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Original Article

Pre-ovulatory follicular cooling correlates positively with the potential for pregnancy in dairy cows: Implications for human IVF

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ABSTRACT

Introduction: In rabbits, pigs, cows and humans, pre-ovulatory Graafian follicles may be more than 1.0 °C cooler than ovarian stroma and both these ovarian compartments are cooler than deep rectal temperatures. This study examines the effect of follicular cooling on the incidence of pregnancy in dairy cows.
Material and methods: Follicular measurements were compiled for cows with one ovulatory follicle (monovular) and cows with one ovulatory follicle per ovary (bi-ovular) and their corresponding uterine horn contents. The study sample consisted of 80 pre-ovulatory follicles in which antral temperatures were measured using a fine thermistor probe.
Results: Mean (± S.D.) follicular fluid temperature of the ovulating follicles was 1.12 ± 0.86 °C significantly cooler ($P < 0.0001$) than rectal temperatures. No significant differences in temperatures were found for non-ovulating follicles. In follicles undergoing cooling ($n = 58$), a one-tenth of a degree drop in temperature with reference to control rectal temperature gave rise to a 3.6-fold increase (odds ratio) in the pregnancy rate ($P = 0.003$). The follicle-rectum temperature differential giving rise to pregnancy ($n = 18$; 1.51 ± 1.15 °C) was significantly greater ($P = 0.004$) than the differential recorded in cooling follicles at that did not result in a subsequent pregnancy ($n = 40$; 0.83 ± 0.57 °C).
Conclusion: Follicular cooling is needed to trigger ovulation and correlates positively with the potential for pregnancy in cows. This finding has interesting implications for human reproductive medicine.

Introduction

Graafian follicles that are just about to embark on the process of ovulation may be more than 1.0 °C cooler than ovarian stroma and both these ovarian compartments are cooler than jugular and deep rectal temperatures [1,2]. This follicular cooling has been described in rabbits, pigs, cows and women. However, original observations on temperature gradients in ovarian tissues have not been seen as having wide implications due in part to the use of anaesthetics and open surgery (laparotomy). Recently, antral pre-ovulatory follicle temperatures have been measured directly in cows not subjected to any type of sedation, anaesthetic or surgical procedure using a fine thermistor probe [3,4]. The probe was introduced via the vagina into the antrum of a pre-ovulatory follicle under ultrasonographic monitoring. Antral fluid temperatures were significantly cooler than rectal temperatures in all cows that ovulated, whereas no significant temperature gradient was recorded in cows that failed to ovulate [3,4].

The extent of cooling of pre-ovulatory follicles varies considerably among individual animals. For example, follicular antral temperatures may be 0.6–5.5 °C cooler than rectal temperatures in cows that become pregnant [3,4]. Progress on the mechanism of follicular cooling has been hindered by an inadequate population size for determining possible relationships between that extent of cooling and chances of subsequent pregnancy. In addition, previous studies on the role of follicular cooling have been performed on monovular [3] or bi-ovular [4] cows showing different patterns of cooling. In essence, the mean follicular temperature drop recorded in bi-ovular cows (−0.93 °C) was smaller than that obtained in monovular cows (-1.54 °C). These differences were measured in follicles that subsequently ovulated.

Members of the bovine genus have two uterine horns and a small uterine body. Cows are predominantly monovular and following fertilisation the embryo passes into the uterine horn ipsilateral to the ovulatory ovary. Embryo redistribution, that is the presence of an embryo in the uterine horn contralateral to the corpus luteum, is a very rare phenomenon, with reported rates of

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0% (zero of 643) [5], 0.1% (one of 1000) [6] and 0.2% (seven of 3094) [7] of pregnancies. This retrospective study assembled a large number of follicular measurements from cows with one ovulatory follicle (monovular) and cows with one ovulatory follicle per ovary (bi-ovular) and their corresponding uterine horn contents. The larger data set increased the likelihood of finding previously unreported aspects of the follicular temperature gradients associated with pregnancy.

The study sought to determine whether the extent of follicular cooling influences the occurrence of ovulation and incidence of pregnancy in lactating dairy cows.

Materials and methods

Experimental animals, temperature measurements and data source

Data were obtained from the records of two studies reported in 2017 (16 cows with one pre-ovulatory follicle) [3] and 2018 (24 cows with one pre-ovulatory follicle per ovary) [4]. Additional data were obtained from further 16 cows with one pre-ovulatory follicle kept under the same experimental conditions as those in the first study [3]. All data derived from a commercial dairy herd of Holstein-Friesian lactating dairy cows (*Bos taurus*) in north-eastern Spain from July to September 2016 [3], 2017 [4] and 2018 (new cows in the same herd). Only healthy cows producing more than 30 kg milk per day, free of detectable reproductive disorders and free of clinical diseases during the study period (days -5 to 28 of insemination) were included. Cows were synchronised according to a progesterone-based protocol for fixed-time insemination, and selected for temperature measurements at the time of insemination [3]. By means of ultrasonography and manual rectal palpation combined, it was confirmed that a cow was in oestrus and ready for insemination [8]. Only cows with one pre-ovulatory follicle larger than 10 mm in diameter per ovary, in the absence of a corpus luteum (CL), were included in the study. It was also confirmed that the uterus was highly tonic and contractile to the touch, and that there was copious and clear vaginal discharge. The final study sample consisted of 80 pre-ovulatory follicles.

Temperature measurements and experimental design

The temperature within each pre-ovulatory follicle and approximately 20 cm deep within the rectum was measured using a fine thermistor probe [3]. The probe was made with a 0.5 x 520 mm thermistor bead with a 20 μm diamel-coated silver wire to 0.75-m-long polythene-coated wire (ThermaData^{TM} Logger TCD; E.T.I. Electronic Temperature Instruments, West Sussex, UK). The probe was connected to a thermistor amplifier with a single scale from 30 to 40 °C and accurate to 0.04 °C and programmed for temperature readings each 12 s. The thermistor bead was introduced into a sterile 17 G 50-cm long needle with an echogenic tip (COVA needle type-A; Misawa Medical Industry CO., Ltd, Tokyo, Japan) for follicle puncture [3]. To guide the probe, a portable B-mode ultrasound scanner (E.I. Medical IBEX LITE; E.I. Medical Imaging, Loveland CO, USA) equipped with a convex 5–10 MHz (E.I. Medical IBEX MC8.0 10-6 Microconvex; E.I. Medical Imaging, Loveland CO, USA) transducer for transvaginal use was employed.

Immediately before the temperature measurements, the vulva and perineal region of the cow were washed with disinfectant solution. The transducer probe coated with a sterile preservative and needle containing the thermistor probe were positioned and pre-warmed for 60 s in the dorsal vaginal fornix, to the left or right of the cervix depending on the side of the pre-ovulatory follicle to be measured. The ovary with the pre-ovulatory follicle was then positioned trans-rectally against the tip of the transducer probe so that the follicle was separated only by the vaginal wall. The vaginal wall was pierced in a cranial direction through the fornix with the needle and introduced into the follicular antrum as previously described [3]. The needle with the thermistor probe was maintained for 36 s to take three readings. Immediately after the third reading, the needle was carefully removed from the follicular antrum and, further measurements made in the contralateral ovary in the case of bi-ovular cows. Finally, the needle was positioned within the tip of the transducer, just emerging 1–2 mm and the transducer introduced 20 cm deeply into the rectum for 36 s and three rectal temperature readings obtained. The temperatures recorded were the means of three readings. Cows showed no signs of discomfort during intra-follicular puncture.

Cows underwent artificial insemination (AI) immediately after temperature measurements. All cows were inseminated by one technician using frozen-thawed semen from one ejaculate. Ovulation was determined as disappearance of the pre-ovulatory follicle 24 h after insemination. The presence of a CL was assessed seven days post-AI. Pregnancy diagnosis was performed by ultrasonography 28 days post-AI. All procedures were approved by the Ethics Committee on Animal Experimentation of the University of Lleida (license number CEEA.06-01/12.

In our geographical area, a clear negative effect of heat stress from May to September on the reproductive performance of lactating dairy cows has been extensively described [9,10]. In effect, ovulation failure increases dramatically under heat stress conditions [11]. Thus, to increase the number of pre-ovulatory follicles failing to ovulate, this study was performed under heat stress conditions. Heat stress conditions were defined as a maximum temperature-humidity index (THI) higher than 72 determined on the day of AI [12].

Data collection and statistical analysis

Ovarian follicular structures and the absence or presence of one CL were assessed by ultrasonography immediately before AI, and seven days after AI, respectively. The following data were recorded for each pre-ovulatory follicle: follicular size at AI (diameter of the follicles > 10 mm); follicular and rectal temperatures and the corresponding differential; ovulation failure (absence of a CL seven days after AI); ovary in which follicular or luteal structures were recorded (right versus left ovary); and pregnancy after AI, defined as the presence of one embryo in the uterine horn ipsilateral to the corresponding pre-ovulatory follicle.

The reproductive potential of the pre-ovulatory follicle side (right versus left ovary) was assessed in a chi-squared test (percentages) or by one-way ANOVA as implemented in the SPSS software package, version 18.0 (SPSS Inc., Chicago, IL, USA) (means ± SD). The effects of pre-ovulatory size on follicular cooling rate (follicles cooler than rectum) were analyzed by binary logistic regression (logistic procedure of PASW Statistics for Windows Version 18.0, SPSS Inc., Chicago, IL, USA) adjusting for side (right versus left ovary) and follicular co-dominance (monovular versus bi-ovular cows). As no follicle not undergoing cooling ovulated, the effects of cooling on pregnancy were analyzed only for follicles that had shown cooling. A binary logistic regression analyses was performed using pregnancy as the dependent variable. The factors entered in the model as independent dichotomous variables (where 1 denotes presence and 0 denotes absence) were side and co-dominance, and follicular size and follicular temperature (continuous variables) were considered factors in the analyses. The estimates and Wald 95% limits were used to calculate odds ratios and 95% confidence intervals (CI). Explanatory variables were assessed according to the method of Hosmer and Lemeshow [13] using a backward elimination procedure, and the variables that significantly affected pregnancy rate were kept in the model.

Basically, this method consists of five steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all the significant variables arising from the univariate analysis; stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; evaluation of plausible interactions among variables; and assessment of model fit using Hosmer-Lemeshow statistics. Variables with univariate associations showing P values < 0.25 were included in the initial model. Model reduction continued until only significant terms according to the Wald statistic remained in the model at $P < 0.05$. Values are provided as the mean ± the standard deviation (SD).

Results

All measurements were performed under heat stress conditions in the THI range 72–86. No variations were registered in the three consecutive readings performed in each follicle, whereas some variations were registered with values always less than five-hundred of a degree in the three consecutive readings of the rectum. Summarized findings regarding pre-ovulatory and deep rectal temperatures are provided in Table 1. Of the 80 follicles included in this study, 51 ovulated, all of which had undergone cooling. Of the 29 non-ovulating follicles, 7 underwent cooling 0.1 to 0.9 °C cooler than rectal temperatures, while temperatures of the remaining 22 follicles were equal to or higher than their corresponding rectal temperatures. None of the 29 non-ovulating follicles gave rise to pregnancy. In contrast, an embryo was found ipsilateral to its corresponding pre-ovulatory follicle in 18 (35.3%) of the 51 ovulating follicles.

Mean rectal temperature was 38.5 ± 0.38 °C, ranging from 37.8 to 39.2 °C. Follicular fluid temperatures in the ovulating follicles were 1.12 ± 0.86 °C cooler ($P < 0.0001$) than rectal temperatures, ranging from −0.20 to −5.5 °C. No significant differences in temperatures were detected for non-ovulating follicles. Mean temperatures of the ovulating follicles were significantly lower than those of the non-ovulating follicles, whereas follicular diameter was similar for both (Table 1). Significant right-left differences were not found.

For all follicles (n = 80), based on odds ratios, pre-ovulatory size, side (right versus left ovary) and follicular co-dominance (monovular versus bi-ovular cows) had no effects on follicular cooling.

In follicles experiencing cooling (n = 58), a one-tenth of a degree in follicular temperature with reference to control rectal temperature was found to give rise to a 3.6-fold increase in the pregnancy rate at 28 days post-AI (odds ratio: 3.6; 95% confidence interval: 1.25–10; $P = 0.003$; R^2 Nagelkerke = 0.19). The mean follicular fluid-rectal temperature differential for the cooling follicles that resulted in pregnancy (n = 18; 1.51 ± 1.15 °C, ranging from −0.60 to −5.50 °C) was significantly higher ($P = 0.004$) than that for cooling follicles that did not result in pregnancy (n = 40; 0.83 ± 0.57 °C, ranging from −0.10 to −3.10 °C).

Discussion

The measurements reported in this study endorse earlier observations that Graafian follicles undergoing pre-ovulatory cooling are those most likely to produce a potential pregnancy [3,4]. Precisely why pre-ovulatory cooling of the enclosed oocyte is beneficial remains to be analysed at the molecular level, but there should now be sufficient confidence in our results to stimulate such fundamental work. Over and above such aspects, the relevance of this work in cattle to current procedures in human IVF clinics cannot be overlooked. As suggested previously [14,15] and now endorsed vigorously, the selection of oocytes in human IVF procedures on the basis of follicular temperature should be given meaningful trials. The construction of an oocyte aspiration pipette that simultaneously measures follicular temperature would be straightforward and inexpensive with modern technology.

In a recent study, cooling of human oocytes *in vitro* from 37 to 36.5 °C led to a lower fertilization rate, 37 °C being the standard temperature for assisted reproductive technologies [16]. *In vivo*, however, oocyte development and maturation occur below the core body temperature, reinforcing the present findings [1,2].

As to our overall proposals based on this work in cattle, reservations may arise in light of the incidence of pregnancy reported in Table 1. However, the relatively low figures are undoubtedly a reflection of the strongly negative influence of heat stress on fertility, and such stress reached a seasonal high during the period of study [9,10,12]. In any event, the incidence of pregnancy reported here does not contradict our thesis and, although our clinical studies will continue, our principal wish is that human fertility clinics will now take the initiative and perform parallel studies.

Apart from specific temperature measurements, there are various observations in this study of direct interest to colleagues working on physiology of the mammalian ovary. These include potential differences in follicular expression between left and right ovaries, and also the influence of monovular versus bi-ovular situations on temperature measurements. We are attempting a detailed analysis of these features in separate publications, and do not wish them to detract from the major proposal presented here.

As an overall perspective by way of conclusion, and surprising as it may seem to a medical audience, bovine ovaries in diverse regards offer a valuable model for human ovaries. A willingness to appreciate this fundamental point should result in intellectual stimulation for readers who follow this work.

Table 1
Summarized findings concerning pre-ovulatory follicular and deep rectal temperatures (mean values ± S.D.; n between parentheses).

	Right ovary	Left ovary	Both ovaries
Temperature (ºC)			
All follicles	37.7 ± 0.68 (43)	37.8 ± 0.85 (37)	37.7 ± 0.84 (80)
Ovulating follicles	37.5^a ± 0.61 (28)	37.4^a ± 0.85 (23)	37.4^a ± 0.72 (51)
Non-ovulating follicles	38.7^b ± 0.35 (15)	38.8^b ± 0.28 (14)	38.8^b ± 0.31 (29)
Follicles cooler than rectum (differential)	−1.06 ± 0.72 (31)	−1.09 ± 0.85 (27)	−1.07 ± 0.86 (58)
Pregnancies	11	7	18
Follicular diameter (mm)			
All follicles	15.8 ± 2.58 (43)	16.0 ± 2.94 (37)	15.9 ± 2.80 (80)
Ovulating follicles	15.9 ± 2.65 (28)	16.1 ± 3.07 (23)	15.9 ± 2.53 (51)
Non-ovulating follicles	15.6 ± 2.38 (15)	16.0 ± 3.05 (14)	15.8 ± 2.82 (29)

Values with different superscript differ significantly within columns when tested by one−way ANOVA (P < 0.001).

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Conflict of interest

The authors declare that they have no conflict of interest.

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