Differential behaviour of epicardial adipose tissue-secretomes with high and low orosomucoid levels from patients with cardiovascular disease in H9C2 cells

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Abstract

Epicardial adipose tissue (EAT) releases orosomucoid (ORM) with multiple modulatory and protective properties. We tried to identify the effect of EAT-supernatants according to their ORM levels on H9C2 cells. Cardiomyoblasts were cultured with EAT-secretomes at different ORM concentrations ~~(higher or lower ORM than 300 ng/mL)~~ or ORM at 50 or 500 ng/mL on a Real-Time Cell Analyser. Proteins identification on each secretomes subgroup was performed by LC-mass spectrometry. Two of them were validated in EAT-supernatants from 42 patients by ELISA. Protective ORM effect on H9C2 cells, under hypoxic conditions with or without palmitic acid, was determined by flow cytometry using a FITC Annexin-V-FLUOS staining Kit. Our results showed a positive or negative effect of EAT-secretomes with ORM levels lower or higher than 300 ng/mL, respectively ($p<0.01$). ORM itself was not harmful and even, was protective against hypoxic conditions. Our data showed that the classification of EAT-secretomes regarding ORM levels might help us to find proteins with deleterious or protective effect on cardiomyoblasts in hypoxic conditions.

1. INTRODUCTION

Epicardial adipose tissue (EAT) is localized around the coronary arteries and may reach 80% of the heart [1]. Although, EAT was early related with cardiovascular events in 1930 [2], several years later, the imaging techniques have allowed to find an association between thickness or volume and coronary artery disease [3], its associated risk factors [4], atrial fibrillation [5] or heart failure (HF) [6]. EAT is localized over the myocardium, sharing the same microcirculation [7]. These characteristics allow EAT directly interact with myocardial [8] and endothelial cells through *vasa vasorum* [9]. Previous studies have shown the existence of EAT-secreted [8-12] factors, involved in cardiovascular deleterious events [8,13] which might explain the association between EAT and heart failure [6,14]. However, the released proteins might also play a protective role through the prevention of apoptosis and induction of angiogenesis. Thus, in animal models of myocardial infarction upon treatment with epicardial fat flap, there was a substantial decrease of apoptosis and infarct size, improving left ventricular ejection fraction and

vascular connections at the flap-myocardium interface [15]. In this sense, previous findings from our group have demonstrated that Orosomucoid (ORM) is differentially released by EAT in patients with diabetes or coronary artery disease (CAD) and its beneficial effects on endothelial cells [16]. In this sense, ORM can play an angiogenic role and reduce the infarct size. Therefore, one of the main current challenges for identifying therapeutic strategies consists in counteracting hypoxia-induced myocardial cell apoptosis [17]. In part, because the common feature of several cardiovascular diseases, including heart failure, myocardial ischemia and infarction is the loss of apoptotic cardiomyocytes [18]. Our aim was to know the differential behaviour of EAT secretome according their ORM levels on H9C2 cells and to identify EAT-released proteins with specific protector role against hypoxia and/or lypotoxia.

2. MATERIAL AND METHODS

2.1. Subjects

Epicardial fat biopsies were obtained from 62 patients, undergoing valve replacement or coronary artery bypass grafting, provided written informed according to Declaration of Helsinki. The study was approved by the Galician Clinical Research Ethics Committee. Samples were processed as it was described previously [16]. Supernatants of EAT were collected and stored until used. Fourteen, eight and forty two from all secretomes were used for cell real-time, mass spectrometric and ELISA analysis, respectively.

2.2. Cell culture and reagents

Rat ventricular cardiomyoblast cells (H9C2) were used because they are energetically similar to primary cardiomyocytes, maintain the morphological characteristics of immature embryonic cardiomyocytes with electrical and hormonal signal pathway elements of adult cardiac cells [19]. Moreover, they were studied also as a good *in vitro* model of cardiac ischemia-reperfusion injury [20]. H9C2 were cultured in 0.1% gelatine coated plates with DMEM medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS), antibiotics (100 UI/mL penicillin, 100 µg/mL streptomycin) and L-glutamine (2mM), in a 5% CO_2 atmosphere at 37°C. H9C2 were seeded at least 24 hours before treatments unless otherwise indicated and the experimental procedures were conducted upon reaching 80% confluence. Cultured cells were

treated with EAT supernatants, palmitic acid (apoptotic inducer of cardiomyoblasts through lipotoxic pathways[21]) and/or ORM (Sigma-Aldrich) at indicated concentrations. Control groups were treated with respective culture medium and vehicle. Palmitic acid from Sigma-Aldrich was prepared by conjugation with fatty acid-free bovine serum albumin (BSA) by dissolving in ethanol and diluting 100-fold in an aqueous 2% BSA solution to achieve selected concentration, indicated in each experiment, and reduces ethanol proportion. Palmitic solution was freshly prepared for each experiment.

2.3. EAT biopsies and Secretome ORM levels

Biopsies from 62 patients were split into 100 mg pieces and washed in M-199 medium (Sigma–Aldrich, St. Louis, MO, USA) with antibiotics supplementation (100 UI/mL penicillin, 100 ug/mL streptomycin) overnight. Fresh medium was replaced into biopsies and incubated for 6 hours. Then, EAT supernatants were collected and frozen at −80 °C until use. ORM levels on each supernatant were quantified using an ELISA kit (GenWay Biotech, Inc., San Diego, CA, USA) according to the manufacturer's protocol.

2.4. Real-Time Cell Viability and proliferation

The Real-Time Cell Analyzer (RTCA DP Instrument) (Roche Applied Science, Mannheim, Germany) is an impedance-based technology that was used for label-free and real-time monitoring of H9C2 proliferation [22]. The system monitored cellular events in real time measuring electrical impedance across interdigitated micro-electrode integrated in the bottom of the well. The measurements, called Cell Index (CI) dynamic values, were monitored in 10 min intervals from the time of plating until the end of the experiment. Five thousand H9C2 cells were seeded on each well of culture *E-plates 16* with DMEM and 10% FBS (Sigma-Aldrich) during four hours. Then, medium was replaced by DMEM without FBS for 3 hours before treatment with EAT supernatants from 14 patients or ORM (Sigma-Aldrich) at 50 or 500 ng/mL concentrations in a proportion 1:1 with DMEM. Impedance was registered for 12 hours and 30 minutes after treatment. All values were normalized at the time of treatment addition. At the end of the experiment, CI values were represented with respect to CI control group values on each time point. EAT supernatants from each patient were analysed by duplicated and ORM treatments were done thrice by triplicated.

2.5. End-point cell viability and proliferation assay

Cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich). Briefly, H9C2 cells were seeded in triplicate at a density of 6000 cells/well in 96-well plates. Cells were treated with different ORM concentrations (50 or 500 ng/mL) for 24 or 48 hours. After, MTT (0.5 mg/ml) was added and incubated at 37°C during 4 hours. Formazan crystals were solubilized with dimethyl sulfoxide (DMSO). The optical density (OD) was measured at a wavelength of 570 nm using an automated microplate reader. Every treatment was made by duplicated.

2.6. Protein Identification by LC-MALDI

After testing the EAT secretomes on cardiomyoblasts, supernatants with ORM higher than 500ng/mL and lower than 100ng/mL were selected since their extreme effect for liquid chromatography- mass spectrometry analysis. Each group was formed by 2 mL of epicardial fat- supernatants coming from 4 patients (0.5 mL each patient). This volume was concentrated by ultrafiltration with three different columns with <10, 10-50 and >50 kDa cutoff (Amicon-Ultracentrifugal filter units, Millipore Corporation, Darmstadt, Germany). The final volume was 0.03 mL. The protein concentrations were quantified by RC DC Protein Assay (BioRad Lab, CA) and 200 µg of protein were concentrated in one band by SDS-PAGE (10%) as it was described previously [23,24]. The protein bands were visualized by Sypro-Ruby fluorescent staining (Lonza Rockland, Inc., Rockland, ME, USA). After, bands were excised and submitted for in-gel manual tryptic digestion following standard procedure with minor modification [25]. Peptides were extracted thrice by 20 min incubation in 40 µL of 60% ACN in 0.5% formic acid. The resulting peptide extracts were pooled, concentrated in a SpeedVac and stored at -20°C.

Separation of the resulting tryptic peptides mixtures was performed by nanoscale reversed-phase LC-MALDI. The nanoLC Ultra 1D plus (EKsigent, ABSciex Boston, MA, USA) was coupled to a MALDI-spotter (Eksigent). Peptides mixtures were re-dissolved in 0.1% formic acid, 2% ACN and injected into the trapping column (ChromXP nanoLC Trap column 350 µm id x 0.5 mm, ChromXP C18 3 µm 120Å, ABSciex) at a flow rate of 10 µL/min (0.1% formic acid 2% ACN). After 15 min the trapped peptides were separated in a nanocolumn (ChromXP nanoLC column 75 µm id x 15 cm, ChromXP C18 3um 120Å, ABSciex) at a flow rate of 300 nl/min in a linear gradient elution from 95% A (0.1% formic acid, 2% ACN) to 60% B (90% ACN, 0.1% formic acid) in 80 min followed by an increase up to 95% B in 5 min. The eluting peptides were

153 mixed with a matrix solution, consisting of 3 mg alpha-cyano-4-hydroxycinnamic acid (α -
154 CHCA) dissolved in 1 mL of 50% ACN in 0.1% trifluoroacetic acid, and 10 fmol/µL angiotensin
155 (as internal standard) and deposited, onto a Opti-TOF LC/MALDI insert (ABSciex) with a speed
156 of one spot per 12 seconds.
157 Mass spectrometry analysis was made using a 4800 MALDI-TOF/TOF analyzer (ABSciex). MS
158 spectra were acquired in reflector positive-ion mode with a Nd:YAG, 355 nm wavelength laser,
159 averaging 1000 laser shots and using at least three trypsin autolysis peaks as internal
160 calibration. All MS/MS spectra were performed by selecting the precursors with a relative
161 resolution of 300 (FWHM) and metastable suppression.
162 Peptide and protein identification were performed using the Protein Pilot software vs 4.0.80.85
163 (ABSciex) with Paragon Algorithm. MS/MS data was searched against the UniProt/Swiss-Prot
164 database of protein sequences (January 2014; Swiss-Prot, Geneva, Switzerland). Searches
165 were restricted to human taxonomy allowing carbamidomethyl cysteine as a fixed modification
166 and oxidized methionine as variable modification. Both the precursor mass tolerance and the
167 MS/MS tolerance were set at 30 ppm and 0.35 Da, respectively, allowing 1 missed tryptic
168 cleavage site. Only proteins with a threshold >95% confidence (>1.3 unused score) were
169 considered as positive hits.
170 Representation and comparison of EAT proteins regarding groups with high or low ORM levels
171 was done by Venn Diagrams [26].

172 **2.7. Hypoxia induction**

173 Hypoxia experiments were conducted in a hypoxic chamber (Baker Ruskinn's Invivo$_2$200,
174 Bridgwater, UK) at 37°C with 0.1% O_2 (hypoxia concentration triggers cardiomyoblasts death
175 [27]), 5% CO_2 and N_2 balance for 24h hours.

176 **2.8. Flow cytometry**

177 Apoptosis was measured using the FITC Annexin-V-FLUOS staining Kit (Roche Diagnostics)
178 according to the manufacturer´s protocol. H9C2 cells were incubated during 24 hours with
179 500µM palmitic acid, 50ng/ml ORM or both. After, H9C2 cells were collected by trypsinization
180 and centrifuged at 1200 rpm for 5 minutes. Following suspension in binding buffer, cells were
181 labelled with Annexin-V-FITC and Propidium Iodide (PI) according to the manufacturer's
182 instructions. The discrimination between apoptotic and necrotic cells with Annexin V-Fluos were

counterstain with propidium iodide. Thus, while Annexin-V has high affinity for phosphatidylserine (protein of membrane which is translocated from inside to outside), the DNA stain by IP allows the discrimination of necrotic cells from the Annexin-V positively stained cell cluster. FITC and PI were measured by flow cytometry analysis of 10000 gated cells using FACScan and CellQuestPro software from Becton Dickinson (Fullerton, CA, USA).

2.9. Wound healing assay

One hundred fifty thousand H9C2 cells were cultured on 24-well clear-bottom tissue culture plates (VisiPlate-24 Black; PerkinElmer, Waltham, MA, USA) with DMEM medium supplemented with 0.5 % FBS, antibiotics (100 UI/mL penicillin, 100 ug/mL streptomycin) and L-glutamine (2mM), in a controlled environment at 37ºC and 5% CO_2 during 24 hours. After serum starvation for 3h, a wound was made in every well with a tip. Then, cells were treated with different ORM concentrations (50 or 500 ng/mL) in DMEM supplemented medium. Healing was followed and registered by an Operetta high content imaging instrument (PerkinElmer, Waltham, MA, USA). Images were acquired every 90 min over 72 hours by brightfield microscopy at 10x magnification with 36 fields of view/well. Images were analysed with Harmony v3.5 software (PerkinElmer). The open area percentage relative to each initial wound size after 72 hours was plotted. Every condition was repeated six times in two independent experiments.

2.10. Statistical analysis

Categorical data were represented as percentage and continuous were represented as mean ± SEM (standard error of the media) for cell experiments and mean ± SD (standard deviation) for supernatant proteins levels, which were determined by ELISA. Demographic, anthropometric and clinical data of patients with normal distribution were represented as mean ± SD. Statistical analysis was determined by two-way Student's *t*-test or Pearson correlation by using GraphPad Prism 6 Software.

3. RESULTS

3.1. Effect of EAT supernatants in H9C2 cells

A Real-Time Cell Analyzer (RTCA) was used to monitor dynamic changes in the properties of

H9C2 cells treated with EAT secretomes from 14 patients (Table 1) which were classified according their ORM content. EAT secretomes with ORM levels lower than 300ng/mL induced a marked increase in CI relative to control group (~80%). On the contrary, higher ORM levels leads to a marked decrease in CI ($p<0.01$) (Figure 1A). Thus, RTCA monitoring showed a negative correlation ($r= -0.68$, $p=0.007$) between ORM levels and their positive effect in H9C2 (CI values) (Figure 1B).

3.2. ORM effect in H9C2 cells proliferation and viability

A Real-Time Cell Analyzer (RTCA) was used to monitor dynamic changes in the properties of H9C2 cells under different concentrations of ORM (0, 50 or 500ng/mL). CI values after 12 hours and al half of treatment was considered. As it is shown in Fig. 2A and B, low ORM levels induce a 20% increase of CI values ($P<0,001$). No difference was observed under highest ORM levels. However, MTT assay showed that the ORM treatment at the selected doses did not modify the number of viable cells (Figure 2C).

3.3. Proteomic analysis of EAT supernatants regarding ORM levels

While EAT supernatants with low ORM levels reached CI values ~1.8, the treatment with 50ng/mL ORM induced a slight increase of CI ~1.2 on cardiomyoblasts. Contrary, 500ng/mL ORM was not able to decrease the CI values as EAT supernatants with high ORM levels. These results suggested that alternative elements other than ORM might be associated to EAT effects in H9C2. EAT supernatants from 8 patients were selected based on extreme values of ORM levels, higher than 500ng/mL or lower than 100ng/mL (Table 2) aiming to differentially identify the secreted proteins. We found 17 common proteins and 29 or 27 differentially expressed proteins in EAT supernatants with highest ORM or lowest ORM levels, respectively (Figure 3A and 3B). We focus our attention on Omentin-1 (adipokine secreted by visceral adipose tissue, named Intelectin-1, and inversely related with CAD severity [27] and IL-27 (cytokine with anti-atherogenic property [28]) and measured their levels in EAT-supernatants from 42 patients more. Our data showed a negative correlation between ORM and Omentin-1 levels. However, the association was not established between ORM and IL-27 (Supplementary figure 1).

3.4. ORM reduces hypoxia-induced apoptosis in H9C2 cells

Conjunction of propidium iodide and Annexin V defines viable, apoptotic, or necrotic cells through differences in plasma membrane integrity and permeability [28]. ORM (50ng/mL) was

able to protect the hypoxia-induced apoptosis in H9C2 cells. In this sense, this protein increased the viable cells (0.88 ± 0.01 *vs.* 0.80 ± 0.02 fold change with respect to control; p=0.0128) and reduced the early (0.02 ± 0.005 *vs.* 0.06 ± 0.01; p=0.018) and late (0.12 ± 0.010 *vs*. 0.18 ± 0.02; p=0.036) apoptotic cells (Figure 4).

3.5. ORM reduces lipotoxic-induced apoptosis in H9C2 cells under hypoxia

Palmitic acid induced a reduction of H9C2 viable cells (0.56 ± 0.04 fold change with respect to control; p<0.001) and an increment of apoptotic cells (0.32 ± 0.02 *vs.* 0.04 ± 0.01; p<0.001) (Figure 5A) in normoxia conditions. An additive effect of apoptotic cells was observed under hypoxia. Thus, while similar fold change of viable cells were observed regarding control (0.35 ± 0.06; p<0.001), there was an increment of apoptotic cells (0.71 ± 0.07 vs. 0.21 ± 0.02; p<0.001) (Figure 5B). Although ORM was not able to reduce the palmitic acid-induced cells apoptosis, it was under hypoxia condition (0.44 ± 0.07 *vs*. 0.71 ± 0.07; p=0.02) (Figure 5B).

3.6. Wound healing assay

In wound-healing assays, cells were grown to confluence and then monolayers were scratched and cultured in an Operetta instrument for a further 72 h. Control and treated H9C2 cells with ORM at 50ng/mL recolonized the wound area faster than 500ng/mL ORM treated cells (Figure 6).

4. DISCUSSION

Previous data from our group showed that ORM is differentially released by EAT from cardiovascular disease patients of different aetiologies [19]. In the present study, we investigated how differential ORM levels affect myocardial cells. Our data represent the first evidence of a differential EAT supernatants behaviour on cardiomyoblast cells according their ORM levels. Although more proteins were identified on EAT-supernatants, ORM itself had a protective role in H9C2 cells under hypoxic conditions. Interestingly our data showed a protective role against lipotoxicity just in hypoxic environment. Neutralize lipotoxic induced apoptosis of cardiomyocytes under ischemic processes is one of the main challenges to improve the prognosis of ischemic cardiomyopathy [29]. These results suggest that low ORM levels counteract fatty acid metabolism disorders triggered by hypoxic conditions in H9C2 cells.

The benefit or deleterious effect of EAT secretome in H9C2 cells can be visualized by real-time impedance changes-based method which defines exponential positive or negative curves [30]. Both kinds of curves were observed in H9C2 cells exposed to EAT supernatants with high or low ORM levels from patients with cardiovascular disease. These results indicate a negative correlation between ORM concentrations and EAT supernatants positive effects. After analysing the isolated ORM effect in H9C2, we verify that low ORM levels had a protective role on cardiomyoblasts, reducing the necrosis and apoptosis, in hypoxic conditions. However, against high ORM levels in EAT supernatants, high concentration of ORM was harmless. This result suggested that the deleterious effect in H9C2 might be due to alternative factors in EAT supernatants. Thus, the determination and classification of EAT supernatants regarding ORM levels could help us to find EAT-released proteins with beneficial or deleterious effect in the myocardium which can allow us to identify new therapeutic targets. ORM is an acute phase protein, mainly produced by hepatocytes [31], but can also be expressed by adipocytes [32]. In fact, ORM can regulate the secretion of IL-1 receptor antagonist (IL-1Ra), which is highly induced in EAT macrophages [33]. Both proteins can be downregulated by adipogenic drugs [34,35], which can increase the viability of cardiomyocytes and reduce the damage after myocardial infarction [36]. However, further studies are necessary because a paradoxical concept can be associated with ORM. While elevated concentration of ORM was identified as a risk factor for myocardial infarction and stroke in the Malmö Preventive Study [37] and heart failure [38], its administration (50 ng/g body weight), in animal experimental models, reduced the proportion of acute myocardial infarction and improved the myocardial contractile function [39]. These results suggest that ORM may act as a bimodal immunoregulatory protein as pro- or anti-inflammatory cytokines in a concentration dependent manner. Moreover, while concentrations between 50-300 ng/mL of ORM were identified to be involved in angiogenesis, highest concentrations were found to be anti-angiogenic [40]. According to these findings, our results also showed positive effects in H9C2 cells in this range. On the contrary, supernatants with higher concentration of ORM had a deleterious consequence in H9C2 cell. But, this effect cannot be rigorously ascribed by ORM because individualized treatments at high doses do not reproduce these results in H9C2. After identifying several proteins in EAT supernatants with high ORM levels, we focus our attention on omentin-1 because their levels were negatively

associated with CAD severity [41] and is expressed by non-fat cells of EAT [42]. Omentin-1 can prevent the contractile dysfunction and insulin resistance of cardiomyocytes [11] and its administration in mouse models of ischemia/reperfusion reduces the myocardial infarct size and decrease apoptosis [43]. Omentin-1 measurement in EAT supernatants from 42 patients has determined its high presence in low ORM levels supernatants. Meaning that Omentin-1, present in the EAT supernatants with low ORM levels, might maximize the protective role of ORM in H9C2 cells. Our data shows that ORM levels quantification in EAT supernatants can help us to classify EAT from patients as protector or deleterious tissue against myocardium damage.

5. Conclusions

Our data showed that low ORM levels have a beneficial effect preventing hypoxic induced apoptosis and suggest that counteract lipotoxic-induced disorders in hypoxic H9C2 cells. The knowledge of EAT-released proteins and their functions might define new therapeutic targets against cardiovascular diseases.

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Conflict of interest

Non declared

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Figure legends

Figure 1. Viability and proliferation of ventricular cardiomyoblast cells exposed to EAT supernatants (A) RTCA-SP viability and proliferation ventricular cardiomyoblast cells exposed to EAT supernatants from 14 patients. Cell index (CI) of H9C2 cells with EAT supernatants treatment for 12 hours and 30 minutes expressed as fold change relative to control group (vehicle) is represented as a scatter-plot regarding higher or lower ORM levels than 300 ng/mL. (B) Correlation between EAT-released ORM levels from patients and ventricular cardiomyoblast viability. Scatter-plot diagram of H9C2 CI exposed to EAT supernatants and their ORM supernatant levels. Data are expressed as mean ± SEM of duplicate values by supernatant. Significant linear relationship between ORM levels and CI fold change was determined by Pearson's correlation test.

Figure 2. Viability and proliferation ventricular cardiomyoblast cells exposed to ORM (A) RTCA-SP of H9C2 cells with ORM treatment at 50 (black triangles) or 500 (grey diamonds) ng/mL. CI is expressed as fold change relative to control group in each time point. (B) Endpoint fold change related to control group at indicated concentrations. (C) MTT cell viability assay 24 h and 48h after ORM treatment. Data are expressed as mean ± SEM of at least triplicate values from three independent experiments. Differences between groups were compared by two-way Student's *t*-test. ** $P<0.01$ *vs.* control.

Figure 3. Differential EAT- supernatants proteins regarding high or low ORM levels (A) Venn diagram represents differential identified proteins in EAT supernatants with higher ORM levels of 500 ng/mL and lower ORM levels of 100 ng/mL. (B) Common and differential identified proteins between two groups.

Figure 4. ORM pretreatment in hypoxic-induced apoptosis in ventricular cardiomyoblast Two hundred and fifty thousand H9C2 cells were seeded and treated with ORM under normoxic or hypoxic (0.1 O2%) conditions. Apoptosis was measured by Annexin V flow cytometry analysis. Histogram represents the viable, apoptotic and necrotic rate of H9C2 cells in presence or absence of ORM 50ng/mL relative to normoxic conditions. Data are expressed as mean ± SEM of three independent experiments. Differences between groups were compared by two-way Student's t-test. *, ** and ***, P<0.05, P<0.01 and P<0.001 *vs.* normoxic; $^{\#}$, P<0.05 *vs.* hypoxic.

Figure 5. ORM pretreatment in lypoxic-induced apoptosis in ventricular cardiomyoblast Two hundred and fifty thousand H9C2 cells were seeded and treated with 50ng/mL ORM and/or palmitic acid under normoxic and hypoxic conditions. Apoptosis was measured by Annexin V flow cytometry analysis. Histograms represent the viable, apoptotic and necrotic rate of palmitic acid treated H9C2 cells under normoxic (A) or hypoxic (B) conditions in absence or presence of ORM. Data are expressed as mean ± SEM of three independent experiments. Differences between groups were compared by two-way Student's t-test. ** and ***, P<0.01 and P<0.001 *vs.* control; $^{\#\#}$, P<0.01 *vs.* palmitic.

Figure 6. Wound Healing assay in ventricular cardiomyoblast cells exposed to ORM (A). Wound Healing assay in ventricular cardiomyoblast cells exposed to ORM at 50 or 500 ng/mL concentration in medium with 0.5% FBS for 48 h. The wound healing of cells was photographed at the indicated times. Histogram represents the open area relative to initial wound between 16 h/0 h, 32 h/0 h and 48h/0. Black and grey bars show 50 or 500 ng/mL, respectively. Data are expressed as mean ± SEM of six individual values by experiment. Each experiment was made at least by duplicated. Differences between groups were compared by two-way Student's t-test. *** P<0.001 *vs.* control; $^{\#\#\#}$ P<0.001 *vs.* ORM 50ng/mL treated group.

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