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Shirasaka, Tomonori

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A slow-releasing form of prostacycline agonist (ONO1301SR) enhances endogenous secretion of multiple cardiotherapeutic cytokines and improves cardiac function in rapid-pacing induced canine heart failure model

徐放性プロスタサイクリンアゴニスト（ONO1301SR）は高速ペーシングによるイヌ心不全モデルにおいて心筋保護作用を有する多様な内因性サイトカインを誘導し心機能を改善する

白坂 知識, 宮川 繁, 福嶌 五月, 斎藤 充弘, 塩崎 元子,
河口 直正, 松浦 成昭, 中谷 敏, 酒井 芳樹, 大門 貴志,
澤 芳樹, 大北 裕

神戸大学大学院医学研究科医科学専攻
心臓血管外科学
（指導教員：大北 裕 教授）

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A slow-releasing form of prostacycline agonist (ONO1301SR) enhances endogenous secretion of multiple cardiotherapeutic cytokines and improves cardiac function in rapid-pacing induced canine heart failure model

-Short title: Shirasaka et al. Intramyocardial prostacycline agonist for CHF-

Tomonori Shirasaka[1]; Shigeru Miyagawa[2]; Satsuki Fukushima[2];

Naomasa Kawaguchi[3]; Nariaki Matsuura[3]; Satoshi Nakatani[4];

Yoshiki Sakai[5]; Takashi Daimon[6], Yutaka Okita[1] and Yoshiki Sawa[2]

[1]Division of Cardiovascular Surgery, Kobe University Graduate School of Medicine, Kobe, Japan

[2]Division of Cardiovascular Surgery, Osaka University Graduate School of Medicine, Suita, Japan

[3]Department of Molecular Pathology, Graduate School of Medicine and Health Sciences, Osaka University Graduate School of Medicine, Osaka, Japan

[4]Division of Functional Diagnostics, Department of Health Sciences, Osaka University Graduate School of Medicine, Osaka, Japan

[5]Ono Pharmaceutical Company Ltd, Osaka, Japan

[6]Division of Biostatistics Hyogo College of Medicine, Hyogo, Japan

- Correspondence to **Professor Yoshiki Sawa**

Osaka University Graduate School of Medicine, Suita, Japan

E1: 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

E-mail: sawa@surg1.med.osaka-u.ac.jp

- 3309 words and 25 references
- 5 figures

Abstract

Background; Cardiac functional deterioration in dilated cardiomyopathy (DCM) is known to be reversed by intramyocardial upregulation of multiple cardioprotective factors, while a prostacycline analogue, ONO-1301, has been shown to paracrinally activate interstitial cells to release a variety of protective factors. We here hypothesized that intramyocardial delivery of a slow-releasing form of ONO1301 (ONO1301SR) might activate regional myocardium to upregulate cardiotherapeutic factors, leading to regional and global functional recovery in DCM.

Methods and Results; ONO1301 elevated mRNA and protein level of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and stromal-derived factor-1 (SDF-1) of normal human dermal fibroblasts in a dose-dependent manner *in-vitro*. Intramyocardial delivery of ONO1301SR, which is ONO1301 mixed with polylactic and glycolic acid polymer (PLGA), but not that of PLGA only, yielded significant global functional recovery in a canine rapid pacing-induced DCM model, assessed by echocardiography and cardiac catheterization (n=5 each). Importantly, speckle tracking echocardiography unveiled significant regional functional recovery in the ONO-1301-delivered territory, consistent to significantly increased vascular density, reduced interstitial collagen accumulation, attenuated myocyte-hypertrophy and reversed mitochondrial structure in the corresponding area.

Conclusions; Intramyocardial delivery of ONO1301SR, which is a PLGA-coated slow-releasing form of ONO-1301, up-regulated multiple cardiotherapeutic factors in the injected territory, leading to region-specific reverse LV remodeling, consequently a global functional recovery in a rapid pacing-induced canine DCM model, warranting a further pre-clinical study to optimize this novel drug-delivery system to treat DCM.

Ultra mini-abstract

In vitro, ONO1301, a prostacycline agonist, enhances secretion of multiple cardiotherapeutic cytokines. In vivo, Intramyocardial administration of ONO1301SR, a slow-releasing form of ONO1301, attenuated the cardiac function in the canine DCM heart failure model. Especially, region specific effects of ONO1301SR were proved by speckle tracking echocardiography and histopathological study.

Background

Dilated cardiomyopathy (DCM) is characterized by progressive and severe deterioration of cardiac function, eventually leading to advanced heart failure necessitating surgical interventions such as cardiac transplantation[1] or mechanical assist devise implantation[2], despite maximum currently-available medical therapy including angiotensin converting enzyme inhibitor[3] or beta-blocker[4]. Despite a variety of etiologies in DCM, the pathologies consistently include pathological hypertrophy of cardiomyocytes associated with mitochondrial dysfunction, increased interstitial fibrosis and limited regional blood flow[5-7]. These pathological LV remodeling is reportedly at least in part reversed by cell transplantation that intramyocardially up-regulates multiple cardiotherapeutic cytokines in a constitutive manner[8-9]. However, cell therapy is limited in clinical arena due to availability of cell processing centre or ethical issues. Therefore, synthetic reagents which yield similar cardiotherapeutic effects to cell transplantation have been sought.

Prostacycline is an endogenous factor released by endothelial cells, activating endothelial cells, fibroblasts or smooth muscle cells in an autocrine and paracrine manner to release multiple growth factors or cytokines, consequently producing local and systemic anti-inflammatory, anti-fibrotic, pro-angiogenic and anti-thrombotic effects. However, clinical use of synthetic prostacycline or prostacycline analogues, such as epoprostenol and beraprost, for chronic pathologies is hampered by its chemical instability[10-11], and therefore the delivery method.

ONO-1301 is a synthetic prostacycline analogue having a unique structural feature to maintain chemical stability, possibly allowing slow-releasing system[12]. Of note, ONO-1301 reportedly activates fibroblasts to release multiple factors such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF)[13], both of which are known to be

cardiotherapeutic[14-15]. Nakamura et al reported that direct intramyocardial injection of ONO-1301 yielded cardiotherapeutic effects in a mice acute myocardial infarction model[13]. On the other hand, Hirata et al. reported that subcutaneous injection of ONO-1301 improves global cardiac function associated with globally reduced fibrosis and increased capillaries in a hamster DCM model[16]. But it remains unclear that intramyocardial delivery of ONO-1301 would produce therapeutic effects on DCM heart failure model in large animal.

We therefore hypothesized that intramyocardial injection of ONO-1301 might activate regional interstitial cells including fibroblasts in the injected area to locally up-regulate multiple therapeutic factors, leading to region-specific functional recovery in DCM. Thus, we investigated therapeutic effects of local administration of a slow-releasing form of ONO-1301 on regional cardiac function of DCM heart by using the canine rapid-pacing induced that is an established DCM model[17-18].

Methods

Animal care

All studies were performed with the approval of the institutional ethics committee in Osaka University Graduate School of Medicine. All animals were treated in compliance with the Principles of Laboratory Animal Care (the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (NIH publication). Human dermal fibroblasts were treated in compliance with the principles outlined in the Declaration of Helsinki. All procedures and analysis were carried out in a blinded manner. The authors had full access to and take full responsibility for the integrity of data and agree to the manuscripts as written.

Culture of human dermal fibroblasts with ONO1301 added

Human dermal fibroblast cell-line (NHDF, CryoNHDF Neo, Lonza, Switzerland) was cultured in FBM (FGM-2 Bulletkit, Lonza) containing 2% FBS. ONO1301 (0.1-1.0 μmol/ml) was added for 72 hours after serum-free culture for 24 hours.

Generation of a slow releasing form of ONO1301

A slow releasing form of ONO-1301 (ONO1301SR, Ono Pharmaceutical Co.Ltd, Osaka, Japan) was created by polymerization of ONO-1301 with PLGA as described previously[19]. Briefly, ONO-1301 (5 mg) was mixed with 100 mg of PLGA in 0.1% polyvinylalcohol with equal molar ratio of lactic acid/glycolic acid. Releasing time of ONO1301SR *in-vitro* was between about 14 days to 25 days, as determined by measuring residual ONO-1301 in the pellets by liquid-chromatography.

Generation of canine DCM model and intramyocardial* ONO1301SR *injection

Beagles weighing 10 kg (Oriental Yeast Co. Ltd, Tokyo, Japan) were endotracheally intubated, supported by mechanical ventilation under general anesthesia using intravenous sodium pentobarbitual (6 mg/kg) for induction and inhaled sevoflurane (1-2 %) for subsequent maintenance. We maintained the adequacy of anesthesia evaluated by giving the dogs electrical stimuli every 30 minutes and this evaluation was performed during each operation for each procedure. Heart was exposed *via* left 5th intercostal spaces and two bipolar pacing leads (FINELINE II EZ STEROX, Boston Scientific, Boston) were attached on the free wall of the right ventricle, connected to a pulse generator (INSIGNIA I, Boston Scientific) placed in subcutaneous pocket. The ventricle was continuously paced at 240 beats per minute (bpm) for 8 weeks[18].

Four weeks after rapid-pacing commenced, either ONO1301SR or PLGA polymer only was injected with 26 gauge needle at 5 points of lateral wall of the left ventricle (LV) at regular intervals (total 15 mg of ONO-1301 or PLGA polymer were injected, ONO1301SR group and Control group, n=5 each). Rapid-pacing was temporally discontinued during the injection procedure, while it was set back at 240 bpm the following day after each operation. Dogs kept on rapid-pacing for 8 weeks were sacrificed by under general anesthesia aforementioned with using overdose intravenous sodium pentobarbitual (18 mg/kg) to achieve complete sedation followed by administration of potassium-based solution intravenously to assure for the complete euthanasia. The hearts were retrieved at 4 weeks after injection of either ONO1301SR or PLGA only. We here defined lateral LV wall where ONO1301SR was directly injected as 'target site' while septal wall as 'remote site'.

Conventional and speckle-tracking echocardiography and cardiac catheterization

Transthoracic echocardiography (Altida, Toshiba Medical Systems Corporation, Tochigi,

Japan) was performed under general anesthesia by 1% sevoflurane inhalation. End-diastolic and end-systolic LV dimensions (Dd and Ds, respectively) and end-systolic and end-diastolic wall thickness (ESWT and EDWT respectively) of target site and remote site were measured at mid-LV short axis view by conventional echocardiography. LV ejection fraction (EF) was calculated with biplanar Simpson's rule from apical 4-chamber view. E/E', an indicator of diastolic function, was calculated by measuring peak doppler velocities of early trans-mitral filling wave (E) and the peak early diastolic velocity of the mitral annulus (E').

Speckle tracking echocardiography and an off-line software (Altida extend, Toshiba Medical Systems Corporation) were used to measure radial, circumferential, transverse and longitudinal strains, to quantitatively assess regional LV wall motion[20]. Radial and circumferential strains were measured from mid-LV short axis view, while transverse and longitudinal strains were from apical 4-chamber view.

Cardiac catheterization was performed under general anesthesia using 1% sevoflurane inhalation. Three Fr micromanometer-tipped catheter (SPR-249, Millar Instruments, Houston) was inserted through the LV apex to measure heart rate, LV maximal-systolic pressure (BPmax), maximal rate of the LV pressure change (dP/dt max) evaluating systolic preload- dependent LV function, and time constant of LV relaxation (τ) evaluating diastolic load- dependent function.

Real-time PCR

Total RNA was retrieved from NHDF by using RNeasy Mini kit (Qiagen, Venlo, Netherlands) and treated with RNase-Free DNase Set (Qiagen). TaqMan probes were designed using Primer Express software (Applied Biosystems, California, USA). Real-time PCR was performed using a 7500 Fast Real-Time PCR System with TaqMan Universal PCR Master Mix (Applied Biosystems). Concentration of HGF, VEGF, and SDF-1 in the culture

supernatant of NHDF was measured by using ELISA kit (Procarta Cytokine Assay kit, Panomics, Santa Clara, CA).

Histological analysis and electron microscopy

The extracted dog hearts were transversely cut, fixed with 10% buffered formalin and embedded in paraffin. The heart sections of 10 μm thickness were stained with hematoxylin and eosin, Masson-trichrome, picro-sirius red and Periodic acid-Schiff (PAS). The heart sections were also labeled by anti-von Willebrand factor (vWF) antibody (Dako EPOS) visualized by HRP (DakoCytomation, Glostrup, Denmark). Fibrotic area was calculated in the picro-sirius red-stained sections by using a planimetric method with morphometry-analyzer (NIS-elements D, Nikon, Japan) on 5 optical fields that were selected randomly for each sample. Extracted dog heart tissues were fixed with 2.5% glutaraldehyde, and stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 electron microscope. (HITACHI High- Technologies, Tokyo, Japan)

Statistical analysis

All data are presented as the mean ± SEM. The analyses were performed using non-parametric methods because the sample sizes are too small to allow checking of the assumptions of parametric methods. Expression of mRNA in vitro analyzed by PCR and ELISA was analyzed by Jonckheere-Terpstra test for assuring dose-dependent effect of ONO1301. Hemodynamic data obtained from conventional echocardiography, cardiac catheterization, and speckle-tracking echocardiography as well as histopathological findings such as %fibrosis, cell diameter, and vascular density at the target and remote sites of Control group or ONO1301SR group were analyzed by nonparametric repeated measures analysis. Statistical significance was defined as having a value of $p< 0.05$. Statistical analyses were

performed with the R program (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna).

Results

Effects of ONO1301 on expression of endogenous cytokines in vitro

Effects of ONO-1301 on expression of HGF, VEGF, and SDF-1 in the NHDF *in-vitro* were examined by real-time PCR and ELISA. Relative expression of mRNA for HGF, VEGF, and SDF-1 was up-regulated in the NHDF with ONO1301 added in a dose-dependent manner (Figure 1a-1c), which was consistent to the release of HGF, VEGF, and SDF-1 into the supernatants (Figure 1d-1f).

Global recovery of the DCM heart by injection of ONO1301SR

Serial changes in global systolic and diastolic cardiac function were assessed under general anesthesia by conventional echocardiography at 3 time points; 0 weeks (prior to commencement of rapid-pacing), 4 weeks after the commencement of rapid-pacing (just before injection of either ONO1301SR or PLGA only) and 4 weeks after injection of either ONO1301SR or PLGA only. Cardiac performance was markedly deteriorated including increased Dd/Ds and E/E', and decreased EF, ESWT, and EDWT at 4 weeks, when either ONO1301MS or PLGA only were intramyocardially injected.

At 4 weeks after PLGA injection, both systolic and diastolic cardiac functions were further deteriorated.

On the other hand, EF, and ESWT/ EDWT at both target site and remote site at 4 weeks after ONO1301SR injection were significantly greater than those at 4 weeks after PLGA injection (EF; 39±1.7 *vs.* 19±2.0 %, $P<0.05$, Fig2e, ESWT/ EDWT at target site; $1.3\pm 3.0\times10^{-2}$ *vs.* $1.1\pm2.0\times10^{-2}$, p=0.01, Fig 2c, ESWT/ EDWT at remote site 1.2 ± 0.1 *vs.* $1.1\pm 3.0\times10^{-2}$, p=0.04, Fig 2d) although the impact of the recovery was stronger in the target site.

Ds was significantly smaller post-ONO1301MS injection than post-PLGA injection (Ds; 23±2.4 *vs.* 31±1.7 mm, P<0.05, Figure 2b), while Dd showed a trend to be smaller post-ONO1301SR injection than post-PLGA injection without statistical significance (Figure 2a). E/E' post-ONO1301SR injection was significantly smaller than that post-PLGA injection (E/E'; 11±1.2 *vs.*16±0.5, *P*<0.05, Figure 2f).

Cardiac catheterization, carried out at 4 weeks after either ONO1301SR or PLGA injection, revealed that τ was significantly smaller post-ONO1301SR injection than post-PLGA injection (τ; 32±0.9 *vs.* 55±5.8, *P*<0.05). Heart rate, BP max, and dp/dt max did not show any significant difference at 4 weeks after injection of either ONO1301SR or PLGA.

Regional functional recovery post-ONO1301SR injection

Serial changes of regional systolic cardiac function were assessed under general anesthesia by speckle tracking echocardiography at the same 3 time points as the conventional echocardiography. At 4 weeks after the commencement of rapid pacing, all strain values at both target and remote sites were decreased compared to those prior to rapid-pacing. At 4 weeks after the PLGA injection, the absolute values of peak systolic radial, circumferential, transverse and longitudinal strains at both target and remote sites further decreased compared to those prior to the PLGA injection. In contrast, at 4 weeks after the ONO1301SR injection, strain values of radial, transverse and circumferential were greater at the target site than those after the PLGA injection (radial strain: 36±4.7 *vs.* 8.3±1.4 %, *P*<0.05, transverse strain: 39±9.3 *vs.* 9.5±2.1 %, *P*<0.05, circumferential strain; -11±1.3 *vs.* -3.9±0.6 %, *P*<0.05, Figure 3a-3c), though longitudinal strain was not different between the hearts with and without ONO1301SR treatment (Figure 3d). On the other hand, only radial strain was significantly improved at the remote site post- ONO1301SR injection compared to that post-PLGA injection (Figure 3e-3h).

Histological findings of reverse LV remodeling post- ONO1301SR injection

Gross myocardial structure, assessed by hematoxylin/eosin staining and Masson Trichrome's staining, showed a thicker LV wall and a smaller LV cavity 4 weeks post- ONO1301SR administration (Figure 4h-4k) than that post-PLGA injection (Figure 4d-4g).

Quantity of interstitial fibrosis at the target site, evaluated by picro-sirius red-staining, was significantly less at 4 weeks post-ONO1301SR administration compared to that post-PLGA injection (% fibrosis at the target site; 9.9±0.7 *vs.* 23±0.9 %, $P<0.01$, Figure 4a). Of note, distribution of interstitial fibrosis was significantly restricted at the target site than that at the remote site post-ONO1301SR administration (9.9±0.7 *vs.* 16±1.2 %), whereas PLGA injection did not produce such an uneven distribution (23±0.9 at the target site *vs.* 23±0.8 % at the remote site).

Mean transverse cellular diameter of cardiomyocytes (Figure 4b) at the target site, measured by PAS-stained sections, was also significantly smaller at 4 weeks post-ONO1301SR administration compared to that post-PLGA injection (12±0.6 *vs.* 15 ±0.8 mm, $P<0.01$). The diameter of cardiomyocytes at the target site was smaller post-ONO1301SR administration compared to that at the remote site (12±0.6 *vs.* 14±0.3 mm, $P<0.01$), whereas such an uneven distribution in the myocyte-size was not observed post-PLGA injection.

Vascular density (Figure 4c), assessed by counting the number of factor VIII-positive cells in the fields, was significantly greater at the target site at 4 weeks post-ONO1301SR administration compared to that post-PLGA injection (998±70 *vs.* 467±33 /mm^2, $P<0.01$). The vascular density at the target site was greater post-ONO1301SR administration compared to that at the remote site (998±70 *vs.* 491±24 /mm^2), whereas such an uneven distribution of vascular density was not observed post-PLGA injection.

Electron microscopy revealed that the cardiomyocytes at 4 weeks post-PLGA-injection showed a prominent swelling or disruption of mitochondria, intracellular or

perinuclear edema, and sarcoplasmic vacuoles resulting from dilation of sarcoplasmic reticulum (Figure 5a). However, marked loss of myofilaments, and alterations of characteristic sarcomeric structure were not observed in any groups. While the interfibrillar space in the myocardium post-ONO1301SR injection was slightly widened, the mitochondria were compact and showed a densely packed cristae (Figure 5b) compared to those post-PLGA injection.

Discussion

We here demonstrate that ONO1301 dose-dependently up-regulated expression of multiple cytokines, such as HGF, VEGF and SDF-1 in fibroblasts *in-vitro*. Histological reverse LV remodeling, such as attenuated fibrosis and swelling of cardiomyocytes, increased vascular density and recovered mitochondria structure, in the target area but not significantly in the remote area were consistent to the regional functional recovery, assessed by speckle tracking-echocardiography, which was more prominent at the target area than that at the remote area following the ONO1301SR- injection. Such regional recovery at the target area post-ONO1301SR injection resulted in recovery of global function, including systolic and diastolic function.

Iwata et al. reported that local administration of prostacycline analogue may induce HGF production followed by VEGF expression via cAMP- mediated pathway and that elevation of HGF or VEGF may mediate the favorable effect in the treatment of ischemic heart failure[21]. We here showed that ONO1301 directly activates fibroblasts *in vitro* and release not only HGF and VEGF as reported previously[13, 16, 21], but also SDF-1 which has been thought to be a representative therapeutic stem cell homing factor in ischemic heart[22]. In the present *in vivo* study, we used the slow-releasing form to deliver ONO1301 and importantly, deliver ONO1301SR directly into the myocardium of canine DCM heart in the aim to elevate regionally ONO1301 level thus maximizing the effects on the cardiac fibroblasts to release cardiotherapeutic factors. Consequently, pathological and functional effects of intramyocardial ONO1301SR injection were markedly prominent in the target area (area surrounding the injection sites) compared to the remote area, suggesting that cardiac fibroblasts residing the target area might have played a key role in locally upregulated cardiothrapeutic cytokines.

In addition, it was noted that the typical structural features of cardiomyocytes in the severely ischemic heart, such as swelling of mitochondria, intracellular or perinuclear edema and sarcoplasmic vacuoles referred to by a phenomenon, `permeability transition`[23], was reversed post- ONO1301SR injection in this study. Based on these findings, targeted injection of ONO1301SR into the damaged myocardial area might maximize therapeutic effects of ONO1301 which upregulates cardiothrapeutic cytokines in a regional concentration-dependent manner.

Use of slow releasing form in administrating ONO1301 directly into the heart includes concerns related to the initial burst which might have an adverse effect on hemodynamics[24]. In this study, there is no hemodynamic compromise during or immediately after the procedure despite the poor cardiac function, suggesting that the protocol used here in injectin ONO1301SR might be appropriate in treating DCM heart. Further study for dose-dependent hemodynamic change immediately post-ONO1301SR administration would be needed in translating this treatment into the clinical arena.

Intramyocardial delivery of ONO1301 might be achieved by direct injection, intracoronary artery injection or attachment on the epicardial surface. Injection area- specific recovery, demonstrated in this study, would suggest that direct injection of ONO1301 might be more effective in the myocardium which has heterogeneous pathology such as ischemic cardiomyopathy, as reported by Iwata et al[21]. Combination with coronary artery bypass grafting would also be a clinically- applicable strategy for this purpose. On the other hand, homogeneous pathology such as DCM might gain more therapeutic benefits by diffusely attaching ONO1301 on the epicardial surface compared to direct injection, though further basic investigation will be needed to establish this strategy. Intracoronary injection is known to diffusely deliver reagents or cells into the myocardium[25], however, intracoronary injection of ONO1301SR whose diameter is more than 20 micrometers will cause coronary embolism

and ischemic myocardial damage.

This study is limited by the use of canine model, which is not exactly relevant to the clinical DCM pathologies and has limited reagents for mRNA or protein investigations available.However, large animal model is essential in investigating cardiac performance by the latest technology used in the clinical arena, such as speckle- tracking echocardiography used in this study, whereas rodent models with or without genetic modifications would be useful in showing the mechanistic insights of this treatment.

As mechanistic insights have been reported by several studies , main focus of this study was to test the hypothesis that intramyocardial injection of ONO 1301 induces region- specific and global functional recovery in dilated cardiomyopathy. In addition, this study investigated the mechanisms of this treatment to show the consistency with the previous studies which used rodent models to prove the mechanisms of this treatment.

Injection to the anterior wall and use of the posterior wall as the control was an option; however, in the surgical view, injection into the lateral wall produced consistent, reproducible and safe injections compared to that into the anterior wall. Therefore, the reagent was injected into the lateral wall and septal wall was used as the control in this study. However, pathophysiology of the septum are substantially influenced by the performance of the RV.

In summary, we quantitatively evaluated region- specific pathological and functional effects of ONO1301SR, a slow- releasing form of prostacycline agonist, on rapid pacing canine DCM model. Multi- therapeutic endogenous cytokines induced by intramyocardial ONO1301SR injection may be responsible for the improved cardiac performance and

ultrastructure. ONO1301SR is a promising therapeutic drug for enhancing myocardial regeneration on the impaired myocardium.

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Disclosure

Yoshiki Sakai is an employee of ONO pharmaceutical Co Ltd. There are no other financial or relations that could lead to a conflict of interest.

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Figure legends

Figure 1. PCR and ELISA analysis in vitro showed that mRNA levels for HGF (1a,1d), VEGF (1b,1e), and SDF-1 (1c,1f) increased in the dose-dependent manner in NHDF cultured with ONO1301; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; SDF-1, stromal-derived factor 1; NHDF, normal human dermal fibroblast. Mean±SEM, respectively.

Figure 2. Echocardiography (2a- 2f) showed ONO1301SR significantly improved distressed cardiac function. Note that ESWT/EDWT, reflecting on the radial strain of myocardium, were significantly recovered at the target site in the ONO1301SR group compared to that in the Control group, while that at the remote site didn`t show significant differences between the groups; Dd, endo- diastolic dimension of left ventricle; Ds, endo- systolic dimension of left ventricle; Ant wall, anterior wall of left ventricle ; post wall, posterior wall of left ventricle; EF, ejection; ESWT, End- systolic wall thickness; EDWT, End- diastolic wall thickness. Mean±SEM, respectively. Ind: induction of rapid- pacing. Pre: pre- treatment of PLGA or ONO1301SR. Post: post- treatment of PLGA or ONO1301SR.

Figure 3. Speckle tracking echocardiography showed that the absolute values of peak radial, circumferential, and transverse strains at the target site in the ONO1301SR group were significantly higher than in the Control group (3a- 3d) while all of them but radial strains at the remote site in the ONO1301SR group and that in the Control group were not significantly different (3e- 3h), which implied that ONO1301SR had influence on the cardiac-performance especially at the very site where ONO1301SR was administrated; Radial, radial strain; Circumferential, circumferential strain; Transverse, transverse strain; Longitudinal,

longitudinal strain. Target site is defined as the area in which ONO1301SR or PLGA is injected while remote site as non-injection area. Ind: induction of rapid-pacing, Pre: pre-treatment of PLGA or ONO1301SR, Post: post- treatment of PLGA or ONO1301SR. Mean±SEM, respectively.

Figure 4. Histopathology; quantitative evaluation of interstitial fibrosis (4a), mean cell diameter (4b), vascular density (4c), and representative micrograph of the Control group (4d-4g) and the ONO1301SR group (4h- 4k). 5d, 5h; Hematixylin- Eosin-staining. 4e, 4i; Masson- trichrome staining, 5f, 5j; Sirius red staining, 5g, 5k; staining with Anti-Human-Von Willebrand Factor. At the target site in the ONO1301SR group, the amounts of fibrosis and mean cell diameter were significantly smaller and vascular density was significantly higher than those in the Control group and those at the remote site in the ONO1301SR group. Mean±SEM, respectively.

Figure 5. Electron microscopy revealed that the myocardium of the Control group (5a) showed prominent swelling or disruption of mitochondria, intracellular or perinuclear edema, and sarcoplasmic vacuoles resulting from dilation of sarcoplasmic reticulum (arrowhead). On the other hand, mitochondria in the ONO1301SR group (5b) were compact and showed densely packed cristae.

Figure 1

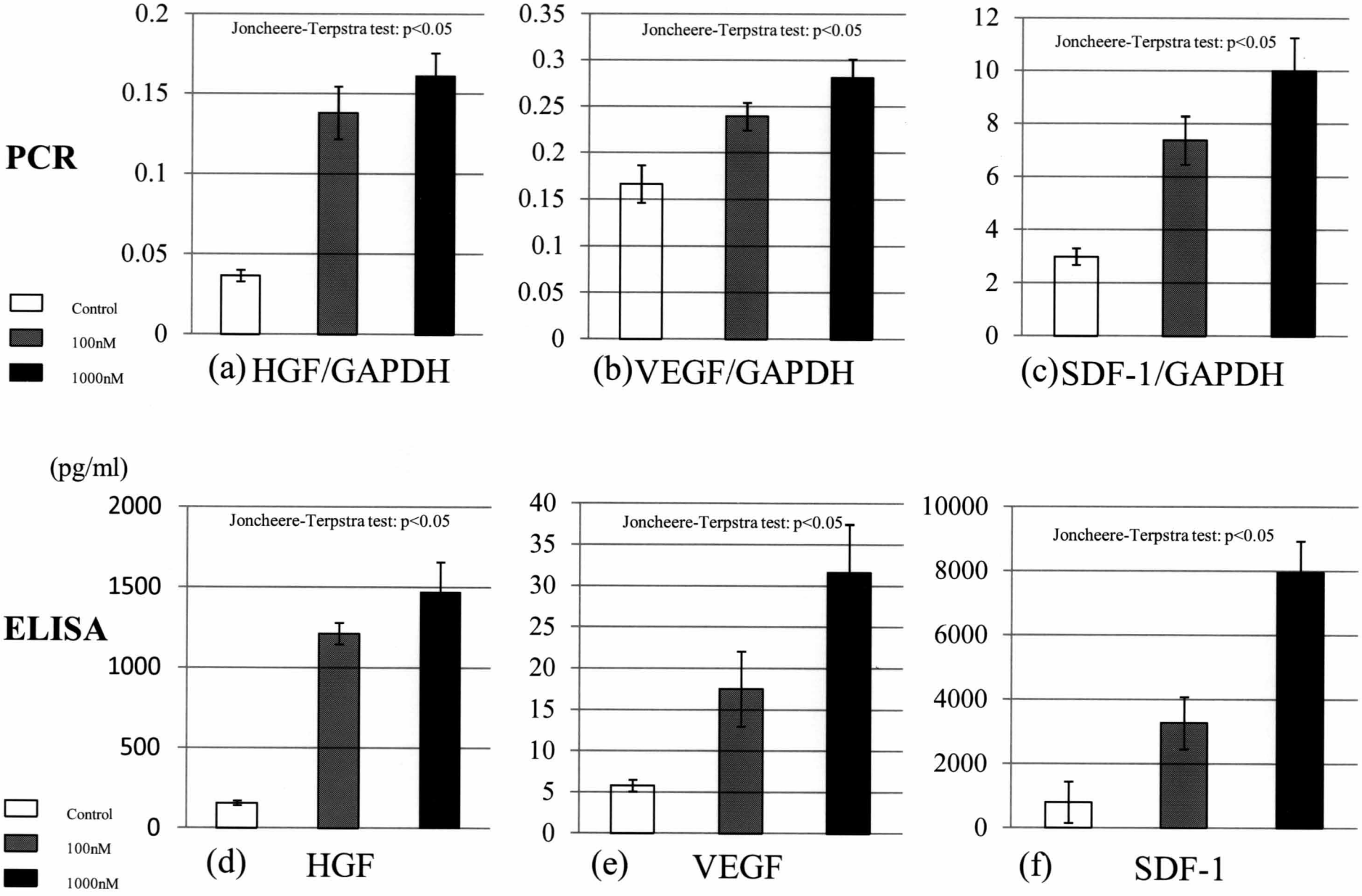

PCR
Joncheere-Terpstra test: p<0.05
0.2
0.15
0.1
0.05
0
Control
100nM
1000nM
(a) HGF/GAPDH
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
(b)VEGF/GAPDH
12
10
8
6
4
2
0
(c)SDF-1/GAPDH
(pg/ml)
ELISA
2000
1500
1000
500
0
(d) HGF
40
35
30
25
20
15
10
5
0
(e) VEGF
10000
8000
6000
4000
2000
0
(f) SDF-1

Figure 2

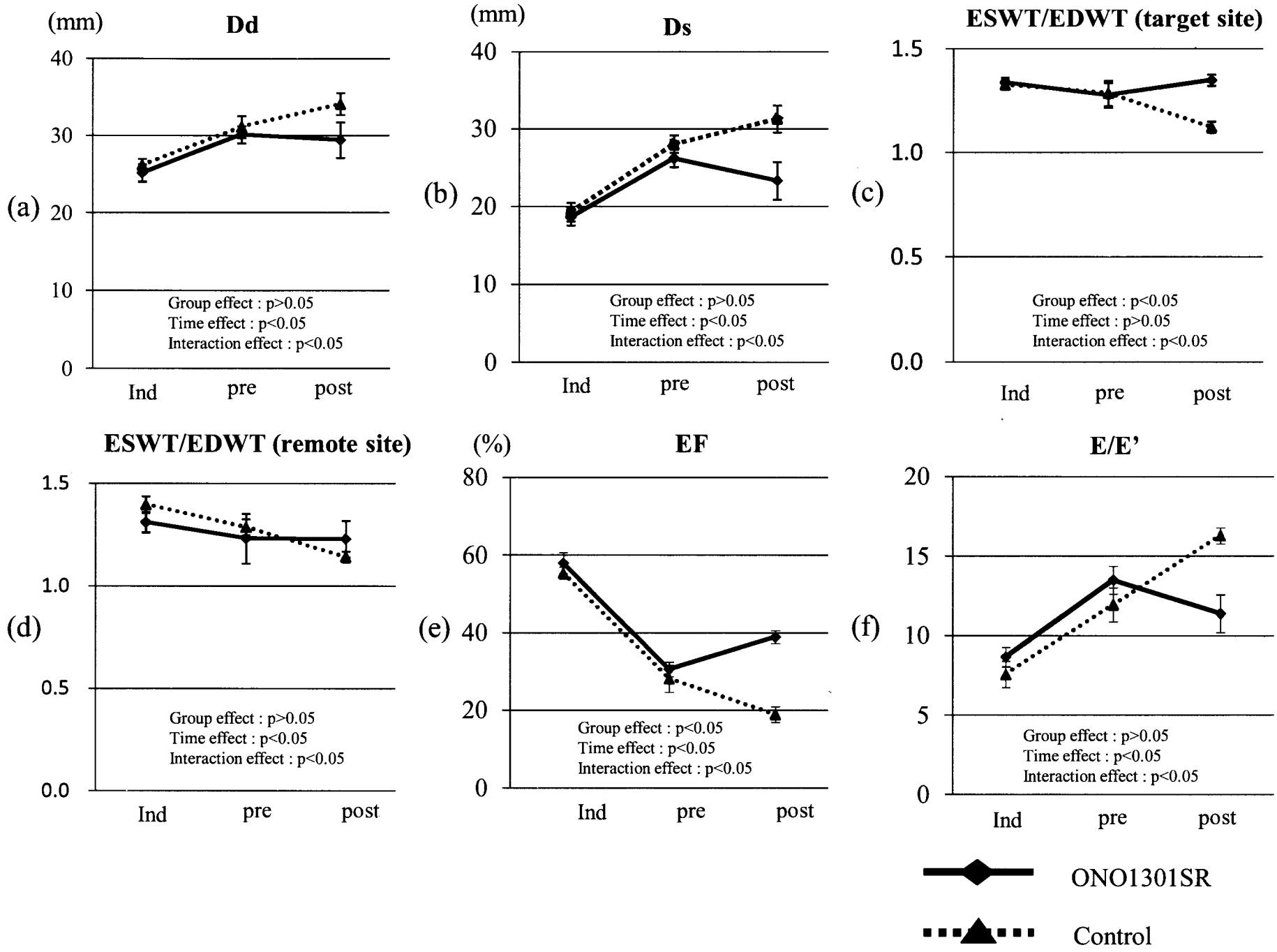

(mm)
Dd
40
30
20
10
0
(a)
Group effect : p>0.05
Time effect : p<0.05
Interaction effect : p<0.05
Ind
pre
post
(mm)
Ds
(b)
Group effect : p>0.05
Time effect : p<0.05
Interaction effect : p<0.05
ESWT/EDWT (target site)
1.5
1.0
0.5
0.0
(c)
Group effect : p<0.05
Time effect : p>0.05
Interaction effect : p<0.05
ESWT/EDWT (remote site)
(d)
Group effect : p>0.05
Time effect : p<0.05
Interaction effect : p<0.05
(%)
EF
80
60
40
20
0
(e)
Group effect : p<0.05
Time effect : p<0.05
Interaction effect : p<0.05
E/E'
20
15
10
5
0
(f)
Group effect : p>0.05
Time effect : p<0.05
Interaction effect : p<0.05
ONO1301SR
Control

Figure 3

Speckle tracking echocardiography

(upper; target site, lower; remote site)

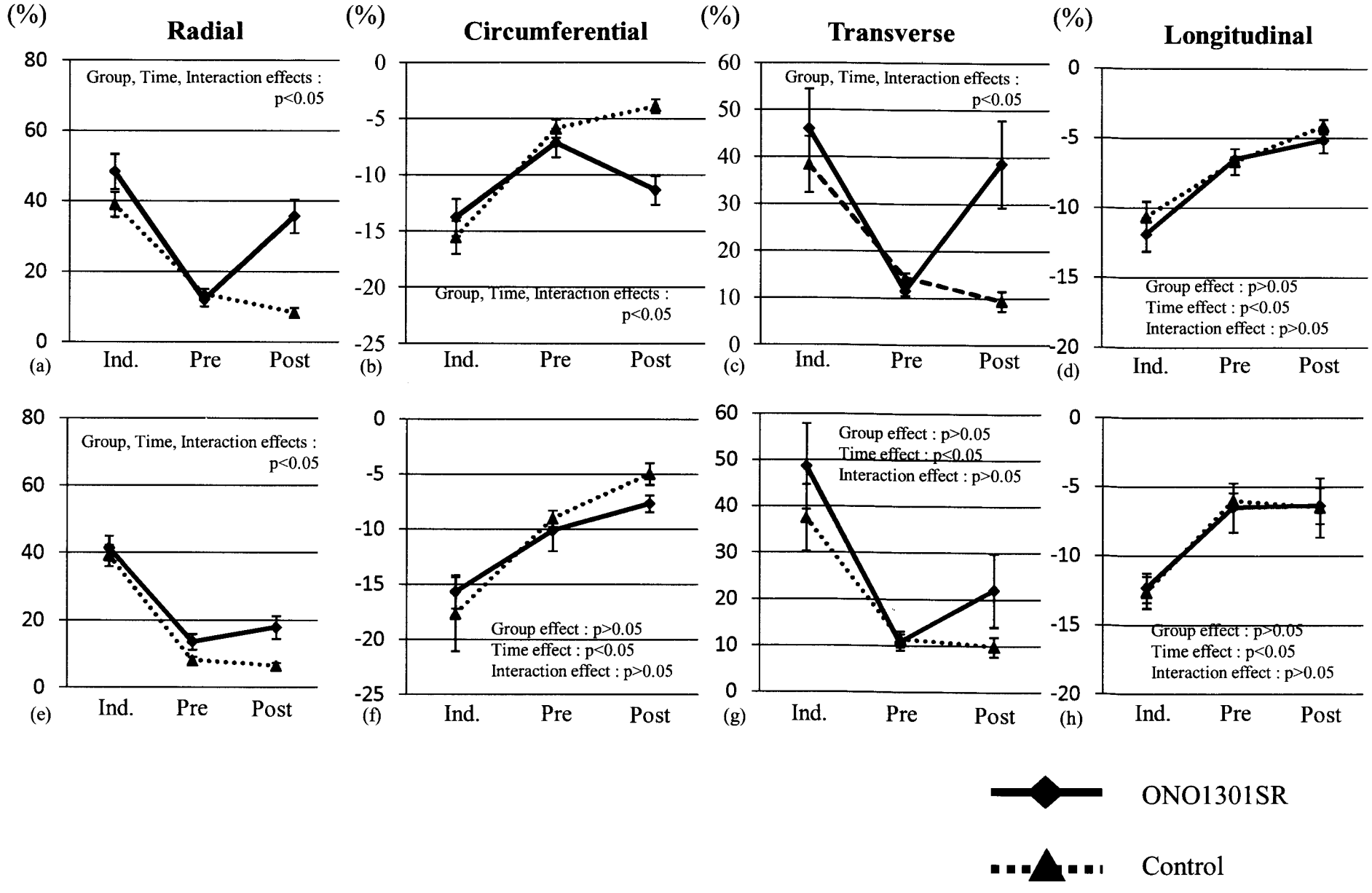


Figure 4

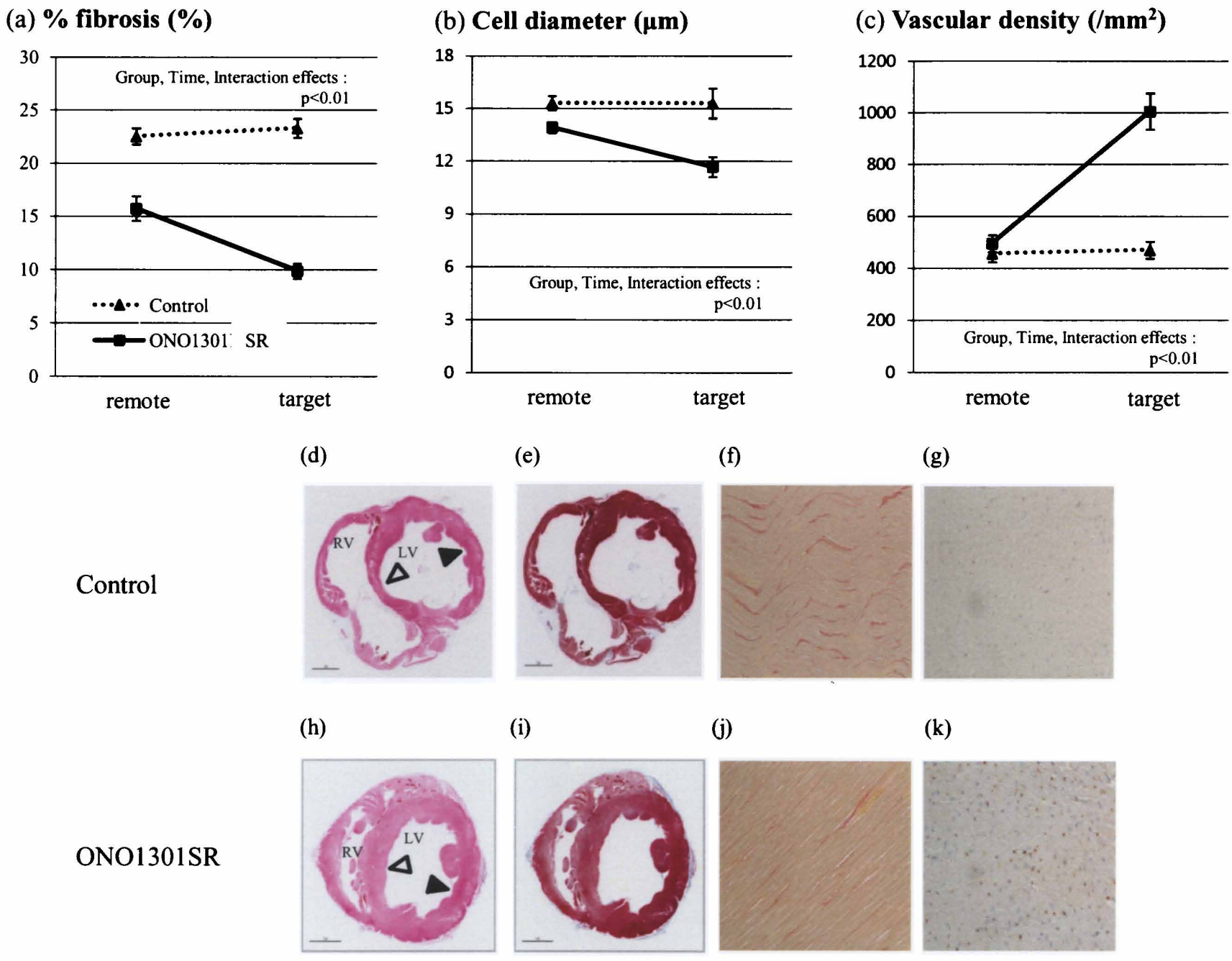
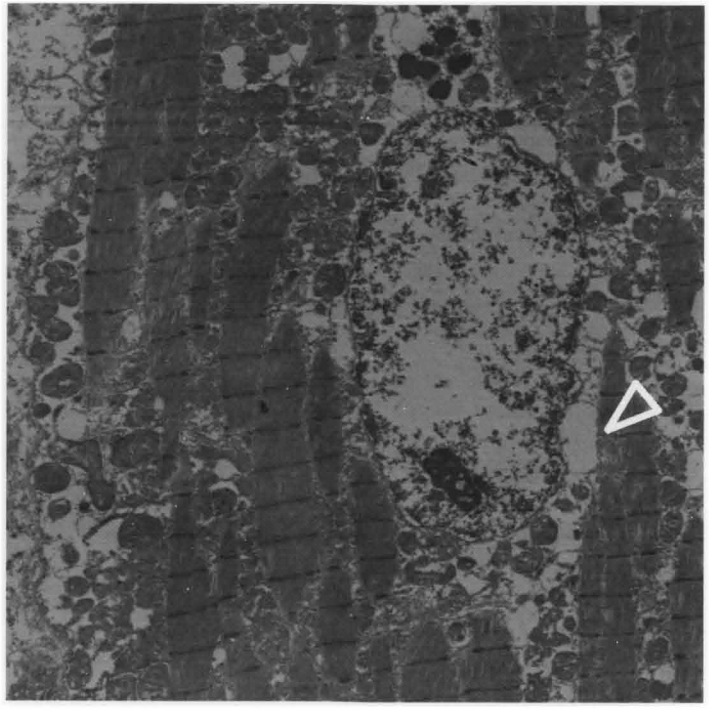

(a) % fibrosis (%)
30
25
20
15
10
5
0
Group, Time, Interaction effects :
p<0.01
Control
ONO1301 SR
remote
target
(b) Cell diameter (μm)
18
15
12
9
6
3
0
Group, Time, Interaction effects :
p<0.01
remote
target
(c) Vascular density (/mm²)
1200
1000
800
600
400
200
0
Group, Time, Interaction effects :
p<0.01
remote
target
(d)
(e)
(f)
(g)
Control
RV
LV
(h)
(i)
(j)
(k)
ONO1301SR
RV
LV

Figure 5

(a)



(b)