Comparison of *in vivo* with *in situ* mobile bag and three step enzymatic procedures to evaluate protein disappearance of alfalfa hay and barley grain

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Summary

Ruminal, post-ruminal and total tract crude protein disappearance of alfalfa hay and barley grain were measured using *in vivo*, *in situ* mobile bag and three-step *in situ*/*in vitro* enzyme procedures (3-step). For *in vivo*, four Baluchi lambs (49.4 ± 3.5 kg, body weight) were used in a 2 × 2 Latin square design within 2 periods (24 days of each period). Experimental diets were made of two alfalfa hay: barley grain ratios (DM basis) as 1.0:0.0 and 0.5:0.5. Diets were fed to animals for 24 days, with 7 days of feces collection. *In situ* mobile bag technique was followed by 12 h rumen incubation and then intestinal movement of the bags in rumen and post-rumen cannulated Holstein steers. The three-step procedure was followed by rumen incubation of samples for 12 h (using polyester bags) and enzymatic incubation of ruminal undegradable samples. *In vivo* total tract crude protein disappearance of alfalfa hay and barley grain (0.74 and 0.69, respectively) was significantly (P<0.01) lower than *in situ* mobile nylon bag (0.89 and 0.96, respectively) and the 3-step procedure (0.81 and 0.89, respectively). Total tract crude protein disappearance from *in situ* mobile bag was significantly (P<0.01) higher than the 3-step technique. Post-ruminal disappearance of ruminal undegradable crude protein from alfalfa hay and barley grain using *in situ* mobile bag method (0.69 and 0.86, respectively) was significantly (P<0.01) higher than the 3-step enzymatic method (0.49 and 0.56, respectively). Results of the present study showed that there was a significant difference between *in vivo*, *in situ* mobile bag and 3-step methods when total tract crude protein disappearance of barley grain and alfalfa hay was evaluated.

Key words: Protein disappearance, 3-step procedure, Mobile bag, *In vivo*

Introduction

To have an accurate estimation of the protein value of feed the data of rumen and post-rumen digestibility is required (Sniffen *et al*., 1992; AFRC, 1993; NRC, 2001). These data can be achieved by *in vivo*, *in situ* mobile bag and/or *in vitro* methods. However, *in vivo* techniques to measure intestinal and total tract digestion of protein are laborious and expensive. Recently, various easy and quick methods have been suggested in some laboratories to estimate protein dis-appearance in the rumen and intestine (Calsamiglia and Stern, 1995; McNiven *et al*., 2002; Danesh Mesgaran and Stern, 2005; Gargallo *et al*., 2006; Danesh Mesgaran *et al*., 2008). It was also suggested that the ruminal and post-ruminal crude protein (CP) and amino acid disappearances of a wide range of feedstuffs can be measured using the mobile nylon bag (MNB) method (Hvelplund, 1985; De Boer *et al*., 1987; Taghizadeh *et al*., 2005; Fathi Nasri *et al*., 2008; Riasi *et al*., 2008). Calsamiglia and Stern (1995) suggested a three-step *in situ*/*in vitro* enzymatic procedure (3-step). It has been successfully

applied by some researchers to numerous feeds (Calsamiglia and Stern, 1995; Yu *et al*., 1999; Danesh Mesgaran and Stern, 2005; Jahani-Azizabadi *et al*., 2007). Data of the 3-step procedure had a high correlation with other methods used to estimate post-ruminal digestibility (Calsamiglia and Stern, 1995). Compared with *in vivo* and mobile nylon bag procedures, for determination of intestinal and total tract disappearance of CP, the 3-step procedure can lead to a substantial reduction in cost and labor, and could be routinely used for estimating intestinal and the total tract disappearance of protein in ruminants (Calsamiglia and Stern, 1995). Few experiments have been done to compare MNB and the 3-step procedures with *in vivo* trails. The aim of this experiment was to compare the *in vivo* protein disappearance of alfalfa hay and barley grain with the 3-step and MNB procedures.

Materials and Methods

Experimental feeds and chemical analysis

Feed samples were alfalfa hay and barley grain. They were dried using a forced-air oven at 60°C for 48 h. All feed samples were ground to pass through a 2-mm screen and then analyzed for dry matter (DM), organic matter (OM), crude protein (CP) and ash (AOAC, 1990). Crude fiber (CF), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were determined using the method of Van Soest *et al*. (1991), and acid detergent insoluble nitrogen (ADIN) was determined as proposed by Licitra *et al*. (1996).

Disappearance procedures

In vivo procedure

Four Baluchi lambs with an average weight of 49.4 ± 3.5 kg were used in a 2 × 2 Latin square design within 2 periods (24 days of each period). The animals were housed in individual metabolical cages (0.5 × 1.2 × 1 m) and had free access to salt and fresh water throughout the experiment. Dietary treatments consisted of alfalfa hay to barley grain ratio as 1.0:0.0 ($A_{1.0}B_{0.0}$) and 0.5:0.5 ($A_{0.5}B_{0.5}$), dry matter (DM) basis. Each period included 7 days of adaptation, followed by 10 days of *ad-libitum* feed intake (DMI) measurements. The final 7 days were used as the faecal collection phase. Alfalfa hay was chopped, with a 2-3 cm cutting length, and barley grain was coarsely ground. During the faecal collection phase, the lambs were fed a diet restricted to 90% of their lower average dry matter intake (DMI) of the adlibitum phase (Cassida *et al*., 1994).

Mobile nylon bag technique

Disappearance of protein from the samples was determined using the *in situ* mobile bag procedure as described by Subuh *et al*. (1996). Four Holstein steers (450 ± 20 kg Body weight) fitted with ruminal fistulae and T-shaped intestinal cannula were used. Animals were fed 5.1 kg of DM of alfalfa hay, 3.2 kg of DM maize silage and 2.5 kg of DM concentrate (170 g CP kg^{-1} of DM) per head per day, two times per day at 8.00 and 18.00 h. Samples of alfalfa hay and barley grain were ground to pass a 2-mm screen. Approximately 6 g DM of each sample (16 bags per each feed) were placed into a polyester bag (9 × 17 cm, with pore size of 50 µm) and incubated in the rumen for 12 h. All bags were placed simultaneously in the rumen before the morning feeding. After removal from the rumen, they were washed with tap water and subsequently dried using a forced-air oven (60°C, 48 h). A part of the feed residual of each rumen incubated bag was taken and 1 g was placed in a mobile bag (3 × 6 cm; 52 µm pore size; 8 bags per sample). Bags were closed by heat sealing and inserted into the small intestine *via* the intestinal cannulae at the rate of one bag every 30 min, and then removed from the voided faces and rinsed in tap water. The bags were dried using a forced-air oven (60°C, 48 h) and weighed to determine DM disappearance. Nitrogen (N) concentration of un-incubated, rumen and post-rumen incubated samples was determined by the kjeldahl method (Kjeltec 2300 Auto analyzer, Foss Tecator AB, Hoganas, Sweden).

Three-step procedure

This stage of the experiment followed the procedure of Calsamiglia and Stern (1995). The sample from the ruminal residue

after 12 h incubation (as described in mobile bag technique) was taken for N analysis using the kjeldahl method (Kjeltec 2300 Auto analyzer, Foss Tecator AB, Hoganas, Sweden). The sample of ruminal un-degradable CP was weighed into a 50 ml polypropylene centrifuge tube (each sample contained 15 mg of N). Two blank tubes were also prepared to correct the N contribution of the enzymes. Ten milliliters of pre-warmed (37°C) HCl-pepsin solution [2 g of pepsin (Merck M-785) dissolved into 1 l of 0.1 N HCl] was placed in each tube. Tubes were vortexed and incubated for 1 h in a shaking incubator at 38.6°C (Parsazma, Iran). After 1-h of incubation, 0.5 ml of 1 N NaOH solution was added to each tube and then vortexed. The procedure continued by adding 13.5 ml of phosphate-pancreatin buffer (68 g of KH_2PO_4 per 1 l of distilled water, 37°C). The pH was adjusted to 7.8 with strong NaOH, followed by the addition of 6 g of pancreatin (Merck, M-7130)]. Tubes were vortexed and incubated for 24 h in a shaking incubator at 38.6°C. After incubation, 3 ml of trichloroacetic acid (TCA) solution (100 g of TCA/100 ml of distilled water) was added to each tube, then vortexed. The tubes were left for 15 min and then centrifuged at 10,000× g for 15 min. A part of the supernatant (5 ml) was pipetted from each tube to determine the N concentration using the Kjeldahl method (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden).

Statistical analysis

In vivo protein disappearance was calculated as suggested by Church (1988). Calculations as described by Subuh *et al*. (1996) were used for ruminal, post-ruminal and the total tract protein disappearance, using the mobile bag technique. The data of the 3-step enzymatic procedure were calculated as described by Calsamiglia and Stern (1995). Data were applied to the general linear model of SAS (1999) with the following statistical model of: $Y_{ij} = \mu + B_i + C_j + E_{ij}$

Where:
Y = dependent variable
μ= overall mean
B_i = block effect of feed source
C_j = procedure effect
E_{ij} = residual error
assumed normally and were independently distributed. Tukey test was conducted to determine the significant difference of the means ($P<0.05$).

Results

Chemical composition (g kg^{-1} of DM) of alfalfa hay and barley grain is presented in Table 1. Post-ruminal and total tract protein disappearance of alfalfa hay and barley grain measured by different methods are shown in Table 2. In the present study, a significant ($P<0.01$) difference was found between the procedures for estimating the total tract protein disappearance of alfalfa hay (*In vivo* = 0.74, MNB = 0.89 and 3-step = 0.81) and barley grain (*In vivo* = 0.69, MNB = 0.96 and 3-step = 0.89). Total tract protein disappearance of alfalfa hay and barley grain measured using the MNB technique (0.89 and 0.96, respectively) was higher than that of the 3-step (0.81 and 0.89, respectively) and *in vivo* techniques (0.74 and 0.69, respectively). However, the total tract protein disappearance measured with the *in vivo* procedure was lower than the MNB and 3-step procedures (Table 2). *In situ* results of ruminal protein disappearance (12 h incubation) of alfalfa hay and barley grain are shown in Fig. 1. Results obtained from MNB method to estimate post-ruminal and the total tract protein disappearance in this study was significantly ($P<0.01$) higher than the 3-step procedure (Table 2). There was no significant effect of feeds on post-ruminal protein disappearance of rumen undegradable feed protein.

Discussion

The present experiment was conducted

Table 1: Chemical compositions of alfalfa hay and barley grain (g kg^{-1} of DM)

Nutrients	Feeds	
	Alfalfa hay	Barley grain
Crude protein	183	121.7
Acid detergent insoluble nitrogen (ADIN)	2.6	0.8
Crude fat	15	10
As	80	37
Crude fiber (CF)	346.7	67.5
Neutral detergent fiber (NDF)	490	-
Acid detergent fiber (ADF)	400	66.7

Table 2: Post-ruminal and total tract protein disappearance of alfalfa hay and barley grain using *in vivo, in situ* mobile bag and 3-step procedures

Item	Feed	*In vivo*[1]	MNB[2]	3-step procedure	Feed		Procedure	
					SEM[4]	P[5]	SEM	P
Post-ruminal disappearance	Alfalfa hay	-	0.69	0.49	0.027	NS[3]	0.02	0.01
	Barley grain	-	0.86	0.56				
Total tract disappearance	Alfalfa hay	0.74	0.89	0.81	0.014	NS	0.018	0.01
	Barley grain	0.69	0.96	0.89				

[1]: Calculated by difference method from diets containing 1.0:0.0 or 0.5:0.5, alfalfa to barley grain ratio. [2]: MNB = mobile nylon bag. [3]: NS = non-significant at p>0.05. [4]: SEM = Standard error of mean. [5]: P = probability

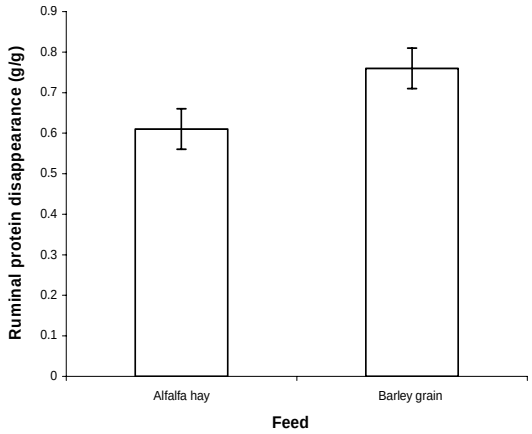

Fig. 1: *In situ* ruminal CP disappearance after 12 h incubation

to evaluate protein disappearance of alfalfa hay and barely grain using *in vivo*, *in situ* and *in vitro*/*in situ* methods. Results of the previous experiments indicated that, compared with *in vivo* and mobile nylon bag procedures, the 3-step procedure might lead to a substantial reduction in cost and labor, and could be routinely used for estimating intestinal and the total tract disappearance of protein in ruminants (Calsamiglia and Stern, 1995). However, results of few experiments demonstrated a significant difference between non *in vivo* methods when protein sources were examined (Danesh Mesgaran *et al*., 2008). In addition, Danesh Mesgaran and Stern (2005) indicated a significant effect of feed sources on protein disappearance using *in situ*, *in vitro* and *in vitro*/*in situ* procedures. Therefore, it is very critical to compare the non *in vivo* methods with the *in vivo* methods using different feeds. The present experiment showed a significant (P<0.01) difference between procedures for estimating the total tract protein disappearance of alfalfa hay (*In vivo* = 0.74, MNB = 0.89 and 3-step = 0.81) and barley grain (*In vivo* = 0.69, MNB = 0.96 and 3-step = 0.89) in the present study, and confirmed the findings of Danesh Mesgaran *et al*. (2008), who compared the results of MNB, 3-step and *in vitro* enzymatic procedures for estimating the total tract protein disappearance of oilseed meals. However, the present results of the total tract protein disappearance of alfalfa hay and barley grain measured with the MNB technique were higher than those of the 3-step and *in vivo* techniques, which did not confirm the finding of Danesh Mesgaran and Stern (2005), who reported that there was no significant difference between the MNB and 3-step procedures when the total tract protein disappearance was measured. The total tract protein disappearance measured with the *in vivo* procedure was lower than the MNB and 3-step procedures (Table 2). This result confirmed the findings of Calsamiglia and Stern (1995) and Titgemeyer *et al*. (1989). The lower *in vivo* protein disappearance of barley grain compared with MNB and the 3-step procedures can be related to the reduction in alfalfa digestibility in the diet containing barley grain. Tejido *et al*. (2002) and Calder (1970) reported that the feeding of donor animals with relatively high concentrate diet (60:40, barley grain: alfalfa hay) showed an *in vitro* reduction of DM digestibility of alfalfa hay and basal diet compared with donor animals fed only alfalfa hay. In addition, increasing the intake of barley grain may be a result of the increase of rumen out flow of barley grain. The supply of carbohydrate to the hindgut, resulted from a high concentrate diet, might enhance metabolic fecal N by increasing microbial protein syntheses (Church, 1988), which reduce apparent disappearance. The feed

class had no significant effect on the total tract protein disappearance.

In the present study, both feed samples had higher ruminal protein disappearance compared with the results of Danesh Mesgaran and Stern (2005). This might be related to varieties, the harvest method or bag materials (NRC, 2001; Danesh Mesgaran and Stern, 2005).

Current feed evaluation systems discovered that the post-ruminal disappearance of protein varied among feed sources (Madsen and Hvelplund, 1987). In recent years, the MNB method has been frequently used for estimating ruminal, post-ruminal and total tract protein disappearance. However, it requires ruminal access and is still time-consuming for the routine evaluation of feeds. Calsamiglia and Stern (1995) indicated that incubation in pepsin pancreatic solution after 12 h incubation gave a high correlation (r^2 = 0.91) with *in vivo* intestinal protein disappearance.

In the present study, higher post-ruminal disappearance of ruminal undegradable protein measured by the MNB method compared with the 3-step procedure confirmed the findings of Danesh Mesgaran *et al*. (2008), who found a significant difference between the results of MNB, the 3-step and *in vitro* enzymatic procedures for estimating post-ruminal and the total tract protein disappearance of oilseed meals. The difference between these methods can be a result of hind gut fermentation in the MNB procedure (Van Straalen *et al*., 1993). Hvelplund (1985) reported that the amount of protein that was digested in the large intestine was 50 and 27% of that leaving the ileum for soybean meal and rapeseed meal, respectively, using the MNB method. In addition, variable results of the 3-step procedure in the present study, compared with the MNB, might be related to the different bag materials, bag pore size, animal, diet, large intestinal fermentation and bacterial contamination (Hvelplund, 1985; Voigt *et al*., 1985; Jirl *et al*., 1996; NRC, 2001). Furthermore, the 3-step procedure estimates the true digestibility of feed protein (NRC, 2001; Danesh Mesgaran and Stern, 2005). Calsamiglia and Stern (1995) reported r value of 0.91 when a pancreatin assay was regressed to *in vivo* intestine estimates (n = 34, P<0.001). Whereas, Danesh Mesgaran and Stern (2005) reported a low correlation (r^2 = 0.26) for post-ruminal rumen undegradable protein disappearance and a medium correlation (r^2 = 0.55) for the total tract protein digestion.

In conclusion, the results of the present study showed that there is a significant difference between *in vivo* with *in situ* mobile bag and the 3-step enzyme procedures when the post-ruminal and total tract of protein disappearance of alfalfa hay and barley grain were considered. However, the 3-step procedure is fast and inexpensive in comparison with that of the MNB and *in vivo* methods. Therefore, modification of this method for estimating feed protein disappearance is proposed.

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