Long-Term Rescue of Dystrophin Expression and Improvement in Muscle Pathology and Function in Dystrophic *Mdx* Mice by Peptide-Conjugated Morpholino

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Abstract

Exon skipping is capable of correcting frame-shift and nonsense mutations of Duchenne Muscular Dystrophy (DMD). Phase II clinical trials in UK and Netherlands have reported induction of dystrophin expression in muscles of DMD patients by systemic administrations of both phosphorodiamidate morpholino oligomers (PMO) and 2'O methyl phosphorothioate. Peptide-conjugated PMO (PPMO) offers significantly higher efficiency than PMO with the ability to induce near normal levels of dystrophin and restores functions in both skeletal and cardiac muscles. Here, we examined one year systemic efficacy of PPMO targeting exon 23 in dystrophic *mdx* mice. LD50 of the PPMO was approximately 85mg/kg. Half life of the dystrophin expression was about 2 months in skeletal muscles but shorter in cardiac muscle. Biweekly injection of 6 mg/kg PPMO produced higher than 20% dystrophin expression in all skeletal muscles, and up to 5% in cardiac muscles with improvement in muscle function and pathology, and reduction in the levels of serum creatine kinase (CK). Monthly injections of 30mg/kg PPMO restored dystrophin to more than 50% normal levels in all of skeletal muscles, but 15% in cardiac muscle. This was associated with greatly reduced serum CK levels, near normal histology, and functional improvement of skeletal muscles. Our result demonstrated for the first time that one year regular administration of PPMO could be safely applied to achieve significant therapeutic effect in animal models.

Introduction

Duchenne muscular dystrophy (DMD) is the most common and lethal muscle disorder with onset in early childhood and no effective treatment is available. DMD is mainly caused by nonsense and frame-shift mutations of dystrophin gene, leading to the lack of functional dystrophin protein in muscles. Becker muscular dystrophy (BMD), an allelic form of DMD, is caused by mutations that typically create shortened, but in-frame transcripts with production of partially functional dystrophin, leading to patients with variable or no symptoms.[1-3]

Antisense oligomer-mediated exon skipping has been demonstrated with high potential for restoration of dystrophin gene open reading frame and induction of BMD-like functional dystrophin. Significant progress in exon skipping for DMD has been made in the last decade.[4-28] Therapeutic potential of exon skipping was first demonstrated in dystrophic *mdx* mice, a model of DMD, by intramuscular injections (*i.m.*).[12] Since then, systemic efficacy with functional improvement in muscles has been demonstrated in both dystrophic mouse and dog with PMO as the chemistry of antisense oligomers.[14,18] Phase I clinical trials targeting human dystrophin exon 51 demonstrated dystrophin expression in muscles injected with specific antisense oligonucleotide (AO) of PMO and 2'O methyl phosphorothioate (2OMePS).[19,20] Systemic administration of antisense drug PRO051 (2OMePS) has been conducted by Prosensa in DMD patients with a weekly subcutaneous injection regimen for 17 weeks. Exon 51 skipping and dystrophin expression were reported in a dose-related manner in all cohorts (0.5 mg/kg, 2 mg/kg, 4 mg/kg, 6 mg/kg).[21] More recently, a Phase II clinical trial with repeated weekly *i.v.* injections of PMO targeting the same

human dystrophin exon 51 reported encouraging preliminary results. Up to 20mg/kg for each injection of AVI-4658 showed a dose-dependent restoration of dystrophin expression.[22] At the dosages so far tested, both of chemistries appeared to be well tolerated although functional consequence and accumulative effect of long-term treatment remain to be demonstrated.

Limitations for use of the two chemistries, PMO and 2OMePS currently on clinical trials, are low efficiency and high variability in exon skipping and dystrophin induction in all muscles as revealed by the results from both animal models and the clinical trials. It is an especial concern for the very low efficiency of exon skipping in the cardiac muscle which is severely affected by the lack of dystrophin expression in DMD boys. Results from animal model studies suggest that a detectable dystrophin induction in cardiac muscle will require the dosage with PMO at higher than 60mg/kg biweekly injection. [23] This has lead to the use of cationic peptides and other polymers to improve the efficiency of PMO delivery.[15,16,24-28] PMOs conjugated with an arginine-rich peptide (PPMO) were able to restore dystrophin expression to near normal levels in body-wide skeletal muscles and to about 50% in cardiac muscle at the dose of 30mg/kg by single injection. Such high levels of dystrophin expression significantly improved functions of both dystrophic skeletal and cardiac muscles.[15] However, it is well documented that the use of positively charged peptides and polymers increases toxicity considerably.[15,16,26-28] This together with the requirement of a life-long AO drug administration for treating DMD necessitates investigation of these modified PMOs for their long-term applicability and efficacy in relevant animal models *in vivo*.

In the present study, we investigated the acute toxicity and dose-related one year efficacy of PPMO treatment targeting mouse dystrophin exon 23 systemically in the dystrophic *mdx* mice. Our results show that PPMO has a high level of acute toxicity with LD50 near 85mg/kg. However, the effective dose for inducing more than 20% dystrophin in skeletal muscles and 5% dystrophin in cardiac muscle requires only 6mg/kg biweekly injection, at which no obvious acute and chronic side effect were detected. PPMO therefore could be an effective chemistry as AO drugs for long-term treatment of DMD.

Material and Methods

Animals, oligonucleotides and in vivo delivery methods

Ten *mdx* mice and C57BL/6 (C57) mice aged 4-5 weeks were used in each group. Experiments were approved by IACUC Carolinas Medical Center. The phosphorodiamidate morpholino oligomer (+07-18) (5'- GGCCAAACCTCG GCTTACCTGAAAT- 3') was used against the boundary sequences of exon and intron 23 of dystrophin gene and conjugated to the peptide $(RXRRBR)_2XB$ (R = arginine, X=6-amino hexanoic acid and B = β-alanine) through a non-cleavage amide linker to form a peptide-PMO conjugate (PPMO) (AVI BioPharma, Bothell, WA). For intravenous administration, PPMO was used in 100 µl saline by retroorbital injections, while control *mdx* mice were injected with 100 µl saline only. Mice were killed at desired time points, and muscles were snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C.

Antibodies and Immunohistochemistry

Sections of 6 µm were cut from at least two-thirds of muscle length of TA, quadriceps, biceps, and gastrocnemius at 100 µm intervals and at least 6 levels from all other muscles including heart, diaphragm, intercostals, and abdominal muscles at 100 µm intervals. The intervening muscle sections were collected for Western blot and RT-PCR analysis. The serial sections were stained with rabbit polyclonal antibody P7 against dystrophin. The primary antibody was detected by goat-anti-rabbit IgGs Alexa 594 (Invitrogen, Eugene, OR). Sections were also stained with hematoxylin and eosin for histological assessment.

Protein Extraction and Western Blot

The collected sections were ground into powder and lysed with 200 µl protein extraction buffer as described previously.[15,16] The protein concentration was quantified by Protein Assay Kit (Bio-Rad, Hercules, CA). Proteins were loaded onto a 4-15% Tris-HCL gradient gel. Samples were electrophoresed overnight at 10 mA at 4°C and blotted onto nitrocellulose membrane overnight at 50 V. The membrane was then washed and blocked with 5% skimmed milk and probed with monoclonal antibody NCL-DYS1 against dystrophin rod domain (Vector Labs, Burlingame, CA) overnight. Antibody against KIM-1 (R&D Systems, Minneapolis, MN) was used for detecting kidney injury molecule-1 (KIM-1) expression in the kidney. The bound primary antibody was detected by horseradish peroxidase-conjugated goat anti-mouse IgG for dystrophin (Santa Cruz Biotechnology, Santa Cruz, CA) or horseradish peroxidase-conjugated donkey anti-goat IgG for KIM-1 (Millipore, Billerica, MA), and ECL Western Blotting Analysis System (Perkin Elmer, Waltham, MA). The intensity of the bands obtained from the AO-treated mice muscles was measured and compared with that from normal muscles of C57 mice (NIH ImageJ 1.42 software).

RNA Extraction and RT-PCR

The collected Sections were homogenized in TRIzol (Invitrogen) by using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). Total RNA was then extracted and 100 ng of RNA template was used for a 50-µl RT-PCR with RT-PCR Master Mix (USB, Cleveland,OH). The primer sequences for the RT-PCR were Ex20Fo 5'-AGAATTCTGCCAATTGCTGAG-3' and Ex26Ro 5'-TCTTCAGCTTGTGTCATCC-3' for amplification of mRNA from exons 20 to 26. A

total of 40 cycles were carried out for the RT-PCR. Bands with the expected size for the transcript with exon 23 deleted were extracted and sequenced. The intensity of the bands was measured with the NIH ImageJ 1.42 and percentage of exon skipping was calculated with the intensity of the two bands representing both exon 23 unskipped and skipped as 100%.

Grip strength test

Grip Strength was assessed using grip strength meter consisting of horizontal forelimb mesh and an angled hind limb mesh (Columbus Instruments, Columbus OH). Five successful hind limb and fore limb strength measurements within 2 minutes were recorded, and data were normalized to body weight and expressed as kilogram force (KGF).

Measurement of serum creatine kinase and other components

Mouse blood was taken immediately after cervical dislocation and centrifuged at 1500 rpm for 3 min. Serum was separated and stored at -80°C. The level of serum components was determined by Charles Riverside Laboratories.

Results

LD50 of PPMO in mdx mice.

Our previous studies have shown that 30mg/kg PPMO was able to induce almost normal level of dystrophin expression in all body wide muscles of *mdx* mice without clearly observable acute or chronic toxicity, even after 6 times of biweekly (fortnightly) intravenous injection.[15] However, acute toxicity remains a serious concern and especially a repeated life long administration is required for treating DMD. We therefore firstly tested the same PPMO targeting *mdx* mouse exon 23 by dose escalation from 6mg/kg to determine the LD50. All survived mice were sacrificed two weeks after injection. As previously reported, all mice injected with 6mg/kg PPMO survived without any observable difference from saline injected control mice. All mice receiving 30mg/kg PPMO also survived without loss of body weight. The only observable change was the reduced activity during the first 2 hours after the injection in 3 of this group of mice. However when the dose of PPMO increased to 60mg/kg, all mice showed reduced activity after recovered from the anesthesia. 2 of 10 treated *mdx* mice died in 5 minutes after the intravenous injection, and another one died within 24 hour. Further increasing PPMO to 90mg/kg and 120mg/kg leaded to the death of 5 and 8 out of 10 mice within 2 days after injection respectively (Figure 1). The results therefore showed that LD50 of PPMO in *mdx* mice was approximately 85mg/kg.

Half life of the dystrophin expression after single i.v. administration of 30mg/kg PPMO

Our previous study demonstrated that single *i.v.* injection of 30mg/kg PPMO induced 100% and 50% normal levels of dystrophin expression in bodywide skeletal muscles and cardiac muscle respectively two weeks after injection. [15] To assess the possible half life of dystrophin expression, we applied the same dose of 30mg/kg of the PPMO targeting exon 23 by single *i.v.* injection into *mdx* mice and examined the levels of dystrophin expression at a series of time points, ranging from 2 days, 2 weeks, 1 month, 1.5 months, 2.5months, 4 months to 5 months after the injections. Two days after *i.v.* injection, nearly 100% efficiency in exon 23 skipping was detected with up to 50% dystrophin positive fibers and approximately 20% normal levels of dystrophin protein expression by western blot in all of the skeletal muscles examined. As high as 40% efficiency in exon 23 skipping was detected in the cardiac muscles. However, dystrophin protein was not convincingly detected by western blot (Figure 2). By two weeks, dystrophin expression in skeletal muscles was detected in nearly 100% muscle fibers with the protein reaching 80% of normal levels or higher.[15] Similar levels of dystrophin protein and percentage of dystrophin positive fibers were maintained up to two and a half months after the single injection. However, the percentage of exon skipping efficiency was significantly reduced, down to about 50% at two and a half months after the injection. The levels of dystrophin decreased to 30% and 10% of normal levels at 4 and 5 months after the injection respectively. In the cardiac muscle, dystrophin expression induced by the single dose PPMO reached peak of proximately 70% of normal levels at 1 month after the injection, and then decreased to less than 50% and 25% at 1.5 and 2.5 months after the injection respectively. No dystrophin was detected at 4 and 5 months in cardiac

muscles by both immunohistochemistry and western blot (Figure 2). The data therefore indicates that PPMO induces dystrophin expression about 2 times more efficiently in skeletal muscles than in cardiac muscle. Furthermore, the half life in the level of dystrophin expression was also longer in the skeletal muscles than in the cardiac muscle. Consistent to our previous results, expression of high levels of dystrophin after single 30mg/kg PPMO treatment improved muscle pathology of the *mdx* mice (Figure 3). The percentage of uncentranucleated muscle fibers increased time-dependently from 20% to 40% from 1 month to 4 months after the single dose treatment and went back to 30% by 5 months in most of the skeletal muscles except diaphragm. Area of muscle degeneration and mononucleocyte infiltration was absent in all muscles, including the TA, Quadriceps, Biceps and gastrocnemius examined between 2 weeks and 5 months after the treatment. In diaphragm, the percentage of uncentranucleated fibers also increased to the highest level by 2.5 months, but then decreased again by 4 months. This was associated with the increase of areas with degenerating fibers and foci of mononucleocyte infiltration. Thus single dose of PPMO treatment provides a shorter period of protection to diaphragm from degeneration than to other skeletal muscles. Serum creatine kinase (CK) levels were reduced significantly and remained at the similar low levels from 1 month to 5 months after the injection (Figure 3).

Biweekly administration of 1.5 mg/kg PPMO for 12 months produced limited dystrophin induction and improvement in muscle function

The high efficiency of PPMO for dystrophin induction, but with a relatively Low LD 50 demands a low dose regimen for treating DMD. We hypothesized that repeated low dose PPMO administration might be sufficient to induce dystrophin expression with functional significance. The *mdx* mice were therefore treated with the PPMO at the dose of 1.5mg/kg with biweekly *i.v.* injection for one year (Figure 4). Immunohistochemistry for dystrophin expression 2 weeks after the final injection showed less than 20% muscle fibers expressing detectable dystrophin, most of them with weak signals in bodywide skeletal muscles. The amount of dystrophin protein examined by western blots was from barely detectable to less than 5% of normal level in any skeletal muscle. This amount of protein was similar to that detected in the muscles after single *i.v.* injection of 1.5mg/kg PPMO, suggesting no significant accumulation of efficiency after one year treatment. No clear difference in pathology including % of fibers with central nucleation and variation in fiber size in skeletal muscles was observed (Figure 5). Consistently the CK levels, the function of the skeletal muscles measured by grip force generation of the treated group were not significantly improved (Figure 5). No dystrophin expression and exon 23 skipping were detected in the cardiac muscle by immunohistochemistry, western blot and RT-PCR (Figure 4). Body weight, serum enzyme tests and histological examination of lung, kidney and liver showed no clear difference between PPMO-treated and control *mdx* mice (Figure 5).

Administration of 6mg/kg PPMO induced high levels of dystrophin expression with improvement of muscle pathology and function

We then examined the long-term effect of higher dosage of 6mg/kg PPMO by regular biweekly *i.v.* injections (Figure 4). Skeletal muscles after 1 year treatment exhibited more than 60% and up to 90% dystrophin-positive fibers in skeletal muscles. Considerable variation in levels of dystrophin staining was also clearly detected in all muscles. The levels of dystrophin expression detected by western blot ranged from 20% to 50% of normal levels. RT-PCR showed the levels of exon 23 skipping from 15% to 50% in the skeletal muscles. Histologically, treated skeletal muscles exhibited clearly detectable improvement with reduced central nucleation and a more homogenous population of fibers in size (Figure 5). However, sporadic foci of degeneration and regeneration and monocyte infiltration remained in all muscles. Significant improvement was also detected in muscle functions with grip force generation (Figure 5). This was supported by the significant reduction in CK levels when compared to control *mdx* mice, although still higher than that of normal C57 mice (Figure 5). Expression of dystrophin in cardiac muscle was clearly detectable in a proportion of fibers with weak and discontinuous membrane staining by immunohistochemistry, but was less than 5% of normal levels (Figure 4). As described above in the 1.5mg/kg PPMO treated group, 6mg/kg PPMO did not show clear difference in body weight, serum enzyme tests and histological examination of lung, kidney and liver when compared with control *mdx* mice (Figure 5). These results suggest that the effect of PPMO treatment at this dosage can achieve measurable therapeutic outcome in skeletal muscles without detectable toxicity.

30mg/kg PPMO monthly treatment restores near normal levels of dystrophin with correction of muscle pathology and function

With the demonstration that the half life of dystrophin induced by single *i.v.* injection of 30mg/kg PPMO was about 2 months, we designed one year treatment of 30mg/kg PPMO with a monthly *i.v.* injection. One year treatment was able to achieve dystrophin expression in near 100% muscle fibers in body-wide skeletal muscles. Western blot detected more than 50% of normal levels of dystrophin protein in most skeletal muscles although as low as 25% was also detected (Figure 4). Muscle pathology was clearly improved with almost no degenerating fibers in any skeletal muscles examined (Figure 5). The fibers within the same muscle became highly uniform and with no clear inflammatory cell infiltrations. Foci of degeneration and regeneration and monocyte infiltration, which remained in muscles after lower dose treatment, were absent in the diaphragm. Consistently, CK levels were significantly reduced when compared to control *mdx* mice (Figure 5). Muscle functions as measured by grip force generation were significantly improved (Figure 5). The levels of dystrophin expression in the cardiac muscle were considerably lower than that detected in the skeletal muscles, but dystrophin expression with clearly detectable signal was observed in more than 50 % cardiac muscle fibers and the amount of dystrophin protein reached 15% in all heart of the PPMO treated mice (Figure 4). The mice under this dosage treatment showed no sign of abnormal body weight change when compared to the control *mdx* mice. No pathologic change of the liver, kidney and lung was observed by H&E staining (Figure 5). This was supported by the serum tests showing normal levels of creatinine, total bilirubin, alkaline

phosphatase, Gamma-glutamyltransferase, and Blood Urea Nitrogen (BUN) (Figure 5). We also examined the levels of kidney injury molecule-1 (KIM-1) expression in the kidney of the PPMO treated mice by western blots. All the kidneys from both 1.5mg/kg and 30mg/kg PPMO treated mice showed barely detectable levels of KIM-1 expression similar to those from untreated mdx and C57 control mice (Supplementary Figure 1).

Discussion

Cationic peptides have been widely used for gene and oligonucleotide delivery and nick named cell penetrating peptides as they are highly efficient for gene and oligonucleotide delivery in cultured cells. However, successful applications of such polymers *in vivo* have been limited. One of the barriers for this limitation is their toxicity *in vivo*.[15,24-28] Data from previous studies in mouse models showed that dosages inducing near normal levels of dystrophin expression by the PPMO (containing cationic peptide, $(RXRRBR)_2XB$ sequence (R = arginine, X = 6-aminohexanoic acid and B = β alanine) did not cause obvious short-term toxicity.[15] PPMO showed no toxic effect at either 20 mg/kg 6 times weekly injections to the wild-type mice or 30 mg/kg 3 month biweekly injection to *mdx* mice.[15, 27] At these dosages, the side effect was detected in some mice including the acute lethargy which can last up to a few hours after systemic injection. There was no clear toxicity in the kidney and liver assessed by both histology and the levels of serum enzymes at the end of the PPMO intervention. However, toxicity of PPMO has been reported as weight loss and tubular degeneration in kidney.[26-28] The results from the present study confirmed that PPMO at 30mg/kg by monthly injections only caused mild lethargy in some mice immediately after the systemic injection. However, further increase of the dose of the PPMO to 60mg/Kg leaded to some death of the treated mice within 48 hours and the remaining mice showed clear lethargy within 2-4 days. More than 80% animals died when the dose was 120mg/kg by a single injection. This result suggests that LD50 of the PPMO was about 85mg/kg. All the data together supports the notion raised by Moulton et al. that there seems to be a dose

threshold for the toxicity of PPMO: below the threshold, the acute toxicity can not be clearly observed; above it, the severity of toxicity increases rapidly in a dose-dependent manner. The severity of toxicity is also suggested to be dependent on the dose frequency.[27] One specific concern for PPMO is its potential chronic toxicity to kidney. The same peptide conjugated PMO, AVI-5038, as used in this study but targeting human exon 50 , was reported to cause tubular degeneration (although mild) in the kidneys of monkeys after 4 times of only 9 mg/kg weekly injections.[27,28] Elevated BUN levels were also reported in rat treated with a similar peptide-conjugated PPMO. [28] However, no clear kidney damage was indicated by histology, tests of serum enzyme including BUN and detection of KIM-1 in this study. While reason(s) for such discrepancy is not understood, two factors are most likely involved. The current study examined the kidney 2 weeks after the last treatment whereas the other studies examined the serum and kidney within a week after PPMO treatment. A longer interval might provide sufficient time for some damage to recover. Perhaps more importantly, different species may respond to the drug differently as the reported studies used three different animals. Nevertheless,, the low LD50 of the PPMO established in this study and possibly higher sensitivity of higher animals including human to the peptide raise serious concern for the safe use of the PPMO in clinics, especially for repeated injections which are essential to maintain therapeutic levels of dystrophin in bodywide muscles to DMD patients. Thus, further studies in higher animal with different dose regimes are essential before moving the chemistry to the clinic and the biggest challenge for PPMO as antisense drug chemistry is to determine safe and effective dosages.[27]

Threshold effect may also apply to the efficiency of exon skipping with dosing of PPMO. AVI-5038targeted to skip human exon 50 is currently in preclinical development for DMD patients. Initial efficacy of the PPMO in 4 healthy cynomolgus monkey showed that weekly i.v. injection of 9 mg/kg for 4 weeks induced an average of 40%, 25%, and 2% exon-skipping effect in diaphragm, quadriceps, and heart respectively.[27] However, little exon skipping product was detected with the same schedule at a lower dose of 3 mg/kg. A similar trend was observed from the current study. Nearly all skeletal muscles expressed 20% or higher levels of dystrophin protein by western blot and more than 60% fibers clearly stained for dystrophin by immunohistochemistry with up to 5% in the cardiac muscle in the *mdx* mice treated with 6mg/kg PPMO. However, exon skipping was hardly detectable by RT-PCR and dystrophin protein was detected in less than 20% muscle fibers with 1.5mg/kg PPMO treatment. Similarly, dystrophin protein was barely demonstrated in most muscles although near 5% expression was detected in some skeletal muscles by western blots. No dystrophin was detected in cardiac muscle. These results indicate a non-linear relationship between the dose and the efficiency of dystrophin induction with systemically delivered PPMO. Therefore a threshold dosage of PPMO may be required to achieve significant levels of dystrophin production, thus functional improvement for DMD patients in clinics.

Results from the current study with 3 different dosing regimes provided a baseline for us to assess a possible therapeutic window for PPMO treatment to DMD. The results showed that 30mg/kg monthly treatment was effective for maintaining the near normal levels of dystrophin in all muscles with highly significant rescue effect

on muscle pathology and functions. Similar to what we observed previously in the study of short-term effect with biweekly PPMO administration[15], this dosing regimen appeared to be safe for long-term administration without observable chronic toxicity indicated by the normal levels of serum enzymes for liver and kidney. No pathology was revealed by histology of all skeletal muscles, liver, kidney, heart and lung. This is further supported by the normal behavior and life span of the treated mice. However, despite the high efficacy associated with only mild lethargy immediately after the injection, this monthly dosage is apparently too close to the LD50 and requires to be tested in higher animals before being considered for clinic applications.

At the dose of 6mg/kg with a biweekly injection, PPMO induced more than 20% dystrophin in all skeletal muscles. Despite clear variation in the distribution and levels of dystrophin expression between and within muscles, functional improvement was evident by the demonstration of serum CK levels and histopathology. Also importantly, this dosage induced significant amount (up to 5%) of dystrophin in cardiac muscle in the majority of muscle fibers. This dosage of PPMO is at least 10 folds less than the LD50, therefore could be envisaged with potential for long-term treatment to DMD in clinics.

Efficacy of antisense therapy to individual DMD patient, however, depends on many other factors, especially the efficiency of individual antisense oligomer and the functionality of the truncated dystrophin created by exon skipping. AOs targeting individual human dystrophin exon needs to be identified, thus they are inevitably of different efficiency in targeted exon skipping. Selection of AO targeting human and mouse dystrophin exons uses different cells and animal models. Thus care has to be

taken in extrapolating the effective dosage obtained in this study to PPMO applications targeting human dystrophin exon in clinics. A more accurate estimation in exon skipping efficiency of individual PMO may become possible when efficiency of the first PMO drug currently under clinical trials becomes available. This may be achieved by comparing new AO drugs with the PMO of known exon skipping efficiency side by side in the same testing system(s), preferably in both cell culture and *in vivo* animal model systemically.

The functionality of the truncated dystrophin protein created by skipping of different exon must also be taken into account for establishing relevant therapeutic dosage. Unfortunately, functionality of most of those anticipated truncated dystrophins remains to be determined. Such investigations are required for the therapy to apply effectively to defined DMD populations and perhaps more important in some cases to determine the choice of exon(s) to skip for highest functionality of the restored dystrophin.

In summary, PPMO as antisense drugs has demonstrated the highest efficacy for long-term treatment of DMD. LD50 of the PPMO was about 85mg/kg, significantly lower than PMO which has been tested safely with the dose of up to 3g/kg in the *mdx* mice. However, the low LD50 may be mitigated by its high efficiency, permitting the safe use of lower doses which are still capable of achieving significant long–term therapeutic effect.

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Conflicts of interest The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. LD50 of PPMO in *mdx* mice. No death was recorded in the groups treated with 6mg/kg PPMO and 30mg/kg PPMO; 3, 5 , and 8 mice were dead from the groups (10 mice total for each group) treated with the PPMO at 60mg/kg , 90mg/kg, and 120mg/kg. Analysis of Probits was used to calculate the LD50 from mortality rates.

Figure 2. Half life of the dystrophin expression after single i.v. administration of 30mg/kg PPMO in *mdx* mice. **A:** Detection of dystrophin by immunohistochemistry with rabbit polyclonal antibody P7 against dystrophin. Blue nuclear staining with DAPI. **B:** Detection of exon 23 skipping in muscles by RT-PCR. Lane 1, size marker. **C:** Western blot showed levels of dystrophin expression in muscles. **D:** α-actin as loading controls. C57-TA, TA muscle from normal C57 mouse; Cont-TA, TA muscle from control *mdx* mouse. 2 days up to 5 months are time points by which the muscles were examined after single i.v. 30 mg/kg PPMO treated *mdx* mice.

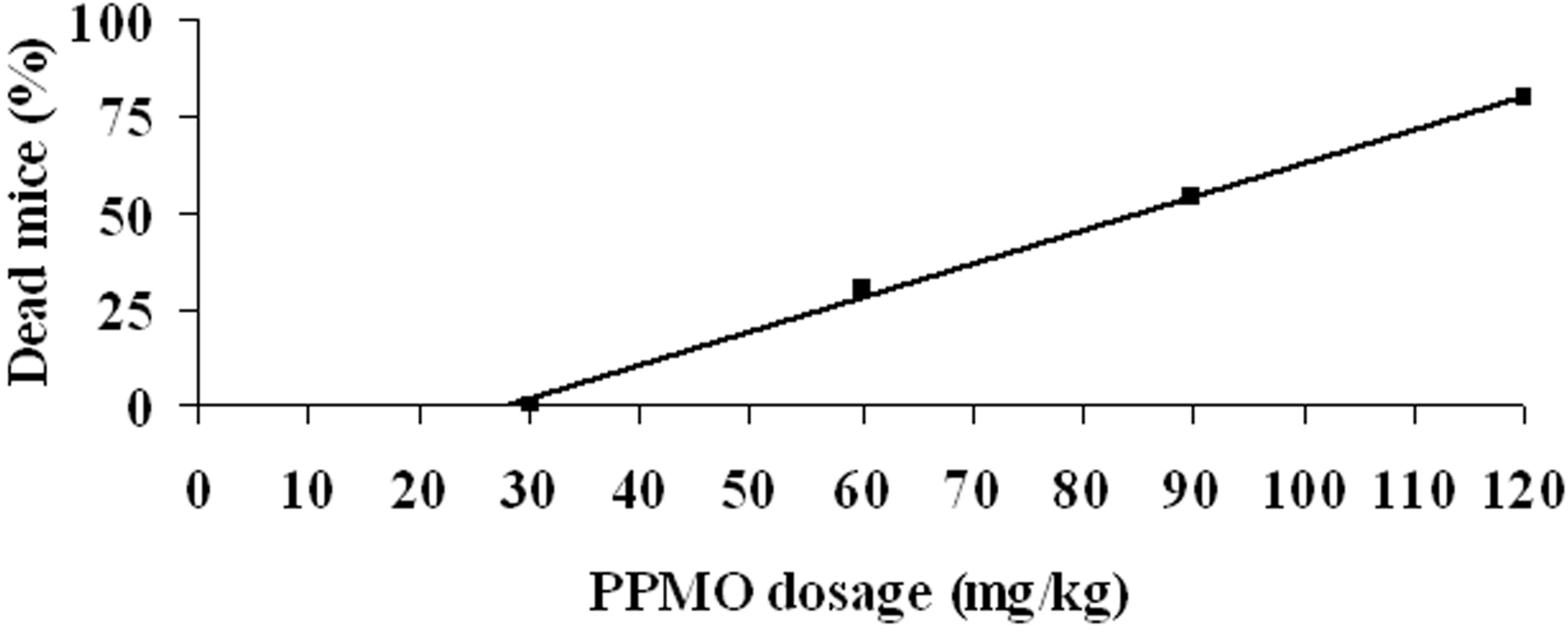
Figure 3. Examination of Pathology, serum and percentage of centranucleated muscle fibers at different time points after single i.v. administration of 30mg/kg PPMO in *mdx* mice. **A:** Histology (H&E staining) of diaphragm, TA and quadriceps from 1 month, 2.5 months, 4 months, 5 months time points after the PPMO treatment. **B:** Percentages of uncentranucleated muscle fibers at different time points. **C:** The levels of serum enzymes. Creatine kinase (KU/L), creatinine (mg/L),

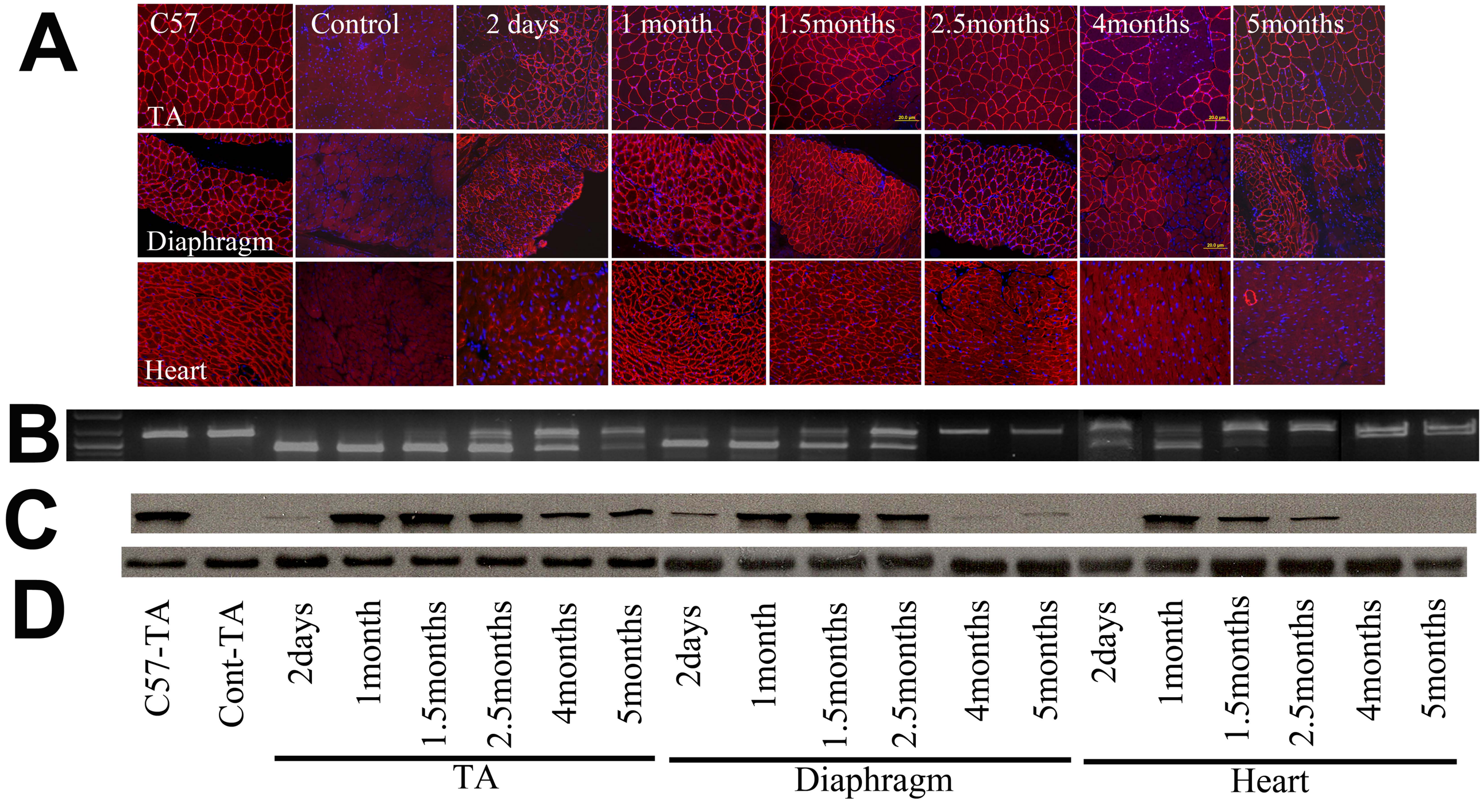
total bilirubin (mg/L), alanine transaminase (ALT) (U/dL), alkaline phosphatase (ALP) (U/dL), Gamma-glutamyltransferase (GGT) (U/L), and Blood Urea Nitrogen (BUN)(mg/ml). The significant reductions in creatine kinase levels were observed at different point in *mdx* mice treated with 30mg/kg PPMO when compared with control *mdx* mice. (n=10; *, $P \leq 0.05$ compared with C57 mice; # $P \leq 0.05$ compared with untreated *mdx* mice. Two-tailed *t* test).

Figure 4. Restoration of dystrophin expression after one year *i.v.*treatment of 1.5mg/kg, 6mg/kg, and 30mg/kg PPMO. **A:** Detection of dystrophin by immunohistochemistry with rabbit polyclonal antibody P7 against dystrophin. Blue nuclear staining with DAPI. **B and E:** Detection of exon 23 skipping in muscles by RT-PCR. Lane 1, size marker. **C and F:** Western blots showed levels of dystrophin expression in muscles. **D and G:** α-actin as loading controls. C57-TA, TA muscle from normal C57 mouse; Cont-TA, TA muscle from untreated *mdx* mouse. 1.5 mg/kg , 6 mg/kg or 30mg/kg iindicate that the muscles were from the mdx mice treated with the PMO at the 3 doses mice.

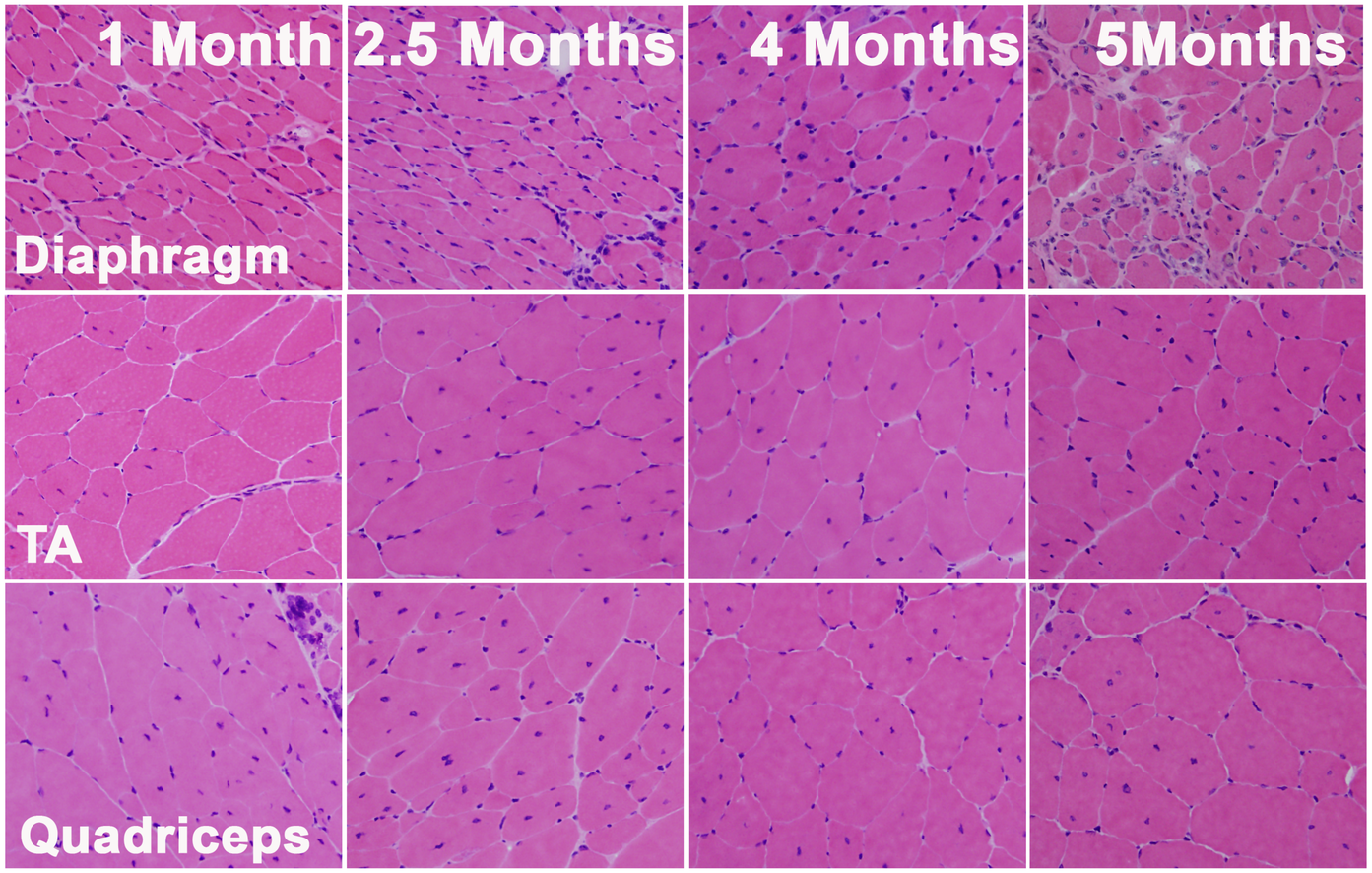
Figure 5. Examination of pathology, serum and skeletal muscle function after one year treatment of 1.5mg/kg, 6mg/kg, and 30mg/kg PPMO. **A:** Histology (H&E staining) of TA, quadriceps, diaphragm, kidney and liver from the normal C57 mice (C57), control *mdx* mice (Control), 1.5mg/kg, 6mg/kg and 30mg/kg PPMO-treated *mdx* mice. **B:** Percentage of uncentranucleated muscle fibers. Control, muscles from control *mdx* mouse. (n=10; # $P \leq 0.05$ compared with control *mdx* mice. Two-

tailed *t* test). **C:** Grip strength measurement. Significant improvement was observed in the mice after treatment with 6mg/kg and 30mg/kg PPMO. KGF, kilogram force. (*n*=10; *, *P*≤0.05 compared with C57 mice; # *P*≤0.05 compared with control *mdx* mice. Two-tailed *t* test). **D:** The levels of serum enzymes. Creatine kinase (KU/L), creatinine (mg/L), total bilirubin (mg/L), alanine transaminase (ALT) (U/dL), alkaline phosphatase (ALP) (U/dL), Gamma-glutamyltransferase (GGT) (U/L), and Blood Urea Nitrogen (BUN)(mg/ml). A significant reduction in creatine kinase levels was observed in *mdx* mice treated with 6mg/kg and 30 mg/kg PPMO when compared with control *mdx* mice.

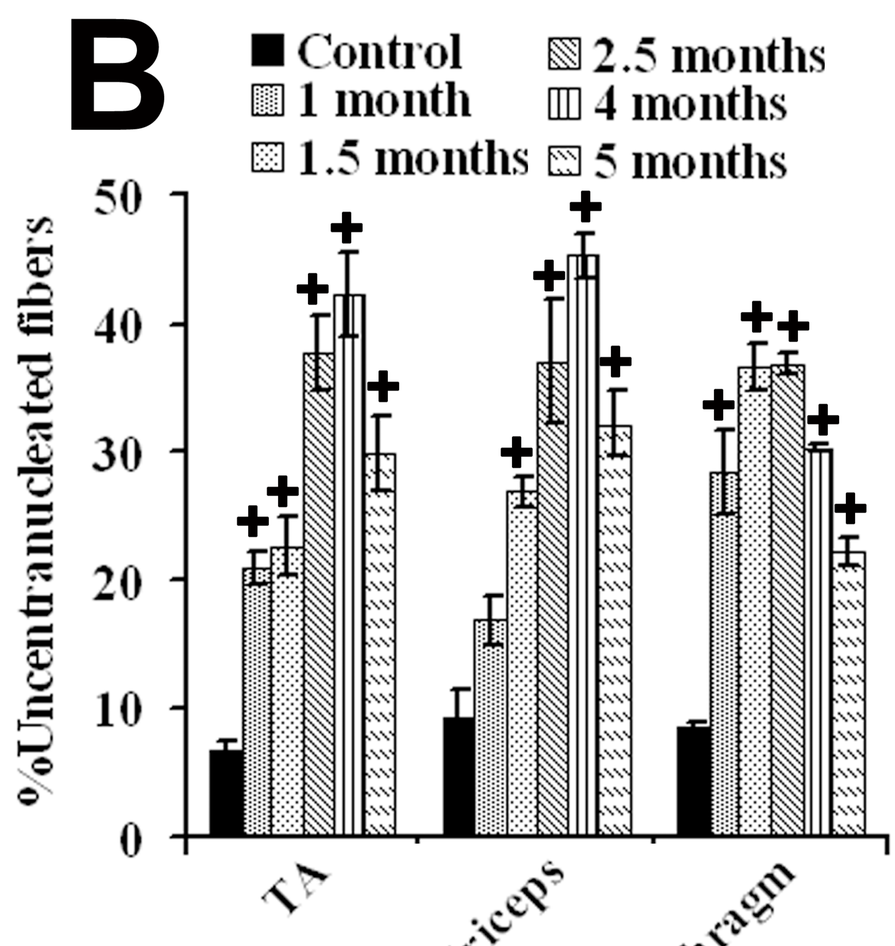

Dead mice (%)
100
75
50
25
0
0
10
20
30
40
50
60
70
80
90
100
110
120
PPMO dosage (mg/kg)


A
C57
Control
2 days
1 month
1.5months
2.5months
4months
5months
TA
Diaphragm
Heart
20.0 µm
B
C
D
C57-TA
Cont-TA
2days
1month
1.5months
2.5months
4months
5months
TA
Diaphragm
Heart

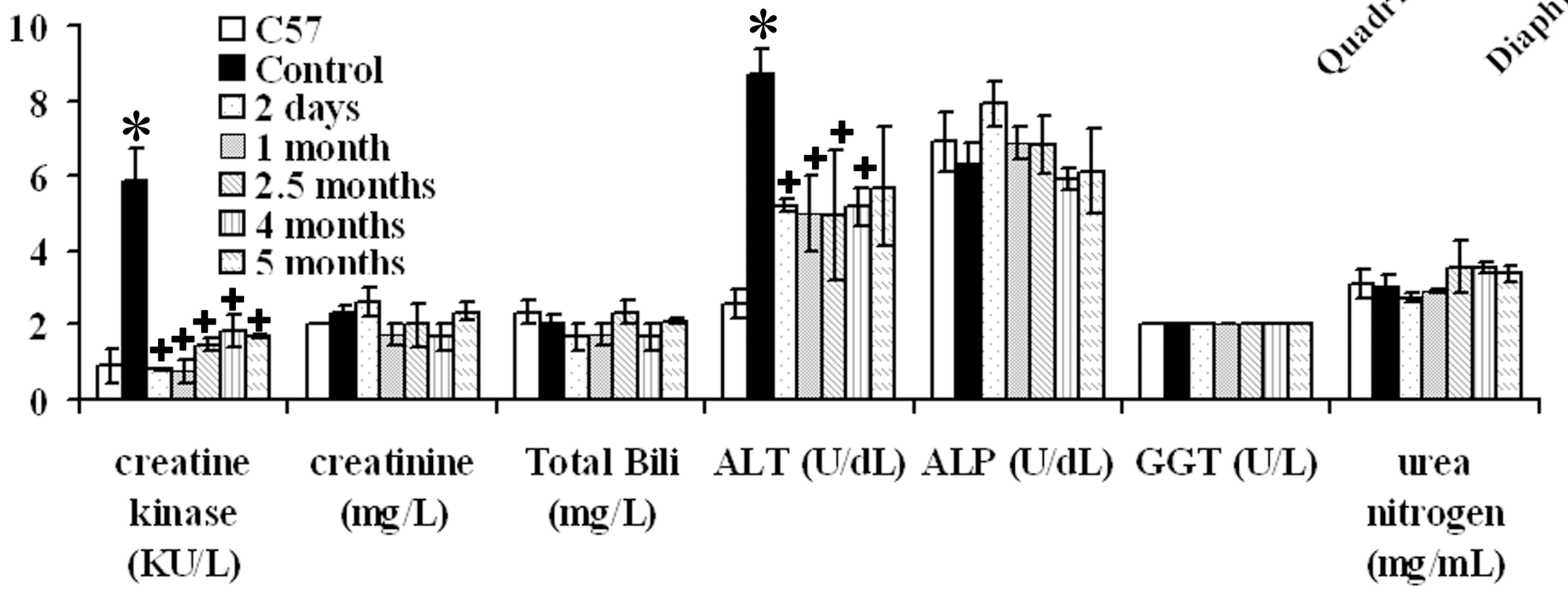
A

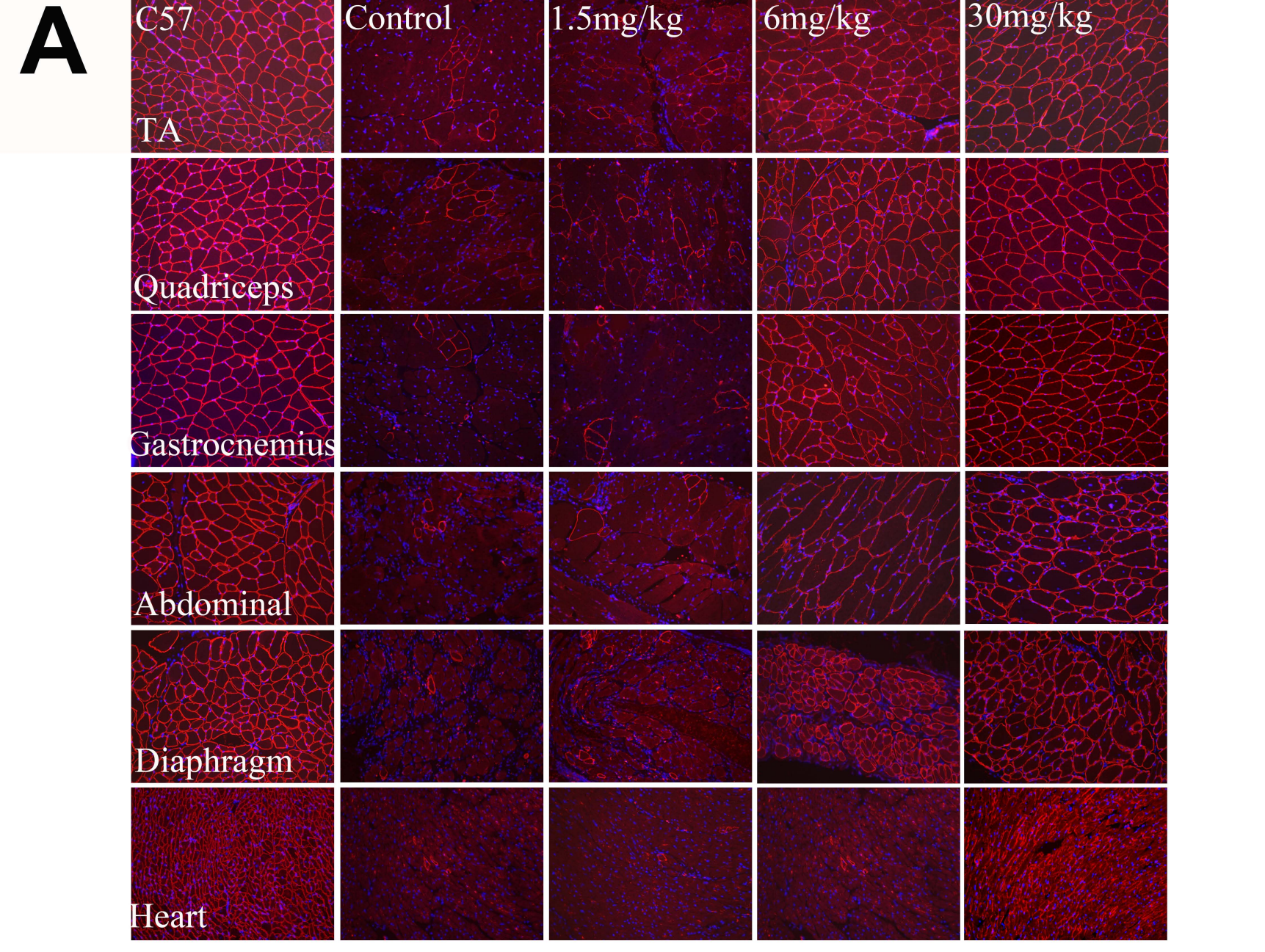

1 Month
2.5 Months
4 Months
5Months
Diaphragm
TA
Quadriceps

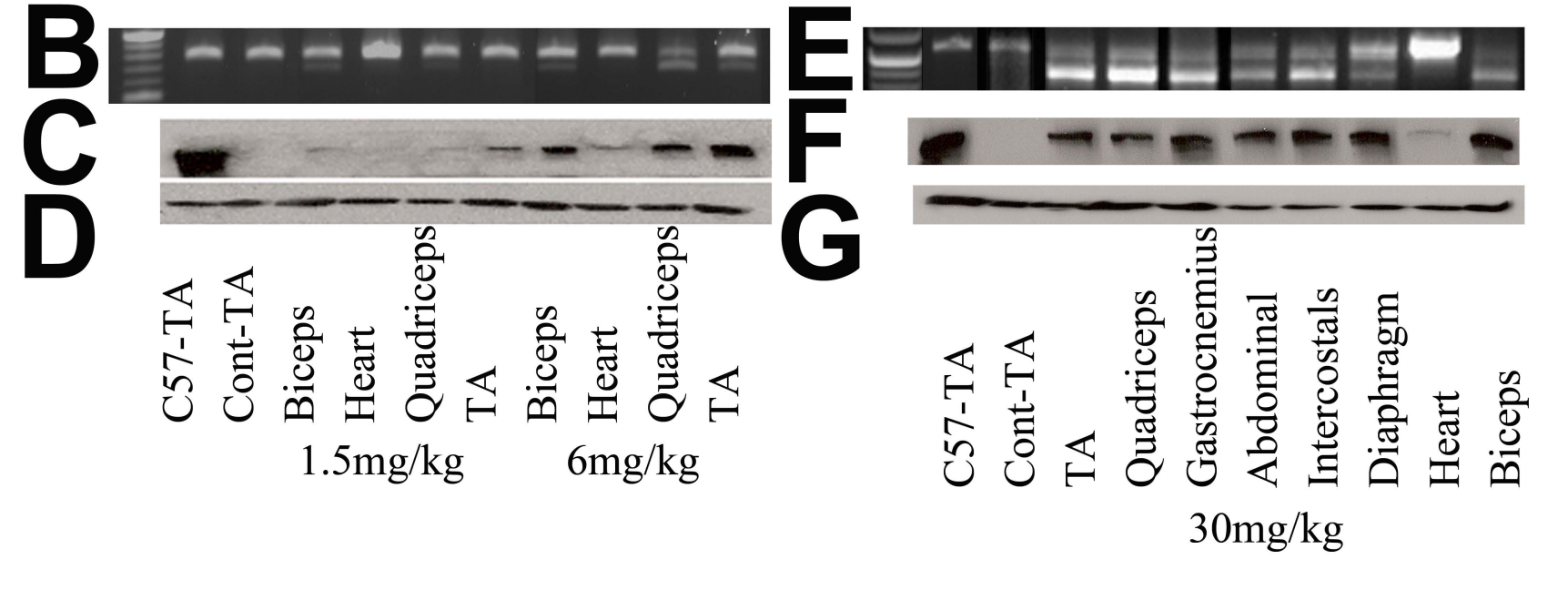
B

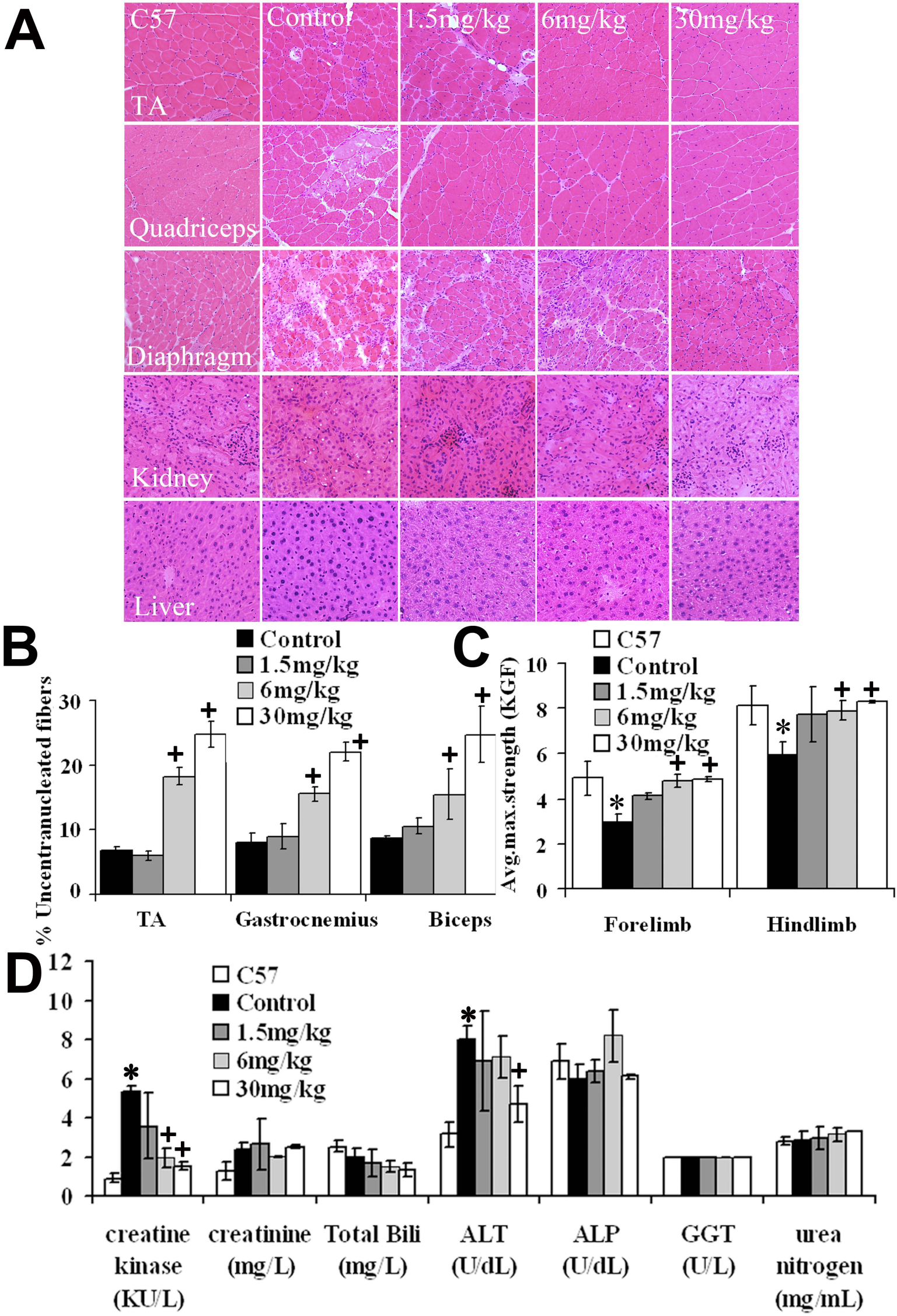

Control
1 month
1.5 months
2.5 months
4 months
5 months
%Uncentranucleated fibers
0
10
20
30
40
50
TA
Quadriceps
Diaphragm

C


C57
Control
2 days
1 month
2.5 months
4 months
5 months
0
2
4
6
8
10
creatine kinase (KU/L)
creatinine (mg/L)
Total Bili (mg/L)
ALT (U/dL)
ALP (U/dL)
GGT (U/L)
urea nitrogen (mg/mL)


A
C57
Control
1.5mg/kg
6mg/kg
30mg/kg
TA
Quadriceps
Gastrocnemius
Abdominal
Diaphragm
Heart


B
C
D
E
F
G
C57-TA
Cont-TA
Biceps
Heart
Quadriceps
TA
Biceps
Heart
Quadriceps
TA
1.5mg/kg
6mg/kg
C57-TA
Cont-TA
TA
Quadriceps
Gastrocnemius
Abdominal
Intercostals
Diaphragm
Heart
Biceps
30mg/kg


A
C57
Control
1.5mg/kg
6mg/kg
30mg/kg
TA
Quadriceps
Diaphragm
Kidney
Liver
B
Control
1.5mg/kg
6mg/kg
30mg/kg
% Uncentranucleated fibers
0
10
20
30
TA
Gastrocnemius
Biceps
C
C57
Control
1.5mg/kg
6mg/kg
30mg/kg
Avg.max.strength (KGF)
0
2
4
6
8
10
Forelimb
Hindlimb
D
C57
Control
1.5mg/kg
6mg/kg
30mg/kg
0
2
4
6
8
10
12
creatine kinase (KU/L)
creatinine (mg/L)
Total Bili (mg/L)
ALT (U/dL)
ALP (U/dL)
GGT (U/L)
urea nitrogen (mg/mL)