Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer

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Antisense oligonucleotide-mediated exon skipping is able to correct out-of-frame mutations in Duchenne muscular dystrophy and restore truncated yet functional dystrophins. However, its application is limited by low potency and inefficiency in systemic delivery, especially failure to restore dystrophin in heart. Here, we conjugate a phosphorodiamidate morpholino oligomer with a designed cell-penetrating peptide (PMO) targeting a mutated dystrophin exon. Systemic delivery of the novel PMO restores dystrophin to almost normal levels in the cardiac and skeletal muscles in dystrophic mdx mouse. This leads to increase in muscle strength and prevents cardiac pump failure induced by dobutamine stress in vivo. Muscle pathology and function continue to improve during the 12-week course of biweekly treatment, with significant reduction in levels of serum creatine kinase. The high degree of potency of the oligomer in targeting all muscles and the lack of detectable toxicity and immune response support the feasibility of testing the novel oligomer in treating Duchenne muscular dystrophy patients.

Mutations in the dystrophin gene underlie two forms of muscular dystrophy: Duchenne and Becker muscular dystrophy (DMD and BMD). DMD is caused mainly by nonsense and frame-shift mutations with little or no production of functional dystrophin protein, leading to disease onset in early childhood with lethal consequences. BMD is caused by mutations that typically create shortened but in-frame transcripts with production of partially functional dystrophin, leading to variable and often overt symptoms (1–3). Most DMD mutations occur within the rod domain, which spans more than half the length of the protein, but seems to have limited functional importance (4, 5). Antisense therapy uses specific oligomers to remove the mutated or additional exon(s) that disrupt the reading frame, thus restoring the expression of shortened forms of dystrophin protein retaining critical functions (6–11).

We previously demonstrated that i.m. delivery of a specific 2′-O-methyl phosphorothioate antisense oligonucleotide (2′OmeAON) was able to skip targeted dystrophin exon 23 in mdx mouse, a model of DMD (9). This mouse carries a nonsense point mutation within exon 23 and lacks dystrophin expression (except in a few rare revertant fibers) in all muscles, including the heart (12, 13). Skipping the mutated exon 23 restored both the reading frame and dystrophin expression, with functional improvement of the treated muscles (14) [supporting information (SI) Fig. S1a]. Recently we showed that a phosphorodiamidate morpholino oligomer (PMO), E23+7-18 targeting the junction of exon 23 and intron 23 of mouse dystrophin (referred to as PMOE23 hereafter), was able to induce up to functional levels of dystrophin expression in some skeletal muscles by regular i.v. injections in mdx mice (15). However, dystrophin expression induced by both 2′OmeAON and PMO required high doses and was highly variable between muscles and myofibers in terms of observed efficacy. Of greater concern, cardiac muscle appeared to be refractory to the antisense therapy, failing to produce detectable dystrophin even after repeated treatment (seven times at ≈60 mg/kg PMO per injection) (15). Both potency and cardiac delivery represent major limitations to antisense therapy as an effective treatment for DMD patients. Because DMD patients live longer owing to improved multidisciplinary patient care, rescuing dystrophin expression in cardiac muscle becomes more critical for their longevity and quality of life (16–22). More importantly, restoration of dystrophin only in skeletal muscles may exacerbate the failure of heart function if dystrophin expression cannot be effectively restored in cardiac muscle (18). It is not understood why AON does not induce dystrophin expression effectively in cardiac muscle, but low delivery efficiency seems to be the most important contributing factor (15). Here we design and examine a cell-penetrating peptide-tagged phosphorodiamidate morpholino oligomer (PMO) and demonstrate the restoration of almost normal levels of dystrophin in cardiac and other types of muscles bodywide in dystrophic mdx mice, with improvement in muscle strength and cardiac function. The latter prevents heart failure under increased workload conditions induced by dobutamine. Repeated treatment maintains levels of dystrophin and ameliorates pathology, with significant reduction in levels of serum creatine kinase without immune response.

Results

PPMO Induces Higher Efficiency of Exon Skipping than PMO. To improve the efficiency of exon skipping in muscles, particularly in cardiac muscle, we designed and examined the effect of several arginine-rich cell-penetrating peptides linked to the same PMOE23. The PMOE23 conjugated with the peptide of the (RXRRBR)2XB sequence (R = arginine, X = 6-aminohexanoic acid, and B = β alanine) (referred as PPMOE23 hereafter) showed the highest efficiency for skipping exon 23 by i.m. injection in the adult (age 4–5 weeks) mdx mouse (Fig. 1A). Strong dystrophin expression was induced in 85% of the fibers in the entire tibialis anterior (TA) muscle after injection of 2 μg of PPMOE23 (Fig. S1). The same amount of unmodified PMOE23 produced only 14% dystrophin-positive fibers. A se-
sequence-scrambled PPMO (with the oligomer sequence not complementary to the dystrophin gene but the same base composition as PPMOE23) showed no effect on dystrophin production. Specific skipping of exon 23 was confirmed by RT-PCR and subsequent sequencing (data not shown). No increases in muscle damage, inflammatory cellular infiltrates, or necrotic fibers were observed microscopically in the muscles injected with any of the PPMOs and PMO (data not shown).

**Single-Dose PPMO Restores Near-Normal Levels of Dystrophin in Skeletal Muscles.** Therapeutic value of the antisense therapy to DMD patients relies on systemic treatment. We therefore injected a single dose of only 30 mg/kg of PPMO i.v. into adult mdx mice. Administration of this amount of unmodified PMOE23 induced dystrophin expression only in 5% or less of muscle fibers of all skeletal muscles and no detectable dystrophin in cardiac muscle when examined 2 weeks after injection (data not shown) (15). In striking contrast, treatment with PPMOE23 produced strong dystrophin expression in 100% of fibers of all skeletal muscles examined, including the TA, quadriceps, gastrocnemius, abdominal, intercostals, diaphragm, and biceps (Fig. 1 B–D). Expression of dystrophin was highly homogeneous throughout the entire length of the muscles (from tendon to tendon). In fact, the levels of dystrophin expression in the muscles of the PPMOE23-treated mice were difficult to distinguish from that in the muscles of normal C57BL mice by immunohistochemical analysis (Fig. 1 B–D). However, variation in fiber size and specifically the presence of central nucleation in most muscle fibers was the unmistakable remaining pathology of the mdx mouse. Consistently, near-normal levels (91–100%) of dystrophin were detected by Western blot (Fig. 1E). The size of the PPMOE23-induced dystrophin was indistinguishable from that of the normal dystrophin. Similarly, dystrophin mRNA with exon 23 skipped accounted for 80–86% of RT-PCR products in all skeletal muscles (Fig. 1G). No off-target skipping of the neighboring exons was observed (Fig. 1G). Precise skipping of exon 23 was confirmed by sequencing (Fig. 1H). Restoration of dystrophin expression also restored the α dystroglycan, α sarcoglycan, and β sarcoglycan on fiber membrane (Fig. S2). Dystrophin expression was not observed in the muscles of the mdx mice treated with scrambled PPMO (Fig. 1 B–G).

**Rescue of Dystrophin Expression in Cardiac Muscle.** Importantly, immunohistochemistry demonstrated membrane-localized dystrophin in 94% of cardiac muscle fibers of mdx mice treated with the single dose of PPMOE23, although the levels of dystrophin varied (Fig. 1D). Dystrophin was expressed at near-normal levels in most areas of the cardiac muscle except in myofibers near the ventricles and papillary muscles (Fig. S3 a and c). A 58% normal dystrophin level was demonstrated by Western blot (Fig. 1E). Consistently, dystrophin mRNA with exon 23 skipped accounted for 63% of the dystrophin transcript by RT-PCR (Fig. 1G). Expression of dystrophin also restored the membrane localization of α dystroglycan and sarcoglycans (data not shown).

**Regular PPMO Treatment Enhances Dystrophin Expression in Skeletal and Cardiac Muscles.** To assess whether regular injections of the arginine-rich peptide can maintain or further enhance dystrophin expression, a group of five adult mdx mice received 3-month treatment with repeated (six times) i.v. injections of 30 mg/kg of PPMOE23 at biweekly intervals. Two weeks after the last injection, dystrophin expression remained in 100% of muscle fibers in all skeletal muscles, including the diaphragm and smooth muscles in...
the small intestine (Figs. 2A–C and 3). The levels of dystrophin expression detected by both immunohistochemistry and Western blot in the PPMOE23-treated mdx mice were again indistinguishable from those in normal C57BL mouse (Fig. 2D). The dystrophin mRNA with exon 23 skipped accounted for nearly 90% (85–92%) of total dystrophin mRNA by RT-PCR in all skeletal muscles (Fig. 2F).

Most significantly, repeated injections of PPMOE23 further enhanced specific exon skipping and dystrophin expression in the cardiac muscle. The dystrophin mRNA with exon 23 skipping increased to 72% of normal levels (Fig. 2F). Immunohistochemistry and Western blot revealed dystrophin expression throughout the whole heart muscle, with levels comparable to that in normal heart (Figs. 2D and 4A–C; and Fig. S3B). Furthermore, strong and homogeneous dystrophin expression was also achieved in the muscles of the atria and in the smooth muscle of large vessels, including the aorta, vena cava, and the pulmonary arteries (Fig. 3F and G).

**Dystrophin Expression Improves Pathology of Skeletal Muscles.** Restoration of near-normal levels of dystrophin in all skeletal muscles after PPMOE23 treatment unambiguously improved pathology in the treated mice. Improvement in cell membrane permeability was observed with Evan’s blue dye and staining for Igs (19, 20). Two weeks after a single 30-mg/kg i.v. injection, the number of dye-stained and Ig-positive fibers was significantly reduced in PPMOE23-treated muscles compared with muscles in the untreated and scrambled PPMO-treated mdx mice. Only occasional

![Fig. 3. Restoration of dystrophin in skeletal and smooth muscles after six cycles of 30-mg/kg PPMOE23 injection. Back thoracic and lumbar muscle (A), digital muscle (B), flexor muscle (C). Smooth muscles (layers between the two arrows) in small intestine of untreated mdx mouse (D) and PPMOE23-treated mdx mouse (E). Arrowhead indicates a revertant fiber. Dystrophin expression in the smooth muscle of aorta and vena cava (F) and arteries and other vessels in the lung (G). Dystrophin was detected by immunostaining with rabbit polyclonal antibody P7. Blue nuclear staining with DAPI. (Scale bars: A–E, 50 μm; F and G, 120 μm.)](image)

![Fig. 4. Dystrophin expression in cardiac muscle after PPMOE23 treatment (30 mg/kg six times at biweekly intervals). Muscles from normal C57BL mouse (A), PPMOE23-treated mdx mouse (B), and scrambled PPMO-treated mdx mouse (C). The muscles were examined 2 weeks after last injection of PPMOs. Dystrophin was detected by immunohistochemistry with rabbit antibody P7 and visualized by Alexa S94 conjugated goat antirabbit Ig. Blue nuclear staining with DAPI. (Scale bar: 100 μm.)](image)
(one or two) muscle fibers were stained positive with the dye and for Igs in all of the muscles of the mice treated with PPMOE23, whereas 20 or more positive fibers, often in groups, were observed in each muscle of the untreated and scrambled PPMO-treated mdx mice (Fig. S4 a and b). The amount of Ig in the extracellular matrix was also considerably reduced in the muscles of the mdx mice treated with PPMOE23, indicating an improved integrity of the vasculature bed. The improved muscle pathology was strongly supported by a significant reduction in serum levels of creatine kinase in PPMOE23-treated mice (Fig. S4c).

The most significant improvement in pathology was seen after 3 months’ regular treatment with PPMO. First, muscle fibers displayed more healthy polygonal shapes and became highly uniform. Fibers of small caliber, a sign of previous regeneration, became rare in all muscles (Fig. 5 A–C and F). This contrasted sharply with muscles of both the untreated and scrambled PPMO-treated mdx mice, in which fiber size varied significantly and groups of regenerating fibers were evident. No foci of mononuclear infiltrates were observed in any muscles of the PPMOE23-treated mice (Fig. 5C). Second, the number of centrally nucleated fibers was significantly reduced in all muscles of mdx mice treated with PPMOE23, indicating decreased degeneration and regeneration (Fig. 5D). The improvement in muscle pathology was most conspicuous in the diaphragm. Untreated and scrambled PPMO-treated mdx mice had prominent degeneration and regeneration, indicated by large variation in fiber size, widespread foci of newly regenerated fibers, and mononuclear infiltrates in the extracellular matrix (Fig. 5 B and F). Degenerating fibers with a rounded shape and condensed cytoplasm were abundant (Fig. 5B). However, PPMOE23 treatment improved homogeneity of fiber size and diminished mononuclear infiltrates (Fig. 5 C and F).

**Restitution of Skeletal Muscle Function.** We evaluated skeletal muscle function with a battery of tests. The forelimb maximum grip force showed substantial improvement in PPMOE23-treated adult mdx mice as early as 2 weeks after two cycles of i.v. injection (Fig. S5a). The group of mdx mice receiving 3 months of biweekly treatment with PPMOE23 continued to show improvement in the maximum grip forces of both forelimbs and hindlimbs (Fig. 5E), as well as improvement in endurance by rotarod tests (Fig. S5b). The muscle functional improvement was supported by significant reduction in serum creatine kinase levels (Fig. 5G).

**Improvement in Cardiac Function Prevents Heart Failure.** Next, we examined whether PPMOE23 treatment could also improve the cardiac function of mdx mice. Consistent with previous reports, microscopic examination and ultrahigh-frequency echocardiography of the PPMOE23-treated mdx mice showed normal histology and ventricular function of the heart (Fig. S5c). However, heart dysfunction of mdx mice can be demonstrated by hemodynamic analysis under dobutamine stress (21–25). We therefore examined 4-month-old male mdx mice 3 weeks after two PPMOE23 treatments (30 mg/kg at biweekly intervals) along with age- and sex-matched untreated mdx and healthy C57BL control mice. A β-adrenergic stimulant, dobutamine, was used to increase cardiac workload and further magnify the dysfunctions in mdx heart (23–25). Under baseline conditions, the untreated mdx heart showed minor deficits, especially in stroke volume, cardiac output, end-systolic pressure, maximal rate of isovolumic contraction (dp/dtmax), and maximal rate of isovolumic relaxation (dp/dtmin) when compared with C57BL control mice. Expression of dystrophin after PPMOE23 treatment improved dp/dtmax, dp/dtmin, and end-systolic pressure (Table S1).

After dobutamine infusion, however, mdx mice revealed significant cardiac dysfunction, with progressive decline in end-diastolic volume, end-systolic pressure, and dp/dtmax and an increase in dp/dtmin (Fig. 6 A–E and Table S2). There was a marked leftward shift of cardiac pressure/volume loops after dobutamine challenge (Fig. 6F) and H), and 60% of the mdx mice did not survive the 30-min procedure (Fig. 6F). Conversely, the PPMOE23-treated mdx mice showed much-improved cardiac function, especially in end-systolic pressure, dp/dtmax, dp/dtmin, and end-diastolic volume, and lack of left-shift of the pressure/volume loop, similar to healthy control C57BL mice (Fig. 6 A–E, G, and H and Table S2). Like C57BL, all treated mdx mice survived the dobutamine challenge (Fig. 6F). Finally, near-normal levels of dystrophin expression in the heart of PPMO-treated mdx mice were confirmed by immunohistochemical staining (Fig. 6 I and J) and Western blot (data not shown) after hemodynamic analysis. These results indicate that PPMOE23-induced dystrophin expression is able to improve car-
diac function of dystrophic heart and prevent acute cardiac decompensation under increased workload conditions.

**Lack of Detectable Toxicity and Immune Response.** Histologic examination revealed no pathologic changes in liver and kidney of mdx mice after both single and repeated PPMO treatment(s) (Fig. S6d). This was consistent with the serum testing results showing that levels of alkaline phosphatase and creatinine were unchanged compared with control mdx mice (Fig. S6c).

We also looked for potential immune response to the PPMOs delivered either locally or systemically. No accumulation of inflammatory cells, including microphages, was detected in any muscles. Consistently, only a few sporadically distributed T lymphocytes were observed by immunohistochemistry in all muscles after PPMOE23 treatment (Fig. S6b and c). Specific humoral immune responses were also examined by ELISA to detect antibodies against PPMO. Serum from mice after single and repeated administration of PPMO showed only background signals similar to serum from normal C57BL and untreated mdx mice (Fig. S6d).

**Discussion**

One major concern that arises with the use of peptides as delivery enhancers is the immune response they might elicit, preventing repeated administration and causing rejection of targeted tissues. This is particularly important given that long-term, repeated administrations are required for the treatment of DMD with antisense therapy. However, we observed no signs of immune response to the PPMOs delivered either locally or systemically. Humoral immune response to the PPMO was also undetectable after repeated PPMO administration. The most compelling evidence was the sustained near-normal levels of dystrophin expression in skeletal muscles and enhanced dystrophin expression in cardiac muscle, with improved muscle histology and function after 3 months of treatment. This is consistent with previous reports showing lack of immunogenicity with similar peptides in animal models (26, 27). The use of unnatural amino acids in the peptide sequence of the PPMO most likely contributes to the lack of immunogenicity. However, only short-term use of arginine-rich peptide has been reported in human clinical trials (28). The principle of precaution together with the fact that immunogenicity varies considerably between species would argue for longer-term studies in other species. Nevertheless, the known sequence and structure of our peptide provide bases for modeling to develop possible nonpeptide polymers with similar or even improved function as effective delivery vehicles.

Strategies of gene and protein replacement therapy for DMD, such as gene and cell therapies as well as antisense therapy, have met the challenge to achieve homogenous protein expression throughout the bodywide musculature (5, 14, 29). Effort has been made to improve antisense effect by modification of AONs with cell-penetrating peptides, but with limited success (30, 31). Fletcher et al. (31) used a PMO tagged with an arginine-rich peptide for exon 23 skipping in mdx mice and reported restoration of near-normal levels of dystrophin in diaphragm by i.p. injections. However, only ≤5% normal levels of dystrophin were produced in all other skeletal and cardiac muscles. The failure to restore dystrophin in cardiac muscle could severely reduce the clinical value of antisense therapy. Of greater concern is that restoration of dystrophin in skeletal muscles but not in cardiac muscle could exacerbate the existing heart dysfunction as a result of increased workload with the improvement of skeletal muscle function (18). Here we show that PPMO as antisense oligonucleotide chemistry can achieve almost full restoration of dystrophin in cardiac and all skeletal muscles. This achievement has several important implications. First, these results prove the principle that cardiac muscles can be effectively targeted for specific exon skipping and rescue of dystrophin expression (32–34). Second, the AON used in our study has been screened and selected using skeletal muscle myoblasts as targets. High-efficiency exon skipping in all types of muscles (cardiac, skeletal, and smooth muscle) in vivo after the same AON treatment suggests that skeletal myoblast cultures may be a reliable system for patient-specific AON selections. This is particularly important for antisense therapies targeting specific exons of human dystrophin for clinical trials, because only skeletal myoblasts but not cardiomcyocytes are available from patients. Third, the finding that PPMOE23 could also effectively enter and correct smooth muscle cells in the vasculature and the digestive system is striking and remains to be further investigated. It has been documented that dystrophin deficiency in blood vessel smooth muscles caused deficits in circulation and could be corrected after dystrophin restoration (35). Finally, effective targeting of cardiac muscle with improved function and prevention of acute cardiac

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**Fig. 6.** Improvement of cardiac hemodynamic function and survival of PPMOE23-treated (two 30-kg/mg i.v. injections, examined 3 weeks after the last injection) mdx mice (aged 4 months) at 15 min after dobutamine challenges. Significant improvement was observed in end-systolic pressure (A), dp/dt max (B), dp/dt min (C), and end-diastolic volume (D). (E) End-systolic volume. (F) Percentage survival of untreated mice (red), PPMOE23-treated mdx mice (green), and C57BL mice (blue) within 30 min after dobutamine challenges. (G) Representative pressure/volume loops in paired mice before dobutamine treatment (baseline). (H) The loop shifts dramatically leftward and downward in the untreated mdx heart (red) but stabilized in the PPMOE23-treated mdx heart (green), similar to the loop in C57BL heart (blue) at 15 min after dobutamine treatment. RVU, relative volume units. (I) Heart from untreated mdx mouse and (J) heart from PPMOE23-treated mdx mouse after hemodynamic analysis and stained for dystrophin expression. One hundred percent of cardiac muscle fibers express dystrophin (J). Blue nuclear staining with DAPI. C57BL, n = 10; PPMOE23-treated mdx mice, n = 8; untreated mdx mice, n = 9. * P < 0.05, t-test.
dysfunction provides the possibility for rescue of cardiac muscle in DMD patients. Furthermore, almost 100% efficiency in targeting genes transcripts systematically in a disease model demonstrates the power of oligonucleotide therapy, not only for DMD but also for many other diseases, such as viral infections and cancers (36).

Materials and Methods

Animals, Oligonucleotides, and Delivery Methods. The mdx mice and C57 Black 10 (C57BL) aged 4–5 weeks and months, respectively, were used in each group. Experiments were approved by the Institutional Animal Care and Use Committee, Carolinas Medical Center. The PMO M23d1(−78)18 (5′-GCCGAACATCATCT-TACCTGAAAT-3′) against the boundary sequences of exon and intron 23 of dystrophin were transfected into the cell line C2C12, and the scrambled oligomers (5′-GCCGAACAC-CTGGGTACCTGAAAT-3′) as a control were used (AVI BioPharma). The PMO was conjugated to the peptide (RXRRBRX8) through a noncovalent amide linker (37) to form a peptide-PMO conjugate (PPMO). For i.m. injections, 2 μg of PMO or PPMO were used in saline for each TA muscle. For i.v. administration, 30 mg/kg of PMO or PPMO was used in 100 μL of saline by retro-orbital injections.

Antibodies and Immunohistochemistry. Serial sections were stained with a panel of polyclonal and monoclonal antibodies for the detection of dystrophin and dystrophin-associated proteins, as described (9, 38). Tissue endogenous IgGs were detected with rabbit antimouse IgGs Alexa 488 (Invitrogen). FITC-labeled monoclonal antibody against mouse CD3 was obtained from EBioscience.

Protein Extraction and Western Blot. Protein extraction and Western blot were done as described (39). The membrane was probed with monoclonal antibody NCL-DYS1 against dystrophin rod domain (Vector Laboratories). The bound primary antibody was detected by HRP-conjugated goat antimouse IgG. The intensity of the bands obtained from the oligomer-treated mdx mice muscles was measured and compared with that from normal muscles of C57BL mice (National Institutes of Health imaging software, gel blotting macros). Alpha-actin was detected by rabbit antiantibin antibody (Sigma) and used as sample loading control.

RNA Extraction and RT-PCR. Total RNA was extracted, and 100 ng of RNA template was used for a 50-μL RT-PCR with the Stratascript One-Tube RT-PCR System (Strategene). The primer sequences for the RT-PCR were Ex20R 5′-CAGAATTCGGATCCATGCGGAG-3′ and Ex26R 5′-TCTTCGACGGTGTCTACAT-3′ for amplification of the mRNA from exons 20–26. Bands with the expected size for the transcript with exon 23 deleted were extracted and sequenced. The intensity of the bands representing mRNAs with and without exon 23 skipping was measured (added together as 100%) by National Institutes of Health imaging software (gel blotting macros), and relative percentage of the mRNA with exon 23 skipping was calculated.

Grip Strength Test. Grip strength was assessed with a grip strength meter consisting of horizontal forelimb mesh and an angled hindlimb mesh (Columbus Instruments). Five successful hindlimb and forelimb strength measurements within 2 min were recorded. The maximum values of each day over a 5-day period were used for analysis. Grip strength analysis for grip strength measurements were collected in the morning hours over a 5-day period, and data were normalized to body weight and expressed as kilogram force.

In Vivo Cardiac Hemodynamics. Hemodynamic analysis on the treated and untreated mdx mice as well as the C57BL mice (aged 4 months) were performed using conductance micromanometer according to previously published methods (23–25). Briefly, the mice were anesthetized by inhalation of 1% isoflurane and 99% oxygen. The mice were then ventilated at 120 strokes per minute with a rodent ventilator (Harvard Instruments) and kept on a warm pad to stabilize the body temperature at 37°C. After thoracotomy and pericardiectomy, a 1.4-F high-fidelity micromanometer catheter (Millar Instruments) was inserted into the left ventricle of each continuous hemodynamic monitoring system. After collection of baseline hemodynamic data, dobutamine infusion was used at a rate of 20 ng per min for 3 min through the jugular vein. Pressure/volume loop data were collected online at 500 Hz. Hemodynamic parameters were analyzed with PVAN3 software (Millar Instruments).

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