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Evaluating the effects of ozonation pretreatment on ruminal fermentation kinetics and nutritive value of wheat straw using *in vitro* techniques

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ABSTRACT

Ozone is a powerful oxidizing substance that increases lignin decomposition and improves cellulose degradation. The aim of this study is to investigate the effect of ozone pretreatment on *in vitro* fermentation kinetics and nutritive value of wheat straw in ruminant animals. In this study, wheat straw was pretreated with ozone for 15, 30, 45, and 60 minutes. Gas production volume was recorded at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h of incubation. The digestibility of dry matter (DMD) and organic matter (OMD), metabolisable protein (MP) and metabolisable energy (ME), as well as the relative feed value (RFV) and relative forage quality (RFQ) of the feed were estimated. The results showed that the pretreatment with ozone, significantly reduced fiber components of wheat straw, including neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL), and increased non-fibrous carbohydrates (NFC), as well as gas production in 12 to 96 hours incubation. Methane production also, significantly increased by ozonolysis pretreatment. Estimated parameters related to nutritive value including digestible energy (DE), metabolisable energy (ME), net energy for lactation (NE_l), OMD, DMD, digestible organic matter in dry matter (DOMD), dry matter intake (DMI), RFV and RFQ were significantly increased by ozonation pretreatment. In an overall conclusion, it seems that ozonolysis pretreatment, particularly at the level of 60 minutes ozonation lead to increasing *in vitro* fermentation kinetics (by 33%) and nutritive value (by 14%) of wheat straw.

Keywords: Gas production, Lignocellulosic biomass, Nutritive value, Ozonolysis, Wheat straw

Agricultural waste form a significant portion of ruminant feed sources. The straws of the different crops, are the main ingredients and are rich in fibers (Ghorbani *et al.* 2021a). The agricultural by-products are less digestible due to the strong physical and chemical bounds between carbohydrates and lignin. In order to fully utilize the potential of straw, the lignin-cellulose structure of the cell walls must be broken or changed (Harper and McNeill 2015, Nayan *et al.* 2019) to simpler molecules. Despite the benefits of the lignin from a botanical point of view, it is an important bottleneck in animal nutrition. In addition to the fact that lignin in the gastrointestinal tract is almost indigestible, it also strictly limits the digestion of other cell wall components by as much as 2.4 times of its weight (Harper and McNeill 2015). For a long time, livestock nutritionists have attempted to solve this restriction and have used various methods to reduce lignin connection with other cell wall components. Given that the level of lignin and the nature of the lignin between different plants are variable, the methods also have different and sometimes contradictory results (Ghorbani *et al.* 2021b). But given that these types of processing are accompanied by time and cost, it is necessary to judge the processing method in relation to the lignin concentration and its nature. Pretreatment is a key step in overcoming biomass resistance, in which a wide range of technologies including physical, chemical, physicochemical and biological processes are used to break the lignocellulosic matrix (Silverstein *et al.* 2007, Sahoo *et al.* 2014, Ballesteros *et al.* 2018). The main purpose of pretreatment is to increase the surface and porosity of the substrate, reduce cellulose crystalline, destruction of cellulose bonding with lignin and thus increase digestion, degradation and fermentation of the lignocellulosic by-products (Das *et al.* 2015). Ozonation is one of the modern technologies to delignify lignocellulosic materials in order to facilitate breakdown of lignocellulosic bond and increase cellulose and hemicellulose biodegradability (Ballesteros *et al.* 2018). The utilization of ozone as a green and environmentally friendly technology has been legally approved in food industry worldwide (Varga and Szigeti 2016). Efficient removal of lignin, without producing toxic substances and inhibitory compounds during the process, performing, lignin destruction with minimal effects on cellulose and hemicellulose at ambient temperatures and pressure, increasing the efficient utilization of released products by microorganisms are of other advantage of

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ozone pretreatment of lignocellulosic materials (Ghorbani *et al.* 2021a, Ballesteros *et al.* 2018).

Several studies reported that ozonation pretreatment resulted in 30 to 86 percent delignification of lignocellulosic materials (Al jibouri 2012, Bule *et al.* 2013, Eqra *et al.* 2015, Ghorbani *et al.* 2021a). Ben Ghedalia and Miron (1981) found that ozonolysis of wheat straw improved the *in vitro* organic matter digestibility by 50%. García-Cubero *et al.* (2009, 2010) showed that ozonation is an efficient pretreatment for delignification of cereal straws. They have concluded that ozonation enhanced lignin degradation, cellulose and hemicelluloses digestion and fermentable sugars production. Ghorbani *et al.* (2022) showed that ozonation of wheat straw generally resulted in a significant increase in the amount of *in vitro* gas production. The researchers also found that the estimated organic matter digestibility, metabolisable energy and short-chain fatty acids increased significantly due to ozonolysis of wheat straw.

The aim of the current study was to evaluate the effects of ozonation pretreatment on ruminal fermentation kinetics and nutritive value of wheat straw using *in vitro* feed evaluation techniques.

MATERIALS AND METODS

Animals and management: This study was performed at the Animal Science Research Institute (ASRI), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran, according to the instruction of Iranian Council of Animal Care (1995). Three fistulated mature Taleshi steers (350 ± 20 kg body weight) were used in current experiment. Diet including 10% higher than the maintenance with 70% forage which contains (alfalfa hay and wheat straw), and 30% concentrate (barley grain, soybean meal and cotton seed, mineral and vitamin supplements) as total mixed ration were offered twice daily at 8:00 and 16:00 hr. The animals had free access to fresh water.

Experimental treatments: Experimental treatments were consisted of: 1) untreated wheat straw (control); 2) 15 minute ozonated wheat straw; 3) 30 minute ozonated wheat straw; 4) 45 minute ozonated wheat straw; 5) 60 minute ozonated wheat straw, Ozonation was performed in the treatments at a concentration of 7 grams ozone per hour and a flow rate of 6 liters per minute.

Chemical composition and fibre components: Chemical composition including dry matter (DM), ether extract (EE), crude protein (CP) and crude ash (CA) content of wheat straw were determined according to AOAC (2005). Neutral detergent fibers (NDF), acid detergent fibers (ADF) and acid detergent lignin (ADL) were measured by procedures proposed by Van Soest *et al.* (1991). Cellulose (C) and hemicellulose (HC) were calculated as: C=ADF-ADL and HC= NDF-ADF. Non-fibrous carbohydrates (%NFC = 100 – (%NDF + %CP + % EE + % CA) were estimated as proposed by NRC (2001).

In vitro gas production procedure: Rumen fluid was collected in a pre-warmed flask from the Three fistulated mature Taleshi steers before morning feeding. Approximately 200 mg samples of dry feed stuff were weighed in triplicate and placed in a 100 mL calibrated glass syringe. Feed samples were incubated *in vitro* with rumen fluid-buffer mixture (30 mL) were transferred into the glass syringes according to the procedure outlined by Menke and Steingass (1988). The samples were incubated at 40°C. Gas production volume was recorded at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h of incubation times and corrected for blank. In order to measure methane (CH_4) production, after reading the syringes at the time of 24 h incubation, 4 mL of NaOH (10 M) was added to syringes and after 10 minutes, the mentioned syringes were read again and removed. Treating the syringes content with sodium hydroxide solution allowed for the absorption of carbon dioxide, and remaining gas considered as methane (Anele *et al.* 2011).

Equations, calculations and statistical analyses: Cumulative net gas production data were fitted to the model described by Ørskov and McDonald (1979) and gas production parameters were estimated by the Fitcurve software version 6: $P = A\ (1-e^{-ct})$

Where, A = potential gas production, c = the gas production rate constant for the insoluble fraction b, t = the incubation time (h), P = the gas production at the time t

The digestible organic matter (DOM), net energy for lactation (NE_l) and metabolisable energy (ME) for differently treated wheat straw were estimated according to equations of Menke and Steingass (1988), short chain fatty acids (SCFA) were estimated using equation of Makkar (2005), and metabolizable protein (MP), digestible energy (DE), dry matter intake (DMI), relative feed value (RFV) and relative forage quality (RFQ) were calculated by the equations of Safaei *et al.* (2023) as mentioned below:

$$\text{DOM (\%)} = 0.889\ \text{GP} + 0.45\ \text{CP} + 0.651\ \text{CA} + 14.88$$

$$\text{ME (MJ/kg DM)} = 0.136\ \text{GP} + 0.057\ \text{CP} + 0.00286\ \text{EE}^2 + 2.2$$

$$\text{NE}_l\ \text{(MJ/kg DM)} = 0.096\ \text{GP} + 0.038\ \text{CP} + 0.00173\ \text{EE}^2 + 0.54$$

$$\text{SCFA (mmol)} = 0.0222\ \text{GP} – 0.00425$$

$$\text{MP (g/kg)} = 19.3{*}\text{OMD}$$

$$\text{DE (MJ/Kg)} = 4.22\text{-}(0.11\times\text{ADF})+(0.332\times\text{CP})+(0.00112\times\text{ADF}^2)$$

$$\text{DMI (g/KgW}^{0.75}) = 2.6+0.49(A)+339(c)+0.17(\text{CP})$$

$$\text{RFV}= -875.540 + 25.109\ \text{GP}$$

$$\text{RFQ}=1.1446\ \text{RFV}- 32.224$$

Where, GP and OMD were gas production volume at 24 h of incubation time (mL/200mg DM) and organic matter digestibility, respectively.

General linear model (GLM) procedure of SAS (2001) software was used in order to statistical analysis of data from gas production. The experiment and statistical analysis designed and performed based on complete randomized design (CRD) with five treatments and three replicates for each treatment. Treatment means were compared by Duncan multiple range tests (p<0.05).

In vitro digestibility technique of Tilley and Terry: Digestibility of DM (DMD) and OM (OMD) and organic matter in dry matter (DOMD) were determined according

Table 1. Chemical composition, cell wall indices and non-fibrous carbohydrate content of ozonated wheat straw (WS) as percent

Treatments	DM	CP	CA	EE	NDF	ADF	ADL	NFC	HC	CC
Untreated WS (Control)	93.1	1.6 [b]	8.0	2.5 [b]	78.0 [a]	47.2 [a]	5.8 [a]	9.9 [c]	30.8 [a]	40.5 [a]
15 minute ozonated WS	93.1	1.6 [b]	7.9	2.5 [b]	77.2 [a]	46.6 [a]	5.6 [b]	10.8 [c]	30.5 [ab]	40.2 [a]
30 minute ozonated WS	93.1	1.8 [ab]	7.8	2.6 [ab]	74.4 [b]	45.9 [a]	5.6 [b]	13.4 [b]	28.5 [b]	39.6 [a]
45 minute ozonated WS	93.1	1.8 [ab]	7.7	2.7 [a]	73.4 [b]	45.1 [ab]	5.4 [bc]	14.3 [b]	28.3 [b]	38.9 [ab]
60 minute ozonated WS	93.1	1.9 [a]	7.3	2.7 [a]	70.6 [c]	42.3 [b]	5.3 [c]	17.6 [a]	28.2 [b]	36.4 [b]
SEM	0.01	0.08	0.29	0.05	0.76	0.95	0.06	0.81	0.71	0.90
Statistical significance	NS	S	NS	S	HS	S	HS	HS	S	S

a-c: different letters in each column, indicated significantly different means ($p<0.05$). NS: no significant, HS: high significant ($p<0.01$), S: low significant ($p<0.05$); SEM: standard error of means, DM: dry matter; OM: organic matter; CP: crude protein; CA: crude ash; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; NFC: non fibrous carbohydrate; ADL: lignin; HC: hemicellulose and CC: cellulose.

to the two-stage technique introduced by Tilley and Terry (1963) and modified by Goering and Van Soest (1970). Rumen fluid was obtained from the rumen of three fistulated Taleshi steers and filtered by cheese clothes. 500 mg of sample was incubated in the solution containing 10 mL rumen liquor and 40 mL artificial saliva. The filled tubes were incubated for 48 hours. At the end of incubation time, 6 mL HCL (20%) and 2 mL pepsin solution (20%) were added into experimental tubes. After 48 h incubation, the solution was filtered by using Whatman No. 41 filter paper and then dry matter and ash content were determined in order to estimation of DMD, OMD and DOMD.

RESULTS AND DISCUSSION

Chemical composition: There are significant differences between untreated wheat straw (UWS) and ozonated wheat straw (OWS) from the view point of CP, EE, NDF, ADF, ADL, HC and CC as well as NFC content (Table 1). The results showed that the amount of NDF, ADF, ADL, HC, CC decreased and NFC content increased by ozonation pretreatment, particularly at the level of 60 minutes ozonation. Changes in the chemical composition of ozonated wheat straw in the present study corresponded to the results of most researchers. Ben Ghedalia and Shefet (1979) showed that pretreatment of ozone reduced the fiber content of cotton and wheat straws. Ben Ghedalia and Miron (1981) reported that the use of ozone in wheat straw reduced lignin and hemicelluloses and increased cell solubles, thereby improving the *in vitro* organic matter digestibility, but had little effect on cellulose content. García-Cubero *et al.* (2009, 2010) also reported that ozonolysis pretreatment decreased lignin and hemicelluloses content and increased the production of fermentable carbohydrate of cereal straws. Ben Ghedalia *et al.* (1980) and Silverstein *et al.* (2007) in the case of cotton straw, also achieved the same results. Ghorbani *et al.* (2022) similarly found that ozonolysis pretreatment (3 g/h for 45 min) led to decrease in NDF, ADF and ADL and increase in CP content of wheat straw. The researchers claimed that decreasing fiber content of lignocellulosic materials by ozonolysis pretreatment may be due to decomposition of lignin and breaking down of hemicelluloses-lignin and cellulose-lignin bonds; which in turn improves enzymes accessibility via increasing lignin and hemicelluloses solubility as well as cellulose liberation. Increasing CP content can be attributed to releasing nitrogen as a result of decreasing acid detergent insoluble nitrogen (ADIN) fraction of ozonated wheat straw (Bono *et al.* 1985, Barros *et al.* 2013, Ghorbani *et al.* 2022).

In vitro gas production parameters: Although the gas production volume (Table 2) at the initial incubation duration (2 to 8 h) was not affected by the ozonation process but was significantly increased at the longer incubation (12 to 96 h); with the highest amount at the 60 minutes ozonation time. The estimated gas production parameters (*A* and *c*) by exponential model as well as methane production, MP and SCFA are presented in Table 3. The significant differences were observed in *A* and *c* parameters of UWS and OWS

Table 2. Gas production volume (mL/200 mg) of experimental treatments at different incubation times (h)

Treatment	2	4	6	8	12	24	48	72	96
Untreated WS (Control)	1.9	2.7	3.3	4.1	5.2[b]	15.2[d]	32.8[c]	35.7[c]	37.8[c]
15 minute ozonated WS	1.9	2.8	3.3	4.5	5.3[ab]	17.2[c]	34.5[bc]	40.1[b]	42.6[b]
30 minute ozonated WS	2.2	2.8	3.6	4.5	5.9[ab]	17.5[c]	35.3[bc]	41.0[b]	43.4[b]
45 minute ozonated WS	2.5	3.0	3.6	5.2	6.3[ab]	19.4[b]	36.3[b]	41.6[b]	44.0[b]
60 minute ozonated WS	3.0	3.1	3.9	5.5	6.6[a]	20.8[a]	39.3[a]	46.5[a]	49.0[a]
SEM	0.46	0.42	0.42	0.47	0.40	0.25	0.86	0.97	1.03
Statistical significance	NS	NS	NS	NS	S	HS	HS	HS	HS

a-c: different letters in each column, indicated significantly different means ($p<0.05$), HS: high significant ($p<0.01$), S: low significant ($p<0.05$), NS: non-significant ($p>0.05$), SEM: standard error of means.

Table 3. Gas production parameters of ozonated wheat straw (WS)

Treatment	*A* (mL)	*c* (/hour)	methane (mL)	MP (g/kg)	SCFA (mmol)
Untreated WS (Control)	42.8 ^{c}	0.0217^{ab}	13.0 ^{c}	78.8 ^{e}	0.33 ^{d}
15 minute ozonated WS	48.0 ^{b}	0.0197^{c}	13.1 ^{c}	82.2 ^{d}	0.37 ^{c}
30 minute ozonated WS	48.5 ^{b}	0.0197^{c}	13.2 ^{c}	83.9 ^{c}	0.38 ^{c}
45 minute ozonated WS	49.2 ^{b}	0.0223^{a}	15.4 ^{b}	87.7 ^{b}	0.43 ^{b}
60 minute ozonated WS	54.4 ^{a}	0.0203^{bc}	19.2 ^{a}	89.6 ^{a}	0.46 ^{a}
SEM	1.37	0.0005	0.83	0.46	0.006
Statistical significance	HS	HS	HS	HS	HS

a-d: different letters in each column, indicated significantly different means (p<0.05), *A*: potential gas production; *c*: the gas production rate constant for the insoluble fraction (b), MP: metabolizable protein, SCFA: short chain fatty acids, HS: high significant (p< 0.01), SEM: standard error of means.

(p<0.01). The *A* parameter was enhanced by ozonation pretreatment with the highest value being observed at 60 minutes ozonation. The methane production, MP and SCFA values of OWS were also significantly higher than that of UWS with the highest amounts at the 60 minutes ozonation treatment.

The results of current study suggested that the gas production was not affected during the initial incubation by the ozonolysis pretreatment but increased as the incubation duration was prolonged; which is in line with Ghorbani *et al.* (2022). Ben'ko *et al.* (2020a,b,c) concluded that ozonation of wheat straw bring about separation of cellulose microfibrils from the lignin carbohydrate matrix and made the cellulose more accessible for enzymes and increasing sugars yield. Higher accessibility of cellulose, hemicelluloses and increasing soluble sugars led to rise in gas production. Rasid *et al.* (2021) also described that ozonolysis increases lignin degradation and total reducing sugar yield. Release of reducing sugars may enhance carbohydrates availability in rumen required to fast microbial colonization and also increase rapid microbial growth (Narimani *et al*. 2014).

The increased methane production by ozonation pretreatment in the current study is supported by Rasid *et al.* (2021). Given that methane comprises 20 and 30% of total gases produced in the rumen (Pishdadi-Motlagh *et al.* 2023), higher gas production may have resulted in higher methane production. In addition, higher production of methane, in the present study, may be related to higher accessibility of cellulose and increasing acetate production. Acetic type fermentation promote methane production in the rumen. On the other hand, increasing forage digestibility increases daily methane emissions because of increased intake (Mirzaei-Aghsaghali and Maheri-Sis 2011).

The MP and SCFA values of OWS in the present study were significantly higher than that of UWS with the highest amounts at the 60 minutes ozonation treatment. Microbial protein are synthesized in the rumen and consequently MP supply are dependent on the energy availability (Souza *et al.* 2021, Santos *et al.* 2021). As the ozonolysis pretreatment resulted in better energy and nitrogen availability (Ghorbani *et al.* 2022), MP increased in current study. In other hand, higher DOM content in feed stuffs can be expressed as higher energy availability for ruminal microorganisms, which can in turn increase MP (Santos *et al.* 2021). Effect of ozonolysis pretreatment on increasing SCFA production was in line with Hamano *et al.* (2017) and Ghorbani *et al.* (2022). Ghorbani *et al.* (2022) stated that the increase in cellulose and hemicelluloses accessibility and soluble sugars supply by ozonation increased the production of SCFA and thus more energy production.

Estimated nutritive value: The results of table 4,

Table 4. Estimated energy content (MJ/kg), nutrients digestibility (%), quality indices and dry matter intake of ozonated wheat straw (WS)

Treatment	Energy content			Nutrients digestibility			Quality indices and dry matter intake		
	DE	ME	NE_l	OMD	DMD	DOMD	RFV	RFQ	DMI
Untreated WS (Control)	5.3 ^{d}	4.4 ^{d}	2.1 ^{d}	40.8 ^{e}	33.1 ^{c}	37.0 ^{d}	46.9 ^{d}	21.5 ^{d}	31.2 ^{c}
15 minute ozonated WS	5.6 ^{c}	4.6 ^{c}	2.3 ^{c}	42.6 ^{d}	33.7 ^{b}	38.1 ^{c}	51.7 ^{c}	26.9 ^{c}	33.1 ^{bc}
30 minute ozonated WS	5.6 ^{c}	4.7 ^{c}	2.3 ^{c}	43.4 ^{c}	33.7 ^{b}	38.6 ^{c}	52.6 ^{c}	28.0 ^{c}	33.4 ^{bc}
45 minute ozonated WS	6.0 ^{b}	5.0 ^{b}	2.5 ^{b}	45.4 ^{b}	33.8 ^{b}	39.6 ^{b}	57.5 ^{b}	33.6 ^{b}	34.5 ^{ab}
60 minute ozonated WS	6.2 ^{a}	5.2 ^{a}	2.6 ^{a}	46.4 ^{a}	34.3 ^{a}	40.4 ^{a}	61.0 ^{a}	37.6 ^{a}	36.4 ^{a}
SEM	0.04	0.04	0.03	0.24	0.14	0.16	0.63	0.74	0.71
Statistical significance	HS	HS	HS	HS	HS	HS	HS	HS	HS

a-d: different letters in each column, indicated significantly different means (p<0.05), HS: high significant (p< 0.01), SEM: standard error of means, DE: digestible energy, ME: metabolizable energy, NE_l: net energy for lactation, DMD: dry matter digestibility, OMD: organic matter digestibility, DOMD: digestible organic matter in dry matter, RFQ: relative forage quality, RFV: relative feed value, DMI: dry matter intake (g/Kg, $W^{0.75}$ /day).

demonstrated that the energy content (DE, ME and NE_l) and nutrients digestibility (OMD, DMD and DOMD) of WS significantly increased by ozonation pretreatment (p<0.01). The highest amount of energy content and nutrients digestibility were observed in treatment of 60 minutes ozonation. In accordance to our findings, Ghorbani *et al.* (2022) had also reported that ozonolysis pretreatment (3g/h for 45 min) led to increase OMD, DMD, NE_l and ME content of wheat straw. They have discussed that ozonation process due to increasing accessibility of cell wall carbohydrates and soluble sugars production, conduced to improve energy content and nutrients digestibility. Also, Orduña Ortega *et al.* (2020) concluded that highest sugar conversion yields were achieved by ozonolysis treatment which is in turn can be led to higher energetic and digestibility of lignocellulosic materials. They have stated that duration of ozonolysis had a greater effect on the sugar conversion yield than the ozone concentration used. In line with our results, Ben Ghedalia and Miron (1981) found that ozonolysis improved the organic matter digestibility of wheat straw.

The estimated amounts of forage quality indices (RFV and RFQ) and DMI of UWS and OWS are shown in table 4. Ozonation pretreatment significantly improved DMI, RFV and RFQ; with the best improvement by 60 minutes ozonolysis. In our study, ozonation pretreatment significantly improved estimated amounts of DMI, RFV and RFQ which is in line with Ghorbani *et al.* (2022). Increasing OMD and reducing fiber content improves DMI by livestock and provides the use of ozonated wheat straw for utilization by high producing and fast-growing animals.

In an overall conclusion, this study indicated that *in vitro* gas production volume and estimated amounts of digestible energy, metabolisable energy, net energy for lactation, organic matter digestibility, dry matter digestibility, dry matter intake, RFV and RFQ are significantly (p<0.05) increased by ozonation pretreatment. Based on our results, it seems that ozonolysis pretreatment at the level of 60 minutes can be lead to increase *in vitro* fermentation kinetics (by 33%) and nutritive value (by 14%) of wheat straw for ruminants.

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