The accumulation and clearance of equol in the blood of ewes grazed on either high or low formononetin red clovers

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ABSTRACT

Formononetin is the major red clover phytoestrogen responsible for reproductive dysfunction in sheep. Formononetin is metabolised in the rumen to equol which is significantly more oestrogenic. This study was conducted to characterise the accumulation of conjugated and free equol in the blood of ewes grazed on either high formononetin (cv Pawera, 0.57% of dry matter) or low formononetin (cv G27, 0.25% of dry matter) red clovers over a 1, 2 or 3 week period and the subsequent clearance of equol after removal from the red clover.

Seventy mixed age ewes were divided into 7 treatment groups (n=10). Each group grazed either Pawera or G27 red clover for 3, 2 or 1 week or ryegrass (control group) prior to mating as one flock on ryegrass pasture. All ewes were regularly blood sampled. Once mated, the ewes were laparoscoped and returns to service recorded.

Conjugated equol concentrations in both Pawera and G27 ewes increased over the first 2 days to 23.97 ± 12.04 (SEM) µg/100ml and 8.40 ± 2.65 µg/100ml for Pawera and G27 treatments respectively. From d 2 to 21 of grazing, conjugated equol concentrations averaged 23.74 ± 2.94 µg/100ml in Pawera ewes and 7.83 ± 2.19 µg/100ml in G27 ewes. Conjugated equol concentrations were on average 4 times higher in Pawera than in G27 ewes. All equol was cleared from the blood the day following removal from the red clover.

During the first 4 d of grazing, free equol remained undetectable in the ewes on G27 but increased linearly to 0.48 + 0.35 µg/100ml on d 4 in the Pawera ewes and then averaged 1.04 ± 0.25 µg/100ml until removal from the red clover. Free equol accumulated in ewes grazing G27 after d 4, peaked at 0.71 ± 0.14 µg/100ml on d 14 and then averaged 0.36 ± 0.09 µg/100ml until removal from the redclover. Levels of free equol were on average 3 times higher in Pawera than in G27 ewes. All free equol was cleared from the blood the day after removal from the red clover.

The ovulation rates were 1.3, 1.5 and 1.4 corpora lutea per ewe for Control, G27 and Pawera treatments respectively. Neither ovulation nor return rates were significantly different ($p<0.05$).

Keywords: Equol; formononetin; red clover; breeding ewes.

INTRODUCTION

Clover pastures containing high levels of phytoestrogens can cause infertility in sheep (Barrett *et al.*, 1965). The plant constituents that contribute to the oestrogenic activity in clovers include isoflavones and coumestans (Braden *et al.*, 1965 ; Lindner, 1966; Torbjorn and Lundh, 1990). Formononetin (7-hydroxy-4-methoxyisoflavone) although only weakly oestrogenic itself, is changed by rumen bacteria to equol (7,4-dihydroxyisoflavan), a far more potent oestrogenic compound, which is largely responsible for reproductive dysfunction in ruminants (Shutt *et al.*, 1970).

Davies and Hill, (1989) reported that 81% of ingested formononetin is metabolised to equol and rapidly absorbed into the blood stream through the rumen wall. Once in the blood stream most of the equol is conjugated into gluconuride or sulphide forms rendering it inactive in terms of oestrogenicity.

Shutt *et al.*, (1967) estimated that of the total plasma equol concentration only 1-2% was present in the unconjugated form. It is these unconjugated, or free "active" amounts of equol in the blood which are responsible for reproductive disturbances. Although the amount of free equol is only a small fraction of the total equol, the large amounts of formononetin ingested by animals grazing red clover results in levels of free equol that are significantly higher than the levels of endogenous steroidal hormones.

Pawera red clover has been commercially available for 22 years and is a high yielding summer/autumn producer with persistence and nutritional attributes that surpass many other red clover cultivars (Anderson, 1973). Pawera is, however, high in formononetin (Kelly *et al.*, 1979) which restricts its use for grazing ewes, particularly near the mating period. G27 red clover was developed from a seven generation breeding programme selecting for low formononetin content within the cultivar Pawera.

Low formononetin red clovers, such as G27, require animal evaluation to determine their efficacy in reducing reproductive dysfunction. Effects on reproductive performance of high and low formononetin content red clovers have been reported (Anwar *et al.*, 1993; McDonald *et al.*, 1994). However, there have been no studies comparing the effects of grazing clovers of different formononetin content on the accumulation and clearance of equol once the animal has been removed from the oestrogenic forage.

Given that the levels of unconjugated equol are the major cause of reproductive dysfunction in sheep grazing

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forage containing formononetin, it is necessary to characterise and quantify the accumulation and clearance of equol, in order to identify ways in which low formononetin red clovers, such as G27, can be used safely as forage for ewes

METHODS AND MATERIALS

Animals and treatments

Seventy mixed age Romney ewes were weighed, ear tagged and randomly divided into seven sub-groups (n=10) with a mean ewe liveweight of 56 kg. Six groups were grazed for either 1, 2 or 3 weeks on Pawera or G27 red clover prior to mating on a perennial ryegrass pasture together with the control group.

Animal groups were introduced on a staggered basis that allowed the removal of all ewes from the red clover treatments at the same time.

All ewes were blood sampled at 3 and 6 h after introduction to the red clover diet and on 3 further d per week while on the grazing treatments. Control ewes on the ryegrass pasture were blood sampled at similar times to the ewes on the red clover treatments.

After the grazing period all ewes were removed from the red clover treatments, blood sampled, weighed and grazed on perennial ryegrass pasture with 2 Texel rams fitted with mating harnesses and crayons.

All ewes were blood sampled daily for a further week and mating date and returns to service recorded.

The ewes were laparoscoped within 7 days of mating to determine ovulation rate.

Extraction and assay of equol in blood.

Samples of whole blood were held at -30°C prior to analysis. A 2ml aliquot was taken from each sample for solid phase extraction for free equol and a 1ml aliquot of whole blood for incubation, extraction and total equol determination.

Plastic cartridges (3 ml Bond-elut) with a C-18 plug were conditioned by running 2ml 100% methanol followed by 2ml milli-Q water through them. Two ml whole blood was added and rubber bungs were used to cap the cartridges. The blood was pushed through the C-18 cartridge by injecting air through the rubber bung using a syringe and hypodermic needle, thereby applying positive pressure. Following the blood a wash of 3ml milli-Q water and then further consecutive washes of 20% and 50% methanol were passed through the C-18 cartridge. After the 50% methanol wash the cartridges were centrifuged to dryness, and the equol was eluted with 4ml 100% methanol.

The 1ml whole blood samples were incubated for 20 h at 37°C with 1ml sodium acetate (pH 5.5) and 500µl Snail acetone powder [0.005g/ml] (SIGMA) containing β-glucuronidase, sulphatase and β-d-mannosidase. Following incubation the extraction procedure was as for the unincubated 2ml samples.

The eluate was collected in a 5ml test tube and dried using a Savant speed-vac under vacuum at a chamber temperature of 60°C for approximately 2 h. The residue was taken up in 150µl of 35% acetonitrile and 65% 40mM acetic acid with triethyl amine, (pH 3.38) and samples were then micro filtered into a 4µl HPLC WISP vial (Alltech) with 300µl 6x38mm poly inserts (Alltech).

The HPLC system consisted of a Waters 490E programmable multiwave UV detector, Waters 712 WISP automatic injection system, Waters automatic gradient controller and a Brownlee 220 x 4.6mm C-18 analytical column packed with porous 5µm sorbents.

For the purpose of determining equol concentration the UV wavelength used was 281nm at a sensitivity of 0.02 AUFS.

The mobile phase used was isocratic, 35% acetonitrile: 65% 40mM acetic acid with Triethyl-amine, pH 3.38. The flow rate was 1ml/min. The WISP was set for 20µl injection and a run time of 15 min. A standard of 1µg/ml was injected after every 10 samples and a sample of 4'-o-methyl equol was also injected to compare with other samples. The chromatograms were displayed and saved by computer using PEAK2 software. The software also marked and integrated peaks consistent with equol and 4'-o-methyl equol . The 4'-o-methylequol concentrations in the samples were not calculated as the standard concentration was not known. For the purpose of analysis the integration of the 4'-o-methylequol peaks were used to compare levels in samples.

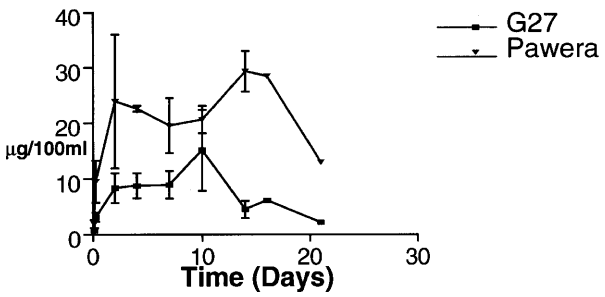
The equol peak was observed at around 9.1 min and the methyl-equol at around 11.1 min with these HPLC specifications. The detection sensitivity allowed concentrations as low as 0.01µg/ml to be detected.

Blood sample composition.

Blood samples taken at a common time and within a group were combined before equol extraction and analysis to give a mean equol concentration for each group at a given time. These mean values were then used to plot the accumulation graph.

In addition to composite samples, the blood samples of two ewes in each of the 3 week and control groups were also analysed to determine free and total equol concentration. The analysis of individual samples showed that the patterns of equol accumulation were similar to those obtained from the composite samples.

FIGURE 1. Conjugated equol concentration of ewes grazing Pawera and G27 red clover. (Mean + SEM)



Formononetin assay

Formononetin concentrations of the red clovers were determined using a modified fluorimetric assay (Gosden and Jones, 1978). Samples of G27 and Pawera red clover were freeze dried and finely ground. A 25 mg of the ground sample was incubated with 1.5 ml of distilled water overnight at room temperature. The following morning 3.5 ml of ethanol was added to each sample, mixed and then centrifuged. A 20 µl sub-sample of the supernatant was placed in 2ml of a basic solution of ethanol and the fluorescence compared to that of a formononetin standard.

RESULTS

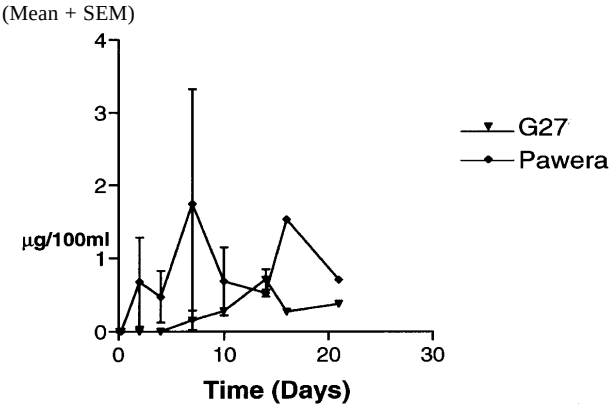
The mean (± SEM) formononetin concentrations of the red clover forages (including leaf, petioles, and flowering stems) were 0.25 ± 0.009 and 0.57 ± 0.091 percent of dry weight for G27 (2 samples) and Pawera (3 samples) respectively.

The accumulation and clearance of conjugated equol

Figure 1 shows the change in conjugated equol concentration over time in ewes grazing either Pawera or G27 red clover. Conjugated equol was detected (2.00 ± 0.71 SEM µg/100ml) in the blood of Pawera ewes after 3 hours of grazing. The accumulation over the first 2 d was very rapid with blood concentrations reaching 24.64 ± 12.65 µg/100ml on the second day of grazing. From d 5 to 21, the levels of conjugated equol in the blood of the Pawera ewes averaged 23.74 ± 2.94 µg/100ml.

Conjugated equol was also detected (0.82 ± 0.29µg/100ml) in the blood of G27 ewes after 3 h of grazing. Conjugated equol concentrations increased rapidly over the first two days of grazing although the rate of accumulation was lower than that observed in the Pawera ewes. Conjugated equol concentration on d 2 was 8.40 ± 2.65µg/100ml which is about half the concentration in the Pawera ewes after the same grazing period. The pattern of conjugated equol accumulation was similar in G27 ewes to that observed in ewes grazing Pawera but the average value 7.83 ± 2.19 µg/100ml between d 5 and 21 was only a third of that in Pawera ewes ($p<0.05$).

FIGURE 2. Free equol concentration of ewes grazing Pawera and G27 red clover.



During the 3 week grazing period the levels of conjugated equol in the Pawera ewes were on average about 4 times greater than those of G27 ewes ($p<0.05$). The general pattern of accumulation of conjugated equol was similar in both Pawera and G27 treatments.

After removal from the red clover treatments the conjugated equol concentrations fell by 95% in the first day and were not detectable by the second day.

Methylequol

Trace levels of free methylequol were detected in only one group from each of the Pawera and G27 treatments.

Conjugated methylequol was detected in all groups during the red clover grazing, at levels much lower than that of total equol. Levels of methylequol were, however, higher in the blood of Pawera ewes than in G27 ewes.

Accumulation and clearance of unconjugated (free) equol

Figure 2 shows the change in free equol concentration over time in ewes grazing Pawera or G27 red clover. In the Pawera ewes the unconjugated or 'free' equol concentrations increased linearly over the first 4 d of grazing to 0.48 ± 0.35 µg/100ml. After d 4 the levels of free equol reached an equilibrium of 1.04 ± 0.25 µg/100ml until d 21. The free equol concentrations in ewes grazing G27 red clover remained undetectable until d 4 of grazing at which point they rose at a similar rate to those during the first 4 d of grazing in the Pawera ewes. The accumulation of free equol in ewes grazing G27 continued linearly from d 4 to a peak concentration of 0.71 ± 0.14 µg/100ml on d 14 at which time levels of free equol in G27 ewes were similar to those in Pawera ewes. After the 14th day the concentration of free equol fell to an average of 0.36 ± 0.09 µg/100ml until d 21 of grazing.

All free equol was cleared from the plasma of both Pawera and G27 ewes within the first day of removal from the red clover treatments.

Free equol was not found in any of the blood samples from the control animals.

Reproductive data

TABLE 1: Live-weight change, ovulation rates and the incidence of returns to oestrus in ewes relative to treatment.

Group	Wt. Change (kg)	Ovulation rate)	Returns %
Pawera			
1 week	3.8	1.50	10
2 week	2.3	1.40	10
3 week	1	1.30	20
G27			
1 week	5.3	1.50	0
2 week	3.8	1.50	20
3 week	3.7	1.60	0
Control	-2.6	1.30	10

There were no significant differences ($p<0.05$) in ovulation or return rates betweenx any of the treatment groups (table 1).

DISCUSSION

In this study the amount of equol accumulated in the blood was proportional to the amount of ingested formononetin. This finding is consistent with results reported previously by Shutt *et al.,* (1967), Davies and Hill., (1989) and Anwar., (1994) where feed containing formononetin was given to ewes and blood equol concentrations measured. The results showed that both conjugated and unconjugated forms of equol accumulated more rapidly in the ewes grazing Pawera (formononetin levels were between two and three times greater) than G27.

The equol concentration in the blood of ewes grazing Pawera was 0 to 6 times higher than in ewes grazing G27 and this was greater variation than that observed in formononetin concentration between the plants.

The results also show that the accumulation and subsequent clearance of both unconjugated and conjugated equol is rapid. Shutt *et al.,* (1970) calculated the mean residence time in the rumen as 1.7 hours, a relatively short time span from ingestion of formononetin to the appearance of equol in the blood stream. This was supported in the present study by the detection of conjugated equol in blood from both Pawera and G27 ewes 3 h after introduction to the feed. Although the appearance of conjugated equol in the blood was very rapid, this was not the case for unconjugated or free equol, and in particular in ewes grazing G27. Free equol was detected in the Pawera ewes 6 h after introduction to the pasture which was only 3 h after the presence of conjugated equol was noted in the same ewes. A far more substantial difference was observed in the G27 ewes where free equol was not detected until the d 4 of grazing. This may be a reflection of more efficient detoxification and excretion of equol by animals ingesting lower quantities of formononetin.

After the initial accumulation over the first 2 d of grazing, the levels of total equol reached an equilibrium which continued to the end of the grazing period. It appeared that a dynamic equilibrium between ingestion of formononetin and excretion of equol was obtained with levels of both free and conjugated equol in Pawera ewes being 3-4 times greater than those in G27 ewes.

With removal of the ewes from the clover treatments there was a rapid clearance of both free and conjugated equol. Within a day after removal from the oestrogenic forage no free equol was detected in either Pawera or G27 ewes. Furthermore only 1-5% of the conjugated equol remained and this was cleared by the following day.

No significant difference was observed in ovulation rate or returns to service between G27, Pawera or control groups. Small differences in reproductive performance are more likely to have been caused by differences in nutrition. A further reason for a lack of any effect of oestrogenic pasture on reproductive data in this trial is that the ewes were removed from the red clover treatments and no equol was detected in their blood at the time of mating. This suggestion is supported by the findings of Kelly and Shackell (1982) who showed a rapid recovery in ovulation rate in ewes once they were removed from oestrogenic forage.

Although there was an attempt to keep feeding levels similar between treatment groups it was not always possible due to the insufficient Pawera forage towards the end of the trial and the nutritional differences between red clover and ryegrass pasture in the summer. These differences are reflected in the weight changes. The ewes grazing G27 and the 1 and 2 week Pawera groups gained significantly more weight than the 3 week Pawera group which had less forage available. The control ewes lost an average of 2.5 kg over the three week period which reflects the poorer qualities of summer ryegrass pasture compared with red clover.

Although conjugated equol was present in the blood of the G27 ewes during the first 4 d of grazing it was unlikely to have affected the reproductive performance of the ewes. The absence of any free equol in the blood of these ewes at this time may be used as a basis for development of a grazing management strategy prior to mating. It should be possible to graze stands of G27 red clover for periods of up to 4 d without undue risk of free equol accumulating. Within a day of removal from G27 the free equol had cleared from the blood and all equol cleared by 2 d. The absence of free equol during the first 4 d and the rapid clearance of free equol once the sheep were removed from the clover would mean that grazing G27 as a component in a pasture mix would have no risk of free equol in the blood and resulting reproductive dysfunction.

Characterisation of the patterns of accumulation and clearance of both free and conjugated forms of equol in the blood of ewes is the first step in the development of grazing management strategies which aim to realise the potential of red clovers as forage of high (quality) feeding value which may be used safely by breeding stock. A next step would be to evaluate an on/off grazing strategy which aims to prevent the build-up of free equol in ewes around mating time when adverse responses to short-term exposure to forages containing high levels of plant and/or fungal produced oestrogenic compounds occur.

ACKNOWLEDGMENT

We are thankful to Anton Erasmuson, National Chemical Residues Analytical Laboratory, Wallaceville, MAF, Upper Hutt; Geoff Lane and Brian Tapper, AgResearch Grasslands Palmerston North for their valued assistance in helping to develop the analysis procedure; and to Robert Abrahams for assistance with animal handling and sample collection.

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