Nomegestrol acetate ameliorated adipose atrophy in a rat model of cisplatin-induced cachexia

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Abstract. Cachexia, a complex disorder that results in depletion of adipose tissue and skeletal muscle, is driven by anorexia, metabolic abnormalities and inflammation. There are limited therapeutic options for this syndrome. Previous evidence has demonstrated that increasing adipose tissue may improve quality of life and survival outcomes in cachexia. Cisplatin, as a chemotherapy drug, also causes cachexia during antitumor therapy due to its adverse effects. To establish a rat model of cachexia, the animals were intraperitoneally treated with cisplatin at doses of 1, 2 and 3 mg/kg, and the rats that responded to cisplatin at the optimal dose were used to test the effect of nomegestrol acetate (NOMAc). Rats that were assessed to be sensitive to cisplatin were randomly grouped and intragastrically administered vehicle, 5 or 10 mg/kg megestrol acetate (MA) or 2.5, 5 or 10 mg/kg NOMAc. The body weights and food consumption of the rats were assessed. Serum IL-6 and TNF-α levels were assessed using ELISA. The protein expression levels of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), peroxisome proliferator activated receptor γ (PPARγ), fatty acid synthase (FASN) and sterol regulatory element-binding protein-1 (SREBP-1) from inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) were evaluated using western blotting. The optimal way to establish a chemotherapy-induced rat model of cachexia demonstrated in the present study was to intraperitoneally administer the rats with 2 mg/kg cisplatin for 3 consecutive days. NOMAc (2.5, 5 mg/kg) and MA (10 mg/kg) were able to significantly ameliorate the loss of body weight in the cisplatin-induced cachectic rats. NOMAc significantly reduced the serum levels of TNF-α at 10 mg/kg. Morphologically, iWAT atrophy, with a remarkable reduction in adipocyte volume, was observed in the cisplatin-induced cachectic rats, but the effects were reversed by administering 5, 10 mg/kg NOMAc or 10 mg/kg MA. Furthermore, 2.5 mg/kg NOMAc markedly reduced the protein expression levels of the lipolysis genes HSL and ATGL, and 5 mg/kg NOMAc markedly enhanced the protein expression levels of adipogenesis genes, including FASN, SREBP-1 and PPARγ in iWAT but not in eWAT. NOMAc was demonstrated to improve cachexia at lower doses compared with MA. Overall, NOMAc is likely to be a promising candidate drug for ameliorating cancer cachexia induced by cisplatin.

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Introduction

Cachexia is a complex disorder accompanied by chronic syndromes. It is characterized by extreme loss of body weight, metabolic disturbance and weakness (1). Aberrant metabolism includes neurohormonal dysregulation, energy expenditure and catabolism increase (2). Patients with cancer, including those with lung, colon, pancreas and stomach cancer, and melanoma usually exhibit cachexia (3). Chemotherapy and radiotherapy are two of the major contributive factors to cachexia (4). Moreover, patients with certain chronic and infectious diseases, including acquired immune deficiency syndrome, tuberculosis and sepsis, also experience cachexia (5). In total, cachexia occurs in 50-80% of patients in the late stages of cancer, which severely affects the survival time and quality of life of the patients, reduces the sensitivity of the treatment and increases the incidence of complications (6,7). Inflammatory factors and metabolic abnormalities such as energy expenditure increase, fat breakdown and decreased protein synthesis serve important roles in the process of cachexia (5,8). The clinical management of cachexia is challenging due to the complexity of multifactorial metabolic dysregulation.

Adipose tissue is generally regarded as a lipid depot for energy stores; however, recent studies have reported that it also acts as a secretory organ that contributes to adjusting the body composition through the regulation of energy homeostasis (9,10). Loss of fat is one of the main features of cancer cachexia and occurs earlier compared with muscle wasting in cachexia. Murphy *et al* (11) reported that accelerated loss of adipose tissue begins 7 months before mortality, and the

average rate of adipocyte loss reaches 29% at 2 months prior to mortality in patients with colorectal and lung cancer. Liu *et al* (12) also demonstrated that accelerated loss of adipose tissue is significantly associated with increased cancer morbidity and mortality. The mechanism of the loss of adipose tissue in cancer cachexia is considered to be due to increased lipolysis (13). High levels of glycerol or fatty acids from lipolysis have been reported in cachectic patients with cancer (14). Lipolysis in adipocytes is activated in patients with cancer, which results in a decline in cellular volume and synthesis of *de novo* lipogenesis (15,16), which may also contribute to adipose wasting. Furthermore, browning of white adipose tissue (WAT) is able to facilitate lipid mobilization and increases energy expenditure, which eventually leads to fat mass reduction (2).

A progesterone-based drug, megestrol acetate (MA), which has been used for cachexia treatment and approved by the US Food and Drug Administration, is able to improve the loss of appetite and increase the body weight of patients (17-19). However, the therapeutic mechanisms of MA for anorexia and cachexia are not well clarified. It has been reported that MA decreases the synthesis and release of cytokines, including IL-1, IL-6 and TNF, and relieves the symptoms of cachexia syndrome (20). Furthermore, a double-blind, placebo-controlled randomized clinical trial suggested that the effects of MA on anorexia and cachexia are similar to those of glucocorticosteroids (21). This may be because MA is a glucocorticoid with weak androgenic activity, which partly contributes to the augment in body weight (22). However, adverse effects induced by MA treatment, including thromboembolic events, edema and adrenal suppression, have been reported (23). Nomegestrol acetate (NOMAc), a 19-norprogesterone derivative, has been used for contraception and treatment of menstrual disorders. It has higher progesterone activity compared with medroxyprogesterone but no androgenic or glucocorticoid properties (23,24).

In a preliminary experiment, the results demonstrated that NOMAc was able to increase body weight in a rat model of endometriosis (data not shown). Therefore, it was hypothesized that NOMAc could serve a role in cisplatin-induced cachexia. The present study established a rat model of cachexia using cisplatin and then assessed whether administrating NOMAc could alleviate the adipose atrophy induced by cisplatin. The effects of NOMAc were compared with those of MA. Furthermore, the effect of NOMAc on the genes responsible for the modulation of adipose degradation and synthesis in cisplatin-induced cachexia was evaluated.

Materials and methods

Chemicals and reagents. Cisplatin was purchased from Qilu Pharmaceutical Co., Ltd. and dissolved in 0.9% NaCl solution. NOMAc was kindly provided by Lijiang Yinghua Biochemical and Pharmaceutical Co., Ltd. MA was purchased from Qingdao GuoHai Biological Pharmaceutical Co., Ltd. MA and NOMAc were dissolved in 0.5% sodium carboxymethylcellulose (CMC-Na). Rat TNFα/Tumor Necrosis Factor ELISA Kit PicoKine® (cat. no. EK0526) and Rat IL-6/Interleukin-6 ELISA Kit PicoKine® (cat. no. EK0412) kits were purchased from Boster Biological Technology. Adipose tissue protein extraction kit (cat. no. HR0049) was purchased from Beijing Biolab Technology Co., Ltd. Anti-adipose triglyceride lipase (ATGL; cat. no. sc-365278) and peroxidase-conjugated goat anti-mouse IgGκ (cat. no. sc-516102) antibodies were purchased from Santa Cruz Biotechnology, Inc. Peroxisome proliferator activated receptor γ (PPARγ; cat. no. abs125245) and sterol regulatory element binding protein-1 (SREBP-1; cat. no. abs131802) antibodies were purchased from Absin Bioscience, Inc. Fatty acid synthase (FASN; cat. no. 3180S), GAPDH (cat. no. 2118s) and peroxidase-conjugated goat anti-rabbit IgG (cat. no. 7074) were purchased from Cell Signaling Technology, Inc. Hormone-sensitive lipase (HSL; cat. no. ab45422) antibodies were purchased from Abcam. BCA Protein Assay kit (cat. no. C503021) was purchased from Sangon Biotech Co., Ltd. Pierce™ ECL Western Blotting Substrate (cat. no. 32106) was purchased from Thermo Fisher Scientific, Inc.

Animals. A total of 125 male Sprague-Dawley rats (body weight, 180±10 g; age, 7-8 weeks) were purchased from Sino-British Experiment Animal (Shanghai Lab Animal Research Center). Animals were housed at a rate of two animals per cage at a temperature of 22±2˚C and 60% humidity with a 12/12 h light/dark cycle and free access to sterilized food and water in specific pathogen free conditions.

Establishment of a rat model of cachexia. A total of 36 male rats were divided into four groups randomly and received interventions via peritoneal injection as follows: Rats in the control group were treated with 0.9% NaCl solution, while the other three groups of rats were treated with 1, 2 or 3 mg/kg cisplatin. The rats were weighed once daily at the same time each day. When their body weight declined by 5% administration of cisplatin was ceased, otherwise the treatment was ended after 5 days. The optimal dose for establishing a rat model of cachexia was evaluated in terms of a decline in the body weight of the animals without observation of major adverse effects. The specific major adverse effects where the experiment would be terminated and the rats would be euthanized were severe diarrhea or ulceration on the limb. If no severely abnormal phenomenon were observed, the rats were euthanized at the end of experiment. All rats were sacrificed by exsanguination of 6-7 ml under anesthesia using 3% pentobarbital sodium solution (30 mg/kg) by intraperitoneal injection on the day after the last administration with cisplatin or 0.9% NaCl solution. Mortality of the rats was confirmed after exsanguination by the absence of heartbeat and respiration for 1-2 min.

Screening of cachectic rats and NOMAc administration. A total of 89 animals were used to establish a rat model of cachexia. The animals were treated with 2 mg/kg cisplatin for 3 days to screen the rat's response to cisplatin and the inclusion criteria were that the body weight of the rats declined >5%. The 21 rats who had not responded to cisplatin were euthanized using exsanguination of 6-7 ml under anesthesia using 3% pentobarbital sodium solution (30 mg/kg) by intraperitoneal injection and mortality was confirmed for 1-2 min after exsanguination by respiratory and cardiac arrest. In total, 69 rats that responded to cisplatin were retained, allowed to

recover for 2 weeks and then randomly divided into 7 groups (n=8-11). The range of group size came from multiple batches of repetition and dose selection. The grouped animals were administered the corresponding treatment via gavage once daily for 11 consecutive days from day 1 (D1) to D11. Rats in the control and model groups received 0.5% CMC-Na, rats in the MA groups received 5 or 10 mg/kg MA and rats in the NOMAc groups received 2.5, 5 or 10 mg/kg NOMAc. Rats in the model and drug treatment groups were intraperitoneally injected with 2 mg/kg cisplatin once daily to induce cachexia 30 min after treatment with 0.5% CMC-Na, MA or NOMAc from D8 to D10. The treatment schedule for the groups is presented in Fig. 1. During cisplatin injection, the body weights of the animals were recorded daily and changes were described as increment in body weight at 24, 48 and 72 h after cisplatin injection against prior to treatment. For example, the body weights of the rats at D9, D10 and D11 respectively minus those at D8. The food intakes were calculated by weighing the leftover food daily. The average food intake of each rat daily was calculated by the remaining forage divided by the number of the animals. On D11, 2 h after the last administration of MA and NOMAc, all 69 rats were anesthetized using 3% pentobarbital sodium solution (30 mg/kg) by intraperitoneal injection and euthanized using exsanguination, collecting 5 ml blood from the abdominal aorta. Rat mortality was confirmed after exsanguination by the absence of respiratory and cardiac arrest within 2 min.

Measurement of serum TNF-α and IL-6 levels using ELISA. Serum collected from the rats was separated by centrifuging at 2,095 x g at 4˚C for 15 min. The serum levels of TNF-α and IL-6 were assessed using ELISA according to the manufacturer's protocol of the aforementioned kits (cat. no. EK0526 and EK0412).

Hematoxylin and eosin (H&E) staining. Inguinal white adipose tissues (iWATs) and epididymal white adipose tissue (eWATs) were collected and immersed in specific fixative for adipose tissue (cat. no. G1119; Wuhan Servicebio Technology Co., Ltd.) for 48 h at room temperature, embedded in paraffin and cut into 5-μm sections. Next, H&E staining was performed at room temperature by immersing the slides into 0.25% eosin alcohol solution for 1 min and 0.2% hematoxylin staining solution for 5 min and then the morphology of adipocytes was assessed using a Leica DM3000 light microscope (Leica Microsystems GmbH). The size and perimeters of adipocytes were evaluated using Image-Pro Plus 6.0 software (version 6.0.0.260; Media Cybernetics, Inc.).

Western blotting. Total proteins were extracted from the iWAT and eWAT of the 7 groups of rats using adipose tissue protein extraction kit (cat. no. HR0049). The protein concentration was assessed using a BCA protein assay kit. The protein extracts (80 μg per lane) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% milk for 1 h at room temperature and incubated overnight at 4˚C with antibodies against ATGL, HSL, PPARγ, FASN, SREBP-1 or GAPDH at a dilution of 1:1,000. Next, the membrane was washed for 30 min with TBS-Tween-20 (0.1%) solution and incubated with peroxidase-conjugated secondary antibodies at a dilution of 1:3,000 for 1 h at room temperature. The bands were visualized using an ECL kit. Images were obtained and semi-quantified analysis of the blots was performed using a ChemiDoc XRS+ Imaging System (version 4.0, Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean ± standard error of mean. Comparisons were performed using one-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism software version 7 (GraphPad Prism software Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of the optimal dose for establishing a rat model of cachexia. The ability of cisplatin to induce cachexia in rats at doses of 1, 2 and 3 mg/kg via intraperitoneal injection for ≤5 days was evaluated. When the rats received cisplatin at 1 mg/kg for 5 consecutive days, their body weight did not decline. The rats received cisplatin at 2 mg/kg for 3 consecutive days, their body weight decreased by 9.32% on the fourth day (Fig. 2). When the rats received 3 mg/kg cisplatin for 3 consecutive days, their body weight decreased by 7.88% on the fourth day (Fig. 2). During the injection, no aberrant symptoms were observed in the rats treated with 1 or 2 mg/kg cisplatin; however, adverse effects including mild diarrhea and ulceration of limbs were observed in the rats treated with 3 mg/kg cisplatin at one day after withdrawal; however, the side effects slowly disappeared after cisplatin withdrawal and no rats were euthanized due to the side effects of cisplatin. These results suggested that 2 mg/kg cisplatin injected for 3 consecutive days was the optimal dose to establish chemotherapy-induced cachexia in rats (Fig. 2).

NOMAc alleviates the loss of body weight induced by cisplatin in cachectic rats. A total of 69 of the rats were selected out for responding to cisplatin and were used in subsequent experiments. Cisplatin (2 mg/kg) was administered to the rats via injection for 3 consecutive days to induce cachexia after 7 days of pretreatment with MA or NOMAc. In the experiment which established the model of cachexia rats using 2 mg/kg/day cisplatin injection and treatment of NOMAc, there were no significant side effects, including limb ulceration and diarrhea observed and no rats were euthanized due to side effects of cisplatin. In all groups, the body weights of the rats demonstrated no significant differences with those before the administration of cisplatin (data not presented). Cisplatin produced a progressive decrease in the body weight of model rats compared with that of the control group at 24, 48 and 72 h after first injection (Fig. 3A-C). In rats pretreated with 2.5, 5 mg/kg NOMAc, the body weight of the rats declined significantly less compared with that of the model group 24 h after first administration of cisplatin (Fig. 3A). Rats pretreated with 2.5 mg/kg NOMAc demonstrated significantly reduced loss in body weight compared with the model group 48 h after first administration of cisplatin (Fig. 3B). The mean body weight loss of rats pretreated with 10 mg/kg MA or 2.5 and 5 mg/kg NOMAc were significantly lower compared with those of the model group 72 h after first administration of

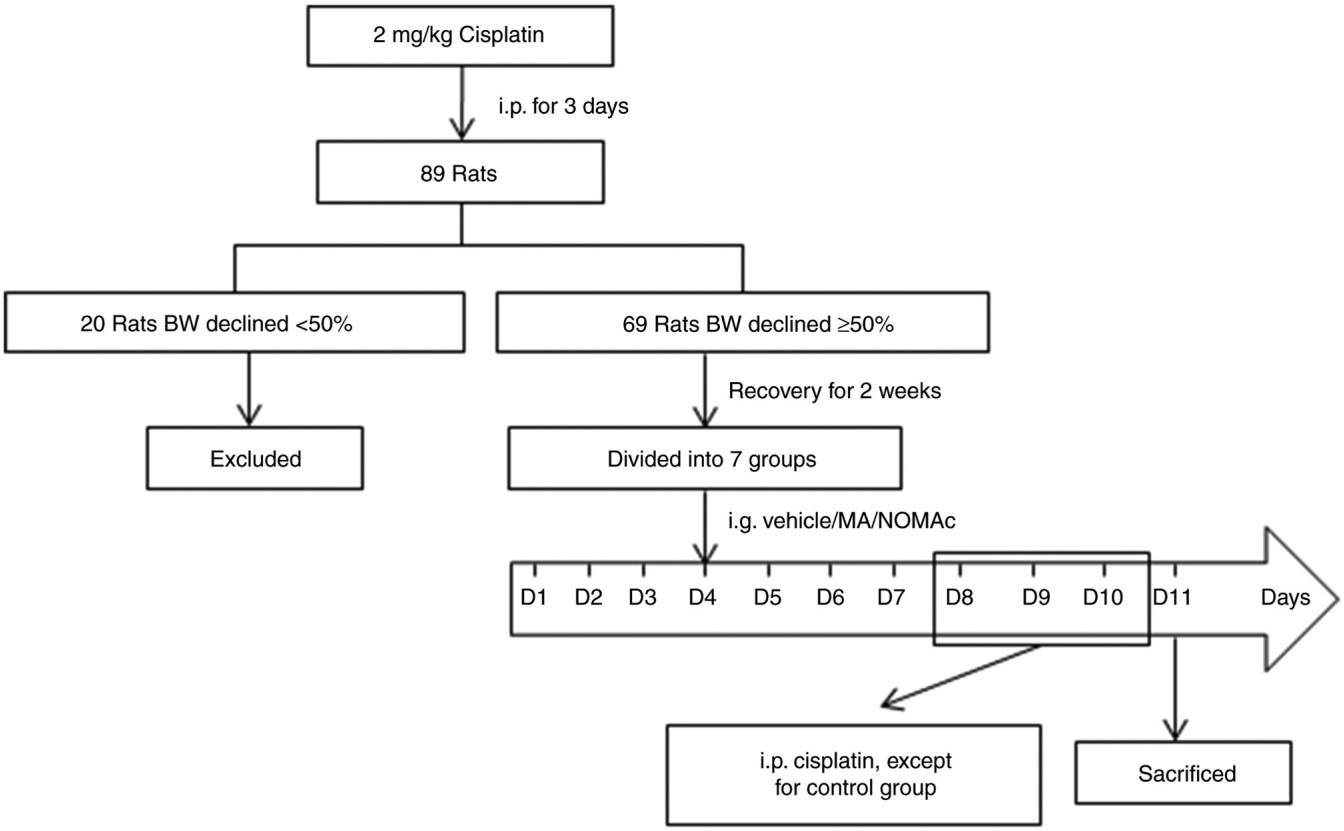

Figure 1. Experimental protocol for screening cachexia-rats and progestin administration. BW, body weight; i.p., intraperitoneal injection; i.g., intragastric administration; MA, megestrol acetate; NOMAc, nomegestrol acetate.

cisplatin (Fig. 3C). During cisplatin injection, no significant differences were demonstrated for the loss of body weight of rats in the 5 mg/kg MA or 10 mg/kg NOMAc treatment group compared with that of rats in the model group (Fig. 3A-C).

Cumulative food intakes were evaluated. The food consumption in the model group was significantly decreased compared with that in the control group (Fig. 3D). The rats in the 10 mg/kg MA and 2.5 and 5 mg/kg NOMAc treatment groups appeared to eat more compared with those in the model group, but the difference was not statistically significant (Fig. 3D).

NOMAc decreases the serum levels of TNF-α and IL-6 in cisplatin-induced cachectic rats. To evaluate the effects of NOMAc on biomarkers associated with inflammatory cytokines and cachexia in cisplatin-induced rats, the serum levels of TNF-α and IL-6 were quantified using ELISA. The levels of serum TNF-α in the model group were 2.15-fold higher compared with those in the control group; however, this was not significantly different (Fig. 4A). With the dosage of MA and NOMAc increasing, the serum levels of TNF-α were decreased in all groups of MA and NOMAc, but the difference was statistically significant between 10 mg/kg NOMAc-treated rats and those in the model group (Fig. 4A).

The levels of serum IL-6 were also evaluated and there was no apparent difference between the control and model groups. The levels of IL-6 in 5 mg/kg MA-treated rats decreased by 77.8% compared with the model group, but this was not statistically significant. The serum levels of IL-6 were decreased by 27.8, 72.8 and 47.8% in the groups subjected to 2.5, 5 and 10 mg/kg NOMAc treatment, respectively, compared with those in the model group; however, these results were not statistically significant (Fig. 4B).

Effects of NOMAc on the sizes of adipocytes in iWAT and eWAT in cisplatin-induced cachectic rats. To assess the effect of NOMAc on adipose tissue in the cisplatin-induced cachexia model rats, the morphological changes of adipocytes in iWAT and eWAT were evaluated. Morphologically, eWAT was characterized by increased blood vessels and a richer blood supply compared with iWAT. The sizes and perimeters of adipocytes were assessed using Image-Pro Plus 6 software. Increased numbers of shrunken adipocytes of iWAT were observed in the rats of the model group compared with those in the control group, while MA and NOMAc treatments reduced the atrophy of adipocytes induced by cisplatin (Fig. 5A). The sizes of adipocytes in 5 mg/kg NOMAc treated rats increased 9.74% and declined 3.85% in 10 mg/kg NOMAc group compared with the same dosage of MA group (Fig. 5A). The cell perimeter of iWAT cells was reduced in model rats compared with that in control rats. NOMAc and MA increased the cell perimeter; however, no significant difference was observed compared with the model group (Fig. 5A). The sizes of adipocytes in eWAT were also evaluated, but no significant difference was demonstrated among all the tested groups. No significant difference was demonstrated among all the tested groups for the cell perimeters sizes of adipocytes in eWAT (Fig. 5B).

Effects of NOMAc on the protein expression levels of lipolysis-related genes in iWAT and eWAT in cisplatin-

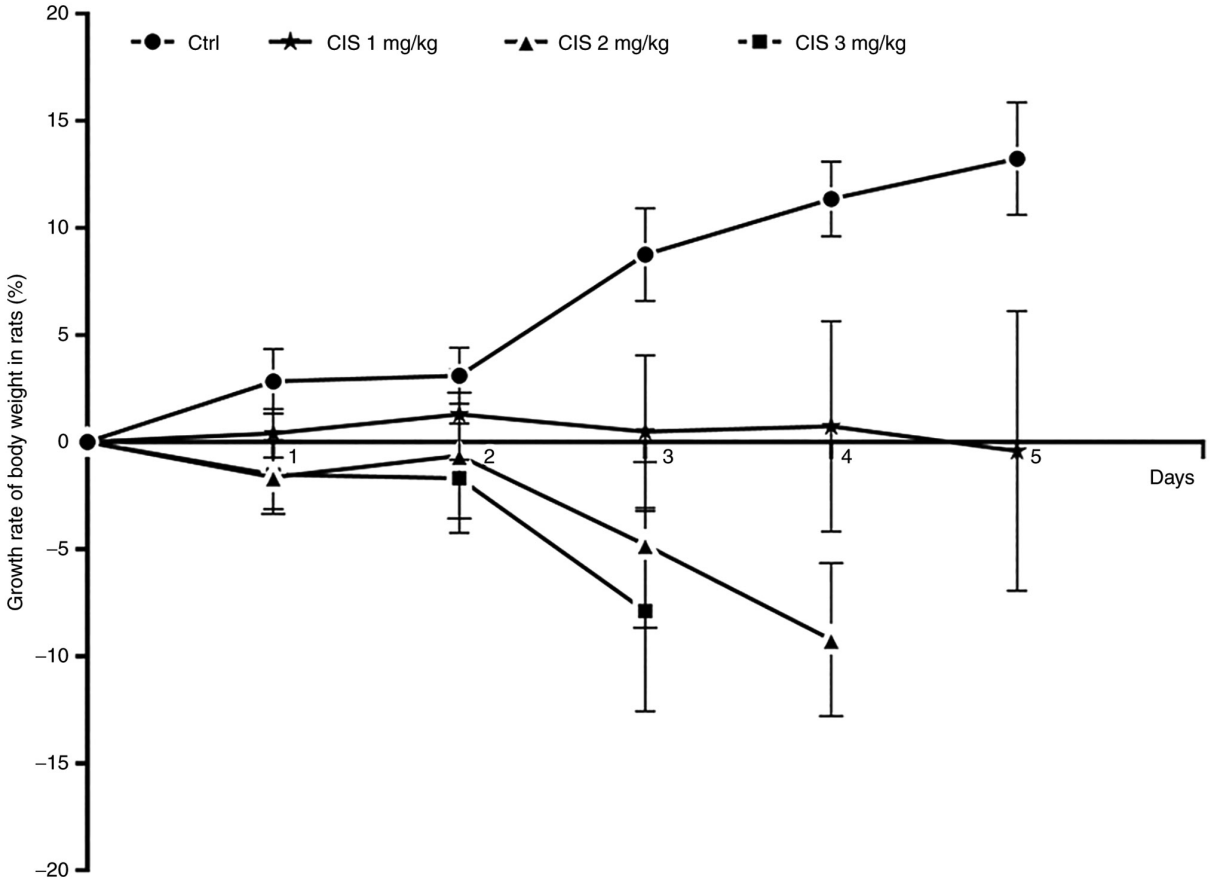

Figure 2. Effects of different doses of cisplatin on the growth rate of body weight in rats. Rats were treated with cisplatin at 1 mg/kg for 5 days, 2 mg/kg for 3 days or 3 mg/kg for 3 days (n=9). Ctrl, control group; CIS, cisplatin.

induced cachectic rats. Biomarkers of lipolysis were assayed to further evaluate the effects of NOMAc on cisplatin-induced rats with cachexia. Cisplatin markedly increased the protein expression levels of HSL and ATGL in iWAT compared with those in the model group (Fig. 6A). The protein expression levels of HSL in the 5 mg/kg MA and 2.5 mg/kg NOMAc treatment groups significantly decreased compared with those in the model group (Fig. 6A); however, 10 mg/kg MA and 5 and 10 mg/kg NOMAc did not influence the protein expression levels of HSL compared with the model group (Fig. 6A). The protein expression levels of ATGL were significantly suppressed by all groups of MA and NOMAc compared with those in the model group (Fig. 6A).

Changes in the protein expression levels of HSL and ATGL in eWAT were evaluated. Cisplatin markedly enhanced the protein expression levels of HSL and ATGL in eWAT compared with those in the control group, but it was not statistically significant. Treatment with 5 and 10 mg/kg NOMAc as well as 5 mg/kg MA decreased the levels of HSL and ATGL in eWAT compared with those in the model group; however, no significant difference was demonstrated (Fig. 6B).

Effects of NOMAc on the protein expression of lipid synthesis-related genes in iWAT and eWAT in cisplatin-induced cachectic rats. The protein expression levels of lipid synthesis-related genes, including PPARγ, FASN and SREBP-1, were semi-quantified using western blotting. The protein expression levels of FASN in iWAT adipocytes in the model group were significantly decreased by 50% compared with those in the control group (Fig. 7A). Compared with those in the model group, the protein expression levels of FASN of the 10 mg/kg NOMAc groups were significantly increased (Fig. 7A). Despite the increasing trend observed, no significant difference in the protein expression level of FASN was demonstrated in the 5 and 10 mg/kg MA or 2.5 and 10 mg/kg NOMAc treatment groups compared with that in the model group (Fig. 7A). No significant difference was observed in the protein expression levels of SREBP-1 or PPARγ between iWAT adipocytes in the model and control groups (Fig. 7A). Compared with that of the rats in the model group, 5 or 10 mg/kg MA had no significant effect on the protein expression levels of SREBP-1 (Fig. 7A). After administering 2.5 and 10 mg/kg NOMAc to the rats, the protein expression levels of SREBP-1 were markedly higher than those in the rats of the model group; however, the result was not statistically significant (Fig. 7A). NOMAc at 5 mg/kg significantly increased the protein expression level of SREBP-1 compared with the model group (Fig. 7A). Compared with those in the model group, the protein expression levels of PPARγ were significantly increased in the rats subjected to 5 and 10 mg/kg NOMAc treatment (Fig. 7A); however, no significant difference was observed in the groups subjected to 5 or 10 mg/kg MA or 2.5 mg/kg NOMAc treatment compared with the model group (Fig. 7A).

The protein expression levels of FASN, SREBP-1 and PPARγ in eWAT adipocytes were also evaluated. Cisplatin did not significantly change the protein expression levels of

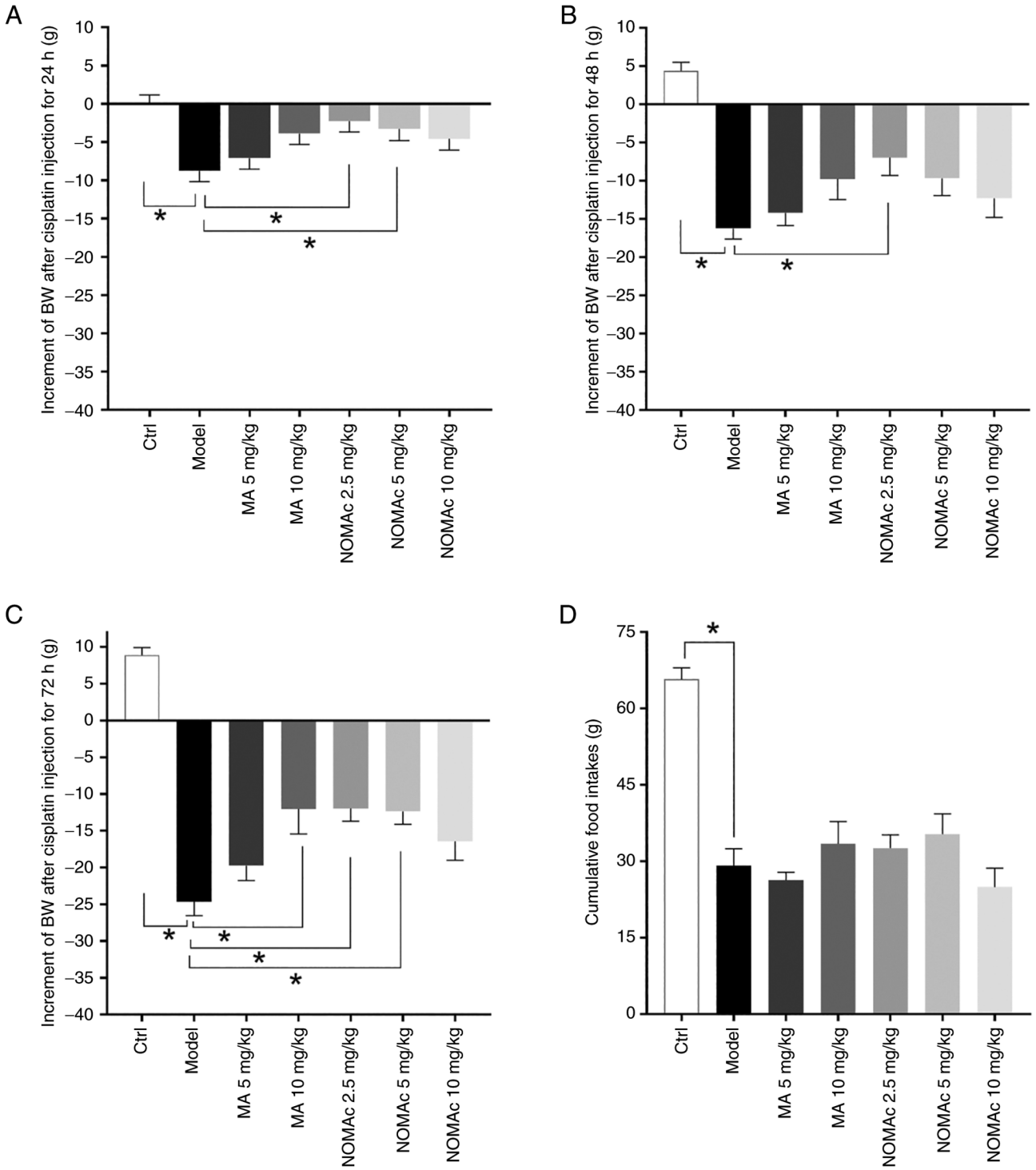

Figure 3. Effects of NOMAc on body weight and total food intakes in cachexia model rats after cisplatin treatment. (A) Change in body weights after cisplatin injection for (A) 24 h, (B) 48 h and (C) 72 h. (D) Cumulative food intake. n=8-11. *P<0.05. Ctrl, control group; BW, body weight; MA, megestrol acetate; NOMAc, nomegestrol acetate.

these genes compared with those of the control group and MA or NOMAc treatment also did not significantly change the protein expression levels of these genes compared with those of the control group (Fig. 7B).

Discussion

One of the prominent features of cancer cachexia is weight loss due to adipocyte lipolysis and muscle wasting. The present study demonstrated that NOMAc exerted protective effects on cisplatin-induced cachexia by preventing the loss of body weight and increasing food intake. Furthermore, NOMAc was able to significantly decrease the serum levels of TNF-α, ameliorate the atrophy of adipocytes, significantly decrease the protein expression levels of ATGL and HSL in iWAT in cisplatin-induced cachectic rats and significantly enhance the protein expression levels of adipogenesis genes associated with cachexia, including FASN, SREBP-1 and PPARγ in iWAT.

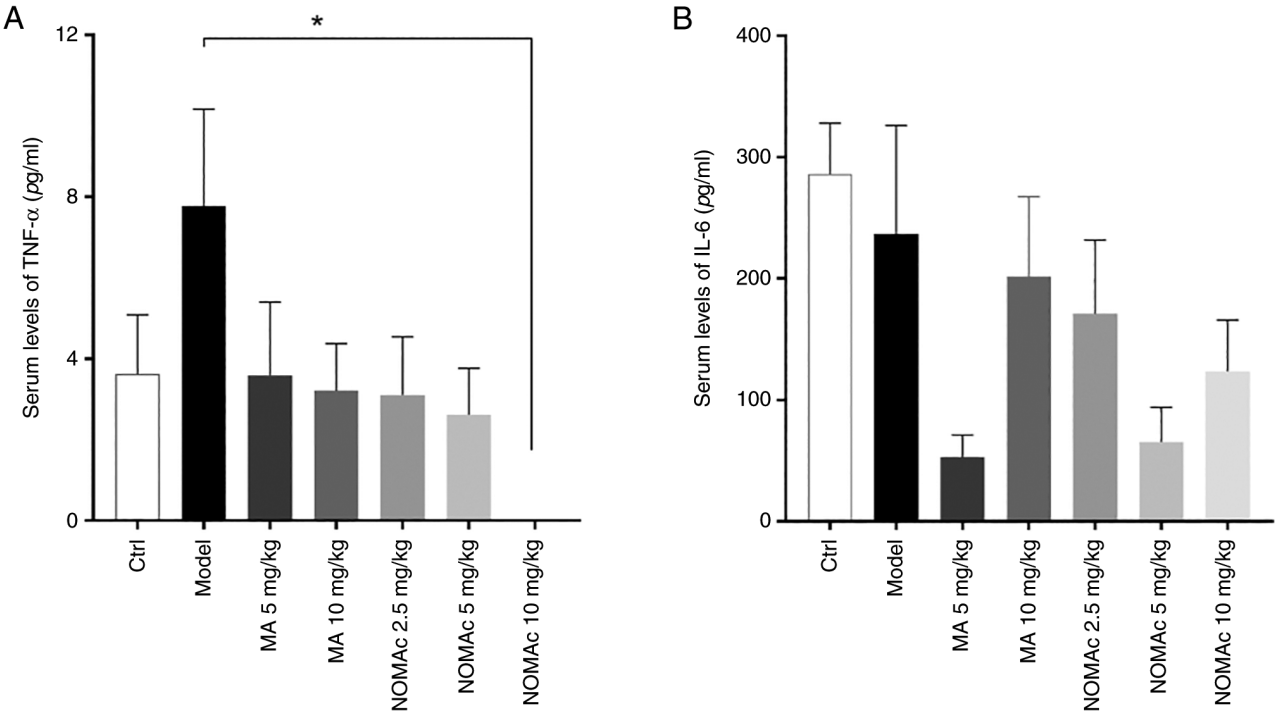

Figure 4. Effect of NOMAc on serum levels of TNF-α and IL-6 in rats. The serum level of (A) TNF-α and (B) IL-6. n=8-11. *P<0.05. Ctrl, control group; MA, megestrol acetate; NOMAc, nomegestrol acetate.

These results suggested that NOMAc has potential to be used for ameliorating cachexia.

Clinically, cachexia is defined as >5% loss of body weight or a body mass index <20 kg/m^2 with ≥2% weight loss over 6 months (2). Establishing models of cachexia require evaluation of the efficacy of a candidate drug. In preclinical experiments, the methods of establishing animal models of cachexia include malignant tumor induction and chemotherapeutic drug injury (25). The presented study established a cachexia model in rats by injecting cisplatin. Cisplatin is a potent chemotherapy drug that is effective against a variety of solid tumors; however, it can cause cachexia during anti-tumor therapy due to its adverse effects (26). As a result, cisplatin-induced animal models of cachexia are considered to be valid models for identifying potential medication for the treatment of cachexia, particularly cachexia associated with chemotherapy (27,28).

The role of cisplatin in chemotherapy-induced cachexia is not well understood. Previously, accumulation of cisplatin has been detected in adipose tissue (29), which suppresses FASN, stearoyl coenzyme A desaturase-1 (SCD1) and carnitine palmitoyl transferase-1 (CPT-1) in WAT to decrease lipogenesis, enhance lipolysis by interacting with HSL and increasing lipid oxidation by regulating food intake (28,30). These previous studies indicated that the effect of cisplatin on adipose tissue may involve both direct and indirect mechanisms. Therefore, different doses of cisplatin were administered to the rats in the present study to assess the optimal dose for establishing the rat model of cachexia in terms of the clinical criteria. The results demonstrated that administering 2 mg/kg/day cisplatin to the rats for 3 consecutive days was the optimal method for decreasing the body weights of the rats by 5% and subsequent experiments were performed using this method.

Previous studies report that the reduction of bodyweight mediated by cisplatin reaches a peak 48-72 h after being administered and that the effects then return to near baseline levels after 16 days (31,32). In the present study, rats in which the body weights declined by <5% after cisplatin injection were first screened out, since not all animals respond to cisplatin induction. To eliminate the effects of cisplatin, the rats selected for use following cisplatin screening were allowed to recover for 2 weeks. Thereafter, the progestins MA and NOMAc were administered for 7 consecutive days prior to injection of cisplatin into the rats again. Following cisplatin administration, the rats in the vehicle-treated model group demonstrated significant weight loss, while 2.5 and 5 mg/kg NOMAc significantly reduced the loss in body weight induced by cisplatin. Weight loss in the 2.5 and 5 mg/kg NOMAc treatment groups was <50% compared with that in the model group, which was similar to the effects of the 10 mg/kg MA treatment. These results suggested that NOMAc exerted a protective effect on weight loss at lower doses than MA in cisplatin-induced cachexia.

Cisplatin-induced cachexia generally causes anorexia accompanied by a decline in food intake (33). In the present study, the observed progressive reduction in body weight was consistent with the decline in food consumption. While there was no statistical difference, an increased trend in cumulative food intake was demonstrated in the rats of the 2.5 and 5 mg/kg NOMAc treatment groups compared with that demonstrated in the model group. This indicated that the protective effect of NOMAc against cisplatin-induced weight loss could partly be attributed to an increase in food intake.

It is known that there is a link between cachexia and inflammatory cytokines. TNF-α and IL-6, as pro-inflammatory cytokines, are able to enhance both systemic and local

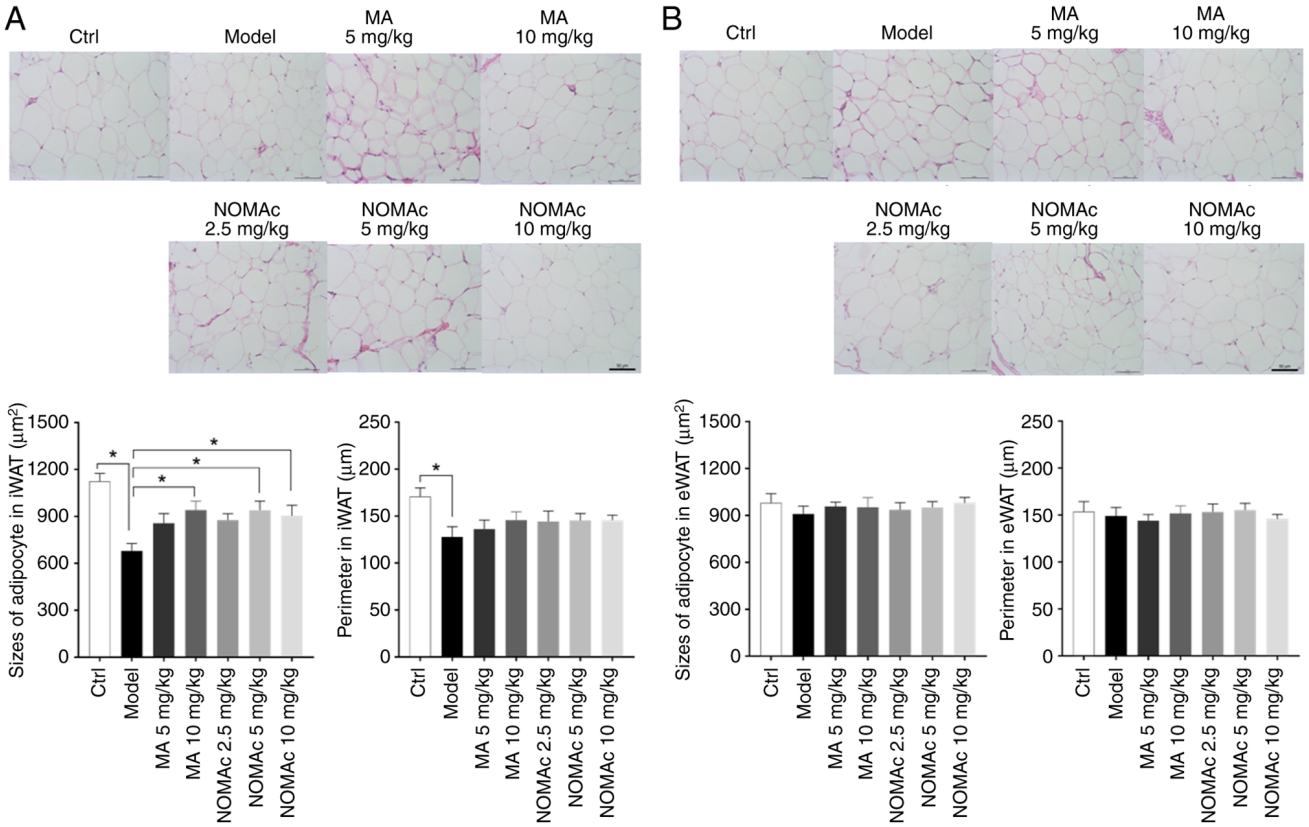

Figure 5. Morphological characteristics of adipocytes in rats. (A) H&E staining of adipocytes in iWAT (magnification, x20), the sizes of adipocyte and perimeters in iWAT. (B) H&E staining of adipocytes in eWAT (magnification, x20), the sizes of adipocyte and perimeters in eWAT. Arrows indicate blood vessels. *P<0.05. Ctrl, control group; MA, megestrol acetate; NOMAc, nomegestrol acetate; iWAT, inguinal white adipose tissue; eWAT, epididymal white adipose tissue; H&E, hematoxylin and eosin.

inflammatory effects in patients with cancer (34). Increased TNF-α expression levels are associated with muscle wasting, loss of adipose tissue and proteolysis in cancer cachexia (35). Sherry *et al* (36) reported that administering an anti-TNF-α antibody can attenuate the development of cachexia in tumor models by preventing the loss of fat, muscle and body weight. In the present study, it was demonstrated that the serum levels of TNF-α increased with a progressive decline in body weight in the model group, which was significantly suppressed by NOMAc treatment. Furthermore, the IL-6 serum level is also considered to be correlated with weight loss and reduced survival in patients with cancer (37). Han *et al* (38) reported that IL-6 was able to enhance the loss in body weight by accelerating the lipolysis of WAT in patients with cancer and cachexia. In the present study, a decreasing trend in the levels of serum IL-6 was observed in the 2.5 and 5 mg/kg NOMAc-treated rats; however, it was not statistically significant. The remarkable decrease in TNF-α levels contributed to the protective effect of NOMAc against cisplatin-induced weight loss, but the effects were not observed in the MA-treated groups.

Previous studies have reported that lipolysis results in depletion of lipid depots in adipose tissue, which reduces the sizes of adipocytes and decreases the rate of *de novo* lipogenesis (19,22). In the present study, the morphological changes in adipose tissues were characterized by iWAT atrophy and a significant reduction in the sizes of adipocytes in cisplatin-treated model rats; moreover, these alterations could be significantly reduced by NOMAc treatment. In eWAT, the sizes of the adipocytes in the model rats did not differ from those in the control group, which indicated that iWAT was more sensitive to cisplatin induction compared with eWAT. Moreover, it indicated that NOMAc could attenuate the atrophy of adipocytes in iWAT but did not in effect eWAT. It suggested that the short time of cisplatin treatment could have affected superficial inguinal fat but not epididymal adipose tissue. Moreover, previous studies have reported that eWAT has a protective effect on gonadal tissue, and loss of adipose tissue can cause a decline in fertility (39,40); therefore, it was hypothesized that the loss of eWAT later compared with iWAT may be to protect the genitals. In addition, it suggests that iWAT could be a better indicator compared with eWAT for evaluating the animal model of cisplatin-induced cachexia and the effects of NOMAc since the results demonstrated that eWAT was insensitive to cisplatin and NOMAc. Various genes participate in the loss of adipose tissue in cancer cachexia. Lipolysis is regulated through ATGL and HSL. ATGL is the rate-limiting enzyme in the process of lipolysis, which converts triacylglycerol (TG) to glycerol and free fatty acids (41,42). In the present study, a significant increase in the protein expression levels of HSL and ATGL was observed in the iWAT of cisplatin-induced model rats, but this augmentation could be markedly reduced by NOMAc. Furthermore, NOMAc reduced the protein expression levels of the aforementioned genes at lower doses compared with

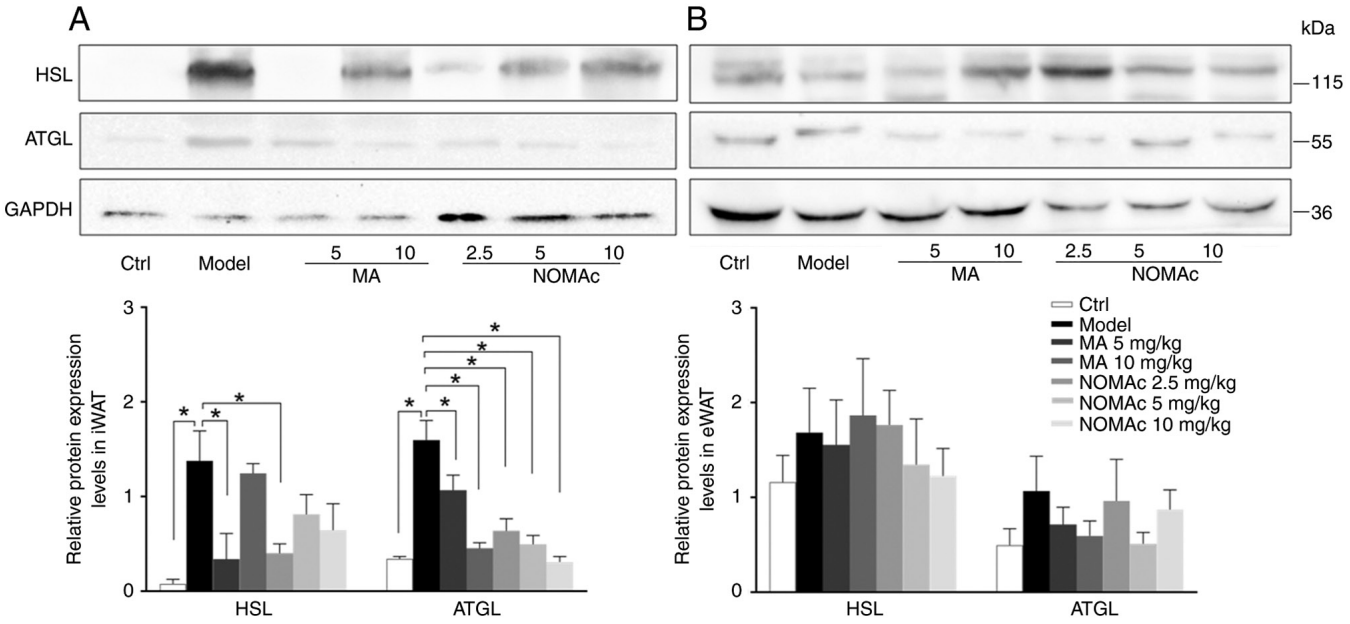

Figure 6. Effects of NOMAc on the protein expression levels of the lipase genes HSL and ATGL in iWAT and eWAT induced by cisplatin. The semi-quantified protein expression levels in (A) iWAT and (B) eWAT, normalized to GAPDH (n=6). *P<0.05. Ctrl, control group; MA, megestrol acetate; NOMAc, nomegestrol acetate; iWAT, inguinal white adipose tissue; eWAT, epididymal white adipose tissue; HSL, hormone-sensitive lipase; ATGL, adipose triglyceride lipase.

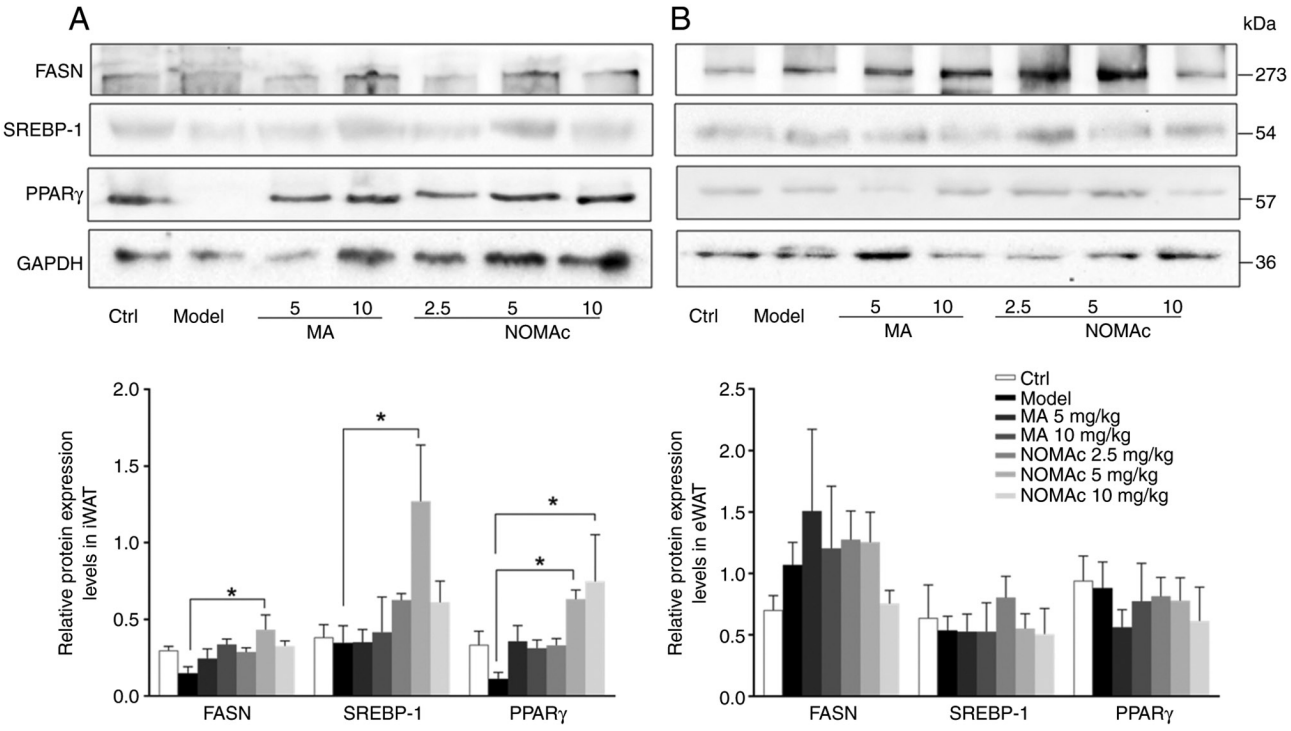

Figure 7. Effects of NOMAC on the protein expression levels of the lipid synthesis genes PPARγ, FASN and SREBP-1 in iWAT and eWAT induced by cisplatin. The semi-quantified protein expression levels of PPARγ, FASN and SREBP-1 in (A) iWAT and (B) eWAT normalized to GAPDH (n=6). *P<0.05. Ctrl, control group; MA, megestrol acetate; NOMAc, nomegestrol acetate; iWAT, inguinal white adipose tissue; eWAT, epididymal white adipose tissue; PPARγ, peroxisome proliferator activated receptor γ; FASN, fatty acid synthase; SREBP-1, sterol regulatory element binding protein-1.

MA, which indicated that NOMAc may have been more effective compared with MA.

In addition, the protein expression levels of lipid synthesis-related genes, including FASN, PPARγ and SREBP-1, were assessed. PPARγ, as a transcription factor, regulates lipid metabolism when activated by ligands and serves a role in adipogenesis and the maintenance of mature adipocyte function (43). Cachexia is able to impair lipogenesis through reducing the levels of PPARγ (44), as well as through inhibiting the expression of fatty acid binding protein 4 (aP2), SCD1, CPT-1α and FASN (28). In the present study, cisplatin markedly inhibited the expression of PPARγ in iWAT, which suggested that cisplatin

may regulate lipid synthesis by affecting the transcription factor PPARγ. Previous studies report that SREBP-1 contributes to the expression of PPARγ and activates PPARγ through the production of endogenous ligands (45,46). SREBP-1c, another transcription factor, is a subtype of SREBP-1, which also regulates the expression of genes involved in lipid metabolism such as FASN (47). As a rate-limiting enzyme, FASN controls the *de novo* conversion of free fatty acid into TG in adipocyte lipogenesis (46). In the present study, the protein expression levels of SREBP-1, PPARγ and FASN were significantly increased in the iWAT of NOMAc-treated rats, which indicated that NOMAc was not only able to reduce lipolysis but also enhanced lipogenesis via activation of the transcription factors SREBP-1 and PPARγ. Lipid metabolism depends on the co-regulation of various transcriptional factors. Whether NOMAc regulates lipid metabolism through other transcription factors requires further investigation. Furthermore, the present study demonstrated that MA has a trend to promote the protein expression of FASN and PPARγ, but did not markedly significance which indicated that MA may ameliorate cachexia via mechanisms other than regulating the expression of these two genes involved in lipogenesis.

The present study semi-quantified the protein expression levels of HSL, ATGL, PPARγ, FASN and SREBP-1 in eWAT, and demonstrated that there were no significant differences among all groups (Figs. 6B and 7B). This indicated that the genes associated with lipogenesis and lipolysis in iWAT were more prone to be affected by cisplatin compared with those in eWAT and that these genes in iWAT were likely to be regulated by progestins. It was hypothesized that the reason could be the short time of the model, which affected superficial inguinal fat but not epididymal adipose tissue. Furthermore, previous studies have reported that WAT has a protective effect on gonadal tissue and that loss of adipose tissue can cause a decline in fertility (39,40); therefore, it could be suggested that eWAT may be lost after inguinal fat in order to protect the gonads.

The protein expression levels of two genes associated with muscle wasting, muscle RING-finger protein-1 and atrogin-1, in skeletal muscles, were assessed in a preliminary study and it was demonstrated that neither MA 5 and 10 mg/kg nor NOMAc 2.5, 5 and 10 mg/kg influenced their protein expression levels at the tested concentrations (data not shown). These results were different from those reported by Busquets *et al* (47), who observed that both genes are downregulated when the dose of MA is increased to 100 mg/kg. Future studies should be performed to evaluate the modulation of progestins in skeletal muscle in cachexia. In the present study, the level of NOMAc in adipose tissues was not determined; however, progestins generally exhibit lipophilic properties (48). Therefore, it was presumed that NOMAc acted on adipose tissues in a direct manner, since it was demonstrated that NOMAc treatment not only improved the appetite of rats but also markedly reduced the protein expression levels of genes associated with lipid degradation and increased lipid synthesis. However, a potential indirect mechanism of action could not be excluded and future investigations are needed.

In summary, the present study demonstrated that NOMAc was able to significantly ameliorate the loss of body weight and reduce the serum level of TNF-α in a rat model of cisplatin-induced cachexia. In particular, NOMAc attenuated the atrophy of iWAT by increasing the volume of adipocytes. The mechanism of NOMAc action not only involved downregulation of the protein expression levels of key factors of lipolysis, such as HSL and ATGL, but also enhancement of the protein expression levels of lipogenesis-associated genes, including SREBP-1, PPARγ and FASN in iWAT but not in eWAT. Furthermore, NOMAc improved the cachexia at lower doses compared with MA. These results suggested that NOMAc was a promising candidate drug for ameliorating cancer cachexia. The present study therefore provided novel ideas for the application of progestins in the treatment of cachexia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RZ designed the study, performed the animal experiments and wrote the manuscript. WY and XG performed the animal experiments. GL and SX performed the experiments to determine protein expression levels in serum. JZ and BR performed western blotting. YZ supervised and designed the study, and revised and approved the manuscript. RZ and YZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Laboratory Animal Ethics Committee of Shanghai Institute for Biomedical and Pharmaceutical Technologies (approval no. 2019-27).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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