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Performing Early Pregnancy Tests in Milk and Their Effect on Cow Welfare and Reproductive Performance Compared to Rectal Pregnancy Tests 40 to 45 Days Post Insemination

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Simple Summary: Dairy cows' fertility is very important for the success of the dairy farm. In addition, welfare issues are very important and can have an effect on cow performance. A pregnancy test is a routine precoder in cows that is usually conducted by rectal palpation performed around 40–45 days from last insemination. Another possibility is a milk test performed 28 days post insemination. The second procedure does not require the use of headlocks. This study compares those two methods and tests the effects on cow welfare and reproductive performance. The milk test resulted in a 16-day reduction in days open for cows identified as non-pregnant on day 28 compared to day 42 (rectal examination). Well-being indices such as rest time and lying ratio of milk-tested cows improved, especially in winter. In addition to fewer days open and economic improvement, early milk tests for pregnancy can free up the practitioner for other tasks on the dairy farm.

Abstract: The main pregnancy test method for dairy cows is rectal palpation performed around 40–45 days from last insemination. This is an invasive examination that disrupts the cow's routine, potentially affecting its well-being. We examined the effectiveness of a commercial kit for performing pregnancy tests on milk samples compared to rectal palpation at 28- and 42-days post insemination. Accordingly, the purpose of the current study was to check if performing early pregnancy test instead of rectal palpation would result in better welfare and improvement of the reproductive performance, at least in part, of the cows. At 28 days, we examined the effect of early pregnancy testing on reproductive performance, especially number of days open. At 42 days, we determined the pregnancy test methods' effects on cow welfare measures and milk production. For the day 28 experiment, cows on one farm were divided into two groups: one milk-tested for pregnancy on 28 to 30 days after insemination, and the other tested by routine veterinary examination (42 to 45 days after insemination). The milk test resulted in a 16-day reduction in days open for cows identified as non-pregnant on day 28 compared to day 42 (rectal examination), potentially improving reproductive performance on the dairy farm. For the day 42 experiment, test effects on cow welfare as seen in pedometer data and milk production were examined on two large dairy farms. Test-day milk production did not differ significantly between milk-tested and rectally palpated groups, but well-being indices of milk-tested cows improved, especially in winter months. The reason for this can be because, in the summer, cows are interrupted due to colling activities. In addition to fewer days open and economic improvement, early milk tests for pregnancy can free up the practitioner for other tasks on the dairy farm.

Keywords: dairy cow; welfare; pregnancy; milk; PAG

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1. Introduction

Reproductive performance is one of the important factors that can affect dairy farms' professional and economical success. Finding new ways to improve fertility parameters

as well as dairy cows' welfare on the farms can lead to an economic improvement. Early identification of non-pregnant dairy cows after Artificial insemination (AI) improves reproductive efficiency and the 21-day pregnancy rate by decreasing the interval between AI services, thereby increasing the AI service rate [1]. Reducing the insemination interval for non-pregnant cows is expected to reduce the time to pregnancy and improve pregnancy rate [2–4]. From 55 to 70% of lactating dairy cows fail to become pregnant after AI and need to be reinseminated [5]. However, non-pregnant cows need to be identified before being subjected to reinsemination to further reduce the interbreeding interval. The diagnosis of pregnancy in cattle is traditionally performed on around day 40 after insemination by transrectal palpation of the reproductive tract for identification of the amniotic vesicle [6] or approximately 26 days after insemination with the aid of ultrasonography [7]. Thus, new technologies to identify non-pregnant dairy cows earlier may play a key role in management strategies to improve reproductive efficiency, dairy cow welfare and profitability on dairy farms. With today's enhanced awareness of cow welfare, using a system that does not require the use of headlock or isolating the cow can have positive value for the cows' well-being. In the current study, we decided to use a leg and neck pedometer for assessing the wellbeing of the dairy cows. This method does not require any interaction with the cow, so it will not have any effect on the result. The major advantages of these methods of pregnancy diagnosis are that a skilled—and therefore relatively costly—examiner is not required on the farm, and they can be performed sooner after breeding compared to rectal palpation. Note that direct palpation of the conceptus early in gestation has also been associated with pregnancy loss in some studies [8,9]. However, it is worth mentioning that recent studies did not find any impact of the rectal palpation on pregnancy loss [10,11]. However, the rectal palpation procedure likely leads to discomfort, resulting in physiological and behavioral stress reactions. Recently, more and more often, attention is paid to the negative impact of stress accompanying the rectal palpation and thus violating animal welfare [12]. Therefore, in Israel, testing all the cows as such is questionable and may need to be reconsidered. Under current law, each breeding animal has the right to freedom, including the right to freedom from: hunger, thirst, pain, stress, injuries, and diseases. Each clinical examination can cause stress in a cow; therefore, the investigator should minimize this effect and not allow pain. Measurements of heart rate and heart rate variability are considered as noninvasive methods for the assessment of the autonomic nervous system activity [13]. Stimulation of this system occurs during stress, and rectal examination is considered stressful. In one of the experiments, the heart rate was measured during the study in both lactating animals and non-lactating cows. An increase in heart rate was detected during rectal palpation in both lactating (+21.4 beats/min) and non-lactating cows (+20.6 beats/min). However, the magnitude of the response to stress and its time was greater in cows in lactation than in nulliparous [14]. Based on this research, it has been stated that the impact of rectal palpation on stress reactions, including cows' heartbeats, may have some influence on animal welfare in dairy farms [14].

Suffering, discomfort, pain and stress are uncomfortable conditions for animals, and animal welfare targets a life without these conditions. Today, the absence of stress responses is accepted as an indicator of welfare in animals [15].

Changes in feeding behavior, development of gastric and intestinal ulcers, hypertension, reproductive dysfunction, malnutrition, electrolyte imbalance and immune deficiency may be related to stress responses [16]. Most veterinary procedures, such as rectal palpation, increase the secretion of cortisol from the adrenal cortex in cattle. Rectal palpation has been shown to affect the animal's physiology and behavior in a manner typical of an acute stress response [17], as evidenced by strong increases in circulating cortisol, total oxidant status and nitric oxide concentrations associated with decreases in total antioxidant status [18]. Increased plasma cortisol concentrations in response to rectal palpation may also enhance oxidative stress [19]. The growing trends of larger herd sizes and fewer veterinarians entering rural veterinary practice [20] call for the development of new methods that will not require a highly skilled operator to be present at the time of testing. The potential

for using those pregnancy detection methods in automated systems that are part of the milking parlor provides additional motivation for their development. Assays for detecting pregnancy-associated glycoprotein (PAG) levels in the maternal circulation originating from mononucleated and binucleated cells of the embryonic trophoblast have been developed and commercialized to determine pregnancy status in cattle [21–23]. One of the main problems that Israeli farmers suffer today is to find good workers, so finding a testing procedure that can reduce workload is a very critical point. Reducing routine work from the practitioner could provide him with extra time for more important things. In this regard, the aims of the current study were to determine whether early diagnosis of non-pregnant cows using the milk test, and early reinsemination, will reduce the number of days open and to test the correlation of the milk test results with the welfare indicators and the results collected by the pedometer.

2. Materials and Methods

2.1. Experimental Design

The experiment was conducted for one year in 2020 on three different commercial dairy farms. Cows received a total mixed ration containing 1.77 Mcal/day, 16.5% protein and 32% neutral detergent fiber, ad libitum. Cows were milked three times daily, yielding an average 12,300 kg milk over 305 days. Two of the dairy farms were located along the coast and the third was in the Jordan valley. All the experimental cows were Holstein breed, and the average number of lactations was between 2.5 and 2.8 in the different farms. The herd structure is 33–36% of primiparous, 25% of 2nd lactation cows and around 40% of multiparous cows. Cows were held in open shaded structures in all herds with dry bedding. Cows add 20–23 square meters per cow. Cows underwent their first AI following detection of estrus according to herd policy, i.e., between 70 and 90 d in milk according to parity and BCS. Cows were milked in all the dairy farms in a parallel milking parlour while standing on metrics during milking as well as while standing in the waiting yard. Cows were cooled during the summer by a sprinkling and ventilation cooling system. Cooling was applied in the waiting area before milking and along the feeding lines. Cows were cooled for about 6 h during a 24-h cycle. The average temperature and humidity in both farms located along the coast and used for the second study were: 9 to 17 °C in the winter and 22 to 32 °C in the summer. The third dairy farm, which was used for the first study, had a higher temperature, ranging between 10 and 18 °C during the winter and 25 and 40 °C during the summer. Detection of estrus occurred twice daily before milking by a computerized pedometric system (Afimilk, Afikim, Israel). A professional technician routinely performed AI once a day in the morning hours. A cow that recorded in estrus during the morning hours was inseminated on the same day; if onset of estrus was recorded in the afternoon or evening, the cow was inseminated the next morning. The first part of the study was performed on one dairy farm to examine the effectiveness of performing pregnancy tests on milk samples as a substitute for rectal palpation tests 28 days after insemination. We examined the effect of early pregnancy testing on various reproductive performance parameters and, in particular, number of days open. A total of 466 cows were divided into two groups: (1) pregnancy test performed on the milk sample 28–34 days after insemination (n = 212) and (2) pregnancy test performed by rectal palpation 42–45 days after insemination (n = 254). Cows in Group 1 that were positive for pregnancy at 32 days were tested again at around 60 days after insemination using milk samples, as in the first test. Cows that tested negative were synchronized for reinsemination. Cows in Group 2 that were positive for pregnancy did not undergo another test. Cows that tested negative were synchronized for reinsemination as in the first group. The synchronization protocol that was used was the OVS protocol, which consisted of first GnRH injection (200 µg of Gonadorelin i.m., Gonabreed, Parnell Laboratories, Alexandria, Australia) followed by PGF2α 7 d later (500 µg of Cloprostenol, Estroplan, Parnell Laboratories) and a second GnRH injection 60 h later, and timed AI 16 h after GnRH. We tested the effect of several fertility parameters, including (i) number of days from negative pregnancy test to reinsemination and (ii) numbers of days open.

The second part of the study was performed on two large dairy farms to examine the effects of pregnancy testing in milk samples 42–45 days after insemination on cow welfare and milk production. In the current study, we decided to use leg and neck pedometer for assessing the wellbeing of the dairy cows. This method does not require any interaction with the cow so will not have any effect on the result. On Farm A, cows (n = 1241) were divided into two groups: (1) pregnancy test performed on milk sample 42–45 days after insemination (n = 547) and (2) pregnancy test performed by rectal palpation 42–45 days after insemination (n = 694). In the two groups, negative and positive cows were treated according to the herd manager's decision. On Farm B, cows (n = 1517) were divided and treated as in Farm A, with n = 323 and n = 1194 for Group 1 and Group 2, respectively. Tested parameters were: (i) number of times locking up the cows for the rectal examination, (ii) length of time from tying to rectal examination by the veterinarian and (iii) total time that the cow was isolated from its group. In addition, we tested milk production and behavioral parameters in both groups.

2.2. Sampling and Analysis

Activity data were recorded by pedometer located on the cow's front leg (Afimilk, Afikim, Israel). Milk samples were collected during milking from healthy quarters into a 25-mL screw-cap tube after elimination of foremilk. The level of PAGs in the milk was assessed using a quantitative milk ELISA (Milk Pregnancy Test Kit, IDEXX Laboratories Inc., Westbrook, ME, USA). Assays were performed according to the manufacturer's instructions using the dedicated software (xCheckPlus, IDEXX Laboratories Inc., Westbrook, ME, USA). Each 96-well ELISA plate included positive and negative controls added in duplicate. A 450-nm wavelength filter was used to read the color change of the serum and for the milk pregnancy test kit. A quantitative result was determined as the sample optical density (OD) minus the mean OD of the negative control in the plate. A cutoff of $\geq$0.25 was used to classify milk ELISA results as positive. A cutoff of <0.1 indicated negative ELISA results. Results between these values were classified as recheck (unless the cow was more than 44 days after last insemination; then results >0.1 were considered positive).

2.3. Statistical Analysis

The test results were documented in the herd management software (NOA, ICBA, Caesarea, Israel). Data on fertility parameters were tested using the Excel program. Distribution of positive and negative dairy cows according to time and pregnancy test method was noted. Differences in days to next insemination or data on days open were analyzed using the Proc Means procedure of SAS (SAS Institute Inc. Version 9.2, Cary, NC, USA, 2009).

The duration for which the cows were tied up or away from their normal group was calculated and is presented as means $\pm$ SE. Milk production and behavioral data (steps per hour, resting time, number of times lying down, lying ratio, average lying time and activity-to-lying ratio) were analyzed by the Proc Mixed procedure of SAS (version 9.2, SAS Institute). The statistical model included: milk production/behavioral data = treatment + herd + parity + season + treatment $\times$ herd + error; where treatment = milk vs. rectal pregnancy test, herd = Farm A or Farm B, parity = 1st lactation cows, 2nd lactation cows and 3rd or more lactation cows, season = winter vs. summer. Cows were served as random effect. Cows were nested within pregnancy method used. To compare levels within a variable, we ran the Bonferroni adjustment for multiple comparisons. Data are presented as least square means $\pm$ SEM.

3. Results

3.1. First Study

Dairy cows with a positive pregnancy diagnosis on days 28–34 (milk test) or days 42–45 (rectal test) did not differ in days open (121 vs. 126, respectively; Table 1). Cows diagnosed as pregnant on days 42–45 had an abortion rate until calving of 8.6%, compared

to milk-tested cows which showed 11.9% abortion from day 32 until calving (Table 1). It is interesting to note that after the second milk test, only 1.6% of the pregnant cows aborted.

Table 1. First positive pregnancy test in both treatment groups.

Days Open	Abortion (%) [3]	Late Embryo Death [2]	Days to 2nd Pregnancy Test [1]	Days to 1st Pregnancy Test	n	Test Method
121	8.65	-	-	42	185	Rectal
126	1.64	11.93	58	34	176	Milk test

[1] Second test was performed only for cows in the PAG group. [2] Between first and second milk test. [3] In the rectal palpation test group—from day 42 until calving; in the milk test group—from day 58 until calving.

Data on cows that tested negative for pregnancy after 28–34 days (milk test) or 42–45 days (rectal test) are summarized in Table 2. Of the total negative cows in the milk test group, 88.8% were reinseminated after 14 days on average and had 188 days open (Table 2). Of the cows testing negative in the rectal palpation group, only 63.7% were reinseminated. The time to next AI did not differ from the milk-tested group and averaged 14 days. However, the days open was longer, with an average of 204 days (Table 2).

Table 2. First negative pregnancy test in both treatment groups.

Days Open	Days to Next AI	Culled (%) [1]	Inseminated Again (%)	Days To 1st Pregnancy Test	n	Test Method
204	14	36	64	42	69	Rectal palpation
188	14	11	89	34	36	Milk test

[1] Cows that were culled from the herd before next AI.

3.2. Second Study

Data on number of cows in each group and percentage of cows in the positive, negative and recheck groups are summarized in Table 3. On both farms, the percentage of negative results was higher in the rectal palpation vs. milk-tested group. The percentage of results that were rechecked ranged between 5 and 8%, but this occurred on only one farm.

Table 3. Descriptive data on pregnancy results according to test method.

Unknown (%)	Negative Result (%)	Positive Result (%)	Test (n)	Test Day	Test Method	Farm
5.3	32.4	62.3	547	45	First milk test	
8	7.4	84.6	325	56	Second milk test	A
-	43.1	56.9	694	43	Rectal palpation	
-	22	78	323	44	First milk test	
-	2.6	97.4	194	58	Second milk test	B
-	36.4	63.6	1194	46	Rectal palpation	

Cow activity measured by automatic pedometer was recorded on Farm A. Figure 1 presents the pedometer data. Number of steps per hour was lower in milk-tested cows compared to rectally palpated cows (Figure 1A; $p < 0.05$). In agreement with this, rest time, number of lie-downs and lying ratio were higher in the milk-tested group compared to the rectally palpated group (Figure 1B–D; $p < 0.05$ to $p < 0.001$). Average lying time did not differ between the groups (Figure 1E). The most interesting finding was a reduction in the activity-to-lying ratio in cows from the milk-tested vs. rectal test group, indicating that the former were less stressed (Figure 1F; $p < 0.005$).

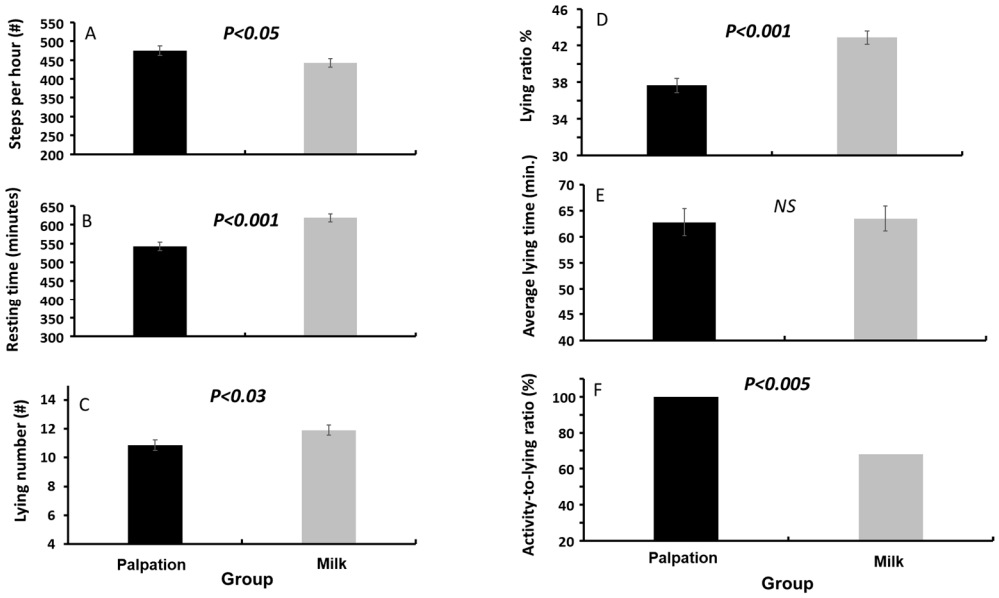

Figure 1. Pedometer data from dairy cows on day of rectal palpation vs. milk pregnancy test. (**A**) Number of steps per hour between two consecutive milking. (**B**) resting time in minute between two consecutive milking. (**C**). number of times the cow is lying between two consecutive milking. (**D**). percentage of lying between two consecutive milking. (**E**). average time for each lying event between two consecutive milking. (**F**). the ratio between activity time to lying time between two consecutive milking. NS—nun significant.

The time for which the cows were locked up or away from their normal group for rectal palpation on Farm A and Farm B is presented in Figure 2. On Farm A, total time away from the group was around 5 h, and the cows were tied up for around 4 h. On Farm B, the time was longer for both measurements: around 6 h locked up and more than 8 h away from the group. Obviously, cows in the milk-test group were not tied up and did not leave their normal group. Milk amount on the test day did not differ between the groups, although, in farm B, cows that were tested by milk had numerically higher milk level during the examination day (41.67 and 41.98; 31.41 and 32.91 for farms A + B palpation group vs. milk group respectively; $p > 0.05$).

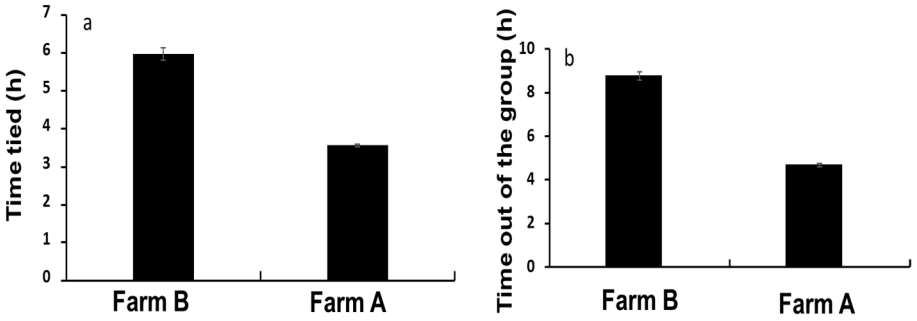

Figure 2. Time cows were tied up (**a**) and away from their group (**b**) while waiting for rectal palpation test in the two different dairy farms A and B.

4. Discussion

We found that performing a pregnancy test on a milk sample instead of by rectal palpation can improve animal welfare and, if conducted early (30 days post-AI), may decrease the number of days to next AI and days open in cows testing negative; this, in turn, can improve reproductive and economic performance of the dairy farm. The profitability of dairy farms strongly depends on the reproductive efficiency of dairy cows [2,24,25]. Many studies have documented that the period during which cows are not pregnant beyond the

optimal time post calving is costly [2,26,27]. The value of a pregnancy depends on several parameters, such as stage of lactation, lactation number, milk yield, persistence of lactation, milk price and breeding, forced culling and replacement decisions.

Rectal palpation for pregnancy is routinely performed in Israel by the veterinarian at around 42 days after AI. This method requires separating the cow from its group and closing them in headlocks for the test; this disruption in the cow's routine can affect its performance and welfare. Reinsemination of non-pregnant cows requires either adequate detection of estrus or an accurate method of pregnancy diagnosis that can be used as soon as possible after finding that the cow is not pregnant [28].

Today, the most common approach for bringing cows back for reinsemination is resynchronization programs, which serve to manage reproduction in dairy cattle with the aim of improving reinsemination rates [29,30], so that time to pregnancy can be shortened. Therefore, establishing methods to perform a pregnancy test as soon as possible after insemination is expected to benefit reproductive performance by reducing the interval between finding the cow non-pregnant and the next insemination, leading to a reduction in days open [3,4,31]. In the current study, cows that were found negative for pregnancy after 30–34 days and were subjected to resynchronization protocols had less days open compared to dairy cows that only tested negative after 42–45 days. However, this potential reduction will only be found in a low percentage of the herd. As an example, for a herd with 100 inseminated cows, approximately 40% will become pregnant after the first AI, leaving 60 cows. Of those 60 cows, about 65–70% (39–42 cows) will go through another estrus and will be inseminated after 24 days, leaving approximately 18% of the cows in which late embryonic mortality can occur between days 26 and 35. Those cows will be the potential non-pregnant cows 28–34 days after insemination, which could reduce their interval to the next insemination and decrease days open. Accordingly, those cows will have a mean interval between inseminations of 14 days less, resulting in a numerical reduction of 16 days open. Sinedino et al. [32] also found a 13-day reduction in the mean interval between inseminations that resulted in a numerical reduction of only 8 open days. Silva et al. [33] reported a 14.5-day reduction in cows resynchronized following a non-pregnancy diagnosis by PAG ELISA on day 27 compared to cows examined by transrectal ultrasonography on day 32 after AI. In general, the benefit of an early resynchronization should be observed when detection of spontaneous estrus and reinsemination are low.

Therefore, benefits of an early pregnancy diagnosis and resynchronization seem to depend on the efficiency of estrus detection: when this is high, such that a large proportion of the cows are reinseminated before pregnancy diagnosis, then the potential benefits of an early method for pregnancy diagnosis and resynchronization are largely diminished.

As anticipated, the cows in the rectal palpation group that were found negative on days 42–45 had the same interval to the next AI but a higher interval for becoming pregnant at 16 days.

Pregnancy testing by transrectal ultrasonography or manual palpation requires regular visits by qualified veterinary personnel, which can disrupt the farm routine. This disruption can affect animal welfare, as seen by activity changes and restrain leading to a stress response [16]. Rectal palpation which requires restriction of the cows in headlocks may result in physiological and behavioral changes that are typical of a stress response [17,34], yet it is a preferred method among veterinarians for physical examination, pregnancy diagnosis and evaluation of reproductive ability in clinical practice [35,36]. In this study, we also found behavioral changes in the cows that were tested for pregnancy by rectal palpation compared to those whose milk was tested, as indicated by pedometer activity. Cows in the rectal palpation group had more steps per hour, less resting time, a lower number of lie-downs per season and a lower lying ratio; in addition, activity-to-lying ratio and a parameter indicating the cow's total activity were higher. Note that reduced lying time can affect rumination time and milk production. However, in this study, we did not find significant differences in milk production, which can lead to the assumption that cows tied for rectal palpation did not suffer from an acute stress response.

5. Conclusions

Early detection of non-pregnant cows 28 to 34 days after AI reduced the number of days open in a subset of cows. Earlier reinsemination of those cows could decrease the amount of non-pregnant cows late in lactation, leading to better planning ability for the farm's milk production, less forced culling of non-pregnant cows and the possibility of economic improvement. Use of the milk test for pregnancy could prevent unnecessary restriction of cows and improve their welfare, as evidenced by a slight improvement in the welfare and well-being indices as reflected by pedometer activity. In addition, use of this method will provide the medical practitioner with more time to deal with other problems on the farm.

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