

Levels of estrogens in muscle tissue of pregnant cows. Comparison with levels found in steers implanted with estradiol 17B.

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## INTRODUCTION

Estrogens are phenolic steroid hormones which play a central role in changes associated with estrus and in the maintenance of pregnancy in mammals. So, they are natural components of animal tissues and are greatly increased in pregnant cows.

Estradiol 17B is a natural estrogen that has been used to improve growth rate and feed conversion of steers (alone or in combination with other compounds).

This natural steroid and its main metabolites, estrone ( $E_1$ ), are normally conjugated to their derivatives glucuronides and sulfates which can be accumulated in tissues (Dunn et al, 1977).

The purpose of this study was to measure the estrogens in muscle tissue of pregnant cows and to compare them with the maximum values found in steers implanted with estradiol controlled released implants.

## MATERIALS AND METHODS

For this trial, samples of sternocervical muscle of 30 pregnant cows at different times of gestation were taken in a slaughter house. The animals were chosen in an aleatory way in order to have the samples well distributed along the period of gestation. The tissues were sealed in plastic bags and stored at  $-15^{\circ}\text{C}$ .

### Determination of 17B $E_2$ and $E_1$ and their conjugates

20 grs. of muscle tissue was blended in an Omni-mixer with acetonitrile : methanol 80:20 and centrifuged at 3500 rpm for five minutes. The supernatant was transferred to separatory funnel and washed with hexane. The hexane was discarded and the solvent was evaporated until water residue. Distilled water was added until 50 ml and pH was adjusted to  $5 + 0.2$ . The sample was divided in two fractions of 25 ml : A and B. 4 mg of glucuronidase/sulfatase were added to fraction A; 5 ml of chloroform were added to both fractions and then were incubated 16 hours at  $37^{\circ}\text{C}$ . After this,  $\text{Cl Na } 10\%$  was added and the samples were extracted with  $\text{Cl}_2\text{CH}_2$ . This solvent was evaporated on a rotary vacuum evaporator. Farther on this step, it was followed by the procedure described by Frank and col.

Final extracts from fraction A contained total  $E_2$  and  $E_1$  (free + conjugated) and those from fraction B, free  $E_2$  and  $E_1$  only.

Measurement of extracted estrogens was made by competitive protein binding radioimmunoassay using specific antisera. Cross reactions of the  $E_2$  and  $E_1$  antibody preparations with related compounds were less than 1%;  $E_2$  antisera cross-reacted 10% with  $E_1$  and  $E_1$  antisera 3% with  $E_2$ .

A liquid Scintillation Spectrometer Beckman Model 7000 was used for radioactivity measurements. Radiolabeled compounds came from New England Nuclear, Boston, Massachusetts 02118, USA. The enzymes used were glucuronidase from *Helix Pomatia* with sulfatase activity (420.000 units/g) and B glucuronidase from bovine liver (920.000 units/g) from Sigma Chemicals.

Final results were compared with a standard response curve over the range 0-200 pg  $E_2$  or  $E_1$ , making reagent blank corrections. The method has a limit of detection of approximately 10 pg estrogen/gr. tissue.

### Efficiency of hydrolysis

The enzyme activity was studied over a range of concentrations of glucuronides and sulfates that could be found in muscle tissue. Muscle extracts were prepared as described before and were spiked with tritium labeled  $E_1$  sulfate and  $E_2$  - 3 - glucuronide before hydrolysis. The same procedure was applied to distilled water (control without extract) to check the real activity of the enzymes and to control which hadn't enzymes in order to determine if conjugates passed to the chloroform phase. In the samples without extract the activity was measured in the chloroform phase and in an aliquot of the final aqueous phase; in the samples with extract this measurement was made after the extractions with dichlorometane due to the impossibility of taking aliquots from the emulsified chloroform phase.

The radioactivity in the final aqueous phase indicated the quantity of conjugates that weren't hydrolyzed by the enzymes and that of the solvent phase the quantity of free estrogens liberated from the conjugates by the enzymes.

### Recovery efficiencies

Crude extracts from muscle were spiked before assayed with 5000 cpm of tritium labeled conjugates  $E_2$  and  $E_1$ . Samples of muscle extract were spiked after the hydrolysis step with 2500 cpm of tritium labeled free estrogens. Radiolabeled recovery was determined in the final extract prior to RIA in each sample of an assay run. If it was under 60%, the samples were reassayed. Final results were adjusted for recovery.

### Determination of the age of the fetus

In this study we used the same parameters as Gjesdal (1969) to determine the age of the fetus. The length occipito-coccigea, the length of metacarpus and metatarsus, the teeth development and the presence of pilose follicles allow to determine the age of the fetus with a difference of fifteen days.

### Statistical Analysis

For this trial, samples were obtained in an aleatory way in the slaughter-house without any statistical model.

## RESULTS AND DISCUSSION

The efficiencies of hydrolysis are presented in the table 1. They are over 88 % in all cases. The samples without extract in the case of the sulfates showed a percentage of hydrolysis a little more higher than the samples with extract, as if the muscle extract contained some inhibitors. But, anyway, the efficiency with extract is very good. The results obtained with the controls without enzymes showed that the proportion of conjugates that passed to the organic phase isn't important. Comparable results were obtained with other steroids by Hoffmann and Rattenberger, 1977.

Recovery efficiencies are presented in table 2. Considering that the analytical procedure isn't a sample one, they are very satisfactory and similar to those obtained with other extraction steps (Henrickx and Torrence, 1978). The recoveries under the whole procedure using radiolabeled conjugates are useful to check the method but they aren't practical, because it is rather difficult to maintain the radiochemistry purity of the conjugates for a long time. So, the samples of pregnant heifers studied were spiked only with  $^3\text{H}$   $\text{E}_2\text{B}$  and  $^3\text{H}$   $\text{E}_1$  to calculate the recovery efficiencies in order to adjust the final results.

The percentage of conjugates changes aleatorily during pregnancy for  $\text{E}_1$  and  $\text{E}_2\text{B}$ , so averages of the percentage of conjugation independent of time of pregnancy were calculated and they are 45.5 % for  $\text{E}_1$  (SD = 18.2; n = 30) and 37.1 % for  $\text{E}_2$  (SD = 15.7; n = 30). Considering this variability the evolution of the contents of estrogens during pregnancy was made measuring the levels of total estrogens (conjugated and free).

From the limits of tolerance it can be inferred that along the pregnancy,  $\text{E}_2$  total/ $\text{E}_1$  total ranged from 5 % to 20 % in the 80 % of the animals sampled (p = 0.98). This rate is very different from those found in non-pregnant cows and it isn't known if it is a consequence of a major production of  $\text{E}_1$  or an alteration of metabolism in favour of  $\text{E}_1$  (Dobson et al, 1974).

Total  $\text{E}_2$  and  $\text{E}_1$  increased considerably during pregnancy following double logarithm models whose estimated equations are:

$$\text{E}_1 \quad \log y = 0.625 + 2.57 \log x \quad (r = 0.89; p < 0.05) \\ \text{CME} = 0.056$$

$$\text{E}_2 \quad \log y = 0.12 + 2.34 \log x \quad (r = 0.87; p < 0.05) \\ \text{CME} = 0.058$$

y being the concentration of total  $\text{E}_1$  and  $\text{E}_2\text{B}$  in ppt and x the month of pregnancy.

A great variability was found in the levels of different animals at the same time, specially in the last months, as in the work of Edquist et al (1973) in plasma. These authors explain it by the physiological differences among animals and the variability of the placental contribution, but in this work we must also consider that the time of pregnancy can't be determined with a high precision.

These results were compared with the maximum levels of estrogens found in steers implanted with estradiol 17B analysed in this laboratory (Fernandez Suarez et al, 1982). The values with a 90 % probability that the 97.5 % of the implanted steers don't exceed them as maximum values were statistically estimated for  $\text{E}_1$  and  $\text{E}_2\text{B}$ , being 47.8 pg/gr and 22.4 pg/gr respectively. To reach these values, it was estimated that 1.5-3.8 months for  $\text{E}_1$  and 2.6-6.5 months for  $\text{E}_2$  are needed (p = 0.9) (Seeber, G.A.F.).

This comparison shows that tissues of non-implanted animals, that can be consumed many times, reach natural levels of estrogens higher than those found as residues in tissues of implanted animals in well-practiced treatments.

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