregnant) can be assessed, depending on the number of germinated seeds. In spite of the reduced number of animals, the accuracy of our method reached 100% value. Germination inhibition and reduced growth of the wheat shoot length due to urine ABA content of pregnant animals indicated the pregnant state of ewes. Within the urinalysis no samples contained ketone bodies or glucose and the pH level varied between 6 and 7. This is the first study with this subject in Romania, and the second one world-wide, with good preliminary results which opens a new topic for research.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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