The effect of anticoagulant, storage time and temperature, and sodium azide on blood progesterone concentrations

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SUMMARY

BLOOD PROGESTERONE *concentrations in zebu cows were measured at ILCA's research station in Debre Birhan, Ethiopia. Progesterone concentrations in unseparated plasma and serum samples declined rapidly, particularly at high storage temperatures (35°C). The commonly used anticoagulants—lithium heparin, sodium citrate and ethylene diaminetetracetate—did not significantly affect the measured concentrations. Once coagulation had taken place, progesterone concentrations were higher in unseparated serum than in unseparated plasma. Assayable progesterone reappeared in unseparated plasma samples kept for 3 to 7 days. When sodium azide was added to plasma with anticoagulant, 90% of the initial progesterone concentration was preserved over a period of 3–7 days.*

INTRODUCTION

Progesterone plays a major role during the cow's oestrous cycle and pregnancy. Measuring blood progesterone concentrations therefore has applications in improving reproductive efficiency, and generally in the study of reproduction.

In dairy cows, however, progesterone measurements in the blood have been overshadowed by techniques measuring progesterone concentrations in milk. The latter have also been shown to reflect accurately progesterone concentrations in plasma (Hunter, 1980).

Milk progesterone cannot be measured in heifers and non-lactating cows. Furthermore, differences have been observed in the milk sampling methods adopted by various researchers, and this can considerably affect the progesterone concentrations measured (Gordon, 1983). While such differences are of little importance where qualitative values are required, for quantitative analysis of progesterone, standardised milk sampling procedures are needed. Measuring progesterone concentrations in the blood helps avoid the problem entirely.

Previous work with blood focused on the effects of:

- *Time* between blood collection and plasma separation (Delahaut et al, 1979; Vahdat et al, 1979, 1981; Owens et al, 1980a, 1980b; Oltner and Edqvist, 1982; Wiseman et al,1982).
- *Storage temperature* before separation (Vahdat et al, 1979, 1981; Owens et al, 1980b; Oltner and Edqvist, 1982; Wiseman et al, 1982).
- *Type of anticoagulant* used for plasma samples (Owens et al, 1980b; Vahdat et al,1981).
- *Use of additives* to prevent progesterone loss (Delahaut et al, 1979; Vahdat et al, 1984; Davies and Fletcher, 1987).

Vahdat et al (1981) and Oltner and Edqvist (1982) reported that measurable progesterone declined between blood collection and plasma or serum separation, but after separation no further decline occurred. According to Oltner and Edqvist (1982), assayable progesterone will re-appear if blood samples treated with heparin are kept at room temperature for 5 to 9 days before centrifugation. Delahaut et al (1979) reported that a final concentration of 5 mg sodium azide per ml of blood would prevent loss of assayable progesterone in unseparated[1] plasma samples.

[1] Unseparated plasma is uncentrifuged whole blood where clotting was prevented by an anticoagulant.

The purpose of the study reported here was to develop practical guidelines for the collection of zebu blood under tropical conditions. The four guidelines given below are based on experiments carried out to determine the effects of such factors as type of anticoagulant, storage time, storage temperature and sodium azide on blood progesterone concentrations.

MATERIALS AND METHODS

Experimental design

Four experiments were designed to measure blood progesterone concentrations. Storage time was a factor in all four experiments. The effects of temperature were tested in two experiments comparing plasma and serum samples. One experiment was carried out to study the effects of three common types of anticoagulant and another to compare progesterone concentrations in plasma with or without the sodium azide preservative.

Experiment 1

In this experiment, the effects of type of anticoagulant and storage time on plasma progesterone concentrations were studied. Collected blood samples were divided into three batches and treated respectively with heparin (lithium heparin), ethylene diaminetetracetate (EDTA) and sodium citrate. Within each batch, samples were kept at 20°C for 30 minutes, 3 hours and 18 hours prior to plasma separation by centrifugation.

Experiment 2

This experiment was carried out to determine the effects of storage temperature and time on plasma progesterone concentrations. Three temperatures (3°C, 20°C and 35°C) were chosen to simulate respectively refrigerator temperature, ambient temperature at a site in sub-Saharan Africa above 1500 m altitude, and ambient temperature at a site below 1500 m altitude. Centrifugation was done within 5 minutes of collection (which would be the ideal situation), and after 3, 6 and 18 hours. To obtain information on developments between 6 and 18 hours, two batches were kept for 12 hours at 3 and 35°C respectively.

Experiment 3

This experiment was designed to study the effects of storage temperature and time on progesterone concentrations in serum. The time intervals and temperatures applied were the same as in experiment 2, but since it is impractical to prepare a serum sample within 5 minutes

of blood collection, a plasma sample was used instead. The results of this experiment were compared with the results of experiment 2 to determine the difference between progesterone concentrations in plasma and serum kept under similar conditions.

Experiment 4

In this experiment, the effects of long-term storage and sodium azide on plasma progesterone concentrations were investigated. Plasma samples were kept at 20–25°C for 5 min, 6 and 18 hours, and 3, 5 and 7 days before centrifugation. Sodium azide (5 mg ml^{-1} blood) was added to half the samples and their progesterone concentrations were compared with those without sodium azide.

Collection tubes

Serum and plasma samples in experiments 2, 3 and 4 were collected into evacuated blood-collection tubes (Vacutainer) which were purchased already prepared with heparin. Collection tubes for citrated and EDTA plasma (experiment 1) were prepared at the laboratory. The amounts of anticoagulant added were as described by Dacie and Lewis (1970). Tubes used in experiment 4 were prepared with sodium azide to produce a final concentration of 5 mg azide ml^{-1} blood.

Sample collection

Blood samples were collected from 27 zebu cows housed at ILCA's research station at Debre Birhan, Ethiopia. In each of the experiments 1, 2 and 3, blood was collected from seven cows, and in experiment 4 from six cows. To obtain maximum initial progesterone concentrations, sampling took place 8–16 days after the onset of oestrus i.e. during the luteal phase of the oestrous cycle. Experiments 1 and 4 included one cow in late gestation.

Cows were restrained in a cattle run and blood was collected by puncturing the jugular vein. Samples to be kept at 20°C were taken first. followed by the 35°C and 3°C samples. The initial (0 hour) sample was taken last and processed immediately after collection, while the others were placed in a waterbath at 20°C or 35°C, or in the refrigerator (3°C).

Sample processing

Following incubation under the selected combination of conditions, samples were centrifuged for 10 minutes at a centrifugal force of 1125 gravities (2500 rotations per minute). The separated[2] plasma or serum was frozen at –20°C.

[2] Separated plasma or serum contains no red bloodcells

Progesterone assay

Plasma and serum samples were analysed for progesterone using Ovucheck Plasma/ Serum Progesterone EIA kits supplied by Cambridge Life Sciences, UK. To minimise inter-assay variation, all samples from the same cow were run in the same assay. Each sample was run in duplicate and its progesterone concentration determined using average absorbance at 405 nm.

A Hewlett Packard 71B calculator was programmed to perform linear regressions on the results obtained with supplied progesterone standards (0.5, 1.0, 5.0 and 10.0 ng progesterone ml^{-1} blood). The corresponding progesterone concentrations for the measured samples were then computed, showing an intra-assay coefficient of variation (CV) of 5.5% .

Analysis of results

An analysis of variance was performed for each experiment to determine the significance of the effects of each treatment. The analysis made it also possible to separate differences due to varying initial concentrations of progesterone from the treatment effects.

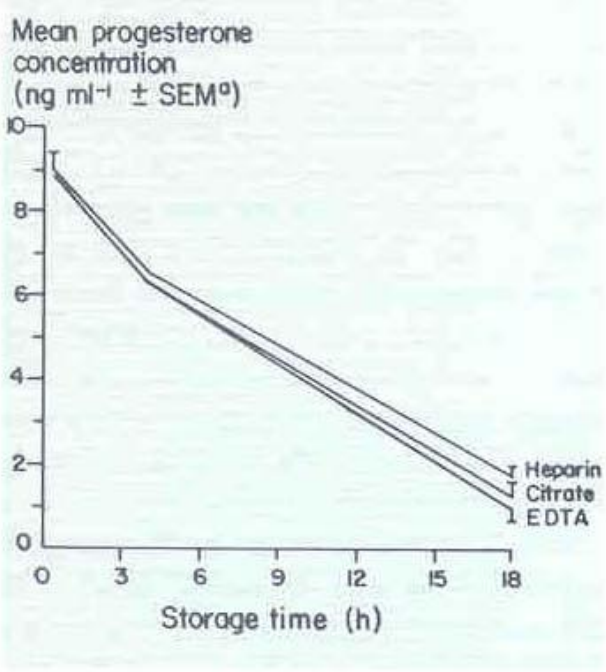
The effects of time were investigated by making multiple comparisons among means of time, using Tukey's method (Sokal and Rohlf, 1981). An honest significant difference (HSD) was calculated, and progesterone concentrations differing by more than the HSD were taken to be significantly different. Plasma and serum progesterone concentrations at different temperatures and times were compared using an unpaired t-test.

RESULTS

Experiment 1

Plasma progesterone concentrations declined rapidly regardless of whether heparin, EDTA or citrate anticoagulant was used (Figure 1). At each time interval (30 minutes, 3 hours, 18 hours), significantly ($P<0.05$) lower concentrations were measured than at the previous time interval. The type of anticoagulant used had no significant effect ($P>0.05$) on the measured progesterone concentrations.

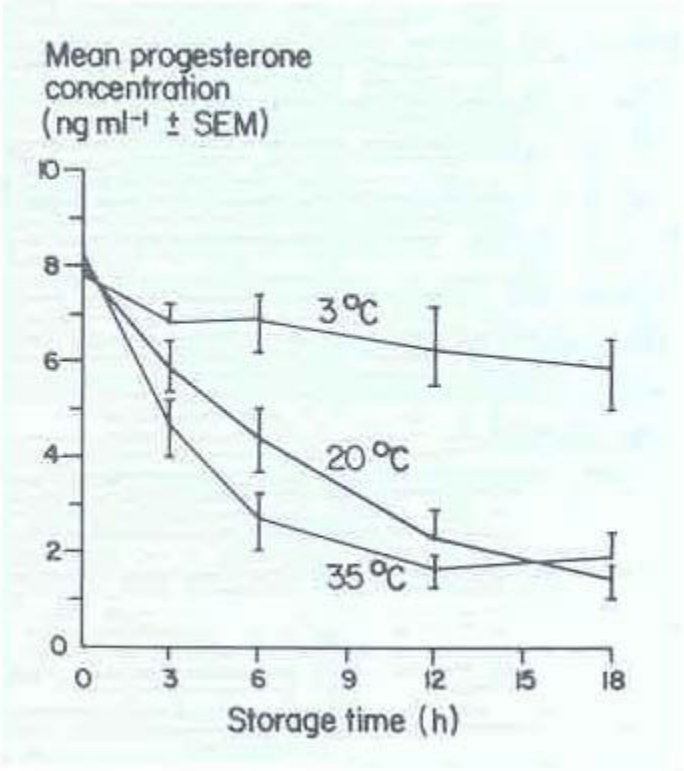
Figure 1. *The effect of type of anticoagulant and storage time on plasma progesterone concentrations, Debre Birhan, Ethiopia, 1986.*



Experiment 2

Plasma progesterone concentrations decreased with time regardless of storage temperature (Figure 2). For samples stored at 20°C and 35°C, a significant decline ($P<0.05$) in plasma progesterone was observed within 3 hours of collection. In samples stored at 3°C a significant decline ($P<0.05$) in progesterone was not found until 6 hours after collection.

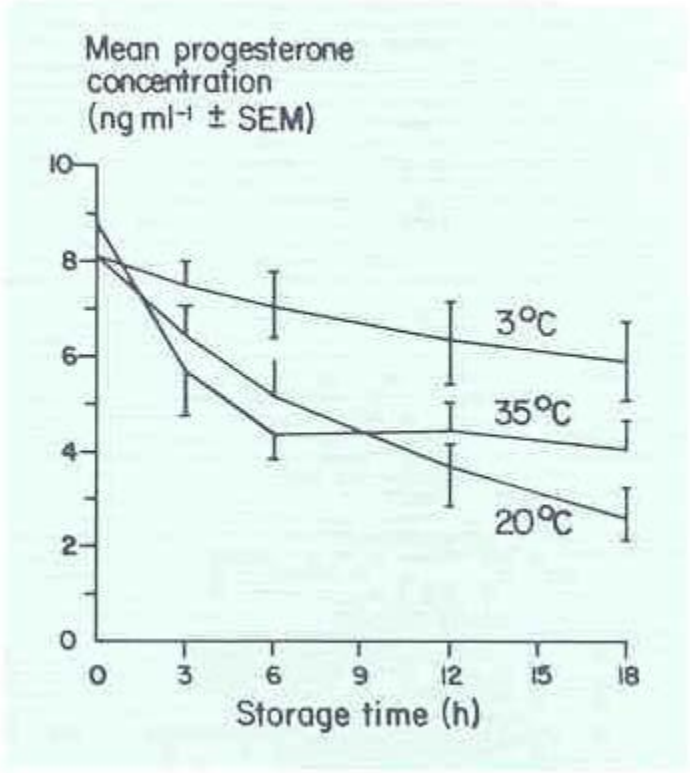
Figure 2. *The effect of storage temperature and time on plasma progesterone concentrations, Debre Birhan, Ethiopia, 1986.*



Experiment 3

Serum progesterone concentrations decreased with time at all three storage temperatures (Figure 3). As in experiment 2, progesterone concentrations in serum samples kept at 20°C and 35°C decreased significantly ($P<0.05$) within 3 hours of sample collection, but in the 3°C samples, a significant decline ($P<0.05$) was not recorded until 12 hours after collection.

Figure 3. *The effect of storage temperature and time on serum progesterone concentrations, Debre Birhan, Ethiopia, 1986.*



To determine whether there was any difference in the decline of plasma and serum progesterone, the results of experiments 2 and 3, expressed as percentages of the initial plasma and serum progesterone concentrations for each cow, were compared. Table 1 shows that significant differences ($P<0.05$) were recorded for plasma and serum samples stored at 20°C for 18 hours and at 35°C for 6 and 18 hours. The highest significant difference ($P<0.001$) was recorded for plasma and serum kept at 35°C for 12 hours. In general, the values for serum were greater than those for plasma.

Table 1. *Progesterone concentrations in serum and plasma at different storage temperatures and times, Debre Birhan, Ethiopia, 1986.*

Temperature(° C)	Time(h)	Percentage of initial concentration remaining in:		Significance level[1]
		Plasma	Serum	
3	3	93.5	93.4	+
3	6	89.4	90.6	+
3	12	80.0	81.8	+
3	18	77.5	78.1	+
20	3	78.5	79.8	+
20	6	55.3	64.7	+
20	12	30.5	46.7	+
20	18	19.6	38.2	*
35	3	56.1	66.7	+
35	6	32.3	53.7	*
35	12	22.1	55.7	***
35	18	26.6	50.2	*

[1] + = no significant difference (P>0.05); * = P<0.05; *** = P<0.001.

Experiment 4

Partial haemolysis was observed in plasma with sodium azide after 5 minutes and 6 and 18 hours of storage. Haemolysis was completed by 72 hours. Samples with azide had significantly (P<0.01) higher progesterone concentrations than those without azide (Figure 4).

Figure 4. *The effect of long-term storage and sodium azide on plasma progesterone concentrations, Debre Birhan, Ethiopia, 1986.*

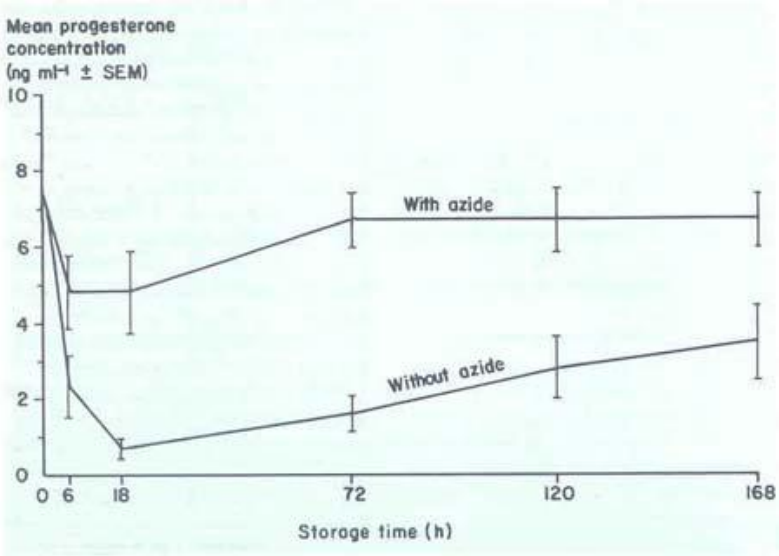


Table 2 shows that progesterone concentration in plasma without sodium azide decreased significantly (P<0.05) from the initial concentration of 7.75 ng ml^{-1} to a low point of 0.66 ng ml^{-1} at 18 hours after collection. At 168 hours measurable progesterone was 3.53 ng ml^{-1} plasma, which was significantly (P<0.05) higher than the 18-hour low value but significantly (P<0.05) lower than the initial progesterone concentration.

Table 2.*The effect of long-term storage with and without sodium azide on plasma progesterone concentrations, Debre Birhan, Ethiopia, 1986.*

Hours:	Mean progesterone concentration[1] (ng ml^{-1})						
	0	6	18	72	120	168	HSD[2]
Plasma							
Without azide	7.74a	2.34bc	0.66c	1.67bc	2.81bc	3.53b	2.40
With azide	7.36a	4.80b	4.80b	6.65ab	6.70ab	6.75ab	2.40

[1] Within each treatment, values followed by the same letter(s) are not significantly different at the 5% level.
[2] HSD = Tukey's honest significant difference at the 5 % level.

In comparison, progesterone concentrations in plasma samples with sodium azide had declined significantly (P<0.05) from the initial concentration of 7.36 ng ml^{-1} to 4.80 ng ml^{-1} by 6 hours after collection: this value was maintained until 18 hours after collection. However, between 72 and 168 hours the concentrations increased to a point where there was no significant difference (P>0.05) from the initial progesterone concentration.

DISCUSSION

The results of this study demonstrate that storage time and temperature affect measurable progesterone in zebu blood. This has important implications for sample collection, particularly where there are no laboratory facilities available, or where accurate determination of progesterone concentrations is required.

The decline in progesterone concentrations in unseparated plasma samples is well documented (Delahaut et al, 1979; Vahdat et al, 1979, 1981, 1984; Owens et al,1980a,1980b; Oltner and Edqvist,1982; Wiseman et al, 1982). No such effect is apparent in separated plasma samples kept at room temperature (Vahdat et al, 1981; Oltner and Edqvist, 1982). Comparing the effects of common anticoagulants, Owens et al (1980a) and Vahdat et al (1981) found, as did this study, that progesterone concentrations in bovine blood are not significantly affected by the type of anticoagulant used.

It was postulated that red blood cells are responsible for the loss of progesterone in unseparated plasma samples (Delahaut et al, 1979). Short (1958) described the conversion of progesterone incubated with bovine blood to a product he identified as 20 β-dihydroprogesterone. Van der Molen and Groen (1968) identified a 20 α-hydroxysteroid dehydrogenase in sheep red blood cells, which catalysed the conversion of progesterone to

20 α-dihydroprogesterone. They showed that the transformation was glucose-dependent and that in the absence of glucose, 20 α-dihydroprogesterone was oxidised back to progesterone.

Oltner and Edqvist (1982) reported that storing unseparated plasma at room temperature for 2 to 9 days results in reappearance of assayable progesterone. This effect was linked to the low concentration of glucose in the samples, which favours progesterone formation. The reappearance of assayable progesterone was confirmed in experiment 4, although glucose concentration was not determined.

The effects of storage conditions on measurable progesterone in unseparated serum have been less widely studied. Nevertheless, it appears that under similar conditions, serum progesterone concentrations are higher than those in plasma (Vahdat et al, 1979; Owens et al, 1980b; Wiseman et al, 1982).

Various preservatives have been used to arrest the decline of progesterone concentrations in plasma, including sodium azide, potassium oxalate/sodium fluoride and potassium dichromate/mercuric chloride. Delahaut et al (1979) found that at an optimum sodium azide concentration of 5 mg ml^{-1} plasma, as much as 90% of the initial progesterone concentration can be preserved in samples kept at room temperature for 4 days. In this study, the same amount of sodium azide preserved 90% of the initial concentration in samples stored for 3 to 7 days. Holdsworth (1980) reported that sodium azide concentrations of 2 mg ml^{-1} and 20 mg ml^{-1} preserved respectively 63% and 76% of the original progesterone concentrations, following overnight storage.

Using a preservative tablet composed of potassium dichromate and mercuric chloride, Holdsworth (1980) abolished the decline of progesterone concentrations in unseparated plasma stored overnight at room temperature. Davies and Fletcher (1987) also reported success with this combination.

Haemolysis occurs with both sodium azide and potassium dichromate/mercuric chloride. Holdsworth (1980) and Davis and Fletcher (1987) used a radioimmunoassay to measure progesterone concentrations and found that these were higher in haemolysed blood samples than in the unhaemolysed ones. Using an enzyme immunoassay, however, Davies and Fletcher (1987) could not find any difference in the measurable progesterone of haemolysed and unhaemolysed samples. This suggests that the products of haemolysis affect blood progesterone concentrations only during the liquid-phase assay, not during the solid-phase assay (Davies and Fletcher, 1987).

Potassium oxalate/sodium fluoride was used by Vahdat et al (1984) to prevent progesterone degradation. Fluoride inhibits glycolysis, and because of this the combination preservative is normally chosen when glucose concentrations are to be determined. In Vahdat's experiment, 68% of the original progesterone concentration remained after 24 hours of storage at 22°C, supporting the assumption that inhibition of glycolysis in the red blood cells slows down progesterone degradation. But glycolysis inhibition may result in high glucose concentrations, which would cause progesterone levels to decline (van der Molen and Groen, 1968). It would therefore appear that fluoride directly inhibits the enzyme responsible for progesterone degradation.

This and other studies suggest that two factors need to be taken into account when formulating guidelines for blood sample collection:

- *Accuracy of results*. For research purposes, quantitative results will be necessary, but when the aim is to detect, for example, the presence or absence of corpus luteum, a qualitative result is likely to suffice.
- *Accuracy of the assay*. A significant decline ($P<0.05$) in progesterone concentrations is indicated when the concentrations decrease by a percentage approximately twice the coefficient of variation (CV) of the assay. (In this study the figure was slightly higher as a result of using Tukey's method for the multiple comparison of means of time.) Where the intra-assay CV is 5.5%, plasma samples would have to be kept at 20°C for 1.3 hours before it could be said with a probability of $P<0.05$ that the concentration of progesterone had declined. This assumes that the two samples were measured in the same assay. Samples measured in two separate assays would have to be kept longer, because the inter-assay CV is usually higher.

Depending on the accuracy of results required, the following guidelines for sample collection should be observed:

- To obtain plasma, blood samples should be centrifuged immediately after collection, preferably in a refrigerated centrifuge.
- When a number of plasma samples are needed, collected blood should be kept on ice before centrifugation. Loss of progesterone in cooled blood samples is unlikely to be significant until after 6 hours.
- Where centrifugation facilities are not available, serum samples can be used, provided that the likely loss of progesterone is taken into account. Serum should be separated as soon as clot retraction has occurred.
- Preservatives may also be used where suitable centrifugation facilities do not exist. The most effective preservatives are sodium azide and potassium dichromate/mercuric chloride, the latter having the additional advantage of being less toxic.

Thus if both highly accurate results and an assay with a low CV are required, the first method of collection will be most appropriate. If a less accurate assay and qualitative results are needed, the other methods listed would probably suffice.

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