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Review

Pitfalls in animal reproduction research: How the animal guards nature's secrets

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ABSTRACT

The estrous cycles of heifers and mares are used for illustrating pitfalls at the animal level in research in reproductive biology. Infrequent monitoring for characterizing the change in hormone concentrations or for detecting a reproductive event can be a pitfall when the interval for obtaining data exceeds the interval between events. For example, hourly collection of blood samples has shown that the luteolytic period (decreasing progesterone) encompasses 24 hours in heifers and mares. Collection of samples every 6–24 hours results in the illusion that luteolysis requires 2–3 days, owing to the occurrence of luteolysis on different days in individuals. A single treatment with PGF2α that causes complete regression of the corpus luteum is an example of an overdose pitfall. A nonphysiological progesterone increase occurs and will be misleading if used for making interpretations on the nature of luteolysis. A pitfall can also occur if a chosen reference point or end point is a poor representation of a physiological event. For example, if on a selected day after ovulation the animals in treatment A are closer on average to luteolysis than animals in treatment B, treatment A will appear to have had an earlier luteolytic effect. Among the techniques that are used directly in the animal, ultrasonography appears to be most prone to research pitfalls. Research during a given month can be confounded by seasonal effects, even in species that ovulate throughout the year. The presence of unknown factors or complex interactions among factors and the sensitivity of the animal to a research procedure separate from the direct effect of a treatment are also research challenges. A hidden factor should be considered nature's challenge to open-minded biologists but a pitfall for the close-minded.

1. Introduction

"Nature guards her secrets well" can be a consoling conclusion after a research project in biology. Biological mechanisms are intricate and exquisite in sharp contrast to our relatively clumsy attempts to unravel them. Biological research therefore is replete with pitfalls. Although some research pitfalls seemingly were designed by nature to mislead the researcher, it is the investigator who carries the responsibility for planning and conducting a research project and therefore for anticipating and minimizing pitfalls. This report describes pitfalls encountered by the author at the animal level during 50 years of scientific inquiry into the mechanisms of the estrous cycle in heifers and mares. The reported examples of pitfalls represent nature's first line of defense against revealing her secrets in that they are activated at the animal level. The research experiences are from a narrow aspect of reproductive biology (estrous cycles in cattle and horses) but relate well with other areas of study (e.g., other mechanisms or research areas, species, gender, geographical location). In this regard, research discoveries in a given research location (e.g., temporal vs. torrid zones) or with a given breed, species, or animal type (e.g., ponies vs. horses; *Bos taurus*

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vs. *Bos indicus*) may require confirmation for other locations or animal breeds or types.

Pitfalls that reflect faulty use of experimental design and the scientific method, the handling of specimens and samples in the laboratory (e.g., *in vitro* techniques, hormone assays), and the processing of data deserve equal consideration, but are not within the whole-animal theme of this report. The reported examples are intended to encourage careful and deliberate thought to potential pitfalls during development, execution, and reflection on the animal portion of research protocols.

2. The infrequent-monitoring pitfall

Characterization of the temporal relationships among events (e.g., hormone concentration changes, beginning of luteolysis, ovulation) is a common initial approach to the study of biological mechanisms. Temporal information provides rationale for developing hypotheses for critical testing. A pitfall may occur if data are obtained at an interval that is greater than the physiological interval between events. For example, inadequate sampling frequency has been a pitfall for decades during attempts to characterize the period of luteolysis or the progressive decrease in progesterone by collecting blood samples every 6–24 hours. Recent reports have shown that the initiation of luteolysis is manifested within 1 hour and is completed in 24 hours in both heifers [1] and mares [2]. To illustrate this pitfall, progesterone concentrations in samples collected every hour from mares are compared with progesterone in the same series, using only the samples that were collected every 6 hours (Fig. 1). The prolonged (~54 hours) illustrated decline in progesterone (apparent luteolysis) using concentrations at 6-hour intervals is a pitfall, owing to the beginning and end of luteolysis at different hours in individual mares. A mean length of luteolysis of 24 hours in heifers [3] and 23 hours in mares [4] on the basis of hourly samples contrasts with the 2 or 3 days in published graphs of progesterone profiles that were on the basis of infrequent sampling in both cattle [5–7] and horses [8–10]. Results on the basis of infrequent sampling that purport to clarify some aspects of a physiological event should be considered with caution, owing to the imprecision in determining the beginning and end of the event.

3. The overdose pitfall

The overdose pitfall involves an experimental challenge with a natural product, but at an excessive unnatural dose. An example is the extensive study for >40 years [11] in many laboratories on the role of PGF2α in the luteolytic process by challenging an animal with a single treatment of PGF2α. A single dose of PGF2α that induces complete luteolysis is an overdose [12,13]. PGF2α is ubiquitous with diverse effects on many animal tissues [14]. Nature has developed several strategies for utilizing circulating endogenous PGF2α for the induction of luteolysis in cattle and horses without activating unnatural effects on luteal and nonluteal tissues. These strategies include (1) rapid clearance of PGF2α from the circulation [15], (2) high affinity of luteal-cell membranes for capturing circulating PGF2α molecules [16], (3) delivery of PGF2α from its source (endometrium) to its target (corpus luteum) by a local route from a uterine horn to the adjacent ovary in some species [17], and (4) secretion of PGF2α in pulses. The pulsatile delivery system precludes an endogenous PGF2α overdose and has evolved apparently in all species that have a PGF2α-driven luteolytic mechanism. Pulsatility is a clever biological solution for the animal but has been an expensive pitfall for investigators.

Scientists have fallen into the overdose pitfall by attempting to unravel the mysteries of the complex luteolytic mechanism by simply exposing the animal to a massive bolus dose of PGF2α. A single luteolytic dose of PGF2α is followed by a nonphysiological progesterone increase that reaches maximum in 10 minutes, and then decreases to below pretreatment concentrations in 1 hour (Fig. 2) [18,19]. In addition, an acute increase and decrease (spike) in progesterone has been found during the first 2 minutes after treatment in mares [19]. Nonluteal tissues also respond to

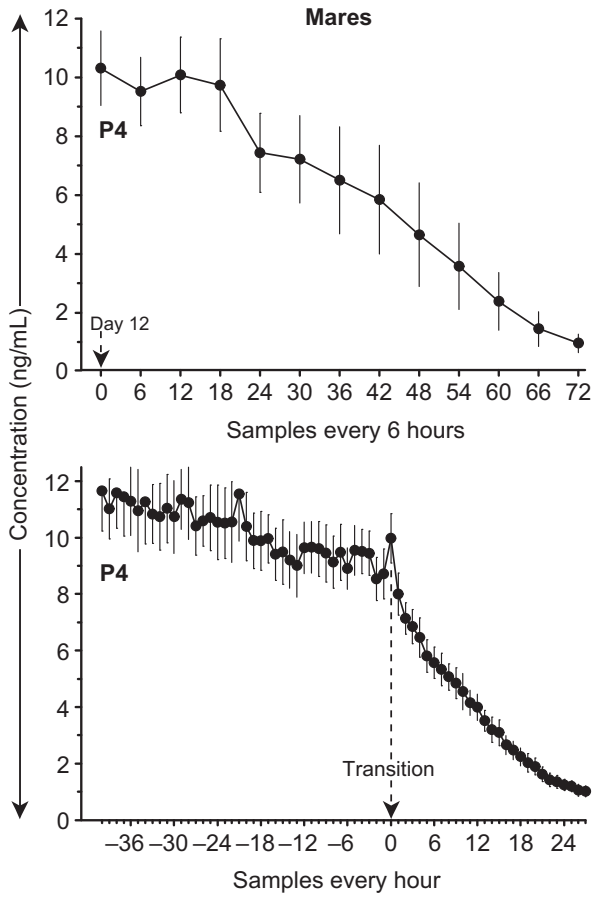

Fig. 1. Mean ± SEM progesterone (P4) concentrations at 6-hour intervals from 12 days postovulation (upper panel) and mean concentrations in hourly plasma samples centralized to the hour of transition between preluteolysis and luteolysis (lower panel). The samples are from the same series in nine mares. The mean luteolytic period is 24 hours for the hourly samples. However, the period appears to be 54 hours for the 6-hour samples, owing to the occurrence of luteolysis on different days in individuals. The prolonged gradual decline in progesterone on the basis of 6-hour sampling is a poor representation of the period of luteolysis. Adapted from [13].