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Synthesis of chromium nanoparticles and evaluation of its impact on ruminal feed fermentation using *in vitro* batch culture technique

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Abstract

The use of mineral nanoparticles as replacer for traditional formulas of mineral dietary supplements for ruminants is a new approach. The main aim of this study was to evaluate effects of dietary Cr nanoparticles (Cr_2O_3-NPs) supplementation at different levels (0.3, 0.6, 0.9, 1.2, 2.1 and 3 mg//Kg DM) on feed fermentation and degradation in the rumen using the *in vitro* batch culture technique. Diet supplementation with Cr_2O_3-NPs at 0.3 mg/kg DM showed the highest ($P<0.05$) values of total gas production (TGP), lactic acid, acetic acid, propionic acid, butyric acid and ammonia-nitrogen (NH_3-N) concentrations, dry matter (DM), organic matter (OM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) degradation. Beyond 0.3 mg/kg of Cr_2O_3-NPs supplementation level, there is an inverse relationship between the dietary level of Cr_2O_3-NPs supplementation and the percentages of DM, OM, NDF and ADF degradation, TGP volume and NH3-N concentration. By comparison with the control, no negative effect of Cr_2O_3-NPs supplementation on all ruminal fermentation parameters up to 1.2 mg/kg DM. Supplementation of Cr_2O_3-NPs up to 3mg/kg DM has no negative impact on concentrations of lactic acid, propionic acid and butyric acid, but significantly decreased the TGP volume, acetic acid NH_3-N concentrations and A/P ratio when compared to the control. It can be concluded that dietary Cr supplementation in nanometric form had a positive effect on ruminal fermentation traits and improving nutrients degradation rates *in vitro*. The optimum level of nano-Cr addition to the ruminants' diet was at 0.3mg/kg DM under the current trial conditions.

Keyword: chromium nanoparticles, dietary mineral, ruminants, feed fermentation and feed degradation.

1. Introduction

Livestock breeders always strive to get the maximum output from their herds. To this end, many feed additives have been used to improve animal's digestion, metabolism, health and productivity[1-4]. Trace mineral as feed additives are of great importance in the diet of ruminant animals because of their essential role in the metabolism pathways[5]. Dietary trace mineral (e.g. chromium) deficiency may cause a defect in the animal health and productivity[6].

Chromium (Cr) is a trace mineral found in livestock feeds in very low concentrations in the form of inorganic compounds or organic complexes[7-8]. The Cr is essential for lipids, carbohydrates, and proteins metabolism and its deficiency may lead to high blood insulin concentration, low glucose tolerance and shortening of productive age in ruminants[6, 9]. Therefore supplementation of ruminant's diets with Cr is essential for maintaining high animal performance and preventing Cr losses during stressful condition[8]. Akers[10] reported that Cr supplementation positively altered production and reproduction rates of ruminants, especially in case of stressed dairy cows.

Many studies have been conducted to determine the optimal formula for adding Cr to ruminant's diets, rate of Cr absorption in the rumen and gastrointestinal tract and how to increase the Cr bioavailability to maximize animal metabolism[11-14]. In living organisms, Cr is predominantly appear in two valence states; hexavalent $Cr6^+$ and trivalent $Cr3^+$. However, $Cr6^+$ is easily reduced to a trivalent $Cr3^+$ in living organisms[15]. Hexavalent Cr sources have numerous industrial applications, while trivalent Cr compounds are used as micronutrients and dietary

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supplements. The Cr dietary supplements including inorganic salts like Cr-chloride or Cr oxide[8], while the organic Cr is mainly appear in Cr-methionine, Cr-nicotinic acid complex, Cr-yeast and Cr-picolinate formula[16].

However, it has been reported that the absorption and bioavailability of organic sources are more than inorganic[17]. According to Forbes and Erdman[18], the bioavailability of inorganic Cr is between 1 and 3% while it ranged between 15 to 30% for organic Cr sources. The low bioavailability of inorganic Cr is may be related to formation of non-soluble Cr oxides as well as slow conversion of inorganic Cr to the biologically active form[19]. Also deficiency of niacin beside interference of Cr with the other minerals may cause its low bioavailability[8].

Nanotechnology as a promising tool for increasing the absorption and bioavailability of trace minerals for animal feeding purpose has attracted a lot of attention[20]. It is noticed that the materials at nano-scale show substantially different electronic, mechanical, thermal, electrical, magnetic and optical properties than in bulk state[21]. Moreover, the nano elements have intriguing properties including; higher absorption and bioavailability, high area surface and surface activity, specific and rapid movement with high catalytic efficacy[22-23]. All these advantages encouraged animal nutritionists to use nanoparticles as replacer for traditional formulas of mineral supplements[24].

Despite the great importance of the Cr in completing the metabolic processes in ruminants, the mechanism of Cr absorption in the rumen has not been yet well understood[8]. Therefore, the main objective of this study was to evaluate the effects of adding Cr to ruminant diets in the traditional (organic) and nanometric forms on the feed digestion parameters as well as the efficiency of the fermentation processes in the rumen (ex; production of total gases and short-chain fatty acids) and determination of the optimal level of adding nano-Cr to the ruminant diets.

2. Experimental

Ammonium hydroxide (liquor ammonia) and chromium sulphate, $Cr_2(SO_4)_3 .12H_2O$ were obtained from Merck, India.

2.1. Preparation of Chromium Oxide Nanoparticles (Cr_2O_3-NP)

The preparation of chromium oxide nanoparticles, aqueous solution of ammonia was added drop by drop continuously to a 500 ml solution of (0.1 M) $Cr_2(SO_4)_3$ with stirring until the pH of the mixture reached 10. The resulting precipitates were filtered using a Buckner funnel and repeatedly washed with distilled water. The precipitates were calcined at 700°C in a muffle furnace for 4 hours after being dried in an oven at 60°C for 12 hours. Chromium oxide nanoparticles was achieved, ground, and sieved using a sieve with 100 mesh size.

2.2. Characterizations of the prepared chromium oxide nanoparticles

Numerous analytical tools were used to assess the fabricated Cr_2O_3-NP. The structure of the created Cr_2O_3-NP was investigated via an X-ray diffraction pattern and a Philips diffractometer (PW 1820 goniometer, PW 1930 generator) standing using source of Cu K radiation (45 kV, 40 mA, with = 0.15418 nm) through the 2 theta range from 20 to 80^o concluded the size of a stage of 0.02 and period of the step of 1s. Utilizing FT-IR spectroscopy (Agilent system Cary 630 FT-IR model), the functional groups of the prepared Cr_2O_3-NP were studied. The potassium bromide powder approach was used in the range of 400 and 5,000 cm^{-1} to evaluate different types of functional groups. With the assistance of a transmission electron microscope (TEM) operating at 80 KV (JEM-1200 EXII, JEOL, Japan), the morphology of the prepared Cr_2O_3-NP was investigated.

2.3. Cytotoxic effect of Cr2O3-NP

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan[25]. All the following procedures were done in a sterile area using a Laminar flow biosafety cabinet Class II A2 (Manufactured by: Labconco). Cells were suspended in DMEM medium, 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B) and 1% L-glutamine and 5% fetal bovine serum at 37 ºC under 5% CO2 using CO2 incubator (Sartorius stedium ,biotech). Cells were batch cultured for 10 days, then seeded at concentration of 10x103 cells/well in fresh complete growth medium in 96-well plastic plates at 37 ºC for 24 h under 5% CO2 either alone (negative control) or with different concentrations of drugs to give a final concentration of (250, 125, 62.5, 31.25,15.625, 7.8125, 3.906, 1.953 ug/ml). After 48 h of incubation, medium was aspirated, 20 ul MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37ºC under 5% CO2. To stop the reaction and dissolving the formed crystals, 200µL of 10% Sodium dodecyl sulphate (SDS) in 0.01M HCL was added to each well and incubated overnight at 37ºC. A positive control which composed of 100µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions[26]. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, Califomia, USA) at 595nm and a reference wavelength of

620nm. Viability = absorbance of drug / absorbance of control x 100. Cytotoxicity = 100- viability.

2.4. Ruminant diet degradation and rumen fermentation characteristics (*In vitro* study)

Batch culture technique was used to study impact of increasing level of nano Cr supplementation on ruminant diet degradation and fermentation characteristics. In this experiment, 400 mg of the tested diet (30% corn grain, 30% Berseem hay, 15% wheat bran, 15% cottonseed meal, and 10% soybean meal) were added to each incubation bottle (120ml). The chemical composition of the tested diet was illustrated in Table (1). Three bottles were allocated for each treatment and the treatments were as follows: 1. control: the tested diet without any addition of chromium, 2. positive control: the tested diet supplemented with 0.3 mg/kg DM of chromium picolinate (CrPic). While the tested diet was separately supplemented with 0.3, 0.6, 0.9, 1.2, 2.1 and 3 mg//Kg DM of chromium oxide nanoparticles (Cr_2O_3-NPs) in the rest of the treatments respectively. The warm rumen fluid (microorganism's inoculum) was collected from the rumen of slaughtered rams in free oxygen plastic jar. Each bottle was filled with 40 ml of mixture of 1:3 (v/v) rumen fluids: buffer solution in anaerobic conditions as described by Ismail et al[27]. All bottles were sealed and incubated at 39 °C in a shaking incubator (90 rpm). After 24 h of incubation, all bottles were filtered in fiber filter bags 25 micron porosity (ANKOM- USA). The residues in the bags were dried at 60° C in oven for 48 h to analyze dry matter (DM), organic matter (OM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility. The chemical composition of the tested diet and its residual was determined according to methods of A.O.A.C. [28]. While the NDF and ADF were analysed according to the method of Van Soest[29]. Rumen fluid pH was measured using pH-meter, the produced gases volume was determined by Hohenheim Syringes (100 ml) as described by Navarro-Villa et al[30]. The ammonia concentration was analysed spectrophotometry as described in kits pamphlet of Biodiagnostic company- Egypt. Lactic, acetic, propionic and butyric acids concentrations were analysed by HPLC Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm) at 30 °C.

Table (1): Chemical composition of the control diet

Diet ingredients	g/kg DM							
	DM	OM	NDF	ADF	CP	EE	Ash	NSC
CFM	90.39	95.14	21.2	12.9	16.07	4.52	4.86	53.35
Egyptian Clover (hay)	90.41	95.07	43.3	28.6	14.43	4.35	4.93	32.99
Basal diet	90.40	95.12	27.83	17.61	15.58	4.47	4.88	47.24

CFM: concentrate feed mixture, DM: Dry matter, OM: Organic matter, NDF: Neutral detergent fiber, ADF: acid detergent fiber, CP: Crude protein, EE: Ether extract and NSC: Non structural carbohydrates.

2.5. Statistical analysis:

Data were statistically analyzed by IBM SPSS Statistics for Windows[31], using the following equation: $Yij = \mu+Ti+eij$

Where, Yij is the parameter under analysis ij, µ is the overall mean, Ti is the effect due to treatment on the parameter under analysis, eij is the experimental error for ij on the observation, the Duncan's multiple range tests[32] was used to test the significance among means using probability level less than 0.05 ($p<0.05$) for significance expression.

3. Result and discussion

3.1. Characterizations of the prepared chromium oxide nanoparticles

The obtained results from the XRD analysis that was applied to determine the crystallinity of the synthesized Cr_2O_3-NP are displayed in (Fig. 1a). As revealed in Figure 1 a, the XRD spectrum of the fashioned Cr_2O_3 nanoparticles displayed nine distinct Bragg's diffraction peaks, at 2 theta = 22º, 32º, 40º, 46º, 50º, 58º, 68º, 73º, and 77º indexing to the crystal planes of (012), (104), (110), (113), (024), (116), (214), (220), and (306), respectively. According to Hao et al[33], the JCPDS 38-1479 diffraction peak matches the Cr_2O_3 nanoparticles very well. The absence of associated contaminants proves the purity of the manufactured Cr_2O_3-NP. The peak's intensity also demonstrated the high degree of crystallinity of the Cr_2O_3 nanoparticles. Furthermore, Figure 1b shows the FT-IR spectra of Cr_2O_3-NP. According to the data, synthesized Cr_2O_3-NP displayed FT-IR peaks for O-H (3388 cm^{-1}), C-H (2882 cm^{-1}), C=O (1725 cm^{-1}), N-H (1654 cm^{-1}), C=C (1525 cm^{-1}), as well as C-O-C (985 cm^{-1}). Moreover, the metal-oxygen bond fabrication was further established by the FT-IR signal at 510 cm^{-1} related to Cr-O[34]. Morphologically well-defined chromium oxide nanoparticles with particle sizes of 19 to 60 nm could be manufactured by hydrothermal method. In addition, as revealed in Figure 1 **c**, the surface morphology of the prepared Cr_2O_3-NP was investigated using TEM. The fashioned Cr_2O_3-NP also possessed spherical forms, as seen by the XRD

pattern in (Fig. 1 a) and the TEM images. In actuality, observed particle sizes from the TEM approach are much more relevant in relation to XRD patterns. TEM micrographs are used to detect particle size that may be polycrystalline, whereas the XRD method is normally used to determine crystal size and shape. According to TEM image, the average mean size of synthesized Cr_2O_3 nanoparticles was around 50 nm.

3.2. Cytotoxic effect of Cr_2O_3-NP on human cell lines

The cytotoxicity of different level of Cr_2O_3-NP (1.953- 250 µg/ml) treatment on human skin fibroblast (HSF) cells was shown (Fig. 2). The results showed that the Cr_2O_3-NP treatment at the lowest level 1.953 (µg/ml) gave the highest percentage of cell viability (100%) and zero % toxicity. The human skin treatments with graded increased level of Cr2O3-NP up to 31.25 (µg/ml) showed zero% toxicity with 100% of cell viability. When level of Cr_2O_3-NP treatment reached the maximum (250 µg/ml), the cell toxicity reached 32.2% with 67.8% of cell viability. Serini et al.[35] stated that the prepared nano-materials may be displayed a significant toxicity when cell viability value was less than 50% compared to the control cells. Accordingly, it is obvious that Cr2O3-NP treatment at such high concentrations is extremely safe for human and animals body.

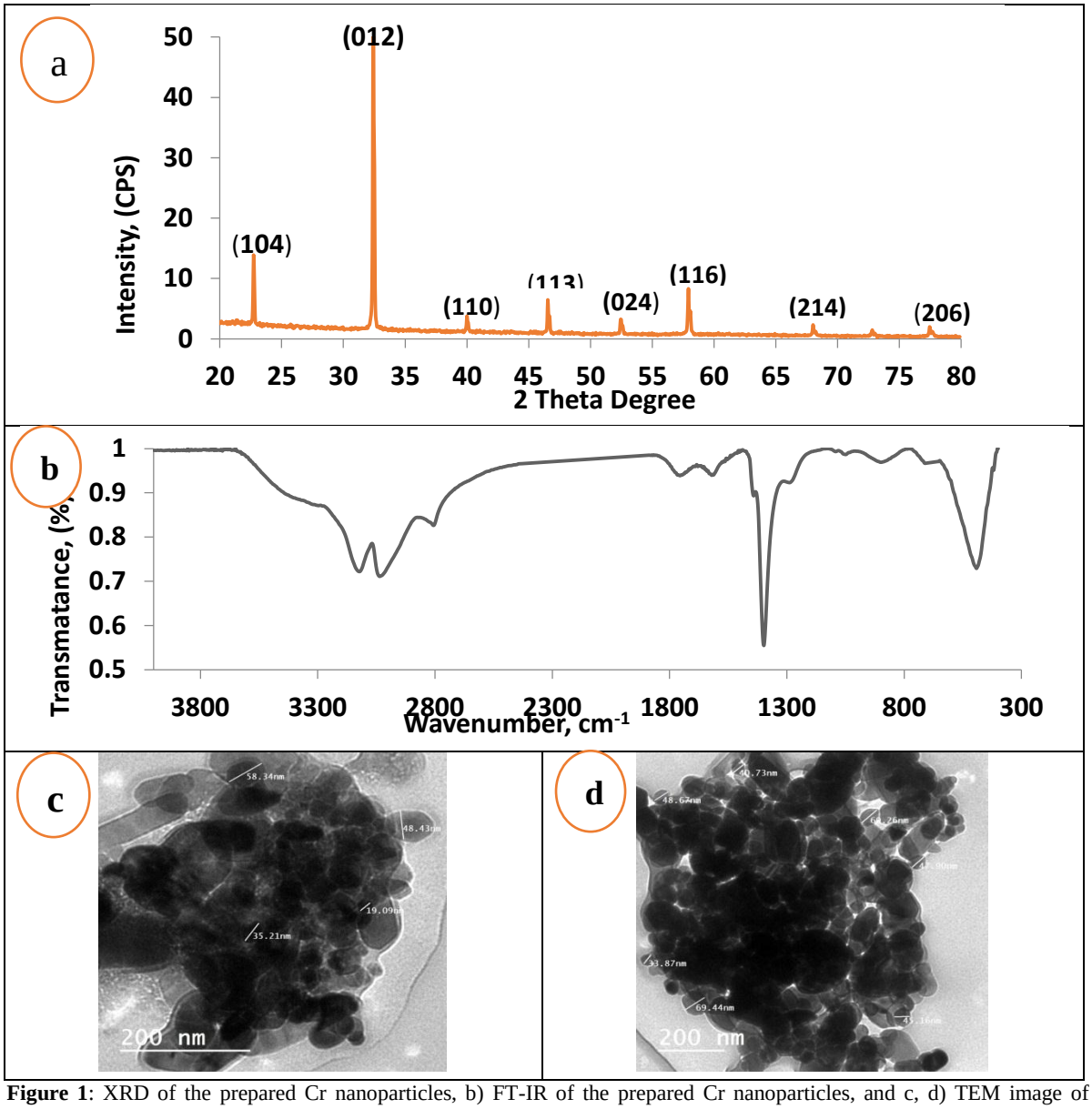

Figure 1: XRD of the prepared Cr nanoparticles, b) FT-IR of the prepared Cr nanoparticles, and c, d) TEM image of the prepared Cr nanoparticles.

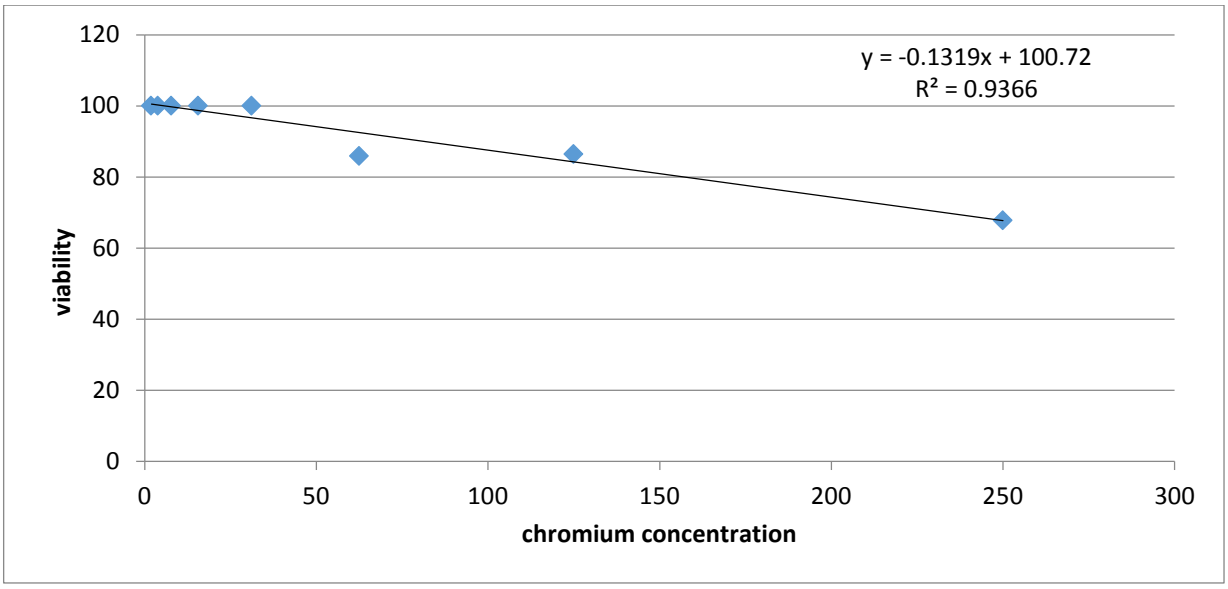

Fig.2. The effect of Cr_2O_3-NPs concentrations on the human skin fibroblast (HSF) cells viability (%)

3.3. The in vitro study

3.3.1. Effect of chromium picolinate (CrPic) or Cr_2O_3-NPs supplementation on ruminal diet degradation

Data of Table (2) illustrated impact of dietary chromium (Cr) supplementation with nanoparticles (Cr_2O_3-NPs) or organic (CrPic) sources on diet degradability by rumen microorganisms (in vitro). Diet supplementation with Cr_2O_3-NPs at 0.3 mg/kg DM gave the highest (P<0.05) values of dry matter (DM), organic matter (OM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) degradability. At the same level of supplementation (0.3 mg/kg DM), it's obvious that Cr_2O_3-NPs is superior over CrPic for improvement of all ruminal diet's degradability parameters. This significant improvement in digestibility is may be due to higher fermentation rate of the feed ingredients in the rumen as a direct result of the positive effect of chromium on the growth, performance and population of rumen microbes[36-37]. Anchal et al[38], reported that supplementation of wheat straw based diet with chromium yeast, chromium picolinate or chromium polynicotinate at 1.0 ppm/kg had shown better in vitro organic matter degradability, and microbial biomass production. Also, higher DM and OM digestibility was observed in Jawarandu goats fed diet supplemented with 1 mg/kg Chromium-yeast compared to goats of control[37]. In addition, Yadong and Yuxiang[39] found that Tan lambs fed diets supplemented with chromium-methionine up to 1.5 mg/head/day showed significant higher dietary DM digestibility than lambs of the control diet.

Table (2): Effect of chromium picolinate or Cr_2O_3-NPs supplementation on ruminal diet degradability parameters

Chromium supplementation level	DMD%	OMD%	NDFD%	ADFD%
Control	51.52^{c}	56.83^{c}	42.21^{b}	32.89^{b}
CrPic (0.3mg Cr/kg)	54.70^{b}	60.01^{b}	43.28^{b}	33.96^{b}
Cr_2O_3-NPs (0.3mg Cr/kg)	59.27^{a}	64.58^{a}	47.09^{a}	37.77^{a}
Cr_2O_3-NPs (0.6mg Cr/kg)	55.91^{b}	61.22^{b}	42.01^{b}	32.69^{b}
Cr_2O_3-NPs (0.9mg Cr/kg)	54.74^{b}	60.05^{b}	39.98^{c}	30.66^{c}
Cr_2O_3-NPs (1.2mg Cr/kg)	50.84^{c}	56.15^{c}	39.55^{c}	30.23^{c}
Cr_2O_3-NPs (2.1mg Cr/kg)	49.28^{d}	54.59^{d}	37.30^{d}	27.98^{d}
Cr_2O_3-NPs (3mg Cr/kg)	46.67^{e}	51.98^{e}	35.58^{e}	26.26^{e}
SE±	0.80	0.80	0.72	0.72

DMD: Dry Matter Degradability, OMD: Organic Matter Degradability, NDFD: Neutral Detergent Fiber Degradability and ADFD: Acid Detergent Fiber Degradability

a, b, c, d and e means at the same column with different superscript are significantly (P<0.05) different.

The superiority of Cr_2O_3-NPs over CrPic in improving degradability of DM, OM, NDF and ADF of the tested diets may be attributed to unique properties of the elements in the nano scale form, which allow for more bioavailability, surface activity and catalytic efficacy[22-23]. Moreover, the different metabolic styles of CrPic and Cr_2O_3-NPs may explain the superiority of nanometric chromium over organic chromium in improvement of dietary NDF & ADF degradability in the current study. Also, ruminal microbes may play a negative role in reduced some of the organic chromium to insoluble forms, and thus decreases its availability and efficacy. However, more research is needed to confirm this hypothesis and to identify other factors that could contribute to this phenomenon.

Beyond 0.3 mg/kg of Cr_2O_3-NPs supplementation level, there is an inverse relationship between the dietary level of Cr_2O_3-NPs supplementation and the percentages of DM, OM, NDF & ADF digestibility, which suggests that 0.3 mg/kg of Cr_2O_3-NP is the optimal level of the Cr supplementation. The addition of Cr_2O_3-NPs at 0.6 mg/kg DM or CrPic at 0.3 mg/kg DM to the tested diets significantly improved the DM and OM degradability with no change in the fiber degradability parameters (NDF and ADF) compared to the control. Chromium nanoparticles supplementation at 0.9mg/kg DM significantly increased the ruminal diet degradability of DM and OM than of the control diet. While fiber degradability parameters (NDF and ADF) were negatively affected by the addition of Cr_2O_3-NPs at the same level (0.9 mg/kg DM) compared to the control. The diet supplementation with Cr_2O_3-NPs above 1.2 mg/kg DM significantly decreased the DM, OM, NDF and ADF degradability compared to the control, which suggested occur suppression of the growth and activity of protozoa and some species of ruminal bacteria.

The gradual decrease of the DM, OM, NDF and ADF degradability with increase Cr_2O_3-NPs supplementation level is may be due to possible toxicity of the high levels of Cr_2O_3-NPs supplementation on ruminal microbial growth, population diversity, and bioactivity which in turn reduces the microbial protein synthesis and carbohydrate digestion[40]. In this context, the toxic potential of CrPic on the ruminal protozoa population has been stated by Dallago et al[41]. The Cr as a heavy metal can damage the DNA of the ruminal microbes especially protozoa, leading to metabolic and reproductive malfunctions and ultimately microbial cell death[42]. It's well known that ruminal protozoa have a fundamental role in cell wall fiber digestion of the feed particles[43]; this may explain that the highest degradation rate of the dietary NDF and ADF was obtained at the lowest level of Cr_2O_3-NPs supplementation in the present study.

3.3.2. Effect of CrPic or Cr_2O3-NPs supplementation on traits of ruminal fermentation

Effects of nanoparticles or organic chromium sources supplementation on ruminal fermentation traits are illustrated in Table (3). Diet supplementation with Cr_2O_3-NPs at 0.3 mg/kg DM gave the highest ($P<0.05$) values for total gas production (TGP), lactic acid, acetic acid, propionic acid, butyric acid and ammonia-nitrogen (NH_3-N) concentrations. While the diet supplementation with CrPic at the same level (0.3mg Cr/kg) had no significant effect on pH, TGP, acetic acid, propionic acid, butyric acid and NH_3-N concentrations compared to the control. These results are consistent with those obtained by Rikhari et al[44] who reported that fistulated male cattle supplemented with chromium picolinate (CrPic) at 0.5 and 1 mg/kg showed no difference in ruminal VFA and NH_3-N concentrations. Also Besong et al[45] observed that Holstein steers diet supplementation with 0.8 mg/kg of CrPic had no effect on molar proportions of ruminal VFA. The toxic effect of the chromium picolinate on protozoa population in the rumen may be the reason for this case[41]. The positive effect of Cr_2O_3-NPs supplementation on the production of VFA and NH_3N in the current study is mostly due to the significant improvement of DM, OM, NDF and ADF digestibility (Table 2) as well as higher synthesis of the microbial protein[46]. It has been thought that Cr_2O_3-NPs supplementation at the level of 0.3 mg / kg DM was not harmful to the growth or activity of the bacterial and protozoa populations in the rumen and maintained a balance between them, which allowed for efficient carbohydrates fermentation.

Ruminant diets supplementation with trace minerals has a fundamental role in improvement of feed digestion process for more production of the volatile fatty acids (main source of energy) and microbial protein synthesis in the rumen[5]. In the present study, it's obvious that TGP volume, propionic acid and butyric acid concentrations were increased numerically by dietary supplementation with CrPic at 0.3mg Cr/kg or Cr_2O_3-NPs up to 0.6 mg/ Kg DM compared to the control. While, acetic acid concentration was increased ($P<0.05$) by dietary supplementation with CrPic at 0.3mg Cr/kg and Cr_2O_3-NPs up to 0.6 mg/ Kg DM compared to the control. These responses are logical due to higher ruminal fermentation rate, higher dietary protein degradation[47].

Table (3): Effect of CrPic **or Cr_2O3-NPs supplementation on ruminal fermentation parameters**

Chromium supplementation level	TGP (ml)	pH	Lactic acid (mg/ml)	Acetic acid (mg/ml)	Propionic acid (mg/ml)	A/P	Butyric acid (mg/ml)	NH3-N (mg/dl)
Control	133.33^{bc}	6.14^{c}	0.88^{b}	0.82^{c}	0.28^{bc}	2.95^{a}	0.21^{bc}	12.81^{bc}
CrPic (0.3mg Cr/kg)	138.33^{ab}	6.12^{c}	1.87^{a}	1.04^{bc}	0.40^{b}	2.64^{c}	0.29^{b}	13.25^{bc}
Cr_2O_3-NPs (0.3mg Cr/kg)	141.67^{a}	6.13^{c}	2.09^{a}	1.40^{a}	0.48^{a}	2.92^{a}	0.36^{a}	13.43^{a}
Cr_2O_3-NPs (0.6mg Cr/kg)	136.67^{b}	6.12^{c}	1.14^{b}	1.17^{b}	0.40^{b}	2.95^{a}	0.30^{b}	13.27^{b}
Cr_2O_3-NPs (0.9mg Cr/kg)	132.67^{c}	6.12^{c}	1.02^{b}	0.97^{bc}	0.33^{b}	2.95^{a}	0.27^{b}	13.22^{bc}
Cr_2O_3-NPs (1.2mg Cr/kg)	131.67^{d}	6.19^{b}	0.58^{b}	0.57^{cd}	0.19^{c}	2.95^{a}	0.15^{c}	12.76^{c}
Cr_2O_3-NPs (2.1mg Cr/kg)	127.67^{e}	6.22^{a}	0.55^{b}	0.45^{d}	0.15^{c}	2.9^{ab}	0.12^{c}	12.48^{c}
Cr_2O_3-NPs (3mg Cr/kg)	121.00^{f}	6.23^{a}	0.51^{b}	0.41^{d}	0.15^{c}	2.72^{bc}	0.11^{c}	11.87^{d}
SE±	1.31	0.01	0.13	0.07	0.03	0.03	0.02	0.11

TGP: Total gas production (ml/24hr), A/P: Acetic acid / Propionic acid ratio
a, b, c, d, e and f means at the same column with different superscript are significantly ($P<0.05$) different.
±SE: standard error

The diet supplementation with CrPic significantly decreased the acetic / propionic (A/P) ratio compared to control. While Cr_2O_3-NPs supplementation up to 2.1 mg/ Kg DM had no significant effect on the A/P ratio compared to the control. This may be due to the detrimental effect of CrPic on ruminal protozoa, the reduction of the ruminal protozoa in the number and activity negatively affects the digestion of fiber and consequently the production of acetic acid[41]. On the other hand, Cr_2O_3-NPs supplementation positively affects the organic matter and fiber digestion which may explains this balance between the production of acetic acid and propionic acid.

By comparison with the control, no negative effect of Cr_2O_3-NPs supplementation on all ruminal fermentation parameters up to 1.2 mg/kg DM. Also, lactic acid, propionic acid, butyric acid, NH_3-N concentrations and A/P ratio were not affected by Cr_2O_3-NPs supplementation up to level of 2.1 mg/kg DM when compared to the control. Supplementation of Cr_2O_3-NPs up to 3mg/kg DM has no negative impact on concentrations of lactic acid, propionic acid and butyric acid, but significantly decreased the TGP volume, acetic acid NH_3-N concentrations and A/P ratio when compared to the control. The negative impact of Cr_2O_3-NPs supplementation on the most of the rumen parameters was detected at the higher levels of supplementation; this may be due to the toxic impact of Cr_2O_3-NPs on some species of ruminal microbes[41]. The higher production of lactic acid after the dietary supplementation with either organic or nano chromium may be due to higher ruminal fermentation rate of the starch content of the concentrate feed mixture (CFM). Kitchalong et al[48] stated that any increase in starch digestion in the rumen should be followed by an increase in lactic acid production.

There is no significant change in the pH values due diet supplementation with CrPic at 0.3mg Cr/kg DM or Cr_2O_3-NPs up to 0.9 mg/ Kg DM compared to the control, but the pH values were increased significantly above 0.9 mg/ Kg DM of Cr_2O_3-NPs supplementation level. Lack of change of the ruminal pH after the diet supplementation with CrPic (0.3 mg/kg DM) or Cr_2O_3-NPs up to 0.9 mg/kg DM is in line with findings of Dallago et al[41] who reported that lamb's ruminal pH was not affected by CrPic treatments at 0.250, 0.375 and 0.500 mg/day. A balance occurrence between organic acids and alkaline ammonia production in the rumen may be the reason for stability of the pH values. In this regard, Swartz et al[49] stated that higher CP content of the diet may have acted as a systemic buffer through catabolism of the feeds' amino acids into ammonia to counteract the high acid loads in the rumen. The observed gradual increase in the ruminal pH with increasing level of nano-chromium supplementation above 0.9mg/kg DM is may be due to reduction of the organic acids production as a result to the toxic effect of Cr on some microbial populations in the rumen[38]. It is worth mentioning that the ruminal pH values of the all treatments were within the normal range, indicating that dietary supplementation with Cr in organic or nano forms did not disrupt rumen homeostasis.

4. Conclusions

Dietary chromium supplementation in organic or nanometric forms had a positive effect on ruminal

fermentation traits and improving digestion rates *in vitro*. The optimum level of nano-Cr supplementation was at 0.3mg/kg DM under the current trial conditions. The Cr_2O_3-NPs showed superiority over chromium picolinate (CrPic) in improvement of diet degradability and ruminal fermentation traits at the same level of supplementation. The ruminal pH values at all Cr_2O_3-NPs supplementation levels were within the normal range of the healthy rumen, indicating that dietary supplementation with Cr in nano form did not disrupt rumen homeostasis. More *in vivo* studies are needed to define the optimal level of Cr nanoparticles supplementation, toxicity, and their effect on digestion, metabolism, meat and milk production by ruminants.

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7. References

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