Research Article

Effect of Intramuscular and Intraperitoneal Injections of conjugated MSTN-siRNA-cholesterol on Inhibition of Myostatin Gene expression

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Received 20 July 2022 Accepted 18 September 2022

Abstract

Myostatin (MSTN) is primarily expressed in skeletal muscle tissue and acts as a negative regulator of skeletal muscle growth by inhibiting differentiation and proliferation of myoblasts. Inhibition of MSTN expression could result in muscular hypertrophy. An effective therapeutic approach based on specific silencing of a target gene is provided by RNA interference. The distribution of biologically active small interfering RNAs (siRNAs) inside the target cells/ tissue, is a significant problem due to the limited stability and delivery of siRNAs. Strategies depending on vector delivery have also a limited clinical utility due to safety concerns. Thus direct application of active siRNAs *in vivo* is the preferred strategy. We described the efficiency of intramuscular and intraperitoneal injections of MSTN-siRNA conjugated with cholesterol into the skeletal muscle of mice. The designed siRNA molecule was complementary to the exon II of the mouse MSTN gene. Mice were injected with a weekly dose of 10 µg/kg conjucated siRNA-cholestrol intraperitoneally or intramuscularly. Our findings suggested that within a few weeks of application, siRNA-treated mice showed a significant increase in muscle mass and suppressed MSTN gene expression. Even though both types of injections increased muscle weight, intramuscular siRNA injections suppressed the MSTN gene more effectively, whereas intraperitoneal RNA injections had a more significant impact on total body weight. The cholesterol-conjugated siRNA platform discussed here may hold promise for treating several skeletal muscle-related diseases, such as atrophic muscle disease, muscular dystrophy, and type II diabetes.

***Keywords*:** Cholesterol conjunction; Gene silencing; RNA interference; Transcription regulation

Introduction

Myostatin (MSTN; formerly known as GDF-8) was discovered in a search for novel mammalian members of the TGF- β (transforming growth factor- β) group of growth and differentiation proteins (McPherron et al., 1997). It is mainly expressed in skeletal muscle and functions as a negative regulator of skeletal muscle growth by inhibiting myoblast differentiation and proliferation (Soleimani, 2019). Because of a combination of muscle fiber hypertrophy and hyperplasia , mice with knockout MSTN gene had nearly a doubling of skeletal muscle mass across the body (McPherron and Lee, 1997). Natural MSTN gene mutations have also been found in cattle (Grobet et al., 1997), dogs (Mosher et al., 2007), sheep (Clop et al., 2006), pigs (Ji et al., 1998) and humans (Schuelke et al., 2004).

RNA interference (RNAi) has already become a standard approach for gene targeting. It is becoming increasingly important for therapeutic knockdown of pathologically important genes due to its considerable effectiveness and specificity. RNA-based gene silencing techniques can be used for various purposes, from validating targets to therapeutic implementations. It offers a viable option to pharmacological drugs, which are frequently associated with toxicity and off-target effects, as well as access to genes that were previously thought to be difficult to target or "undruggable" by pharmacologists. Since the discovery in 2001 showing that small interfering RNAs (siRNA) can be utilized for specific gene downregulation in mammalian cells (Elbashir et al., 2001), numerous researchers have effectively used this technology to knock down genes *in vivo* and *in vitro* (Martin and

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Caplen, 2007. Scherr and Eder, 2007). In cell lines and animal models, siRNAs are considered as practical tools for gene silencing. siRNAs are double-stranded RNAs with a length of 21–23 nucleotides that cause RNA-induced silencing complex (RISC) recruitment, cleavage, and breakdown of target mRNA molecules when they engage with a RISC (Bakhtiyari et al., 2013). Because RNAi works by limiting the expression of a specific target gene, it could be a safer approach for treating a wide range of hereditary and clinical illnesses. RNA interference (RNAi) allows researchers to investigate the consequences of shutting down a gene under controlled settings. Similarly, biologists were inspired to develop plasmids expressing short hairpins to target genes because of the function and structure of miRNAs, which are naturally expressed in form of short hairpins and processed into inhibitory molecules (Zeng et al., 2002. McManus, et al., 2002). Many genes have been effectively targeted using this strategy, including those in skeletal muscle cell lines and muscle tissue (Magee et al., 2006; Payande et al., 2019. Riasi et al., 2022).

On the other hand, recent developments in chemical modification of oligonucleotides led to invention of oligos with improved nuclease resistance, pharmacokinetics, gene specificity, and reduced immunostimulatory effects (Lares et al., 2010). Despite of significant advances and a few studies demonstrating systemic and targeted siRNA delivered *in vivo*, siRNA transport to the most tissues remains a crucial hurdle. Systemic delivery of siRNA has been used for liver, tumor, spleen, and jejunum (Kawakami et al., 2011. Kinouchi et al., 2008). A crucial requirement for successful delivery of systemic RNAi *in vivo* is the introduction of "drug-like" charachteristics, such as longer half-life, tissue bioavailability and cellular delivery, into chemically synthesized siRNAs. In inspecting the potential of synthetic siRNAs to silence desired genes, chemically modified siRNAs, including cholesterol conjugated, 2'-O-methyl sugar modified and antibody-linked siRNAs, have been found to improve pharmacological properties *in vitro* and *in vivo* markedly (Morrissey et al., 2005. Song et al., 2005). Covalent conjugation of cholesterol to siRNA improves the resistant activity of free siRNA to nucleases and facilitates cellular import, which results in the efficient silencing of target genes *in vivo* (Lorenz et al., 2004. Soutschek et al., 2004).

In the current study, we tried to investigate the effect of the type of injection (intraperitoneal or intramuscular) of siRNA on the inhibitory function of MSTN gene expression in a mouse model.

Materials and Methods

Design of siRNA molecule

The mouse MSTN gene sequence with accession number NC-000067.6 was achieved from NCBI database (National Center for Biotechnology Information). The siRNA was designed using the E-RNAi program. The non-specific target was made sure of using the siRNA-Blast online program. Microsynth conjugated RNA with cholesterol in three heads to improve its stability against the endonuclease enzyme (Syntech Co., Switzerland). Myostatin gene expression inhibition was monitored and measured using a negative control (siRNA negative; Sigma, Germany) . This negative control does not complement any part of the mouse genome (Table 1).

Experiment design and tissue preparation

Twenty-four male BALB/c mice were divide into two experimental and two control groups. Animals in this experiment conformed to all relevant animal testing and ethical research requirements. Experiments were carried out with the ethical approval of Ferdowsi University of Mashhad (Approval number 3/52696). The temperature was 22±1°C, with a 12-hour light-dark cycle and free access to water and food (Roozbeh et al., 2019). The experimental group 1 (n=6) received a weekly dose of RNAi of 10 µg/kg intraperitoneally. The same dose of RNAi was administered intramuscularly to the experimental group 2 (n=6). The scramble-RNAi was given intraperitoneally and intramuscularly to the control groups (n=12). Finally, the animals were quickly killed by exposing them to an increasing CO_2 concentration. Then muscles of the right biceps were removed. The tissue was sliced, and the upper half was immediately frozen in RNA shield (Dena Zist Asia, Iran) for RNA extraction, while the lower half was fixed in 10% formaldehyde for histological investigation. Until RNA extraction, all samples were stored at -80 °C. After the tissue was fixed in paraformaldehyde 10%, the biceps muscle was embedded in paraffin. On sections (7mm) cut and mounted on silanized glass slides, hematoxylin and eosin (H&E) staining was utilized (McKinnell et al., 2008). MyoVision software was used to count the cells (Wen et al., 2018).

Table 1. siRNA sequences selected to inhibit MSTN gene

	Sequence	Strand
SiRNA	5' UCAACAGUGUUUGUGCAAAUATT 3'	Sense
SiRNA	5' AAUAUUUGCACAAACACUGUUGA 3'	Antisense

RNA extraction and quantitative PCR

In 2 ml screw-cap tubes containing ten glass beads (3mm), 20-40 mg of biceps tissue was added. The Bioprep-24 Homogenizer homogenized the samples at 3500 g for two 15-second cycles with a 15-second pause period (Allsheng, China). Then, according to the manufacturer's recommendations, total RNA extraction and cDNA synthesis were carried out with the total RNA extraction kit and Easy cDNA synthesis kit (ParsTous, Iran) utilizing oligo d(t) and random hexamers. DNase I (Thermo Fisher Scientific, Austin, TX, USA) was used to treat RNA samples before cDNA synthesis, as directed by the manufacturer. The MIQE (Minimum information for publication of qPCR experiments) guidelines were followed for all qPCR reaction settings (Bustin et al., 2009). In a BioRad CFX96 TouchTM real-time PCR instrument, 2 µl of cDNA, 10 µl of 2X SYBR Green PCR Master Mix (Ampliqon, Odense, Denmark), and 5 pM of each primer were mixed in 0.1-ml 8-strip tubes (Gunster Biotech, Viluppuram, Taiwan) in a final volume of 20 µl. (Bio-Rad Laboratories Inc., Singapore). The experiment performs under the following condition: an initial denaturation step (95°C for 3min); 45 cycles of amplification, including (95°C for 30s, 58°C for 25s, and 72°C for 20s). The specificity of amplicons was validated using melting curve analysis (60 to 95 °C). The slope of the standard curve was used to compute the reaction efficiency (efficiency = (10 (1/slope) – 1) 100). The R^2 correlation coefficients were used to make the decision. The geometric average of the GAPDH and B-actin reference genes was used to normalize the relative replication of MSTN transcript (Ruan and Lai, 2007. Ghanipour-Samami et al., 2018. Danesh Mesgaran et al., 2021). Primer sequences are shown in Table 2.

Statistical analyses

Student's t-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 presented as mean ± standard error (SE).

Results

Relative leg weights

The results of body weight measurement in the fourth week before killing the mice in group 1 showed a 10% difference between the treatment and control groups. In contrast, intramuscular injection (group 2) had no significant effect on increasing body weight. The efficiency of the MSTN-siRNA/cholesterol conjugation molecule and its impact on increasing muscle growth was therefore assessed using the muscle weight/body index (Figure 1).

Histological analysis

Histology images were prepared as previously described. The results of myonuclear counting showed that there was a significant difference at 95% and 99% between groups which received RNAi interaperitoneally and interamuscularly in comparsion to the control group, respectively (Table 3) (Figure 2). The results demonstrated that intramuscular injection of the conjugated MSTN-siRNA/cholestrol induced muscle enlargement by suppressing the MTSN gene expression.

Table 2. Primers used in the quantitative RT-PCR experiments

Gene name	Sequence	Amplicon length (bp)	Accession number
MSTN	F:5' TCCAGAGGGATGACAGCAGT 3' R: 5' GGGCTTTTACTACTTTGTTGTACTGT 3'	177	NM_010834
GAPDH	F: 5' GAGAAACCTGCCAAGTATGATG 3' R:5' CATACCAGGAAATGAGCTTGACA 3'	196	NM_001289726
B-actin	F: 5'CTCTGGCTCCTAGCACCATGAAGA3' R: 5' GTAAAACGCAGCTCAGTAACAGTCC 3'	200	NM_028135

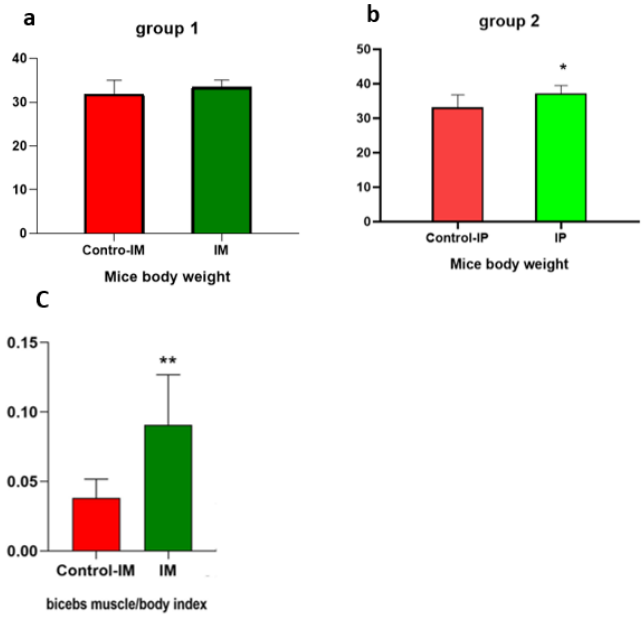

Figure 1. Intramuscular and intraperitoneal administration of the MSTN-siRNA/cholesterol conjugation induces muscle enlargement in the mouse by blocking of MSTN gene expression. After three weeks, the body weight of mice in each group was measured and compared to the control. (a) Body weight change after intramuscular injection, (b) body weight change after intraperitoneal injection, (c) comparison of muscle weight/body weight index between the MSTN-siRNA/cholesterol conjugation and control mice (group 2 0.094±0.038 versus 0.041±0.024g). Graphical representation of data uses the following convention: mean±s.d.; treated muscles in green; control muscles in red.

Figure 2. Muscle fiber density in biceps (a) control (b) right biceps muscles injected with MSTN-siRNA/cholesterol conjugation interaperitoneally (c) right biceps muscles injected with MSTN-siRNA/cholesterol conjugation intramuscularly. Serial sections (7 mm thickness) were cut and stained. (Scale bar, 100 µm)

Table 3. Results of cell counting in biceps muscle tissue of control and treated legs.

Group	mean	*P*-value
RNAi- IP	261	0.0296*
Control- IP	117	
RNAi- IM	360	0.010**
Control- IM	123	

Gene expression

The qPCR reaction results were confirmed by melt curve analysis and agarose gel electrophoresis (Supplementary Figures 1 and 2). The qPCR showed a 66% and 90% decrease in MSTN expression in muscle tissue treated with Mstn-siRNA/cholesterol conjugation interaperitoneally and interamuscular compared to the control (Figure 3). We observed a significant difference in MSTN gene expression when MSTN-siRNA was injected interamuscularlly in mice.

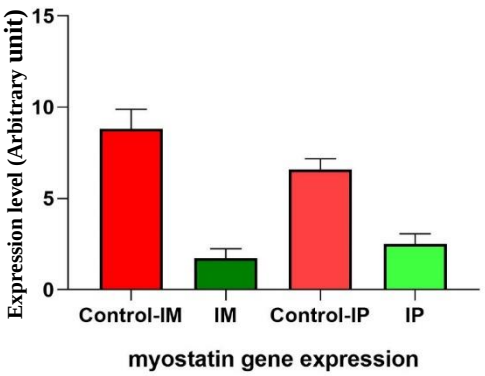

Figure 3. MSTN gene expression: MSTN mRNA expression in groups treated by MSTN-siRNA/cholesterol conjugation interaperitoneally, MSTN-siRNA/cholesterol conjugation intramuscularly and the control. MSTN expression was normalized using the geometric averages of GAPDH and b-actin. **p < 0.01

Discussion

A promising tool for the suppressing of particular disease pathways is the gene-silencing methodology based on RNAi. Due to the possibility of more excellent selectivity and decreased toxicity and side effects, it has the potential to be more effective than conventional pharmacological medications. Skeletal muscles are essential for morphofunctional function, and their atrophy results in severe illnesses like muscular dystrophy. One in every 3500 male births is affected by Duchenne muscular dystrophy (DMD), a severe condition that causes muscle atrophy (Laws et al., 2008). Although there is no treatment at the moment, gene therapy techniques are promising new directions for the creation of medications(Foster et al., 2006). Anti-myostatin-blocking antibodies are one of the therapeutic strategies used to improve muscle mass. These antibodies suppress MSTN (Bogdanovich et al., 2002). However, it requires a considerable amount of time and effort to produce antibodies against recombinant target proteins. MSTN suppression caused by overexpression of the MSTN prodomain12 in mice used as models for limb-girdle muscular dystrophy 1C was demonstrated to minimize muscle atrophy and correct intracellular MSTN signaling (Ohsawa et al., 2006). On the other hand, Magee et al. showed that increasing local skeletal muscle mass by electroporating a plasmid carrying a short-hairpin interfering RNA (shRNA) against MSTN could downregulate MSTN production (Magee et al., 2006). However, techniques based on vector administration may only have limited therapeutic utility due to safety concerns. Applying active siRNAs *in vivo* directly is the preferred strategy. We looked for a more suitable application with higher cholesterol-conjugated siRNAs effectiveness as one of the feasible platforms delivery of siRNA. Other *in vivo* investigations have used cholesterol-conjugated siRNAs to show uptake into the heart, kidney, adipose, liver, small intestine, and lung. Few published studies on the distribution of chol-siRNA to the muscle by systemic dose exist (Soutschek et al., 2004; Wolfrum et al., 2007; Soutschek et al., 2004). Therefore, in the current research, we evaluated local and systematic injections to determine whether the method of administration of DNAi could affect the efficiency of MSTN inhibition.

We made use of the siRNA sequences that were mentioned in previous research by Payande. (Payande, 2019) at Ferdowsi University in Mashhad. They transfected it into C2C12 cell line (a mouse myoblast cell line) that had been induced to express MSTN to demonstrate in that work that the present MSTN-siRNA/cholesterol conjugation could effectively downregulate the MSTN expression. The findings of this study demonstrated the remarkable efficacy of siRNA sequences in reducing gene expression while having no detrimental changes in cell morphology. Then we injected MSTN-siRNA/cholesterol conjugation in mice, as mentioned earlier.

Our weight findings validated the decrease in the muscles treated with MSTN cholesterol-conjugated siRNAs (Figure 1). The muscles in the treated legs are noticeably larger than the controls, although the body weights for the group 1 and its control were 37.20±0.7 and 33.19±0.4 g and for group 2 and its control were 33.45±0.2 and 31.85±0.5, respectively. Khan et al. conducted a study on the systemic administration of cholesterol-conjugated siRNA targeting the muscle-specific MSTN gene. They measured the expression of MSTN in several mouse muscles (Khan et al., 2016). Their findings indicate that MSTN cholesterol-conjugated siRNAs raise body weight by 10% while also increasing leg muscle size by up to 20%. When they measured the shape of the cell, they saw hypertrophy rather than hyperplasia. These confirmed all the results obtained in our research at the time of MSTN-siRNA/cholesterol conjugation intraperitoneal injection. On the other hand, no increase in body weight was seen in the treated mice in group 2, most likely, since the gain in muscle weight offset the reduction in fat formation (McPherron and Lee,

2002). When the body weight varied, we considered the muscle weight/body weight ratio to demonstrate a gain in muscle, similar to a research performed by Kinouch et al. (Kinouchi, et al., 2008). They administered the Mst-siRNA/ATCOL complex intramuscularly to 20-week-old male mdx mice, into the tibial and masseter muscles on the left side. An enormous increase in muscle mass was seen two weeks following the single injection. Morphometric measurements show both hypertrophy and hyperplasia in muscle. These findings from our investigation, comparable to those from the previous study, demonstrated the value of intramuscular injections for promoting muscle growth.

In our model, fiber size analysis and H&E staining of histological preparations reveal both hyperplasia and hypertrophy in the treatment group compared to the control group. Even though, MSTN knockout mice exhibit both muscle hypertrophy and hyperplasia, the differences between our model and the MSTN-knockout model were most likely caused by the nearly complete absence of MSTN throughout the entire development of MSTN knockout mice as opposed to "acute" MSTN inhibition in adult animals for siRNA targeting (Lee and McPherron, 2001). Our findings from siRNA injections into the intramuscular space are in line with several gene knockdown and MSTN inhibition or studies, which also demonstrate only increased muscle growth (Whittemore et al., 2003).

Gene expression data in our study showed that intramuscular injection of RNA into the target tissue was able to silence the gene with a better performance of about 80%. In comparison, intraperitoneal injection inhibited the expression with 60% efficiency. Our gene expression result of interamuscular injection of siRNA was in line with the Khan study. They observe 80-90% MSTN suppression in soleus muscle. It was possible that we would get more similar results if we used different muscle tissues in our study. Alternatively, if we used higher doses of siRNA, the rate of inhibition of gene expression in systemic injection would be higher. In Khan's study, it was noted that using higher doses of siRNA was more effective in inhibiting gene expression. On the other hand, in Kinouchi experiment, systematic delivery of siRNA knockdown MSTN gene by 25% during three weeks. Since the MSTN chol-siRNA used in the current investigation was a simple conjugate that could be injected intramuscularly and interaperitoneally, there is no need for a technically challenging delivery mechanism or complex formulations. The cholesterol conjugate can deliver to skeletal muscles and silence genes there as well. In mice, it demonstrates long-lasting silencing after a single dosage. The cholesterol-siRNA conjugates used in our study were a helpful tool for *in vivo* target validation studies. They may one day be therapeutically effective for treating some of skeletal muscle disorders.

Conclusion

Finally, the results of the current study demonsterated that the use of cholesterol-conjugated siRNA could effectively suppress the MSTN gene in mice within three weeks. However, both types of intramuscular and peritoneal injection led to an increase in skeletal muscle weight and significant inhibition of gene expression. However, it appears that future studies with greater injection doses and more frequent injections are required in order to precisely recommend one of the two methods of intramuscular and peritoneal injection.

Acknowledgments

This study was supported by the Ferdowsi University of Mashhad, grant number: 3/52696. The authors would like to thank the Kavosh Gene Sarvdasht company (Mashhad, Iran) for the technical assistance.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Supplementary Files:

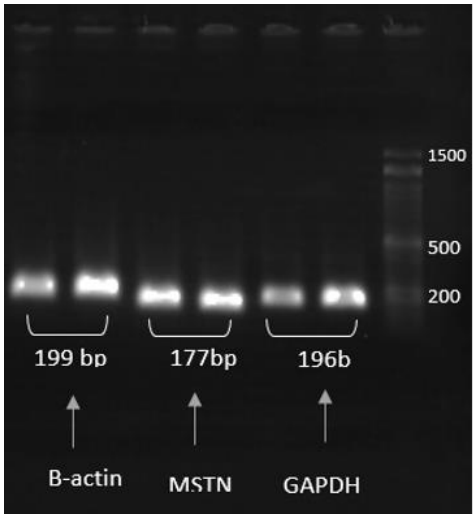

Figure S1. Agarose electrophoresis of qPCR products from GAPDH, MSTN and B-actin genes.

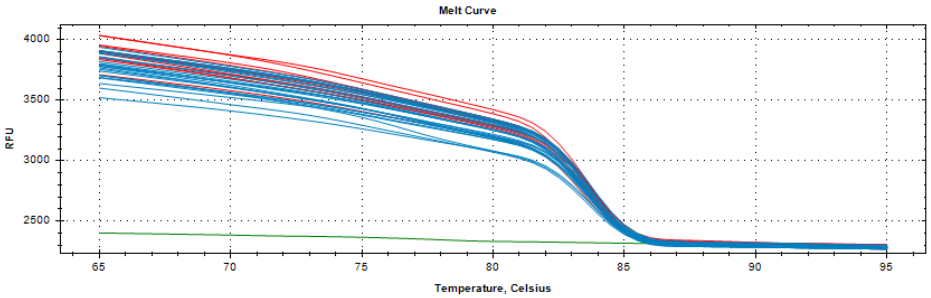

Figure S2. Melt curve analysis of mouse MSTN transcript (standard: red lines, samples: blue lines and negative control: green)