Effect of polyethylene glycol on in vitro gas production kinetics of Prosopis cineraria leaves at different growth stages

Mostafa Yousef Elahi,1 Moslem Moslemi Nia,1 Abdelfattah Z.M. Salem,2 Hormoz Mansouri,3 Jaime Olivas-Pérez,4 Maria A. Cerrillo-Soto,5 Ahmed E. Khoiïfi6 1Animal Science Department, University of Zabol, Iran 2Departamento de Nutrición Animal, Universidad Autónoma del Estado de México, Toluca, Mexico 3Animal Science Research Institute, Karaj, Iran 4Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Guerrero, Mexico 5Facultad de Medicina Veterinaria y Zootecnia, Universidad Juárez del Estado de Durango, Mexico 6Dairy Science Department, National Research Centre, Cairo, Egypt

Abstract

The aim of this experiment was to determine the effect of polyethylene glycol (PEG) on in vitro gas production (GP) kinetics of Prosopis cineraria leaves at different growth stages. The contents of total phenol (TPH), total tannin (TT) and condensed tannin (CT) were determined. Effects on in vitro organic matter digestibility (OMD), metabolisable energy (ME) and effective dry matter digestibility were assessed by PEG tannin bioassay. No significant differences (P>0.05) were observed for TPH content; however, the stage of flowering had the highest (P<0.05) content of both TT and CT. No interaction effects (P>0.05) were observed between the growth stage and PEG addition for in vitro GP and its parameters. Addition of PEG increased (P<0.05) GP, OMD and ME in all stages. In conclusion, adding PEG to P. cineraria leaves can improve their nutritive value and could be considered as a potential feed for ruminants.

Introduction

Tree legume forages play an important role in livestock nutrition in many parts of the tropics. One of the commonly used tree species in south of Iran is Prosopis spp. (mesquite). The genus Prosopis belongs to the family Leguminosae (subfamily Mimosraceae). About 43 species of this genus are known; three of them are commonly found in Iran. These species are P. cineraria (Iranian Kahor), P. koelsziana (Valley Kahor) and P. juliflora (Pakestanian Kahor). Mesquite trees and shrubs are vigorous, drought- and heat-tolerant plants that are able to survive in many arid parts of the world. In some of these regions mesquite leaves and pods are principal sources of forage during dry seasons (Feller, 1979).

Prosopis cineraria is found in the arid areas of Arabia, Indian sub-continent, Iran and Afghanistan where it provides fodder, fuel, shade and improvement of soil and stabilises sand dunes. Kahor leaves and pods are consumed by many animal species. However, Prosopis sp. has been reported to contain levels of anti-nutrients such as tannins. Therefore, the nutritive value of these leaves as feed for ruminants is offset by tannin potential negative effect on protein utilisation. Tannins are known to affect the availability of nutrients by formation of soluble and insoluble complexes. Their effects on the digestibility of nutrients will vary depending on tannin content and astringency (McNeill et al., 1998). As it would take a considerable effort to screen for all possible anti-nutritive factors by conventional chemical methods, there is interest in the possible use of simple bio-assays as potential screening methods. The use of polyethylene glycol (PEG) to neutralise condensed tannins (CTs) has proved useful in further elucidating the specific nutritional consequences of dietary CT as it displaces protein-tannin complexes. As a consequence, CTs interact more strongly with PEG than they do with protein (Mangan, 1988). Thus, supplementation with PEG has been used to alleviate the negative effects of tannins on livestock (Landau et al., 2000). Palmer and Jones (2000) have shown that PEG improved the in vitro digestibility of nitrogen in Calliandra and most other legumes containing tannins. The treatment with tannin binding agents can be highly effective in overcoming the anti-nutritive effects of tannins leading to improved animal performance. However, their effects can be variable which may relate to the nature of the tannins and the nature of the tannin-feedstuff complexes which can form.

Materials and methods

Forages were collected from Sardoeeyie of Jiroft, located in the South-West of Iran. Sardoeeyie region is situated at 57°19’E and 29°9’N. Its average altitude is 2700 m asl. Fresh mesquite leaves were collected from trees planted. Leaves were removed from branches, pooled to five samples per different growth stages. The samples were dried at 60°C in forced air oven for 48 h, except for those samples used for tannins determination that were air-dried in the shade to minimise changes in tannin content and activity (Makkar and Singh, 1991).
**Chemical analysis**

Standard methods as described in AOAC (1990) were used for determination of dry matter (DM) (method #930.15), ash (method #924.05) and N (method #984.13). Ash-free neutral detergent fibre (NDF) was determined using sodium sulfate according to the method of Van Soest et al. (1991), while ash-free acid detergent fibre (ADF) (method #973.18) was determined based on AOAC (1990).

**Total phenols determination**

Samples were analysed for CT using the method of Makkar (2000). Briefly, dried plant material (200 mg) was extracted with acetone:water (10 mL; 70:30, v/v) in ultrasonic bath for 20 min. Contents were centrifuged (4°C, 10 min, 3000 × g), then the supernatant was kept on ice until analysis. Total phenols were determined with the Folin-Ciocalteau reagent and detected at 725 nm. A calibration curve was prepared using tannic acid (Merck GmbH, Darmstadt, Germany). Total phenols were calculated as tannic acid equivalents and expressed as equivalents g/kg DM.

**Tannins determination**

Samples were analysed for total extractable tannin (TT) using HCl-butanol as described in Makkar (2000). Briefly, an aliquot from the above acetone:water extract (0.5 mL) although this extract occasionally needed diluting with the extract of acetone:water, if final absorbance at 550 nm exceeded 0.6 absorbance units plus HCl-butanol (3 mL) plus ferric ammonium sulphate (0.1 mL) reagents were heated in a boiling water bath for 60 min. Absorbance was read at 550 nm. The colorimetric data (in absorbance units) were converted to leucocyanidin equivalents based on the assumption that the color yield of tannic acid was accepted as the NTP (Makkar, 2000). Non-tannin phenols (NTP) were determined using absorption to insoluble polyvinylpyrrolidone (PVPP). The insoluble PVPP (100 mg) was weighed in 100x12 mm test tubes. Distilled water (1 mL) and 1 mL tannin containing extract were added and vortexed. The tube was kept at 4°C for 15 min, vortexed again, then centrifuged (3000 × g) for 10 min, and then the supernatant was collected. The phenolic content of the supernatant was measured by the Folin-Ciocalteau reaction and this was accepted as the NTP (Makkar, 2000).

Total tannins (TT) were calculated as the difference between TPH and NTP. The results were expressed as gallotannin. Protein perceptible phenolics were determined according to Makkar (2000) and results were expressed as tannic acid equivalent.

**Gas production**

Rumen fluid for the in vitro digestibility tannin bioassay was obtained from two mature male native cattle with live weight of 346±11.5 kg fitted with permanent 70 mm rumen canulae. Cattle were fed a ration divided into equal meals at 08:00 a.m. and 04:00 p.m. daily. Cattle had free access to water throughout the experiment. Rumen fluid was obtained from the two cattle in the morning before feeding (07:00 a.m.), flushed with CO₂ filtered through three layers of cheesecloth and mixed (1:2, v/v) with an anaerobic mineral buffer solution as described by Makkar et al. (1995) and revised by Makkar (2000). Preparation of an in vitro mineral buffer media for the gas test was completed as described by Menke and Steingass (1988). Reduced buffer medium composition, per liter, was 70.0 g of NaHCO₃, 4.00 g of NH₄HCO₃, 5.7 g of Na₂HPO₄, 6.2 g of KH₂PO₄, 0.6 g of MgSO₄·7H₂O, 0.52 g of Na₂S, 13.2 g of CaCl₂·2H₂O, 10.00 g of MnCl₂·4H₂O, 1.00 g of CoCl₂·6H₂O, 0.01 g of sodium resazurin, 60 mL of freshly prepared reduction solution containing 580 mg of Na₂S·9H₂O, and 3.7 mL of 1 M NaOH. The mixture was kept stirred, under CO₂ flushing at 39°C, using a magnetic stirrer fitted on a hotplate.

Effects of tannins on in vitro organic matter digestibility (OMD) were assessed by incubating approximately 375 mg (DM bases) of triplicate test feed samples with or without 750 mg PEG with MW 6000 (Merck Schuchardt OHG, Hohenbrunn, Germany). Feed samples were incubated in 100 mL glass syringes based on Menke and Steingass (1988) procedures. The PEG tannin bioassay was performed according to Makkar et al. (1995) and revised by Makkar (2000). Petroleum jelly was applied to the pisovent to ease movement and prevent escape of gas. Syringes were pre-warmed (39°C) for 1 h before addition of 30±0.5 mL of rumen buffer mixture into each syringe, and incubated in a water bath maintained at 39±0.1°C as described by Blümml and Ørskov (1993). Syringes were gently shaken every hour during the first 8 h of incubation. Gas production readings (mL) were recorded at 2, 4, 6, 8, 10, 12, 16, 24, 48, 72 and 96 h for PEG-treated and -untreated samples. Total gas values were corrected for blank with a hay standard with a known GP.

For a more precise estimation of GP throughout the duration of in vitro fermentation, a nonlinear equation was used to analyse the kinetic data (France et al., 2000).

Cumulative GP data were fitted to the exponential equation \( Y = b \left(1 - e^{-ct}\right) \), where \( b \) is the GP from the fermentable fraction (mL), \( c \) the GP rate constant for \( b \), \( t \) the incubation time (h), and \( Y \) the gas produced at time \( t \).

Feed OMD (g/kg OM) and metabolisable energy (ME) in MJ/kg DM were estimated by equations of Menke and Steingass (1988), based on 24 h GP (mL) and crude protein (CP) content (g/kg DM) as: OMD (g/kg DM) = 148.8 + 8.89 net GP + 4.5 CP + 0.651 XA. ME (MJ/kg DM) = 2.20 + 0.136 net GP + 0.057 CP + 0.0029 CP². Where CP is in g/100 g DM; XA in g/100 g DM. Net GP data were converted from 375 to 200 mg after 24 h of incubation.

**Statistical analysis**

Data of chemical composition and tannin content were subjected to analysis using the General Linear Model procedure of SAS (2002), based on the statistical model: \( Y_{ij} = \mu + S_i + e_{ij} \), where \( Y_{ij} \) is the general observation on chemical composition and tannin content, \( \mu \) is the general mean, \( S_i \) is the \( i \)th effect of growth stage on the observed parameters, and \( e_{ij} \) is the random standard error. Means were tested using Duncan test.

For in vitro GP and estimated parameters of digestibility and ME, the following statistical model was fitted: \( Y_{ijk} = \mu + S_i + P_j + (SP)_{ij} + E_{ijk} \), where \( Y_{ijk} \) is the observation on GP and digestibility estimates, \( S_i \) is the \( i \)th effect of growth stage and \( P_j \) is the \( j \)th effect of PEG on the observed parameters. The \( (SP)_{ij} \) term represents \( i \)th and \( j \)th interaction effects of growth stage and PEG on in vitro GP, and \( E_{ijk} \) is the random standard error.

**Results**

**Primary and secondary compounds**

The chemical composition of *P. cineraria* at different growth stages is shown in Table 1. There were variations in chemical compositions between phenological stages of *P. cineraria*. The CP content of the different stages ranged (P<0.05) from 9.26 to 11.79% for seed ripening and vegetative stages, respectively. The NDF and ADF content ranged (P<0.05) from 45.12 to 46.51 and 28.14 to 30.48%, respectively for seed ripening and vegetative stages.

The content of TPH was almost the same (P>0.05) between different stages. However, the stage of flowering had the highest (P<0.05) between different stages. There were variations in chemical compositions between phenological stages of *P. cineraria*. The CP content of the different stages ranged (P<0.05) from 9.26 to 11.79% for seed ripening and vegetative stages, respectively. The NDF and ADF content ranged (P<0.05) from 45.12 to 46.51 and 28.14 to 30.48%, respectively for seed ripening and vegetative stages.

The content of TPH was almost the same (P>0.05) between different stages. However, the stage of flowering had the highest (P<0.05) content of both TT and CT compared to seed ripping and vegetative stages, respectively (Table 1).

**Gas production**

No interaction effects (P<0.05) were observed between the growth stage and PEG addition for in vitro GP and GP parameters.
The cumulative volume of GP increased (P<0.05) with increasing time of incubation (Table 2, Figure 1). Addition of PEG to the stage of maturity increased (P<0.05) in vitro GP only during the first 8 h of incubation without significant (P>0.05) effects from 8 to 96 h of incubation. However, the addition of PEG increased GP (P<0.05) over the time. The insoluble but fermentable fraction (b), the rate of GP during incubation (c), OMD, dry OMD (DOMD), and ME all not affected (P>0.05) by stage of growth with significant (P<0.05) response for PEG addition (Table 2, Figure 1).

**Discussion**

In our study, leaves of *Prosopis cineraria* had CP content more than 80 g/kg DM (range: 92.6 to 117.9 g/kg DM), which according to Norton (2003) should provide ruminal ammonia levels above the minimum required by rumen microorganism to support optimum growth for maintenance, production and optimum activity. However, the CP content of leaves of seed ripping stage was lower than required by microorganisms in the rumen to support optimum activity (Norton, 2003).

In tree leaves, tannins are tightly bound to the cell wall (NDF and ADF) and cell protein (Reed et al., 1990). Tannins may form a less digestible complex with dietary proteins and may bind and inhibit the endogenous protein, such as digestive enzymes (Kumar and Sing, 1984). Tannin can also adversely affect the microbial and enzyme activities (Silanikove et al., 1996a; Makkar et al., 1995). However, in ruminants dietary CT 20 to 40 g/kg DM has been shown to have beneficial effects because they reduce the wasteful protein degradation in the rumen by the formation of a protein-tannin complex (Min et al., 2003). Getachew et al. (2002) concluded that samples containing TPH and TT (tannic acid equivalent/kg DM) up to 40 and 20 g/kg DM, respectively, are not expected to induce an increase in GP with addition of PEG. On the other hand, high levels of CT in tree leaves have been reported to restrict the nutrient utilisation and decrease voluntary food intake,

**Table 1. Chemical composition and phenolic compounds of *Prosopis cineraria* at different growth stages.**

<table>
<thead>
<tr>
<th>Chemical composition, %</th>
<th>Vegetative</th>
<th>Flowering</th>
<th>Seed ripping</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>93.5*</td>
<td>93.5*</td>
<td>93.2*</td>
<td>0.61</td>
</tr>
<tr>
<td>OM</td>
<td>85.5*</td>
<td>86.2*</td>
<td>85.2*</td>
<td>0.80</td>
</tr>
<tr>
<td>Ash</td>
<td>14.5*</td>
<td>13.8*</td>
<td>14.8*</td>
<td>0.78</td>
</tr>
<tr>
<td>CP</td>
<td>11.8*</td>
<td>10.7*</td>
<td>9.3*</td>
<td>0.47</td>
</tr>
<tr>
<td>Ether extract</td>
<td>3.04</td>
<td>4.7*</td>
<td>4.8*</td>
<td>0.35</td>
</tr>
<tr>
<td>ADF</td>
<td>30.4*</td>
<td>29.0*</td>
<td>28.1*</td>
<td>0.51</td>
</tr>
<tr>
<td>NDF</td>
<td>46.5*</td>
<td>45.8*</td>
<td>45.1*</td>
<td>0.39</td>
</tr>
<tr>
<td>Phenolic compounds, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPH</td>
<td>8.83</td>
<td>8.83</td>
<td>8.53</td>
<td>0.231</td>
</tr>
<tr>
<td>TT</td>
<td>7.67ab</td>
<td>7.76a</td>
<td>7.15b</td>
<td>0.262</td>
</tr>
<tr>
<td>CT</td>
<td>3.01b</td>
<td>4.23a</td>
<td>3.68b</td>
<td>0.322</td>
</tr>
</tbody>
</table>

**Table 2. Gas production characteristics of *Prosopis cineraria* leaves without or with polyethylene glycol at different growth stages**

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Without PEG</th>
<th>With PEG</th>
<th>SEM</th>
<th>Stage</th>
<th>PEG</th>
<th>Stage*PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time, h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>11.9</td>
<td>15.6</td>
<td>0.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>16.1</td>
<td>21.6</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>17.9</td>
<td>20.1</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>21.8</td>
<td>31.3</td>
<td>1.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>26.3</td>
<td>37.5</td>
<td>1.74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>29.8</td>
<td>41.2</td>
<td>2.13</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>72</td>
<td>32.7</td>
<td>44.0</td>
<td>2.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>34.5</td>
<td>45.5</td>
<td>2.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b, mL/200 mg dry matter</td>
<td>30.9</td>
<td>42.8</td>
<td>2.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c, mL/h</td>
<td>0.096</td>
<td>0.106</td>
<td>0.0060</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMD, %</td>
<td>52.9</td>
<td>62.9</td>
<td>1.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOMD, %</td>
<td>44.7</td>
<td>53.8</td>
<td>1.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME, MJ/kg DM</td>
<td>6.8</td>
<td>8.4</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PEG, polyethylene glycol; GP, gas production; b, insoluble but fermentable fraction; DM, dry matter; c, rate constant of gas production during incubation; OMD, organic matter digestibility; DOMD, digestible organic matter in dry matter; ME, metabolisable energy. *Means with different letters within stages differ (P<0.05); ns, not significant.
nutrient digestibility and N retention (Kumar and Vaithiyananathan, 1990; Silanikove et al., 1996b). Total tannin content of forages in the range of 60 to 100 g/kg DM depresses intake and growth of animals (Barry et al., 1984). The tannin content of leaves obtained in this experiment fell into this range. Therefore, supplementation of PEG can be recommended to reduce the detrimental effect of tannin in leaves. Pritchard et al. (1998) showed that giving PEG to sheep fed with mulga markedly increased feed intake, weight gain and wool growth. Gilboa et al. (2000) also showed that a single daily oral dose of PEG substantially improve feed intake and efficiency of utilisation by sheep and goats consuming tannin rich forages (Gurbuz, 2007). The effects of tannins seem to be dependent on several factors including forage species, chemical nature and structure of tannins, and biochemical interaction among tannins and proteins, than the tannins level itself. On the other hand, in palm leaves, addition of PEG had small effect despite their relatively high tannin content (Arhab et al., 2009).

The OMD was higher when PEG was added to the different growth stages. Increased in vitro GP and OMD due to addition of PEG suggest a negative influence of tannins on digestibility (Makkar, 2003). Inactivation of tannins through PEG binding increases availability of nutrients resulting in increased microbial activity and GP (Makkar, 2003).

Addition of PEG caused different increments in GP between different growth stages. These variable responses of GP could be due to variations in tannin content between different stages. The increase in the GP in the presence of PEG can be due to an increase in the availability of nutrients to rumen microorganisms, especially N (Bakhshizadeh and Taghizadeh, 2013). Increased degradability of samples at different growth stages treated with PEG was reflected in greater GP at different time intervals.

The leaves of P. cineraria at the stage of flowering provide more soluble fractions, which is a fermentable energy source within time. Gas production is associated with volatile fatty acids production following fermentation of substrate; therefore, more fermentation of a substrate will result in a greater GP (Blümml and Ørskov, 1993). Differences between GP could be explained by the differences in total VFA production and molar proportion of VFA as a result of fermentation (Beuvingk and Spoelstra, 1992). Doane et al. (1997) found a significant correlation between GP and VFA production. Quickly soluble carbohydrates produce relatively higher propionate as compared to acetate and vice versa when slowly fermentable carbohydrates are fermented (Getachew et al., 1998).

The increased GP when samples were incubated with PEG were also reported for different forages by other authors. Basha et al. (2013) noted that the addition of PEG overcomes the inhibitory effect of tannins on rumen microbes. Arhab et al. (2009) evaluated the influence of tannins present in arid zone forages from North Africa including Aristida plumosa, Astragalus gombiformis, Genista saharae, two date palm fractions (leaves and racemes), and vetch-oat hay taken as control on in vitro GP. They found that addition of PEG resulted in an overall increase in GP (20.2%), with the exception of Danthonia and Aristida. However, PEG addition did not influence the rate of GP. Moreover, the increase in GP when samples were incubated with PEG were also reported by others (Baba et al., 2002; Rubanza et al., 2005; Singh et al., 2005). Bakhshizadeh and Taghizadeh (2013) determine the effect of PEG inclusion during in vitro incubation on GP kinetics, OMD and the ME of red grape pomace. Total phenol, T, CT and hydrolysable tannins contents in grape pomace were 4.23, 2.23, 1.16 and 0.89%. Addition of PEG resulted in an increased GP at all incubation times than control; however, there was no significant increase in GP within levels of PEG.

Figure 1. In vitro gas production of Prosopis cineraria leaves with or without polyethylene glycol at different growth stages (P<0.05): a) vegetative, b) flowering, and c) seed ripping stage.

Arhab et al. (2009) stated that addition of PEG can affect partitioning factor (PF) of tannin contain forage. They showed that PEG addition can promote GP but not OMD with decreasing PF of Aristida plumose and palm leaves forages. Whereas, addition of PEG did not alter PF in Genista saharae, Danthonia forskahllii and vetch-oat hay. They returned the different responses between these types of forages to the limited ability of PEG to completely inhibit the negative effects of tannins (Baba et al., 2002; Frutos et al., 2004), which depends mainly both on stereochemistry and chemical structure of tannins. Other factors than tannins, like limited available N for ruminal microbiota, the higher NDF, ADF and lignin contents, and the saponins may limit fermentation (Ndlovu and Nherera, 1997). Blümml et al. (2003) suggested selection of forages for high degradability but proportionally low GP. The theoretical range for PF values for tannins free plants was suggested by Blümml et al. (1997) to be between 2.75 and 4.41. According to Blümml and Becker (1997), plants with high PF are in general highly digestible and the values correlate well with DM intake in ruminants. Thus, these results could suggest that these forages had a potential nutritive value which tends to enhance microbial synthesis rather than GP.

The effect of PEG addition is more pronounced on potential GP, measured at 96 h of incubation. The effects of tannins on nutrient degradability depends essentially on the formation of complexes between tannins and the components of diets, primarily proteins and to a lesser extent with amino acids, polysaccharides and minerals, as well as on their effects on the microbial population and on its enzy-


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