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Original Research Paper

Toxicological effects of diazinon as an organophosphate pesticide on fermentation activity of microorganisms and evaluation of sodium bentonite as a toxin binder by using the *in vitro* batch culture

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ABSTRACT

Rumen microflora may play an important role in biological degradation of organophosphates and other substances with anti-cholinesterase effect. In this experiment the effects of low-term exposure to the diazinon pesticide (0, 100, 250, 500, 750 and 1000 ppm) with sodium bentonite (0, 1 and 2 % of DM) on the ruminal fermentability of microorganisms was investigated using *in vitro* batch culture. The correlation coefficient between gas production parameters and other fermentation parameters was also investigated. Results indicated that by increasing diazinon to the culture, there were some toxicological effects in the microorganisms activities which lead to the decrease of potential digestibility of DM and other fermentation parameters such as 96 h cumulative gas production and NH3-N (p<0.05), but the rumen fluid pH was not affected by adding of diazinon. Although sodium bentonite numerically increase the dry matter disappearance rather than control group, however generally it seems rumen fluid couldn't conceivably be cleaned up the deleterious effects of diazinon at *in vitro* conditions by sodium bentonite as toxin binder.

Key words: Pesticide, Diazinon, *In vitro*, Fermentation parameters, Sodium bentonite.

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INTRODUCTION

Pesticides play a major role in pest management in agriculture. Chemically, organophosphate pesticides (such as diazinon) (OPs) are esters of phosphoric or thiophosphoric acids that can be toxic to ruminants because of their capacity to phosphorylate acetylcholinesterase, causing accumulation of acetylcholine in synapses (Wright et al., 2009; Anjum et al., 2010). Some OPs have been found to have endocrine disrupting properties (Kavlock, 2001). Due to their extensive use and moderate persistence, organophosphate pesticide (OP) residues could be found on commodities at the time of sale and natural waters (Ambrus, 2009; Hernandez-Borges et al., 2009). The impact of OP molecules on the environment result from several factors: the toxicity, the bio-accumulative and long-term effects, and the transport between different compartments (Lu et al., 2008; Palma et al., 2009). Feed and forage offered to animals are often contaminated with pesticide residues (Raikwar and Nag, 2003) and after feeding, these residues pass through the body systems (Prassad and Chhabra, 2001). Pesticides are toxic xenobiotics, which can adversely affect the biological systems in a number of ways. After entry in the animal body, residues are distributed to different organs, tissues and also translocated to milk in the case of Holstein dairy cows. Some residues may also be excreted via the urine and feces (Juliet et al., 1998). The continuous intake of pesticide residues in ruminants is a particularly serious problem in the case of the OPs, which after ingesting; OPs may be absorbed from the intestines into the systematic circulation and tend to concentrate in tissues such as adipose tissue, brain, liver, kidney and milk. Rather, talk about bio-concentration and bio-accumulation of pesticide residues though the food chain. In view of this, many countries have enacted regulations that limit the level of pesticides residues in milk and dairy products. Many studies have examined the pesticides residues in the bovine milk (Sharma et al., 2007; Darko and Acquaah, 2008; Nag and Raikwar, 2008). OPs are extensively included as safe for use on agricultural crops due to their relatively fast degradation rate, which depends on function of microbial components, pH, temperature, hydrolysis, photolysis and other parameters (Gilani et al., 2010). Researcher had shown that pesticides have series deleterious effects on the rumen fluid (Kazemi et al., 2012a).

Hasan (1999) suggest that rumen liquor played an active role in hydrolyzing OPs. Kazemi et al. (2012b) reported that phosalone as an organophosphate pesticide can numerically reduce potentially degradability of dry matter. Wallace and Newbold (1991) demonstrated that bovine rumen fluid hydrolyzed many OPs, particularly parathion.

OPs are metabolized by many different microorganisms, particularly members of the genera Pseudomonas (Hoskin and Walker, I994), Arthrobacter, Streptomyces, and Thiobacillus and by fungi in the genera Asperoillus (Hasan, 1999) and Trichoderma (Dmbrell, 1991; Smith, I993). In order to avoid potential human exposure to OP pesticide residues via food and drinking water, the effective techniques to remove and adsorption pesticide residues for health purposes is badly needed. Moreover, it has recently been determined that by-products of transformation in the environment can play a significant role in defining the impact of pesticides on both human health and the natural ecosystems (Mitsou et al., 2006).

One of these methods have been used for the adsorption of OP residues is application of bentonite as toxin binder in the ration. Sodium bentonite is an expanded lattice clay of the montmorillonite group (belong to minerals) (Rosa et al., 2001) with high ion exchange capacity that binds a wide range of cations specially pesticides and aflatoxins (Saleh and Bonf, 2000).

The objective of this research was to apply the diazinon as OPs with or without sodium bentonite as toxin binder for determination of its toxicological effects on the digestibility parameters of a TMR ration according to *in vitro* batch culture.

MATERIALS AND METHODS

Sample collection and experimental diet

Diazinon as used in this experiment was supplied by fluka company (America, 98.3% of purity). Experimental diet used for batch cultures was a mixture of alfalfa silage (50:50, 250 mg) and concentrate (250 mg) which was ground to pass through 1.5 mm screen. The chemical composition of experimental diet is shown in Table 1. The sodium bentonite was supplied by Ghaen Zarin Khak Company (with trade name of zarin binder). TMR ration was formulated according to NRC (2001) recommendations. The project was conducted in the laboratory of Ferdowsi University of Mashhad.

Batch culture and sampling method

Rumen fluid was obtained from four adult Holstein fistulated steers (420 ± 12kg, body weight), before the morning feeding. Animals were fed 50% of alfalfa silage and 50% of a commercial concentrate. Steers were housed in individual pens, and fed a TMR ration. Ruminal content was immediately strained through four layers of cheesecloth to eliminate large feed particles and transferred to the laboratory in a prewarmed thermos. In an anaerobic condition, 50 ml of buffered rumen fluid [ratio of buffer to rumen fluid was 2:1, buffer were prepared as proposed by Menke and Steingass (1988)] was dispensed with pipetor pump into a 120 ml serum bottle containing 0.5 g DM of the experimental diet. Treatments were the basal diet without diazinon pesticide (as control) and basal diet plus 100, 250, 500, 750 and 1000 µg of diazinon pesticide per ml of the medium and experiment repeated in two consecutive days (two runs, 5 replicates for each treatment) then sodium bentonite was added at three levels to the basal diet at all of treatments (0, 1 and 2% of DM). Then, each bottle was sealed with rubber stopper and aluminum cap and placed in a shaking water bath for 24 h at 38.6ºC. The diazinon was dissolved in ethanol (96%), and the control also dosed the same amount of ethanol. In each run, bottles containing only buffered rumen fluid were provided as blank. To prevent accumulation of gas produced, head space gas pressure of each bottle was recorded using a pressure transducer (Theodorou et al., 1994) at 3, 6, 12, 24, 36, 48, 72 and 96 h of the incubation and then gas released. After 24 h of the incubation, the bottles were respectively transferred to an ice bath to stop fermentation and then opened to measure medium pH using a pH meter (Metrhom pH meter, Model 691). Then, each bottle content was filtered (45 µm pore size) and a 5 ml sample of each filtrate bottle was taken and acidified with 5 ml of 0.2 N HCl and frozen at -20°C. The filtrated residual was oven dried (60°C for 48 h) and used to calculate *in vitro* dry matter.

Chemical analysis

Nitrogen concentration of basal diet and NH3-N concentration of the bottle content were determined using kjeldahl method (Kjeltec 2300 Auto analyzer, Foss Tecator AB, Hoganas, Sweden). Neutral detergent fiber (NDF) and acid detergent fiber (ADF were determined, according to Van Soest et al. (1991). Fat content was determined by ether extraction (AOAC, 1995).

Calculation and statistical analysis

Cumulative gas production data were fitted to the exponential equation $y = b (1 - e^{-ct})$ (Osuji et al., 1993),

Table 1. Ingredient and chemical composition of basal diet.

Composition Ingredient (% of DM)	Amount
Alfalfa silage	50
Barley grain, rolled	13.50
Corn grain, ground, dry	9.70
Soybean meal, solv, 44% CP	9.10
Wheat bran	4.25
Cotton seed meal solvent	6.50
Cottonseed whole with lint	6.95
Chemical composition (% of DM)	
CP	22.4
EE	3.50
NDF	34.9
ADF	23.7
NFC	34.8
ME(Mca/Kg DM)	2.54

Table 2. Effects of different levels of diazinon with different levels of sodium bentonite on dry matter digestibility of TMR ration after 24 h incubation.

Treatment	Levels of [1]D (ppm)	Levels of [2]SB (% of DM)	Digestibility of DM (%)
1	0	0	67.00[a]
2	0	1	66.72[a]
3	0	2	67.96[a]
4	100	0	67.42[a]
5	100	1	67.44[a]
6	100	2	68.96[a]
7	250	0	61.16[bc]
8	250	1	61.08[bc]
9	250	2	61.42[bc]
10	500	0	60.50[bc]
11	500	1	61.96[b]
12	500	2	61.20[bc]
13	750	0	58.16[bc]
14	750	1	59.84[bc]
15	750	2	60.76[bc]
16	1000	0	56.48[c]
17	1000	1	56.92[bc]
18	1000	2	58.14[bc]
		SEM	1.53
Factorial effect			
D			p<0.0001
SB			Ns
SB×D			Ns

a, b and c means in the same column with different superscript differ significantly ($P < 0.05$).
[1]D: Diazinon; [2]SB: Sodium Bentonite.

where b is the gas production from the readily soluble fraction and the insoluble fraction (ml); c is the gas production rate constant (ml/h); t is the incubation time (h) and y is the gas production at time of t (ml). A 6×3 factorial design was applied for data analysis with a completely randomized design for experimental basal using the Statistical Analysis System (SAS) program General Linear Model procedure (SAS, 9.1). Significant means were compared; using the Duncan's multiple range tests. Mean differences were considered significant at $P < 0.05$. Standard errors of means were calculated from the residual mean square in the analysis of variance.

RESULTS AND DISCUSSION

Effects of different levels of diazinon with different levels of bentonite on dry matter digestibility of TMR ration after 24 h incubation are presented in table 2. In comparison with control group, adding of diazinon to batch culture resulted in a significant decrease ($P<0.05$) in dry matter degradability. Many experiment were done by researchers about toxicological effects of different pesticide on *in vitro* dry matter digestibility, for example Kazemi et al. (2012a) reported that concentration of 5.6 mg diazinon inhibited significantly ($P<0.05$) *in vitro* potentially dry matter degradability. Greeballah et al (2005) reported that application the high levels of some organophosphate pesticides can decrease the dry matter digestibility, which was agreed with results of this experiment. There was no significance difference for dry matter digestibility by using the levels of sodium bentonite, but application of 2% sodium bentonite numerically leads to increasing of dry matter digestibility. Saleh and Bonf (2000) reported there was an improvement of increased dry matter digestibility after application of bentonite. Influence of diazinon with or without sodium bentonite on pH and NH3-N after 24 hours incubation was shown in table 3 and 4. Compared with the control, ammonia concentration was reduced ($P < 0.05$) at higher levels of diazinon. There was not a regular relationship (decrease or increase) between treatments for pH. Organophosphates can be metabolized by many different microorganisms (Hasan, 1999). With increasing of diazinon levels (especially at high levels, 750 and 1000) to the batch culture, NH3-N content significantly reduced ($p<0.0001$).

Different levels of sodium bentonite had not a significant effect on pH and NH3-N. Greeballah et al (2005) reported that all levels of malathion as an organophosphate pesticide, were not significantly ($p< 0.05$) effected on pH of rumen fluid, which was agreed with results of this experiment. There was no significant effect on rumen ammonia concentration in sheep fed with bentonite (Murray et al., 1990). In this experiment, it seems higher levels of diazinon had a toxicologically effect on ammonia producing bacteria. At an *in vivo* study, it has been shown that bentonite alone has the ability to maintain rumen pH when included in the diet at 4% of the DM (Ehrlich and davison, 1997). There was a trend for a more moderate increase in pH in 2% sodium bentonite rather than 0 and 1 % group alone, but generally no differences were observed.It appears that bentonites may act as buffers (Saleh and Bonf, 2000) to prevent very rapid reductions in rumen pH during starch fermentation, and can modify the pattern of rumen fermentation to reduce the molar proportion of propionate relative to acetate (Erwin et al., 1997).

Responses to bentonite have been variable however; Helal and Abdel-Rahman (2010) reported increasing in dry matter digestibility when diets supplemented with bentonite.

According to Wallace and Newbold (1991), bentonite interferes with the efficiency of protozoa ciliary's motion and thereby reduces the activity of ciliate protozoa. Because of the huge surface area of bentonite and the electrical charges on its surface, it slows the capture rate of microbes by protozoa allowing higher bacterial and fungal populations to remain within the ruminal fluid (Heijnen et al., 1991; Wallace and Newbold, 1991). By increasing the levels of diazinon, all parameters of gas production (such as cumulative gas production after 24, 48 and 96h incubation) were significantly less than control group.

Also there was a reduction for these parameters with increasing levels of sodium bentonite to batch culture. Certain OP pesticides were shown by Williams et al. (1963) to stimulate gas production *in vitro* by rumen holotrich protozoa, whereas these compounds had no appreciable effect when rumen bacteria served as the inoculums source.

Feed additives are a group of feed ingredients that can cause a desired animal response in a non-nutrient role such as pH shift, growth, or metabolic modifier (Hutjens, 1991). Particularly, sodium bentonite is used to manipulate ruminal fermentation. Fibrolytic enzymes can increase fiber digestibility (Sheppy, 2001). Whereas, bentonite participates by shifting VFA patterns, slowing passage rates, exchanging mineral ions and inactivating mycotoxins and other toxins (Kabak et al., 2006). Much of the variation in response to bentonite may be attributable to the very variable structural and chemical properties of the different types of bentonite clays mined throughout the word.

Kazemi et al. (2012b) reported that there was not any significant effect for potentially dry matter degradability according to *in situ* procedure. Correlation coefficient (r) between *in vitro* gas production parameters and some fermentation parameters are shown in Table 5. There was a significant positive correlation between *in vitro* gas production parameters, NH3-N and dry matter digestibility ($p<0.0001$), but the correlation between *in vitro* gas production parameters and pH was insignificant. The highest correlation was observed for cumulative gas production at 48 h after incubation and NH3-N.

Table 3. Influence of diazinon with or without sodium bentonite on pH and NH3-N after 24 h incubation.

Treatment	**Levels of [1]D (ppm)**	**Levels of [2]SB (% of DM)**	**pH**	**NH_3-N**
1	0	0	6.70^{ab}	18.88^{a}
2	0	1	6.73^{ab}	18.26^{abc}
3	0	2	6.75^{a}	17.80^{bcde}
4	100	0	6.73^{ab}	18.42^{ab}
5	100	1	6.71^{ab}	18.33^{abc}
6	100	2	6.70^{ab}	18.13^{abcd}
7	250	0	6.72^{ab}	17.67^{bcde}
8	250	1	6.74^{ab}	17.42^{cde}
9	250	2	6.70^{ab}	17.19^{def}
10	500	0	6.71^{ab}	17.53^{bcde}
11	500	1	6.70^{ab}	16.93^{ef}
12	500	2	6.69^{ab}	17.01^{ef}
13	750	0	6.73^{ab}	17.092^{ef}
14	750	1	6.69^{ab}	16.87^{ef}
15	750	2	6.68^{ab}	16.36^{gf}
16	1000	0	6.72^{ab}	15.78^{g}
17	1000	1	6.77^{a}	15.74^{g}
18	1000	2	6.66^{b}	15.93^{g}
		SEM	0.03	0.3
		Factorial effect		
D			Ns	P<0.0001
SB			Ns	<0.05
SB×D			Ns	Ns

a, b, c, d, e, f and g means in the same column with different superscript differ significantly (P < 0.05).
[1]D: Diazinon; [2]SB: Sodium Bentonite.

Table 4.Effect of diazinon with or without sodium bentonite on gas production parameters.

Treatment	**Levels of [1]D (ppm)**	**Levels of [2]SB (% of DM)**	**C_{gas} (ml/h/500 mg DM of TMR)**	**B_{gas} (ml/500mg DM of TMR)**	**Cumulative gas production (24 h after incubation to ml)**	**Cumulative gas production (48 h after incubation to ml)**
1	0	0	0.066^{bcd}	108.92^{a}	87.13^{a}	106.12^{a}
2	0	1	0.068^{abc}	105.29^{ab}	84.59^{a}	100.75^{a}
3	0	2	0.053^{gf}	97.21^{cd}	71.81^{b}	89.73^{b}
4	100	0	0.073^{a}	107.07^{a}	87.20^{a}	87.20^{b}
5	100	1	0.058^{ef}	100.14^{bc}	75.63^{b}	85.65^{b}
6	100	2	0.047^{h}	90.10^{def}	63.80^{c}	74.02^{c}
7	250	0	0.063^{cde}	92.07^{de}	72.97^{b}	72.97^{cd}
8	250	1	0.045^{h}	87.06^{ef}	59.38^{cd}	69.59^{cd}
9	250	2	0.044^{h}	84.21^{fg}	57.35^{cde}	66.62^{de}
10	500	0	0.069^{ab}	90.78^{def}	72.74^{b}	72.74^{cd}
11	500	1	0.046^{h}	76.54^{hi}	52.82^{ef}	60.28^{ef}
12	500	2	0.043^{hi}	76.97^{hi}	51.81^{ef}	60.35^{ef}
13	750	0	0.061^{de}	79.10^{gh}	60.37^{cd}	60.37^{ef}
14	750	1	0.043^{hi}	76.03^{hi}	50.17^{f}	59.00^{f}
15	750	2	0.034^{j}	71.11^{ij}	41.10^{g}	48.58^{g}
16	1000	0	0.048^{gh}	78.64^{gh}	54.17^{def}	54.17f^{g}
17	1000	1	0.038^{ij}	70.63^{ij}	43.00^{g}	49.42^{g}
18	1000	2	0.038^{ij}	66.48^{j}	41.52^{g}	47.26^{g}
		SEM	0.002	2.33	2.14	2.29
			Factorial Effect			
D			p<0.0001	p<0.0001	p<0.0001	p<0.0001
SB			p<0.0001	p<0.0001	p<0.0001	p<0.0001
SB×D			p<0.0001	Ns	<0.01	Ns

a, b, c, d, e, f and g means in the same column with different superscript differ significantly (P < 0.05). [1]D: Diazinon; [2]SB: Sodium Bentonite

Table 5. Correlation coefficient (r) between in vitro gas production parameters and some fermentation parameters.

Gas production and theirs parameters	Fermentable parameters.		
	Degradability of DM	**NH3-N**	**pH**
12	0.53^{****}	0.69^{****}	0.13^{ns}
24	0.55^{****}	0.72^{****}	0.10^{ns}
48	0.62^{****}	0.74^{****}	0.10^{ns}
[1]$\mathbf{b}_{gas}$	0.58^{****}	0.73^{****}	0.09^{ns}
[2]$\mathbf{c}_{gas}$	0.38^{***}	0.59^{****}	0.13^{ns}

[1]Cumulutive gas production for 96 hours after incubation.
[2]Constant rate of gas production.
$^{***}p<0.001$ and **** $p<0.0001$

CONCLUSION

Microbially mediated decomposition is the major, and sometimes the only, mechanism of partial removal or modification of organophosphates in rumen. Rumen is the major place for prevent of toxicological effects of pesticides. Hence, it is necessary to i) determination of toxicological effects of all of pesticides (for example diazinon) for rumen microorganisms by using the different levels of it ii) experiment of some material as toxin binder for decreasing of toxicological effects of theses pesticides. The results of this experiment indicated that inclusion of diazinon at high levels can be toxic for microorganisms. Also some *in vitro* parameters reduced. It appears that the microorganisms can bear the deleterious effect of diazinon up to 100 pp. also sodium bentonite couldn't reduce the effects of diazinon poisoning. The different patterns of fermentation observed *in vitro* indicate the very variable nature of different types of bentonite clays available in the entire world.

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