Protective Role of Perivascular Adipose Tissue in Endothelial Dysfunction and Insulin-Induced Vasodilatation of Hypercholesterolemic LDL Receptor-Deficient Mice

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Background: Endothelial dysfunction plays a pivotal role in the initiation of atherosclerosis. Vascular insulin resistance might contribute to a reduction in endothelial nitric oxide (NO) production, leading to impaired endothelium-dependent relaxation in cardiometabolic diseases. Because perivascular adipose tissue (PVAT) controls endothelial function and NO bioavailability, we hypothesized a role for this fat deposit in the vascular complications associated with the initial stages of atherosclerosis. Therefore, we investigated the potential involvement of PVAT in the early endothelial dysfunction in hypercholesterolemic LDL receptor knockout mice (LDLr-KO).

Methods: Thoracic aortas with and without PVAT were isolated from 4-month-old C57BL/6J (WT) and LDLr-KO mice. The contribution of PVAT to relaxation responses to acetylcholine, insulin, and sodium nitroprusside was investigated. Western blotting was used to examine endothelial NO synthase (eNOS) and adiponectin expression, as well the insulin signaling pathway in aortic PVAT.

Results: PVAT-free aortas of LDLr-KO mice exhibited impaired acetylcholine- and insulin-induced relaxation compared with those of WT mice. Both vasodilatory responses were restored by the presence of PVAT in LDLr-KO mice, associated with enhanced acetylcholine-induced NO levels. PVAT did not change vasodilatory responses to acetylcholine and insulin in WT mice, while vascular relaxation evoked by the NO donor sodium nitroprusside was not modified by either genotype or PVAT. The expression of insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), AKT, ERK1/2, phosphorylation of AKT (Ser473) and ERK1/2 (Thr202/Tyr204), and adiponectin was similar in the PVAT of WT and LDLr-KO mice, suggesting no changes in PVAT insulin signaling. However, eNOS expression was enhanced in the PVAT of LDLr-KO mice, while eNOS expression was less abundant in PVAT-free aortas.
Conclusion: These results suggest that elevated eNOS-derived NO production in aortic PVAT might be a compensatory mechanism for the endothelial dysfunction and impaired vasodilator action of insulin in hypercholesterolemic LDLr-deficient mice. This protective effect may limit the progression of atherosclerosis in genetic hypercholesterolemia in the absence of an atherogenic diet.

Keywords: perivascular adipose tissue, endothelium, LDL receptor deficiency, hypercholesterolemia, nitric oxide, insulin, adiponectin

INTRODUCTION

Hypercholesterolemia is the main risk factor for the development of atherosclerosis by enhancing low-density lipoprotein (LDL) retention within the vessel wall (Stapleton et al., 2010). Atherosclerosis is a progressive disease in which inflammation, fat deposits, and cells and extracellular matrix accumulation in the artery result in the occlusion of the vessel lumen, underlying a major cause of clinical cardiovascular events worldwide (World Health Organization, 2014; Mozaffarian et al., 2016). One of the initial steps of the atherosclerotic process is the development of endothelial dysfunction, which precedes atherosclerosis development in humans (Guzik et al., 2000; Heitzer et al., 2001).

LDL receptor knockout mice (LDLr-KO) is a model of human familial hypercholesterolemia (FH). Although young adult LDLr-KO mice fed with standard low-fat diet only develop small spontaneous atherosclerotic lesions in the aortic root (Dorighello et al., 2016, 2017), these animals present impaired endothelium-dependent relaxation in the aorta (Rabelo et al., 2003; Langbein et al., 2015; Guizoni et al., 2016). This finding is consistent with reduced endothelium-dependent vasodilation in human hypercholesterolemia (Creager et al., 1990, 1992). In this model of genetic hypercholesterolemia, endothelial dysfunction is associated with the reduced gene and protein expression of endothelial nitric oxide synthase (eNOS) (Guizoni et al., 2016; Langbein et al., 2016), and reduced dimerization of this enzyme, resulting in impaired endothelial nitric oxide (NO) release (Guizoni et al., 2016).

Nitric oxide (NO) is an endothelium-derived vasodilator factor with antiatherogenic properties, including the inhibition of platelets, and monocyte adhesion to the endothelium, platelet aggregation, smooth muscle proliferation, and oxidation of LDL (Vanhouthe, 2003). Consistently, the administration of L-arginine, as eNOS substrate and NO precursor, improves the endothelial function in hypercholesterolemic patients (Creager et al., 1992; Siasos et al., 2007), whereas the lack of NO accelerates the progression of atherosclerosis (Kauser et al., 2000). Genetic hypercholesterolemia is also associated with the impaired secretion of insulin (Souza et al., 2013), a hormone stimulating NO production (Potenza et al., 2009). In addition to endothelium-derived NO, NO release from perivascular adipose tissue (PVAT) has recently been demonstrated as an important adipocyte-derived relaxation factor (ADRF) in the aorta (Vicentio et al., 2016; Xia et al., 2016), but whether or not changes in PVAT eNOS function are involved in hypercholesterolemia-induced vascular dysfunction remains unknown.

Perivascular adipose tissue (PVAT) surrounds most blood vessels and releases numerous factors and adipokines with paracrine effects on both vascular structure and function (Akoumianakis et al., 2017). Higher levels of proinflammatory adipocytokines and lower levels of adiponectin were found within the PVAT after artery injury (Takaoka et al., 2010), and an inflamed PVAT results in neointima formation (Moe et al., 2013). In addition, increased amounts of angiogenic factors release by PVAT may have a pathological relevance for atherosclerosis development (Chang et al., 2013). However, the thermogenic properties of PVAT have been demonstrated as anti-atherogenic (Chang et al., 2012; Brown et al., 2014). Nevertheless, the role of PVAT in the development and progression of this vascular disease is still unclear. Because PVAT regulates endothelial dysfunction and NO bioavailability, we hypothesized a role for this fat deposit in the vascular complications associated with the initial stages of atherosclerosis. Therefore, the present study was designed to evaluate the potential influence of PVAT in the early endothelial dysfunction of hypercholesterolemic LDLr-KO mice.

MATERIALS AND METHODS

Animals

LDL receptor knockout (LDLr-KO) mice and respective C57BL6/J wild-type (WT) mice were purchased from the Jackson Laboratory, and the strains were maintained by breeding at the Multidisciplinary Center for Biological Research (CEMIB-UNICAMP, Campinas, SP, Brazil) with genotypic control. The mice were housed at 22 ± 1°C on a 12:12 h light:dark cycle with free access to a standard rodent chow diet (Nuvital CR1, Colombo, Paraná, Brazil) and water. At 4 months old, the mice were weighted and anesthetized with urethane (5 g/kg body weight, i.p.) to collect blood samples and isolate thoracic aorta.

All experimental protocols were approved (protocol # 3639-1) by the Ethics Committee on Animal Use of the University of Campinas (CEUA-UNICAMP, Campinas-SP, Brazil) and carried out in accordance with the ethical principles for animal experimentation adopted by the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA).

Plasma Biochemical Analysis

Blood samples were obtained from the tail vein for blood glucose measurement (Accu-Chek Advantage, Roche Diagnostics, Sao Paulo, Brazil). Subsequently, the mice were anesthetized (5 g/kg...
of urethane, i.p.), and blood samples were collected by cardiac puncture and centrifuged (8,000 g for 15 min at 4°C); the serum supernatants were subsequently collected for biochemical analysis. Total cholesterol and triglyceride levels were measured using standard commercial kits (Chod-Pap, Roche Diagnostic GmbH, Mannheim, Germany).

Vascular Reactivity in the Presence or Absence of PVAT

The thoracic aorta was isolated and cut into cylindrical segments (~2 mm in length) with or without surrounding PVAT. The segments were mounted in a tissue chamber bath (Panlab Harvard Apparatus, Cornellà-Barcelona, Spain) containing Krebs–Henseleit solution (in mM: 118 NaCl, 4.7 KCl, 25 NaHCO3, 2.5 CaCl2-2H2O, 1.2 KH2PO4, 1.2 MgSO4-7H2O, 11 glucose and 0.01 EDTA; pH 7.4, 37°C) with a resting tension of 0.5 g stabilized for 1 h, as previously demonstrated (Davel et al., 2012; Guizoni et al., 2016). Subsequently, aortic rings were exposed to 125 mM of KCl to test vascular integrity and assess maximal contraction, and no differences were found among groups (data not shown). Following washing, the aortic rings were contracted with a submaximal concentration of the thromboxane A2 receptor agonist (U-46619, 70% of maximal contraction to 125 mM of KCl) and relaxation curves to acetylcholine (0.1 nmol/L to 10 µmol/L, Sigma-Aldrich, Saint Louis, MO, USA), insulin (0.1 to 10 nmol/L, Humulin R – rDNA origin, Lilly USA, Indianapolis, USA), or the NO-donor sodium nitroprusside (1 µmol/L to 0.1 µmol/L Sigma-Aldrich, Saint Louis, MO, USA) were performed.

PVAT Homogenization and Western Blotting

Thoracic aorta and respective PVAT were separately pulverized in N2 liquid and homogenized in cold RIPA lysis buffer (Merck Millipore, Billerica, MA, USA) containing PMSF (1 mM), Na3VO4 (1 mM) and 2 µL/mL of protease inhibitor cocktail (PIC, Sigma-Aldrich) to obtain total protein extract (Victorio et al., 2016). The proteins were quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) to determine the protein concentration values of the samples (PVAT: WT = 4.49 ± 2.94 and LDLr-KO = 5.74 ± 1.98 mg/ml; aorta: WT = 6.13 ± 3.09 and LDLr-KO = 7.46 ± 1.98 mg/ml). Then, 40 µg of PVAT or aorta extracts and the 1 µL of plasma were electrophoretically separated on 7.5 or 12% acrylamide SDS–PAGE and then transferred to PVDF membranes using a Mini Trans-Blot Cell system. Non-specific binding sites were blocked with 5% non-fat milk solution, and then the membranes were incubated for 12 h at 4°C with the following primary antibodies: anti-insulin receptor substrate-1 (IRS-1), anti-p85 subunit of phosphatidylinositol 3 kinase (PI3K), anti-Akt1/2/3, anti-phospho (Ser473)-Akt1/2/3 (1:1,000; Santa Cruz Biotechnology), anti-eNOS (1:1,000; BD Transduction, Franklin Lakes, NJ, USA), anti-ERK1/2, anti-phospho (Thr202/Tyr204)-ERK1/2 (1:1,000; Cell Signaling, Danvers, MA, USA), and anti-adiponectin (1.5 µg/mL, Novus Biologicals, Littleton, CO, USA). Additionally, α-tubulin (1:1,000; Santa Cruz Biotechnology) was used as an internal control protein for PVAT, α-actin (1:1,000; Abcam, Cambridge, MA, USA) was used as an internal control protein for aorta, and Ponceau S staining for adiponectin expression in plasma and PVAT. After washing (10 mM of Tris, 100 mM of NaCl and 0.1% Tween-20), the membranes were incubated for 90 min with the specific secondary antibody conjugated to peroxidase. Immunocomplexes were detected using a luminol peroxidase chemiluminescence kit (ECL Plus, Amersham) and visualized using photographic film (Hyperfilm ECL, Amersham) in a dark room. The intensity of the immunoblots was quantified using ImageJ 1.46p software (National Institutes of Health, Bethesda, MD, USA).

PVAT NO Release

NO in PVAT was detected with 4,5-diaminofluorescein diacetate (DAF-2 DA) (Xia et al., 2016). Aortic PVAT cryostat sections (20 µm) were loaded with DAF-2 DA (8 µM) in the presence of acetylcholine (10 µM) at 37°C for 30 min. Then, fluorescence imaging was performed with a microscope (Eclipse Ti-S, Nikon, Tokyo, Japan) equipped with a fluorescence filter. DAF-2 DA was excited by an interference filter at 465–495 nm, and fluorescence emitted between 515 and 555 nm was collected (Zhou and He, 2011). The images captured under a 10X objective were analyzed using ImageJ 1.46p software (National Institutes of Health), the mean optical density of the fluorescence was measured, and the results were normalized by the PVAT area (µm²). Fluorescence intensity was quantified by the subtraction of acetylcholine-treated samples from basal conditions.

Statistical Analysis

Data were expressed as the means ± SEM. Two-way ANOVA was used to analyze the vasorelaxation response curves. When ANOVA showed a significant effect, Bonferroni’s post-hoc test was used to compare individual means. Unpaired Student’s t-test was used for two-group comparisons. For each concentration-response curve, Rmax and the negative logarithm of the concentration of the agonist that produced half of Rmax (−LogEC50) were calculated using non-linear regression analysis. GraphPad Prim Software 5.0 (San Diego, CA, EUA) was used for statistical analysis and −LogEC50 and Rmax calculation. P < 0.05 values were considered significant.

**TABLE 1 |** Body weight and total serum cholesterol, triglycerides, and glycemia in wild-type (WT) and LDLr knockout mice (LDLr-KO).

|                  | WT      | LDLr-KO |
|------------------|---------|---------|
| Body weight (g)  | ± 28.6  | ± 27.6  |
| Blood glucose (mg/dL) | ± 135 ± 14 | ± 143 ± 23 |
| Total cholesterol (mg/dL) | ± 84.6 ± 4.6 | ± 262.3 ± 14.3* |
| Triglycerides (mg/dL) | ± 54.3 ± 4.1 | ± 145.4 ± 11.5* |

*Data are expressed as mean ± SEM (N = 10–14). Unpaired Student’s t-test: *P < 0.05 vs. WT.
RESULTS

As expected, LDLr-KO mice exhibited dyslipidemia characterized by elevated plasma levels of total cholesterol and triglycerides (Bonfleur et al., 2011), with no changes in blood glucose or body weight (Table 1).

The vascular reactivity study revealed an impaired relaxation to acetylcholine and insulin in aorta of LDLr-KO mice in the absence of PVAT (Figures 1A,B), with a reduced \( R_{\text{max}} \) to both agonists (Table 2). These data suggest an impaired endothelial vasodilatory response and vascular insulin resistance. However, the presence of PVAT prevented the impaired vasodilatory response to both acetylcholine and insulin in LDLr-KO associated with increased \( R_{\text{max}} \) while not affecting vasorelaxation in WT (Figures 1A,B; Table 2). Changes in acetylcholine- and insulin-induced relaxation were not the result of a smooth muscle defect, as the relaxation response to the NO donor sodium nitroprusside was similar between WT and LDLr-KO mice, with or without PVAT (Figure 1C, Table 2).

Considering that NO is the main endothelium-derived vasodilator factor mediating acetylcholine- and insulin-induced relaxation in the murine aorta (Wu et al., 1994), we investigated vascular and PVAT eNOS expression. Interestingly, while eNOS expression was reduced in aortic tissue from LDLr-KO mice (Figure 2A), aortic PVAT showed more abundant eNOS expression (Figure 2B), associated with enhanced PVAT NO levels (Figure 2C). These data suggest increased eNOS-derived NO production as a mechanism involved in the protective effect of aortic PVAT in dyslipidemic LDLr-KO mice.

To investigate insulin signaling in PVAT, we evaluated the protein expression of IRS-1, PI3K, and total and phosphorylated Akt and ERK. However, as shown in Figure 3, no differences between groups were observed.

Finally, because adiponectin is an important adipokine that stimulates NO production and facilitates endothelium-dependent relaxation (Margaritis et al., 2013), we examined PVAT and circulating adiponectin expression (Figures 4A,B). No significant changes were found between WT and LDLr-KO mice.

![Figure 1](image1.png) Figure 1 | Perivascular adipose tissue (PVAT) avoids endothelial dysfunction in LDLr knockout mice. Relaxation curves to acetylcholine (A), insulin (B), and sodium nitroprusside (C) in aortic rings with (+) and without (−) PVAT from wild-type (WT; circles) and LDLr knockout mice (LDLr-KO; squares). Two-way ANOVA, \( P < 0.05; * \) vs. WT/PVAT−; + vs. LDLr-KO/PVAT−.

![Table 2](image2.png) Table 2 | Maximal relaxation response (\( R_{\text{max}} \)) and potency for acetylcholine, insulin, and sodium nitroprusside in aortas with (+) or without (−) perivascular adipose tissue (PVAT) from wild-type (WT) and LDLr knockout mice (LDLr-KO).

Data are expressed as mean ± SEM (\( N = 4–9 \)). 2-way ANOVA followed by Bonferroni’s post-hoc test, \( P < 0.05; * \) vs. WT/PVAT−; + vs. LDLr-KO/PVAT−.
DISCUSSION

The results from the present study showed that in LDLr-KO mice, the presence of PVAT protected against impaired endothelium-dependent relaxation to acetylcholine and insulin, in association with enhanced eNOS protein expression and NO levels in thoracic aortic PVAT. Since this genetic model of FH exhibited only small aortic-root lesions in mice maintained on a standard low-fat diet (Dorighello et al., 2016), these data reveal a protective role for PVAT in an early phase of vascular injury induced by hypercholesterolemia.

We (Guizoni et al., 2016) and others (Rabelo et al., 2003; Langbein et al., 2015) have demonstrated a slight but significant reduction in acetylcholine-induced relaxation in LDLr-KO mice, as evidenced by a reduced maximal response to this agonist (Hofmann et al., 2017). Similarly, we found a reduced maximal relaxation to acetylcholine in aorta without PVAT from LDLr-KO fed a standard diet, indicating endothelial dysfunction in this genetic model of FH. This endothelial dysfunction became more evident in the presence of high fat/high cholesterol diets or the upregulation of lectin-like oxidized LDLr-1 (LOX-1) (Hofmann et al., 2017). However, Western-type diets also induced a range of secondary factors, such as inflammation, insulin resistance, and obesity, which synergistically interact to increase atherosclerosis. A high-fat diet per se upregulates proinflammatory gene expression in PVAT depots (Chatterjee et al., 2009). Inflamed fat surrounding the vessels resulted in enhanced atherosclerotic lesions and exacerbated endothelial dysfunction (Öhman et al., 2011). Therefore, PVAT may have pathological relevance in advanced atherosclerosis, contributing to plaque complications (Chang et al., 2013). In the present study, we investigated the role of PVAT in endothelium-dependent relaxation in the early stages of vascular injury in LDLr-KO mice fed a standard diet. Interestingly, the presence of PVAT improved relaxation responses to acetylcholine and insulin in the aortas of LDLr-KO mice, suggesting a protective role for this tissue in the present genetic model of FH by improving endothelial function. In healthy mouse aorta, the presence of PVAT did not affect the endothelium-dependent relaxation induced by acetylcholine, as previously demonstrated (Ketonen et al., 2010; Li et al., 2011).

Gil-Ortega et al. (2010) showed adaptive NO overproduction in PVAT during the initial steps of high-fat diet-induced obesity in mice. PVAT-derived NO may contribute to the anticontractile effect of PVAT independently of the endothelium (Aghamohammazdeh et al., 2016). Since eNOS is expressed in aortic endothelium and PVAT (Victorio et al., 2016; Xia et al., 2016), we investigated whether the protective role of PVAT in endothelium-dependent relaxation responses in LDLr-KO mice was associated with changes in eNOS expression. The results demonstrated reduced eNOS expression in the vascular wall of LDLr-KO mice, as previously demonstrated, associated with reduced NO production (Guizoni et al., 2016). Lower eNOS and nNOS activation is also related to...
endothelial dysfunction in apolipoprotein E-deficient mice (Capettini et al., 2011). In accordance with these data, lower NO production is observed in endothelial cells exposed to the serum of hypercholesterolemic patients (Feron et al., 1999). However, in contrast to lower endothelial eNOS expression in the vessel wall, increased eNOS expression
and NO levels were observed in the aortic PVAT of LDLr-KO mice. Therefore, higher eNOS-derived NO production in surrounding fat may be the mechanism enhancing endothelium-dependent relaxation in the aortas of LDLr-KO mice.

In addition to reduced relaxation to acetylcholine, the aortas from LDLr-KO mice without PVAT exhibited impaired relaxation to insulin. Insulin is an important stimulus in endothelial cells for eNOS-derived NO production (Potenza et al., 2009). Therefore, lower eNOS expression in the vascular wall should be a mechanism involved in the impaired relaxation to this hormone in hypercholesterolemic mice. Since relaxation response to sodium nitroprusside was not different among groups, changes in smooth muscle responsiveness may not be involved in the reduced vasodilatory responses observed in LDLr-KO mice. High plasma levels of cholesterol in LDLr-KO mice impairs beta-cell pancreatic function reducing insulin secretion, even in the absence of metabolic factors induced by Western-type diets, suggesting that genetic hypercholesterolemia increases the risk of diabetes development (Bonfleur et al., 2011; Souza et al., 2013). This finding is consistent with the slight increase in fasted plasma glucose previously observed in LDLr-KO mice (Bonfleur et al., 2011; Souza et al., 2013), although we did not find significant differences in non-fasted animals. In the present study, the reduced vasodilatory effect of insulin suggests vascular insulin resistance. Insulin enhances the expression and activity of eNOS in endothelial cells by upregulating the PI3K/Akt pathway, which induced endothelium-dependent relaxation (Montagnani et al., 2002; Potenza et al., 2009). However, hyperinsulinemia and insulin resistance favor the insulin-dependent activation of MAP-kinases (MAPK), which is associated with the elevated expression of proinflammatory and atherogenic factors (Eringa et al., 2004; Cersosimo et al., 2012; da Silva Franco et al., 2017). The presence of PVAT prevented the reduced insulin-induced relaxation in aorta of LDLr-KO mice. Thus, we hypothesized that an enhanced insulin signaling in PVAT could be a mechanism opposing the vascular insulin resistance in these animals. However, no differences were found for IRS-1, PI3K, and phosphorylated and total Akt and ERK, suggesting that this signaling pathway may not be involved in the protective effect of PVAT. However, we cannot exclude changes in the insulin molecular pathway, other than those evaluated in the present study.

Adiponectin has been described as an ADRF with antiatherogenic properties (Fésüs et al., 2007; Li et al., 2015). The upregulation of adiponectin is observed in PVAT of internal mammary arteries of obese patients with coronary artery disease and might reflect a compensatory mechanism to preserve endothelial function (Cybularz et al., 2017), as previously demonstrated in obese diabetic mice (Liu et al., 2014). In addition, adiponectin improves insulin sensitivity in major insulin target tissues (Ruan and Dong, 2016) and enhances NO production through eNOS activation via PI3K/Akt phosphorylation and eNOS coupling by increasing BH4 bioavailability (Margaritis et al., 2013). Thus, we investigated the expression of adiponectin in PVAT and plasma. However, no differences were observed between WT and LDLr-KO mice, suggesting that the effect of PVAT on improving endothelial function in LDLr-KO mice is not associated with modifications of local or circulating levels of this ADRF.

Thoracic PVAT acts as a buffer against toxic levels of fatty acids in arterial circulation and clears fatty acids via inducing thermogenesis (van Dam et al., 2017). Interestingly, enhanced thermogenic activity of PVAT improves endothelial function by inducing the increased release of prostacyclin, whereas impaired PVAT thermogenesis causes atherosclerosis (Chang et al., 2012). Therefore, PVAT adaptive thermogenesis has a beneficial impact on endothelial function protecting against vascular injury. We cannot exclude that this protection exerted by PVAT on endothelial function could be lost in aging. One limitation of the present study is that we did not evaluate PVAT thermogenesis, which is a potential mechanism of endothelial protection in standard-diet fed LDLr-KO mice, as Western-type diets induced whitening and impaired thermogenesis in PVAT (Chang et al., 2013). Recently, Srikakulapu et al. (2017) demonstrated that harboring of B-1 cells by PVAT provides atheroprotection to the aorta, suggesting an additional mechanism for the beneficial role of PVAT in the vasculature.

CONCLUSION

Taken together, the results of the present study suggest that aortic PVAT is protective for endothelial dysfunction in LDLr-KO mice, a genetic model of FH. Therefore, this adaptive mechanism in PVAT may protect endothelial function and maintain normal endothelium-dependent relaxation in the early stages of atherosclerotic disease.

ETHICS STATEMENT

All experimental protocols were approved (protocol # 3639-1) by the Ethics Committee on Animal Use of the University of Campinas (CEUA-UNICAMP, Campinas-SP, Brazil) and carried out in accordance with the ethical principles for animal experimentation adopted by the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA).

AUTHOR CONTRIBUTIONS

NB designed and executed the experiments, analyzed the data, wrote the manuscript. DG assisted in the experiments, data analysis and interpretation, read and revised the manuscript. JV performed DAF-2 DA fluorescence analysis. AD conceived the study, guided the experimental design, data analysis and interpretation, read and revised the manuscript.

FUNDING

This work was supported by grant from Sao Paulo Research Foundation - FAPESP (#2014/22506-6 and # 2016/14461-8).
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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