Journal of Cell and Molecular Research (2025) 16 (2),113-120 DOI: 10.22067/jcmr.2025.90056.1100

Research Article

The Effects of the DNAi Molecule on Growth and Muscle Weights in Male Wistar Rats

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Received 1 Oct 2024 Accepted 4 Mar 2025

Abstract

The growth and development of skeletal muscle tissue is largely regulated by myostatin during tissue development in embryos. This tissue may overgrow if myostatin expression is deficient. Gene expression may be regulated in a particular way by oligonucleotide antisense molecules. It has been demonstrated that a new DNA-based oligonucleotide can downregulate myostatin expression in a rat model. The purpose of this work was to evaluate the impact of a DNAi-based myostatin inhibitor on the visceral fat and leg muscle weights of Wistar rats undergoing strength training. Three groups of male rats, with an average weight of 203g ± 10.5, were chosen at four weeks of age. These cohorts comprised: 1) DNAi group had resistance training in addition to receiving 10 mg/kg of rat body weight of DNAi. 2) Resistance exercise and saline injection group. Group for injection of saline. Then, weight measurements for the carcass, heart, liver, left kidney, right kidney, spleen, visceral fat, twin muscles, soleus muscle, and left leg were made for each group. Histological assessment of the soleus muscle section was performed. One-way ANOVA was then used to examine the results, and means were compared using Tukey's test. As the data show, the proposed molecule did not significantly contribute to an increase in body weight, in contrast to previous assumptions. Nonetheless, the twin muscles' relative and absolute weights increased significantly with visceral fat decreased with DNAi injection ($P<0.05$). Although weekly body weight increase and the final weights were not affected by DNAi injection, this could be explained by the loss of fat tissue during the experiment. This molecule is promising in increasing muscle tissue growth; however, further prolonged experiments and evaluating myostatin gene expression are recommended in future experiments.

***Keywords*:** DNAi; gene silencing; phosphorothioates; Wistar rats; resistance training

Introduction

Myostatin (MSTN), belonging to the TGF-β/BMP family, significantly restrains muscle growth (McPherron & Lee, 1997). Its actions involve binding to cell receptors, inhibiting cell proliferation, myoblast differentiation, and influencing protein synthesis in mature myotubes (Langley et al., 2002; Taylor et al., 2001). During muscle atrophy, myostatin expression notably increases (Carlson et al., 1999). Mutations lowering myostatin activity induce remarkable muscle hypertrophy across species like mouse, dog, pig, human, and cow (Soleimani et al., 2019). Conversely, overexpressing myostatin leads to a substantial decrease in mouse muscle mass (Reisz-Porszasz et al., 2003). These findings highlight the significant impact of myostatin expression and activity on regulating muscle tissue growth (Riasi et al., 2023; Riasi and Javadmanesh, 2025).

The dynamic changes observed in muscle mass, whether in response to aging or pathological conditions, are intricately linked to the equilibrium between protein synthesis and breakdown rates within muscle fibers. This equilibrium is highly contingent upon the nature, intensity, and duration of physical activity undertaken (Mitchell et al., 2013; Phillips & McGlory, 2014). The nature and extent of muscular engagement serve as critical determinants in shaping the delicate balance between muscle anabolism and catabolism, thereby exerting profound effects on overall muscle health and functionality (Cermak et al., 2013; Egan & Zierath, 2013). Understanding the complexity of these interactions is pivotal in devising strategies to preserve, restore, or augment skeletal muscle

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integrity, particularly in the context of age-related changes or pathological circumstances (Tieland et al., 2018).

Exercise training is well recognized to have anti-inflammatory properties, including the ability to decrease the expression of local cytokines and raise anti-apoptotic proteins (Gielen, 2003). This advantageous metabolic impact leads to an increase in exercise tolerance. Myostatin's function in this situation is still unknown. The impact of exercise training on myostatin expression in healthy individuals varies depending on the type of muscle and the type of exercise (Kim et al., 2005; Matsakas et al., 2006). Nevertheless, research has not yet discovered how antisense oligonucleotides and exercise interact to suppress the expression of the myostatin gene in rats.

There are a very limited number of studies on the effectiveness of using DNAi molecules in inhibiting MSTN expression in animal models. The purpose of the present study is to investigate the effect of the designed DNAi molecule on increasing muscle weight and visceral fat in male Wistar rats with resistance training.

Materials and Methods

Design of the DNAi molecule

The promoter sequence of mouse MSTN was retrieved from the NCBI (accession number NC-00006706). The following DNAi sequence was designed complementary to a region downstream of the TATA box. The DNAi sequence is as follows: 5'TATA(x)AAAAGCCA(x)CTTGGAATAC(x)A GTA'3. To increase stability against nucleases, phosphorothioate modifications were used in three positions marked by X (Riasi et al, 2023).

Animals

Eighteen male Wistar rats (203 grams ± 10.5, four weeks old) were randomly divided into three groups, each comprising six rats: (i) DNAi group received 10 mg/kg rat body weight of DNAi along with resistance exercise, (ii) Saline injection group with resistance exercise (iii) Saline injection group. All rats were housed in groups of six. They were maintained in controlled conditions with a 12-hour light-dark cycle, provided ad libitum access to standard rat chow and water. All animals in this study adhered to ethical research guidelines for animal testing (Roozbeh et al., 2019). Ethical approval for animal handling and experimental procedures was obtained from the Ethics Committee of the Ferdowsi University of Mashhad Research Centre (Code: IR.UM.REC.1401.183).

Resistance training protocol

During a week-long familiarization with resistance exercises on a one-meter ladder with two-centimeter rungs at an 85-degree incline, the rats were positioned at the bottom of the ladder for ascending without any weights. Following this familiarization period, the main exercises consisted of three sessions per week, each comprising three sets of five repetitions, conducted for a duration of five weeks. The rest time between repetitions was one minute, and between sets was two minutes. The weight used was initially set at 50% of each rat's weight in the first week of the training period, gradually increasing by 10% each week. The weight of each rat was measured weekly (Lee & Farrar, 2003).

Injection procedure and tissue collection

Intraperitoneal injections were performed three times a week. The rats' weekly body weights were measured, and after five weeks, all rats were euthanized in accordance with ethical guidelines. Then mice were sacrificed by exposure to a rising concentration of CO_2 (Baumans, 2004). The Carcass weight, heart, liver, kidney (Average of left and right), spleen, visceral fat, left leg without skin, twin, and soleus muscle weights were measured. The relative leg weight, the twin and soleus muscle (Ratio of organ weight to body weight), was also calculated (Kinouchi et al., 2008).

Histological preparation

Soleus muscles embedded in paraffin after being fixed in paraformaldehyde. Hematoxylin and eosin (H&E) staining was used on sections ($7\mu m$) cut and mounted on silanized glass slides (Parise et al., 2008). Cell counting was performed by MyoVision software (Wen et al., 2018).

Statistical analyses

All data were analysed by one-way ANOVA and means were compared using Tukey's test (SAS,v 9.4, SAS Institute Inc., Cary, USA) was used to evaluate the statistical significance of the data, and P-values less than 0.05 were considered statistically significant. All data were expressed as mean ± standard error.

Results

The results of the weekly weight of the rats did not show any significant difference between groups (Figure 1). Similarly, the weight of the heart, liver, kidney, spleen, adipose tissue, soleus muscle, and left leg showed that there was no difference between

the groups. But the weight of the twin muscle in group i was significantly higher than the other two groups, which can be an indication of the efficiency of the designed DNAi molecule (Table 1).

In the current study, similar to Kinuchi et al.'s study (Kinuchi et al, 2008), the left leg and muscle (twin and soleus) relative weights were calculated. The results of this index also showed that the relative weight of the twin muscle was significantly higher in DNAi injected animals in contrast to solues muscle, which showed no significant difference (Figure 2).

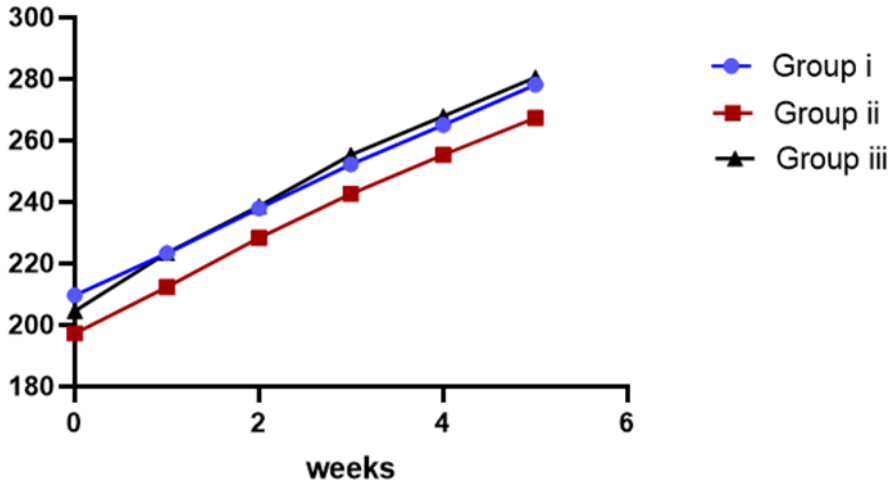

Figure 1. Profiles of the body weights (g) of rats, measured weekly. Groups (i) DNAi group, received 10 mg/kg rat body weight of DNAi along with resistance exercise, (ii) Saline injection group with resistance exercise, (iii) Saline injection group without any exercise.

Table 1. The results of body and organ weights in the three experimental groups. (i) The DNAi group received 10 mg/kg rat body weight of DNAi along with resistance exercise. (ii) Saline injection group with resistance exercise (iii) Saline injection group without any exercise. Different letters indicated a significant difference ($P < 0.05$).

Weight (g)	**Group** (i)	**Group (ii)**	**Group (iii)**	**P value**
Weight Day-1	204.50 ± 7.40	209.67 ± 9.56	197.25 ± 8.27	0.61
Pre-surgery	278.13 ± 10.82	267.35 ± 9.37	280.59 ± 8.38	0.56
Heart	0.88 ± 0.03	0.88 ± 0.3	0.95 ± 0.03	0.10
Liver	8.07 ± 0.37	7.71 ± 0.32	8.42 ± 0.28	0.27
Kidney	0.97 ± 0.04	0.99 ± 0.04	1.07 ± 0.03	0.15
Spleen	0.82 ± 0.08	0.93 ± 0.07	0.98 ± 0.06	0.29
Visceral fat	5.62^{a} ± 0.57	6.43^{ab} ± 0.46	7.75^{b} ± 0.53	0.04*
Twin muscle	2.00^{a} ± 0.11	1.62^{b} ± 0.09	1.65^{b} ± 0.09	0.03*
Soleus muscle	0.09 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.32
Left leg	4.08 ± 0.23	4.24 ± 0.19	4.21 ± 0.20	0.85

Histological analysis of leg muscles

The results of counting the muscle fiber density show that there was no significant difference between the experimental and control groups (Figure 3).

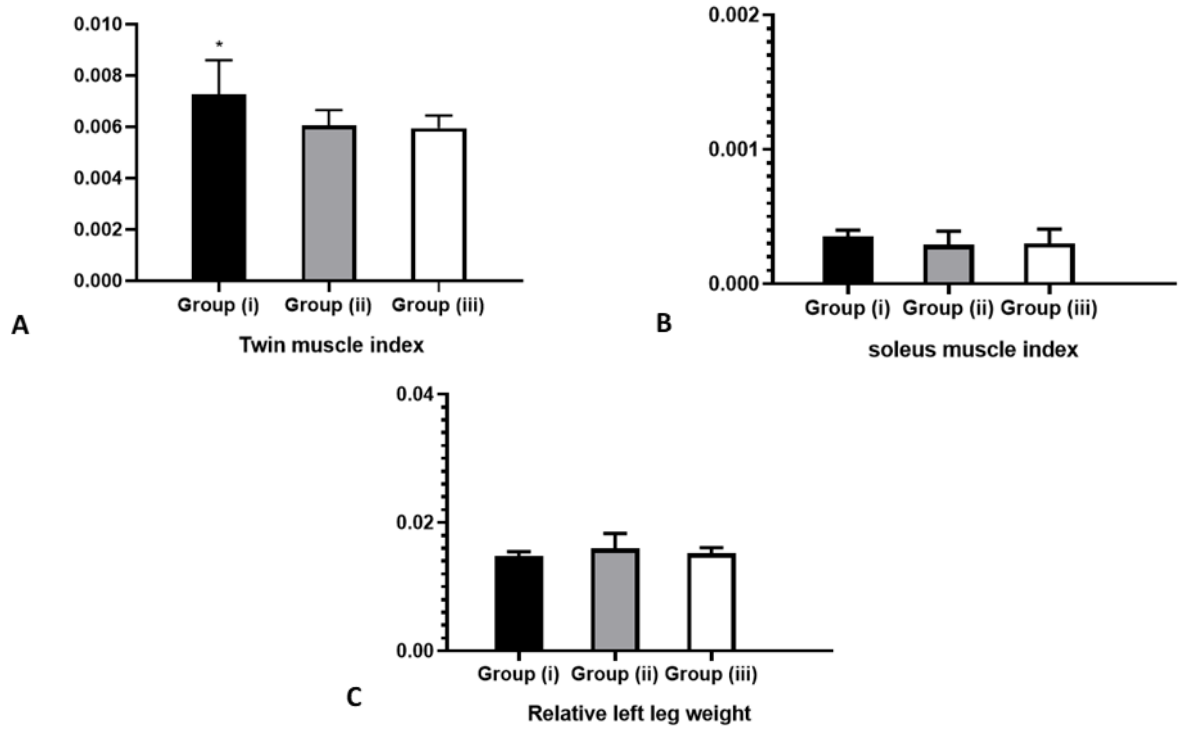

Figure 2. Relative weights in experimental groups. A: The Twin muscle. B: The Soleus muscle. C: The Left leg. Group (i): DNAi with saline injection group with resistance exercise, Group (ii) Saline injection group with resistance exercise (iii) Saline injection group without any exercise. Error bars indicate standard error. *: Significant with $P < 0.05$.

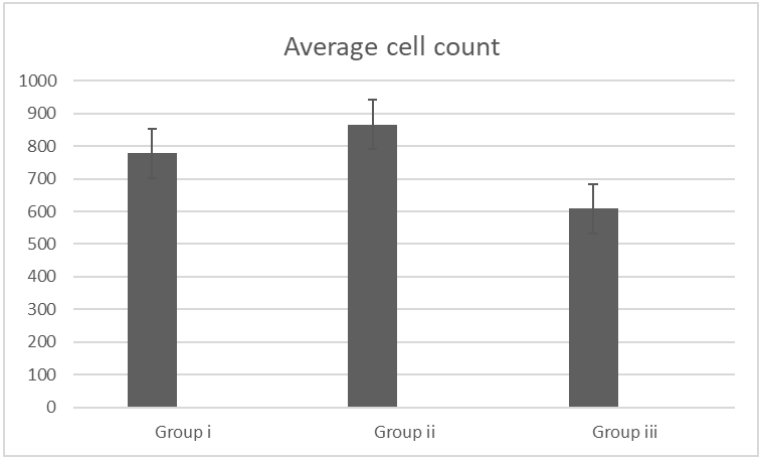

Figure 3. Muscle fiber density in twin muscle in the experimental three groups. Group (i): DNAi with saline injection group with resistance exercise, Group (ii) Saline injection group with resistance exercise (iii) Saline injection group without any excercise.

Although at the time of slaughter, we observed that carcass fat in group (i) was relatively lower than other groups (Figure 4). In the current study, we did not perform any evaluation on intramuscular fat.

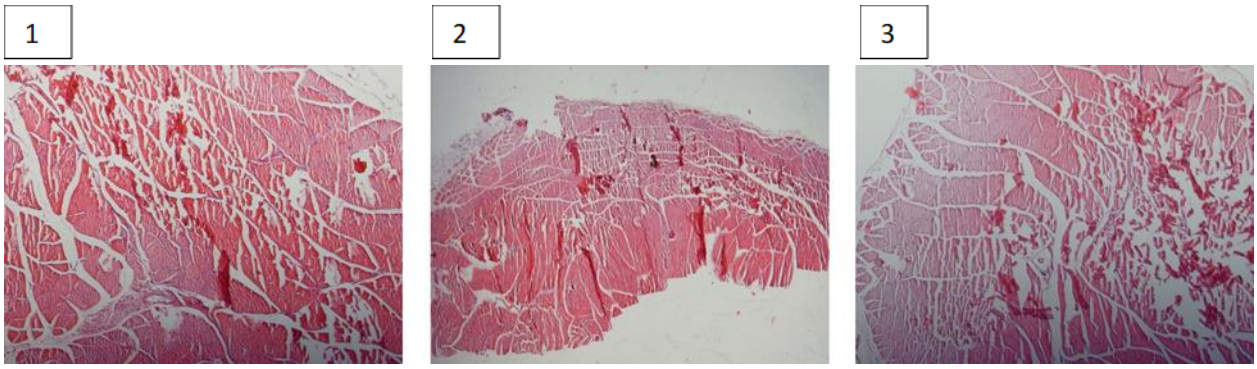

Figure 4. Soleus muscles histology of mice treated with DNAi along with resistance exercise (1), Saline injection group with resistance exercise (2), and Saline injection group (3)

Discussion

Silencing genes using oligonucleotides resembling DNA fragments is a promising strategy for inhibiting pathways related to specific diseases. Due to its reduced toxicity, side effects, and increased specificity, it has the potential to surpass standard drugs. DNA interference (DNAi) is a novel method of gene silencing. In this method, PCR products are injected to induce gene silencing. Therefore, DNAi is a simple and cost-effective approach because the preparation of PCR products is more straightforward than that of double-stranded RNA in the RNAi method (Omotezako et al., 2013). However, studies on DNAi are generally limited. Therefore, this study examines the effects of blocking MSTN transcription using DNAi with exercise on abdominal fat and leg muscle weight in male Wistar rats.

Previous studies have confirmed that DNAi inhibits myostatin activity under laboratory conditions. Payandeh et al. (2019), for the first time, investigated the inhibition of myostatin gene expression using the DNAi method in murine C2C12 cells. In this study, the length of DNAi was 29 nucleotides, designed as a complement to the myostatin gene promoter region, and transfected into C2C12 cells using the PEI-PEG carrier (Payande et al., 2019). The level of gene expression inhibition was evaluated at 24, 48, and 96 h post-transfection using qPCR. The results showed that the DNAi technique inhibited gene expression by approximately 70% within 96 h. Additionally, this method blocked myostatin gene expression by 80% and 50% at 48 h and 24 h, respectively, indicating the inhibition of myostatin activity by DNAi under in vitro conditions.

It is difficult to deliver oligonucleotides to target tissues, and they are easily digested by exo- and endonucleases in vivo, which are the main limitations of oligonucleotide (ON)-based therapeutics. To ensure that oligonucleotides are resistant to nuclease degradation without losing specificity, they must be structurally modified (Buck et al., 2002). These modifications should improve absorption, distribution, metabolism, and excretion properties, and they should be stable and non-toxic (Kontturi et al., 2019). Several modifications, such as methylphosphonate or phosphorothioate, have been proposed to confer resistance to nuclease digestion. The oligonucleotide phosphate backbone was replaced with sulfur instead of a non-bridging oxygen atom (Li et al., 2020; Odeh et al., 2019).

In a study by Riasi et al. (2023), the efficacy of phosphorothioate-modified (PS) and unmodified (WPS) DNAi molecules in inhibiting myostatin gene expression in mice was investigated (Riasi, Mozaffari-Jovin, et al., 2023). These researchers reported that intraperitoneal administration of modified DNAi could suppress myostatin expression by up to 70%. Leg weight and histological analysis demonstrated that the modified DNAi significantly suppressed myostatin gene expression in mice. Considering previous studies, the present study also utilized modified DNAi to enhance performance. In another study on silencing MSTn by exon skipping, weekly intravenous administrations of 100 nmol antisense phosphorodiamidate morpholino oligomers (PMO) did not reduce myostatin transcription; therefore, no significant effects were reported (Eilers et al., 2021). The injection duration in this study was five weeks. Intramuscular injection of PMO did affect MSTN expression significantly (Eilers et al., 2021). In recent years, ten anti-sense-based therapies have received market approval, nine of them were currently on the market. The majority of these anti-sense medicines target transcripts in the liver, because this tissue is the primary site of anti-sense oligo accumulation after systemic administration (Geary et al., 2015). Targeting of other tissues and transcripts has necessitated local and target-specific delivery. Therefore, injecting intraperitoneal might

not be as effective as intramuscular in the current research.

Several researchers have demonstrated that MSTN inactivation leads to increased skeletal muscle mass in mice following the intravenous and oral administration of specific MSTN oligonucleotides (Kinouchi et al., 2008; Li et al., 2008), which aligns with the results of the present study (Liu et al., 2008). Notably, the effect of increased weight gain in mice was particularly significant in the twin muscles. Researchers have suggested a potential explanation for the enhanced growth observed when therapeutic oligonucleotides are used with exercise, attributing this to increased serum follistatin, a potent regulator of skeletal muscle hypertrophy (Mosler et al., 2014).

Additionally, researchers have reported that blocking myostatin concurrently with exercise may produce synergistic effects, thereby enhancing the therapeutic benefit of myostatin inhibition in muscular disorders and metabolic diseases. However, scientific data to substantiate this claim are still scarce, and further investigation is required. Matsakas et al. (2010) stated that fiber hypertrophy, oxidative capacity, and glycolytic phenotype in myostatin-deficient muscles can be modulated by exercise training (Matsakas et al., 2010). Researchers claimed that the cross-sectional area of hypertrophic myofibers in Mstn−/− mice was reduced to wild-type levels in response to exercise. However, exercise training increased muscle force in Mstn−/− mice (Matsakas et al., 2012). In the present study, a potential explanation for the increased weight gain effect in the DNAi-treated group could be the protective mechanism against excessive hypertrophy. Similar results were observed by Mosler et al. (2012) in exercise-trained mice treated with metandienone (Mosler et al., 2012). This aspect warrants further investigation.

In the present study, a reduction in fat mass was observed, indicating that myostatin blockade could be used as a treatment for muscle-wasting diseases. Researchers have demonstrated that in addition to increasing muscle mass, myostatin inhibition leads to a decrease in fat mass in mice and double-muscled cattle (Lin et al., 2002; Wilkes et al., 2009), suggesting the potential application of myostatin inhibition in the treatment of diseases such as obesity and diabetes (Lin et al., 2002; Wilkes et al., 2009). Furthermore, researchers showed that taken together, oligonucleotide-mediated MSTN knockdown in combination with exercise stimulated skeletal muscle hypertrophy, decreased visceral fat, and modulated lipid metabolism (Mosler et al., 2014). It has been proposed that myostatin blockade may play a role akin to resistance exercise for individuals unable to exercise, as both resistance and endurance training have been shown to improve diabetes in humans (Castaneda et al., 2002). To improve outcomes, myostatin inhibition with exercise training was combined, and further prolonged research could provide a novel solution for metabolic disorders. Assessments of myostatin gene expression in different tissues could be explanatory.

Conclusion

In summary, the data from the present study suggest that muscle tissue growth via DNAi might be an effective strategy, particularly when combined with exercise training. Myostatin inhibition may offer novel therapeutic options and have implications in anti-doping research. Additionally, myostatin inhibition induces metabolic changes such as a reduction in body fat content and may have an impact on serum lipid profile. This highlights a potential for further investigation using myostatin inhibition techniques, such as DNAi, to stimulate skeletal muscle growth and treat muscle-related disorders.

Acknowledgments

This study was supported by the Ferdowsi University of Mashhad, grant number: 3/52696.

Conflict of Interest

The authors declare that there is no conflict of interest.

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