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Autologous fat transplants to deliver glitazone and adiponectin for vasculoprotection.

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ABSTRACT

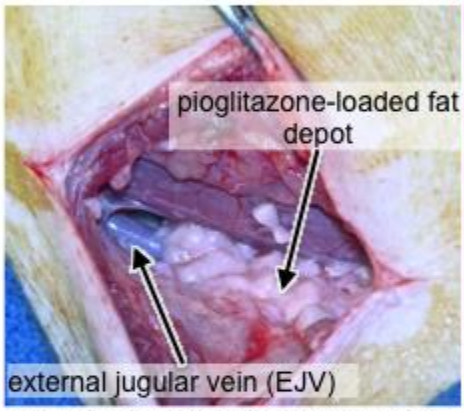
The insulin sensitizing glitazone drugs, rosiglitazone (ROS) and pioglitazone (PGZ) both have anti-proliferative and anti-inflammatory effects and induce adipose tissue (fat) to produce the vaso-protective protein adiponectin. Stenosis due to intimal hyperplasia development often occurs after placement of arteriovenous synthetic grafts used for hemodialysis. This work was performed to characterize the *in vitro* and *in vivo* effects of ROS or PGZ incorporation in fat and to determine if fat/PGZ depots could decrease vascular hyperplasia development in a porcine model of hemodialysis arteriovenous graft stenosis.

Powdered ROS or PGZ (6-6000 µM) was mixed with fat explants and cultured. Drug release from fat was quantified by HPLC/MS/MS, and adiponectin and monocyte chemotactic protein-1 (MCP-1) levels in culture media were measured by ELISA. The effect of conditioned media from the culture of fat with ROS or PGZ on i) platelet-derived growth factor-BB (PDGG-BB)-stimulated proliferation of human venous smooth muscle cells (SMC) was measured by a DNA-binding assay, and ii) lipopolysaccharide (LPS)-induced human monocyte release of tumor necrosis factor-alpha (TNFα) was assessed by ELISA. In a porcine model, pharmacokinetics of PGZ from fat depots transplanted perivascular to jugular vein were assessed by HPLC/MS/MS, and retention of the fat depot was monitored by MRI. A porcine model of synthetic graft placed between carotid artery and ipsilateral jugular vein was used to assess effects of PGZ/fat depots on vascular hyperplasia development.

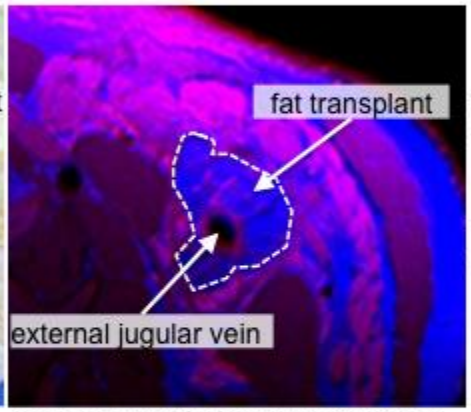
Both ROS and PGZ significantly induced the release of adiponectin and inhibited release of MCP-1 from the fat. TNF production from monocytes stimulated with LPS was inhibited 50-70% in the presence of media conditioned by fat alone or fat and either drug. The proliferation of SMC was inhibited in the presence of media conditioned by fat/ROS cultures. Fat explants placed perivascular to the external jugular vein were retained, as confirmed by MRI at one week after placement. PGZ was detected in the fat depot, in the external jugular vein wall and in adjacent tissue at clinically relevant levels, whereas levels in plasma were below detection. External jugular vein exposed to fat

incorporated with PGZ had increased adiponectin expression compared to vein exposed to fat alone. However, the development of hyperplasia within the arteriovenous synthetic grafts was unchanged by treatment with fat/PGZ depots compared to no treatment.

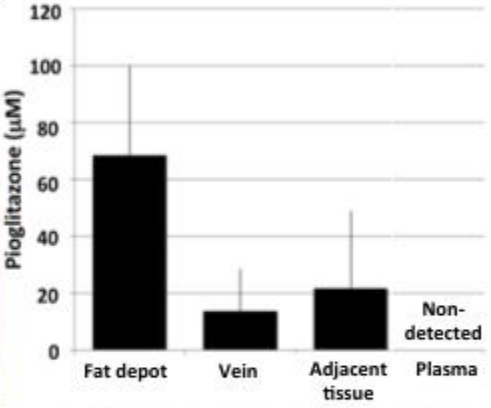
Graphical Abstract



Autologous fat/glitazone depot after transplant to EJV in swine.



MR image of fat/glitazone depot in swine; fat signal assigned blue color.



Glitazone in tissues one week later

INTRODUCTION

Stenotic lesions in the vasculature promote thrombosis. In the arterial system, stenotic lesions are commonly due to atherosclerosis where oxidative stress and inflammation trigger cholesterol deposition and cell proliferation in the intima [1]. Stenosis also occurs in the venous system, typically due to previous trauma such as placement of in-dwelling catheters [2], cardiac rhythm transvenous devices, synthetic arteriovenous hemodialysis grafts [3] and creation of native hemodialysis fistulas[4, 5]. The stenotic lesions that occur in the venous system differ from arterial atherosclerotic lesions as they typically do not have cholesterol plaques, necrotic cores or foam cells, and consist mostly of smooth muscle and/or myofibroblast cells [6]. However, inflammation is a contributor to lesions in both vein and artery. Venous stenosis is a particularly pernicious problem for synthetic grafts used for hemodialysis vascular access, where approximately 50% of arteriovenous grafts fail in the first year due to thrombosis, primarily because of underlying stenosis at the vein-graft anastomosis [7]. As of yet, there are no clinically used treatments to inhibit stenosis in this setting.

Percutaneous transluminal angioplasty (PCTA) is commonly used to treat arterial as well as venous stenotic lesions but PCTA triggers endothelial injury and inflammation and is often associated with restenosis and clotting. The vein wall is more compliant than the artery and PCTA may stretch the vein without compressing the lesion making the venous stenotic lesions challenging to treat. Placement of bare-metal stents, covered stent grafts, and drug-eluting stents are targeted therapies often used in conjunction with PCTA in both artery and vein. A prospective multicenter trial has shown that stent grafts (metal stents covered with a polymeric sheath) were associated with better lesion patency over PCTA alone for treating the stenoses occurring in the vein-graft anastomoses of synthetic hemodialysis accesses [8]. Nevertheless, the thrombosis rates were the same between the stented and un-stented groups. Better treatments are needed.

Drug administration via a locally applied, sustained delivery system has benefits over oral delivery as it decreases systemic exposure to the drug and the “first-pass effect”, where active drug concentration in the circulation is greatly reduced by liver and gut metabolism. Also, local delivery can

be a means to administer drugs that are unsuitable for oral ingestion due to poor solubility, instability or limited gastrointestinal uptake. Of note, localized delivery of drug is particularly attractive to treat the stenotic lesions in the hemodialysis synthetic grafts as such lesions typically occur at the readily accessible anastomosis of vein and graft. Intraluminal delivery of drugs that target cell proliferation and/or inflammation has been investigated [9-12]. However, intraluminal application of drug by means of drug-eluting balloons can damage the endothelium that acts as a barrier to thrombosis and hyperplasia.

The perivascular application of drug (i) targets the outer layers of the vessel wall that contain the adventitial fibroblasts and smooth muscle cells that are known to populate the stenotic lesions[13], and (ii) does not involve physical disruption of the protective luminal endothelial layer. We have previously reported the successful use of perivascular administration of sirolimus, an anti-proliferative immunosuppressant, using a biodegradable synthetic polymer to inhibit arteriovenous graft stenosis [14, 15]. However, swelling and exudate occurred at the site of polymer-drug administration in almost 17% of animals, suggesting the polymer-drug mixture may have triggered inflammation.

The ideal properties of a locally applied perivascular delivery system include biocompatibility, sustained drug release, ability to remain at the site of application, and low cost. Adipose tissue is a natural reservoir for a number of drugs and we hypothesized that autologous fat transplants could act as a biocompatible, sustained drug delivery system. The insulin-sensitizing thiazolidinediones, including pioglitazone (PGZ) and rosiglitazone (ROS), are lipophilic thus may be slowly released from fat. Both ROS and PGZ have direct anti-inflammatory, anti-thrombotic and anti-proliferative properties [16-20] that target important mediators of hyperplasia development. Furthermore these drugs induce fat to produce the protein adiponectin. Adiponectin, a multimeric protein produced almost exclusively by adipose tissue, also has salutary effects on the vasculature, preserving production of the vasodilator nitric oxide by endothelial nitric oxide synthase (eNOS) [21, 22], decreasing oxidative stress [23, 24], and inhibiting monocyte adherence to endothelial cells [25, 26]. As such, ROS or PGZ

may suppress hyperplasia, via direct actions or through stimulation of adiponectin, and both laboratory and clinical investigations support that PGZ, taken orally, can inhibit in-stent restenosis [27-30] and interpositional vein graft stenosis [31]. Although ROS and PGZ share many characteristics such as binding to peroxisome proliferator activated receptor-gamma (PPARgamma) to induce gene transcription, they also have differences that may impact their utility when delivered by a fat depot. For example, ROS has a lower lipophilicity than PGZ. Other differences may influence efficacy. ROS is more selective for activating PPARgamma whereas PGZ also activates PPARalpha. ROS and PGZ have been reported to have some differences in binding of protein from mice heart as determined by a chemical proteomic screen[32]. Of note, systemic delivery of ROS and PGZ is contraindicated in patients with congestive heart failure, since these drugs are known to promote fluid retention and aggravate heart failure [33]. Localized delivery of ROS or PGZ would avoid this drawback.

Here we performed *in vitro* studies to test whether fat mixed with ROS or PGZ could provide sustained local delivery of drug and serve as a biofactory for production of adiponectin. Further *in vivo* studies in swine were performed to examine drug pharmacokinetics, fat depot retention and tolerability and finally effect on intimal hyperplasia development in a model of arteriovenous graft stenosis.

MATERIALS AND METHODS

Materials. ROS, PGZ and the deuterated internal standards (IS), rosiglitazone-D3 and pioglitazone-D4, were purchased from Toronto Research Chemicals (North York, ON, Canada). Acetonitrile (ACN), methyl-tert-butyl ether, and 1-chlorobutane, all of HPLC grade, were obtained from Sigma-Aldrich (St. Louis, MO).

In vitro culture studies with human subcutaneous fat explants. Subcutaneous adipose tissue collected from the abdominal wall that was unused after breast reconstruction surgeries and would

normally be discarded was collected in a de-identified fashion under a protocol approved by the Institutional Review Board at the University of Utah (IRB no. 10924). The tissue was rinsed in sterile 1X phosphate-buffered saline (PBS), and the fat was minced into approximately 3 mm^3 pieces under sterile conditions. Powdered ROS or PGZ was added to the fat by weight to achieve the various final molar concentrations of drug used in these studies. One gram of fat equaled approximately 1 mL volume and the weight-to-volume conversion was used for molarity concentration calculations. After addition of the powder, the fat was mixed thoroughly with a sterile spatula. In control experiments in which no drug was added, the fat also underwent physical mixing. The fat was then aliquoted by weight into separate tissue culture wells, covered with media (DMEM low glucose, supplemented with 0.01 mg/mL bovine transferrin, 10 nM insulin, and 1X gentamicin/amphotericin B, (Life Technologies)) and incubated at 37 ^oC/5% CO_2 for the durations indicated. The media was removed and replaced every 2-3 days by aspiration. The fat depot floated in the tissue culture well and the media was aspirated from beneath the fat. The media was strained through a sterile 100 µm nylon mesh and portions of fat that had been inadvertently aspirated were transferred back into the culture dish.

Human monocyte isolation and culture. Peripheral blood samples were obtained in heparin from normal adult volunteers using protocols approved by the Institutional Review Board of the University of Utah and Veterans Affairs Salt Lake City Healthcare System (IRB no. 10-0398). Peripheral blood mononuclear cells (PBMC) were isolated by an adherent method previously described[34]. The PBMC were cultured in 96-well culture plates (2.5×10^4 cells/0.3 cm^2) with fat/PGZ or fat/ROS culture conditioned media diluted in half with SFM media (Gibco Life Technologies, ThermoFisher Scientific) supplemented with 20% serum. Conditioned media was collected from ROS-treated, PGZ-treated, or untreated (no drug) fat cultures. The conditioned media was diluted in monocyte media prior to use in the monocyte cultures. The monocytes were stimulated using LPS (10 ng/mL) (Sigma-Aldrich) for 24 hrs at 37 ^oC/5% CO_2 then the PBMC-conditioned media was collected and stored at -80^oC. Media

from PBMC without LPS stimulation served as controls. Media was assayed for tumor necrosis factor-α (TNFα) by ELISA following manufacturer's instructions.

ELISA. Total adiponectin (R&D Systems), MCP-1 (PeproTech) and TNFα (R&D Systems) levels in the conditioned media was assessed by commercially available ELISAs following the manufacturer suggested protocols.

Smooth muscle cell proliferation assay. Human venous vascular smooth muscular cells, initially isolated and cultured in-house from donor peripheral veins (IRB no. 00031132), were seeded in 96-well tissue culture plates at 5000 cells/well in SMC media (Cascade Biologicals, ThermoFisher Scientific) without serum for 48 hr to quiesce the cells. The quiescence media was replaced with media conditioned with cultured fat/ROS, fat/PGZ, or untreated human subcutaneous fat explants (no drug) that had been collected at various time points. The conditioned media was diluted 1:10 with the SMC media. Platelet-derived growth factor-BB (PDGF-BB, 50 ng/mL) (R&D Systems) was added to stimulate cell proliferation and cells were incubated for 48 hr whereupon cell proliferation was measured by a fluorescence-based DNA-binding assay following manufacturer guidelines (CyQuant, Invitrogen, ThermoFisher Scientific). Cells cultured with and without PDGF in SMC media (no conditioned media) served as additional controls.

Drug release from human subcutaneous fat explants. To compare the release profiles of powdered ROS and PGZ, either drug was interspersed as a powder into human fat explants by thoroughly mixing with a sterile spatula to yield a final concentration of 200 μM. ROS or PGZ was directly applied as a powder to the fat to avoid the use of deleterious solvents. The fat/ROS or fat/PGZ depots were placed in tissue culture media that was removed and replaced with fresh media every 2-3 days. The conditioned media was frozen immediately at -80 ^{o}C until assayed for drug release by HPLC MS/MS.

To further characterize drug release, experiments were also performed in 1XPBS plus 0.2% Tween20 as release media but this was not used for conditioned media experiments.

To assess the release of ROS or PGZ from fat explants with different concentrations of drug, powdered drug was added to human fat to achieve incremental concentrations of drug (6 to 6000 μM) and the fat/drug depots were cultured in media that was collected and analyzed for ROS or PGZ content as described above.

Porcine model. Swine were used for the *in vivo* portions of this study, which were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Utah (IACUC Permit Number 11-08008). Female Yorkshire cross domestic swine (~30 kg) (Sigma Livestock, Salt Lake City, UT) were sedated with intramuscular injection of a Telezol® cocktail (ketamine 3-5 mg/kg (Hospira Inc.), tiletamine/zolazepam 3-5 mg/kg (Fort Dodge Animal Health), xylaxine 6-10 mg/kg (Lloyd Laboratories, Shenandoah, IA)) and then anesthesia was maintained with isofluorane gas (1-3%) inhalation as previously described by us [15, 35, 36]. All surgeries were performed under sterile conditions. Subcutaneous adipose tissue was surgically excised from the dorsal aspect of the neck and rinsed in normal saline. The fat was minced into approximately 3-mm^3 pieces then weighed. Powdered PGZ was added to the fat by weight to achieve the desired final concentrations of drug. The dorsal wound was sutured closed and the animal was turned over and the ventral neck area was prepared for surgery.

For pharmacokinetic studies, the external jugular vein was dissected to create a space for the fat transplant while the fat was prepared. The minced fat was placed perivascular to the vein and the wound was sutured closed and the animals were allowed to recover. For efficacy studies, a porcine model of arteriovenous graft that has been described in detail previously [14, 15, 35, 36] was used. In sedated animals, under sterile conditions, a spiral-reinforced expanded synthetic

polytetrafluoroethylene (PTFE) graft (7-cm length, 6-mm internal diameter) was placed between the common carotid artery and the ipsilateral external jugular vein such that blood flow from the carotid was partially shunted into the vein via the interposing graft. Profound hyperplasia from in-growth of medial vascular smooth muscle and adventitial fibroblasts occurs almost invariably at the anastomosis of the vein and graft within 4 weeks in this model[13]. After graft placement, 10 g of fat collected from the neck and mixed with PGZ (6000 µM) was placed perivascular to the anastomosis of graft and jugular vein (see **Supplemental Figure 1**) and the wound was sutured closed. In control animals, the wound was closed without addition of a fat depot. Post-operative analgesia was provided to all animals by intramuscular injection of buprenorphine hydrochloride (0.01 mg/kg; Reckitt Benckiser Healthcare Ltd., Hull, England) and application of a fentanyl patch (50 mg) (Watson Laboratories, Inc. Corona, CA). The control and treated pigs with graft were maintained for four weeks whereupon they were imaged by MRI to obtain a measure of the open lumen area at the anastomosis of graft and vein as previously described[36].

Serum chemistries. Peripheral blood samples were collected for complete blood counts and comprehensive metabolic panel chemistries (Antech Diagnostics) from fasting animals prior to experimental treatment and just prior to euthanasia. The following biochemical and hematological measurements were obtained: aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, blood urea nitrogen (BUN), creatinine, glucose, cholesterol, triglyceride, white blood cell count (WBC) red blood cell count (RBC), hemoglobin, insulin and hematocrit.

PGZ or ROS extraction from porcine tissue harvest. Plasma was collected from anti-coagulated blood samples taken prior to surgery and at various times post-operatively. At time of euthanasia, the following tissues were collected to assess drug release from fat transplants: fat transplant tissue, external jugular vein, sternocleidomastoid muscle, tissue adjacent to fat transplant and blood. Prior to

extraction, the jugular vein, adjacent tissue and sternocleidomastoid muscle were carefully scraped of any attached fat. The tissue was stored at -80 ^{o}C prior to drug extraction.

High Performance Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS) for drug measurements. The sample extraction protocol was modified from previously published protocol [37, 38]. The conditioned media samples were spiked with ROS-D3 (IS) or PGZ-D4 (IS) at known concentrations to monitor recovery efficiency. The ROS, PGZ, and corresponding IS were extracted from the conditioned media by adding 1 mL 0.1 mM ammonium acetate (pH 6.0 for ROS and pH 4.0 for PGZ), shaking vigorously with 1:1 MTBE:1-chlorobutane for 25 min, and centrifugation at 25 ^{o}C at 2000xg for 15 min. The organic layer was collected and solvent evaporated at 42 ^{o}C overnight then reconstituted in acetonitrile-deionized H_2O (60:40, v/v) running buffer containing 10 mM ammonium acetate and 0.02% trifluoroacetic acid for HPLC/MS/MS analysis. Cumulative drug release from the fat explants was calculated by the summation of the total amount of drug released at each time point and expressed as the fraction of the total initial amount of drug loaded into each explant. For extraction of drug from porcine tissue, tissue was weighed, spiked with the appropriate deuterated drug to track extraction efficiency, then homogenized in 0.1 M ammonium acetate (pH 4.0 for PGZ and pH 6.0 for ROS) (2 mL/g tissue) using disposable rotor stator generator probes (Omni-Tips, Omni-International) to prevent drug carryover between samples. A 1:1 solution of tert-butyl methyl ether:1-chlorobutane (5 mL) was added to each sample then the samples were shaken vigorously on an orbital shaker for 20 min. The samples were centrifuged at 2500 *x g* at r.t. for 20 min and each upper organic layer was transferred to a clean glass tube which was then evaporated under vacuum. The dessicated extracts were resuspended in 2 mL acidified methanol (pH 4.0) by vigorous vortexing for 2 min. The samples were further shaken for 20 min on the orbital shaker (300 rpm) then centrifuged (2500 x *g*) for 20 min then the upper layers transferred to clean glass tubes. The solvent was evaporated and the precipitate reconstituted in 1 mL HPLC/MS/MS buffer (60% acetonitrile, 40% dI water, 10 mM ammonium acetate, 0.02% trifluoracetic acid) by vortexing. The samples were

centrifuged again and an aliquot of the liquid was transferred to polyspring inserts in slit cap glass tubes for analysis by HPLC/MS/MS.

The analytical separation of ROS and the IS ROS-D3 or PGZ and the IS PGZ-D4 was performed using an Atlantis dC_{18} column (2.1 x 30 mm, 3.0 μm) (Waters Corp.), protected by a 4 mm x 2 mm C_{18} packed guard column (Phenomenex) at 25 ^{o}C on an Acquity UPLC H-Class system (Waters Corp.) equipped with a refrigerated autosampler. The mobile phase consisted of acetonitrile–deionized H_2O (60:40, v/v) containing 10 mM ammonium acetate and 0.02% trifluoroacetic acid run isocratically at a flow rate of 0.3 mL/min. Sample run times were 4 min followed by a needle wash process to prevent possible carryover. The ROS and ROS-D3 eluted at 0.48 min and PGZ and PGZ-D4 eluted at 0.5 min.

A triple quadrupole tandem mass spectrometer (Acquity TQD, Waters Corp.) equipped with an electrospray ionization (ESI) interface was used for analytical detection. Quantification was performed in positive-ion mode using multiple reaction monitoring (MRM) of the transition masses of ROS (m/z 358.1 → 135.1) and ROS-D3 (m/z 361.1 → 138.1) or PGZ (m/z 357.1 → 134.3) and PGZ-D4 (m/z 361.1 → 138.1). Instrument parameters were optimized for simultaneous detection of both ROS or PGZ and corresponding IS. The capillary energy was set at 1.25 kV for ROS or 3.5 kV for PGZ, cone voltage at 45 V for both, extractor at 4 V for both, source block temp at 100 ^{o}C for both, desolvation temperature at 200 ^{o}C for both, and the optimal collision energy was determined to be 36 V for ROS and 34 V for PGZ. Calibration curves for both ROS and ROS-D3 or PGZ and PGZ-D4 were established using standard samples at seven concentrations ranging 5-1000 ng/mL. The coefficient of determination (r^2) from a least squares linear regression was found to be 0.999 for both drugs and matching IS. Validation for accuracy and precision of the HPLC MS/MS method was performed using spiked samples of media, plasma, or tissue. Sample recovery of >75% was attained for ROS and recovery >65% was achieved for PGZ, as determined by percent recovery of the IS.

Immunohistochemistry. External jugular vein was explanted from swine and fixed for 24 h in 10% zinc formalin. The fixed tissue was processed and paraffin-embedded using standard techniques. Tissue slices (5 μM) were deparaffinized and rehydrated and antigen-retrieval performed in 10 mM sodium citrate using the EZ-Retriever System (BioGenex Laboratories). Nonspecific binding was blocked with 2% goat serum and tissue slices were incubated overnight at 4 ^{o}C with a rabbit anti-porcine adiponectin antibody (1:125 dilution) (Xeno Diagnostics, LLC). Antibody binding was detected with anti-rabbit IgG biotinylated secondary antibody (1:200 dilution, Vector Laboratories) and its binding was detected with avidin/biotinylated peroxidase enzyme complex (Vectastain ABC system, VECTOR NovaRed peroxidase substrate, Vector Laboratories).

Magnetic resonance imaging (MRI) of autologous subcutaneous fat/drug transplants. At one week after autologous fat transplant placement, animals were sedated and placed supine in the bore of a 3 Tesla MRI scanner (TIM Trio 3T, Siemens Medical Solutions). An overlap-decoupled 16-channel transmitted radiofrequency (RF) coil mounted on a fiberglass support molded to fit the porcine neck, or two sets of bilateral paired-array RF coils with eight total coil elements, were placed over the surgical area to obtain MR images as previously described[12]. To image fat, two sets of 2D or 3D black-blood Turbo Spin Echo (TSE) sequences (fat saturation and no fat saturation scans) were performed. In the fat saturation scan, a chemical-shift fat saturation pulse was applied to eliminate fat signal. To visualize the fat transplant signal, the 2D or 3D TSE sequence was repeated with no fat saturation pulse (No fat sat). The imaging parameters of 3D TSE sequences were: variable refocus flip angles, TE/TR=23/700 ms, ETL=52, voxel dimension=0.5x0.5x2.0 mm. The MR imaging was performed without intravascular injection of MRI contrast agent.

OsiriX medical image processing software (Osirix v. 3.6.1), an open-source software (http://www.osirix-viewer.com/downloads.html), was used for MR image analysis. The 2D black-blood sequences with fat suppression and the sequences without fat suppression were synced so that the same cross-sectional image slices within the animal could be viewed simultaneously.

Comparing the images with fat suppression and without fat suppression allowed the detection of the autologous fat transplant. The fat transplant could be differentiated from native surgically undisturbed fat by its bright but non-homogenous signal, occurring because the transplant consisted of minced fat. Native undisturbed fat had a bright homogenous signal. To highlight fat signal, the black-blood images captured without fat signal suppression were fused into the fat signal suppression image using the fuse tool in Osirix and setting the fat signal as blue.

Statistical analyses. Differences in serum chemistries between preoperative and euthanasia time points in the same animals were evaluated using paired t-tests. The differences in serum chemistries between the drug-treated and control groups at either the preoperative and euthanasia time points were evaluated using unpaired t-tests. Differences in MCP-1 released from fat treated with the drug were evaluated using a two-way ANOVA with bonferroni post test. The differences in quantity of adiponectin released from fat treated with ROS or PGZ, TNFα release from monocytes treated with the fat-conditioned medias, and the differences in proliferation of smooth muscle cells treated with the fat-conditioned medias were evaluated using a one-way ANOVA with a Tukey post hoc test. Differences were considered significant at $p \leq 0.05$.

RESULTS

Induction of adiponectin release from fat explants by powdered drug. Previous studies have shown that PGZ dissolved in solvent then added to the media of cultured fat induces the fat to express adiponectin [39]. Here, the ability of the *powdered* form of ROS or PGZ to induce adiponectin production within the explanted human fat was assessed. Although high concentrations of adiponectin were detected in the conditioned media of fat explants without drug, similar to the concentrations reported by others [39], powdered PGZ further enhanced adiponectin release (**Figure 1).**

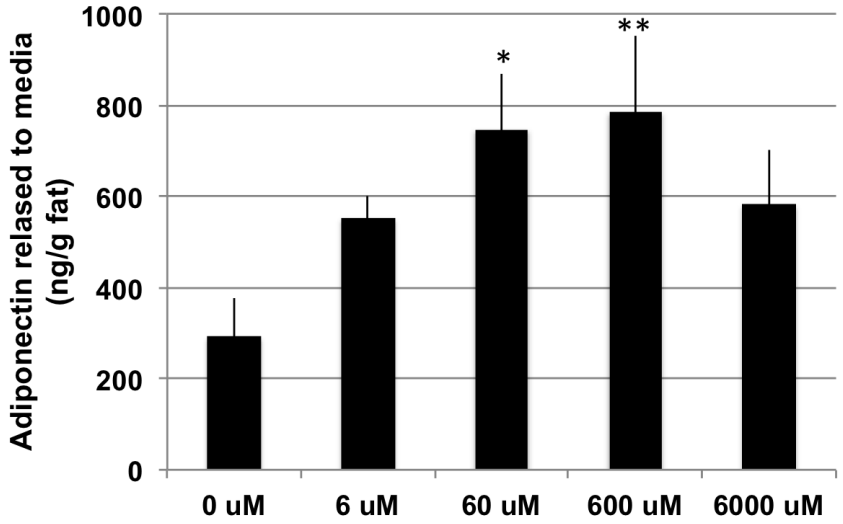


Figure 1. Adiponectin concentrations in media over time after culturing human subcutaneous fat explants incorporated with 0 – 6000 µM pioglitazone (PGZ). Shown is the concentration of adiponectin detected in the media removed at day 4. N = 5 separate experiments using fat from five subjects. *P<0.05, 0 µM PGZ vs 60 µM PGZ; **P<0.01, 0 µM PGZ vs 600 µM PGZ.

Marked variability in adiponectin release was observed from control fat explants (no PGZ) from the five individuals used in these experiments, similar to the highly variable levels of adiponectin that have been reported in human serum. Powdered ROS incorporated into fat explants also induced adiponectin (**Supplemental Figure 2**). The induction of adiponectin was sustained for at least 11 days, the latest time point assessed, in fat treated with either PGZ or ROS (data not shown).

Effects of fat/drug depot-conditioned media on MCP-1 release and cell proliferation. Besides producing the vasoprotective adiponectin, adipose tissue is also a source of inflammatory cytokines that could promote intimal hyperplasia in the vasculature. The effect of ROS or PGZ on adipose inflammation was thus assessed. High concentrations of MCP-1 were observed in conditioned media removed after 2 days from cultures of fat alone, but MCP-1 levels were significantly inhibited by PGZ

and ROS treatment (**Figure 2**). By 6 days of culture, MCP-1 levels were lower in both untreated and drug-treated fat cultures.

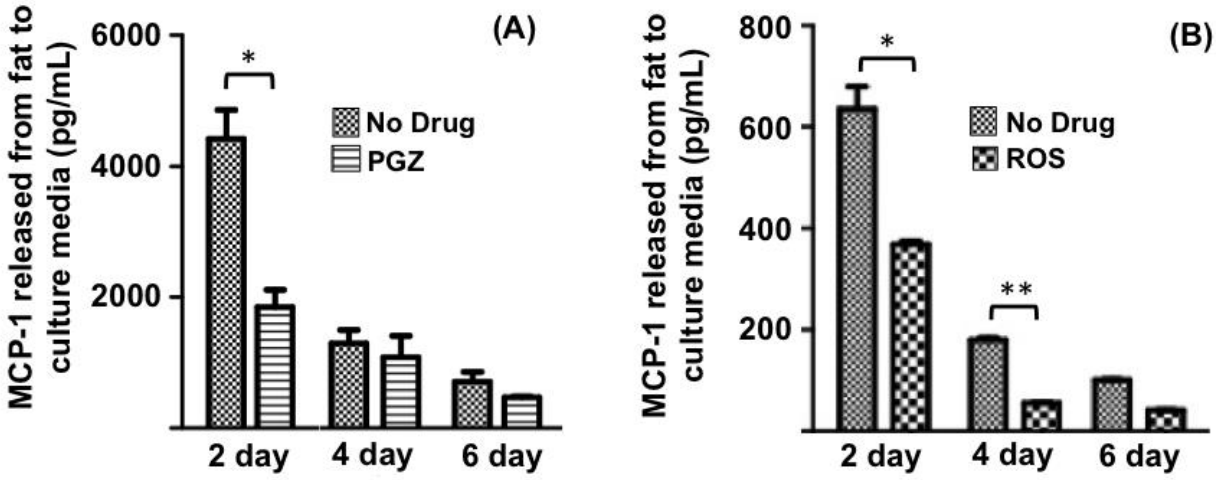


Figure 2. Effects of rosiglitazone (ROS) or pioglitazone (PGZ) on release of monocyte chemotactic protein-1 (MCP-1) from cultured human subcutaneous fat explants. (A) Fat explants alone (stippled bar) or with PGZ added to achieve an initial concentration of 800 µM (striped bar) were cultured. Media was removed and replaced at 2, 4 or 6 days and assessed for MCP-1 concentrations by ELISA. *p<0.001 vs. untreated fat; (B) Fat explants alone (stippled bar) or mixed with ROS to an initial concentration of 1000 µM (checkered bar) were cultured. Media was removed and replaced at 2, 4 or 6 days and assessed for MCP-1 concentrations by ELISA. *p<0.001 vs. untreated fat; **p<0.01 vs. untreated fat. Fat cultured with PGZ was obtained from a different individual than the fat cultured with ROS. Note the different initial concentrations of MCP-1 detected in the culture medias suggesting different levels of baseline inflammation.

The effect of fat/drug culture-conditioned media on LPS-induced monocyte production of TNFα was next assessed. For these experiments conditioned media was removed from fat explants incorporated with or without PGZ or ROS after 4 days and approximately 2 weeks of culture. The monocytes were stimulated with LPS in the presence of the different fat/drug culture-conditioned medias and incubated for 24 h whereupon the monocyte-conditioned media was then assessed for TNFα concentrations by ELISA. The LPS-induction of TNF was markedly inhibited by conditioned media from fat cultured in the absence or presence of PGZ (**Figure 3**). Similar results were observed using conditioned media from fat/ROS cultures (not shown).

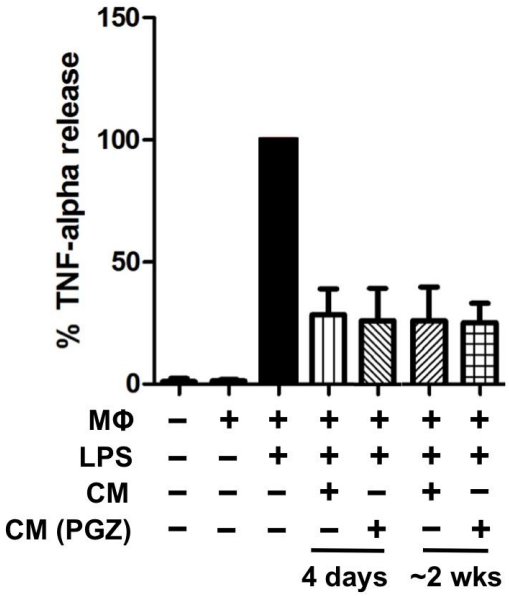


Figure 3. Effect of conditioned media from human subcutaneous fat explants incorporated with powdered pioglitazone (PGZ) or no drug, on tumor necrosis factor-alpha (TNF) release from lipopolysaccharide (LPS)-stimulated human monocytes. Human monocyte/macrophages (MØ) were treated with or without LPS (10 ng/mL) for 24 h while cultured in the presence of different medias conditioned with fat/PGZ. The conditioned media was from fat mixed with powdered pioglitazone (CM (PGZ)) to achieve 800 µM initial concentration, or with no glitazone added (CM). The MØ were cultured in the different conditioned medias that had been removed at 4 days or at ~2 weeks after fat culture began. After culturing the MØ for 24 h in the different conditioned medias, the MØ-conditioned media was then assessed for TNF by ELISA. The first column indicates TNF detected in plain unconditioned media (no fat or MØ). The second column indicates TNF detected in MØ-conditioned media but without LPS stimulation. The presence of conditioned medias, with or without glitazone, significantly inhibited LPS-induced TNF release compared to cells treated with LPS alone ($p < 0.0001$). There was no difference in inhibition between CM and CM(PGZ).

The effect of fat/drug culture-conditioned media on cultured smooth muscle cell proliferation was also assessed. For these experiments human venous smooth muscle cells were treated with or without platelet-derived growth factor-BB (PDGF), an inducer of proliferation, in the presence or absence of media conditioned by fat with or without ROS (**Figure 4**). Smooth muscle cells treated with conditioned media taken from fat/ROS cultures at 8 days showed almost complete inhibition of PDGF-induced proliferation, with proliferation levels similar to basal cultures without PDGF stimulation (white bar). Greater inhibition of proliferation was observed in cultures treated with conditioned media from fat treated with ROS than conditioned media from fat alone. The concentration of adiponectin, as determined by ELISA, was 2.2 ng/mL and 10 ng/mL in the conditioned medias collected on day 2 and day 8 from the naïve fat (no ROS) and 2.5 ng/mL and 40

ng/mL from the ROS-treated fat. The concentration of ROS as determined by HPLC/MS/MS in the day 2 and day 8 conditioned medias from the naïve fat was below detection, and was 1.9 µM and 1.3 µM in the ROS-treated fat.

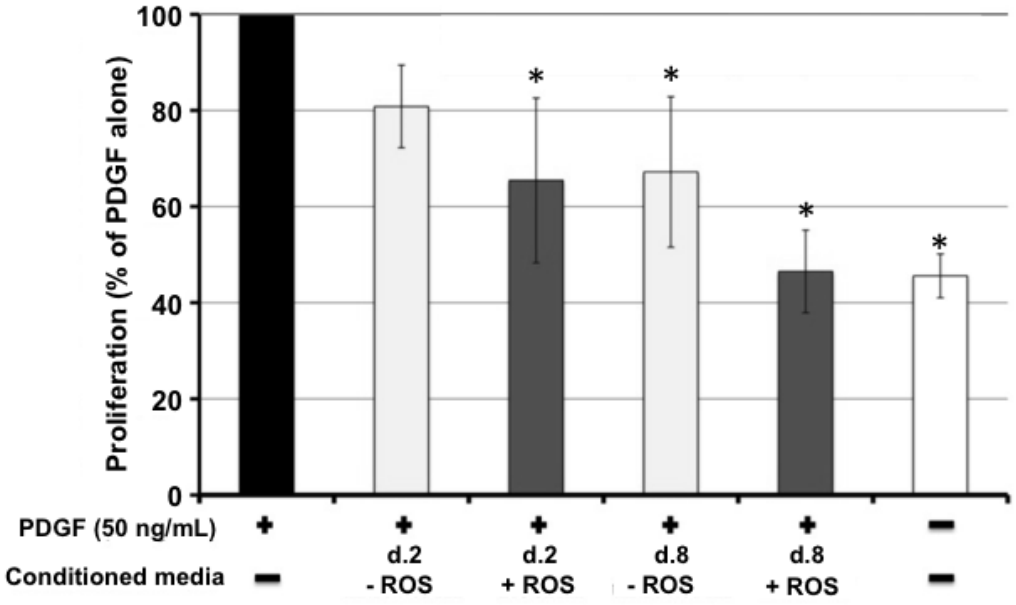


Figure 4. Effect of conditioned media from human subcutaneous fat explants incorporated with powdered rosiglitazone (ROS) on platelet-derived growth factor-BB (PDGF)-induced smooth muscle cell proliferation. Subconfluent human venous smooth muscle cells were stimulated to proliferate with platelet-derived growth factor-BB (PDGF) (50 ng/mL) while cultured in the absence or presence of media conditioned by fat cultured without (-ROS) or with powdered ROS (+ROS, 1000 µM, initial concentration). The fat-conditioned media had been removed from the fat cultures and replaced every 2 or 3 days. The smooth muscle cultures were exposed to the fat-conditioned media that had been removed from the fat cultures at day 2 (d.2) or day 8 (d.8). The last column in the chart indicates growth when smooth muscle cells were exposed to naïve media alone without PDGF. Growth is expressed as percent of growth observed in cells cultured with PDGF alone. *<0.0004 vs PDGF alone.

Release of ROS or PGZ from human subcutaneous fat explants. The fat was repurposed as a drug depot with the intent to provide sustained release, thus high initial concentrations were tested in these studies. Fat was mixed with the powdered form of PGZ or ROS to achieve an initial concentration of 200 µM in the fat depot. At 12 days, approximately 30% of the initial concentration of ROS and approximately 15% of PGZ was released to the media (**Figure 5**). When 0.2% Tween20 in 1XPBS

was used as release media, ~33% of ROS but less than 2% of PGZ was released at 12 days (data not shown).

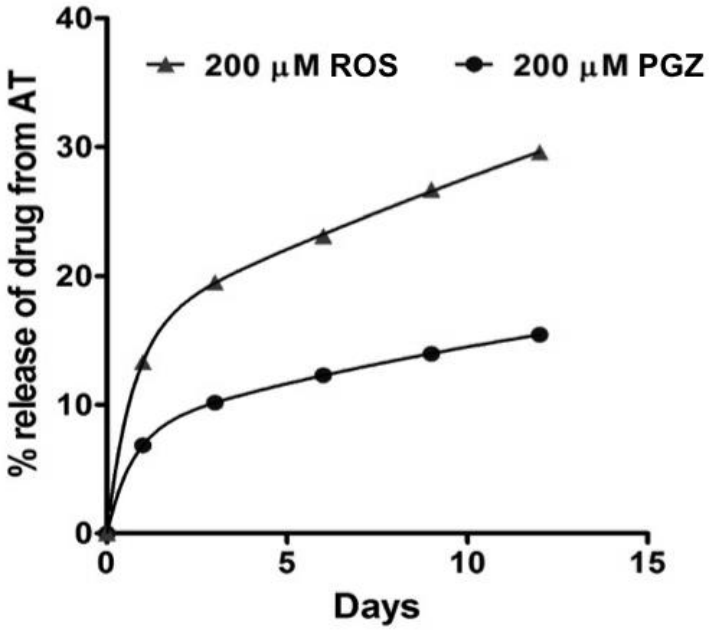


Figure 5. Release profile of ROS or PGZ from human subcutaneous adipose tissue (AT) explants. Release of rosiglitazone (ROS) or pioglitazone (PGZ) from human fat explants after incorporation of 200 μM of either drug. The concentration of drug that was detected at each time point was summed with the previous time point concentrations to yield total percent release of drug. These results are from one experiment.

Since a prolonged release profile was desired for *in vivo* use, and a slower release was obtained with PGZ, the release of PGZ into the media from cultured human fat explants mixed with incremental concentrations of powdered drug was next assessed. The PGZ was released in a sustained, dose-dependent manner (**Figure 6**). Drug concentrations were below detection limit in media from explants embedded with 6 μM PGZ at day 4, 6 and 8. Drug release at day 2 is likely greater because of the initial release of oil from the minced fat which is obtained in the first media collection.

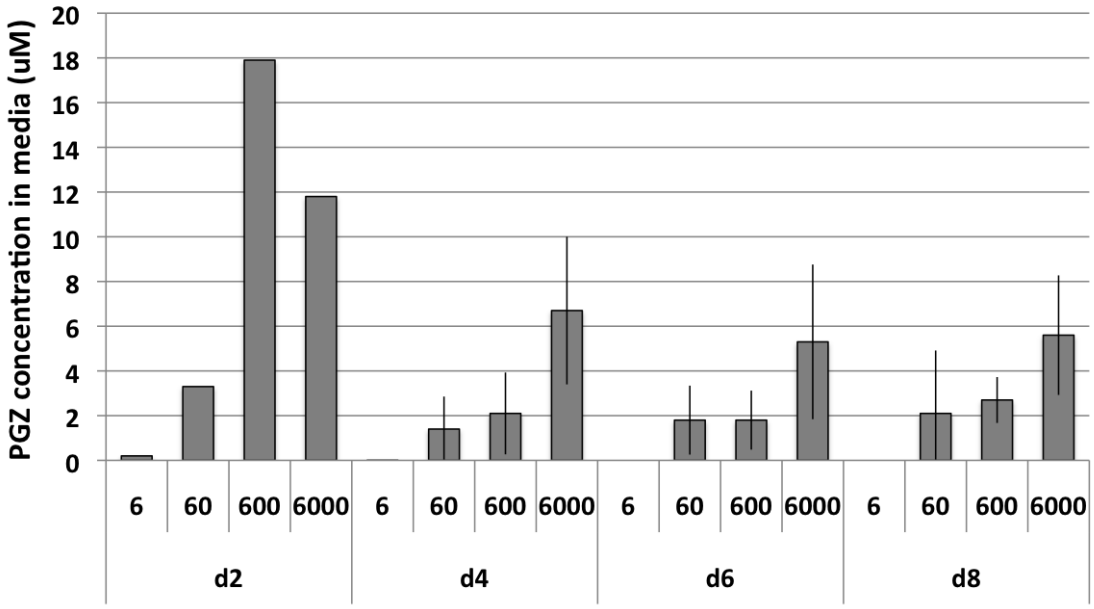


Figure 6. Pioglitazone (PGZ) concentrations in media at day 2, 4, 6 and 8 of culturing human subcutaneous fat explants initially incorporated with 6, 60, 600 or 6000 µM pioglitazone (PGZ). Data for each initial concentration is from 3 or 4 experiments, except day 2 which is from one experiment. The concentrations shown are what were detected in each media aliquot collected at the indicated time.

Safety and pharmacokinetics of fat/PGZ transplants in vivo in swine. For *in vivo* studies, fat was transplanted from the neck of swine to the perivascular area of the external jugular vein (**Figure 7**).

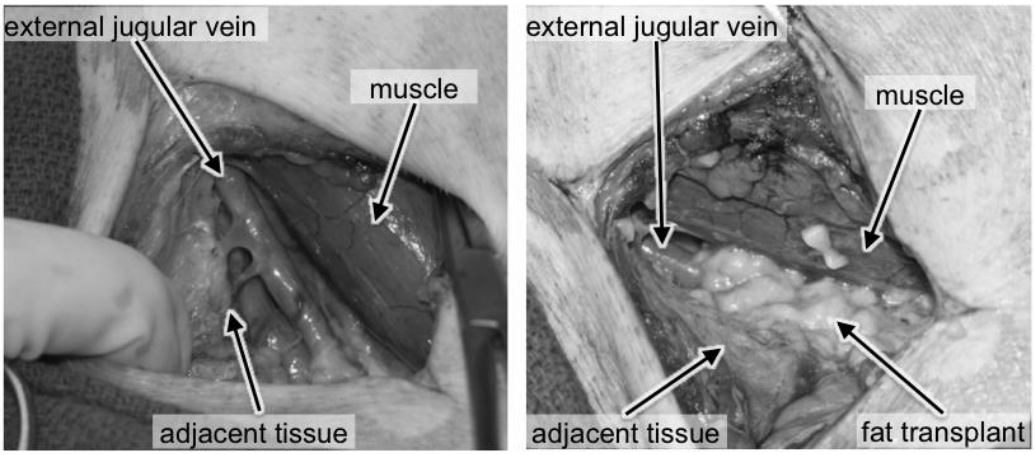


Figure 7. Example of an external jugular vein before and after placement of an autologous fat transplant. The image on the left shows an external jugular vein (EJV) in the neck of a pig after surgical dissection. The location of what was considered "adjacent tissue" and the sternocleidomastoid muscle "muscle" are both indicated. The image on the right shows the external jugular vein after placement of an autologous fat transplant just prior to suturing of the wound.

Initial assessment of the safety of the fat/drug transplants was performed by transplanting 5 g of autologous fat mixed with PGZ (200 μM) (n=1) or ROS (200 μM) (n=2) to the perivascular space of dissected external jugular veins. Fat alone was placed on the contralateral external jugular veins. The fat/drug transplants and fat transplants were well tolerated. No overt swelling, erythema, irritation, infection or delayed wound healing was noted at any time point after placement. External jugular vein, sternocleidomastoid muscle, fat depot, adjacent tissue and blood collected from a peripheral vein were all harvested two weeks after fat transplantation and assessed for drug content by HPLC/MS/MS. Low concentrations of drug were detected in the fat retrieved from the fat/drug depots (PGZ - 1.1 μM; ROS 0.1 ± 0.13 μM). No drug was detected in the target jugular vein of the pig treated with fat/PGZ depot but an average of 0.06 ± 0.06 μM was detected in the animals treated with fat/ROS depots. Drug was also detected in the contralateral untreated side of pigs treated with a fat/ROS depot, but not in the animal treated with a fat/PGZ depot. No drug was detected in plasma collected from a peripheral vein from any animal. Since ROS has a lower Log P, a measure of drug lipophilicity, and because ROS was detected in non-target tissue away from the site of implant, further *in vivo* experiments were only performed using PGZ.

The amount of fat used for the depot was increased to 10 g while the PGZ concentration remained at 200 μM. In tissue collected at one week from three pigs treated with 10 g of fat with PGZ (200 μM), the average concentration of PGZ remaining in the fat depot was 68.5±31.5 μM, the concentration present in the treated external jugular vein was 13.7±14.9 μM, and in the adjacent tissue was 21.7±27.2 μM (n=3 pigs) (**Figure 8)**. No drug was detected in plasma.

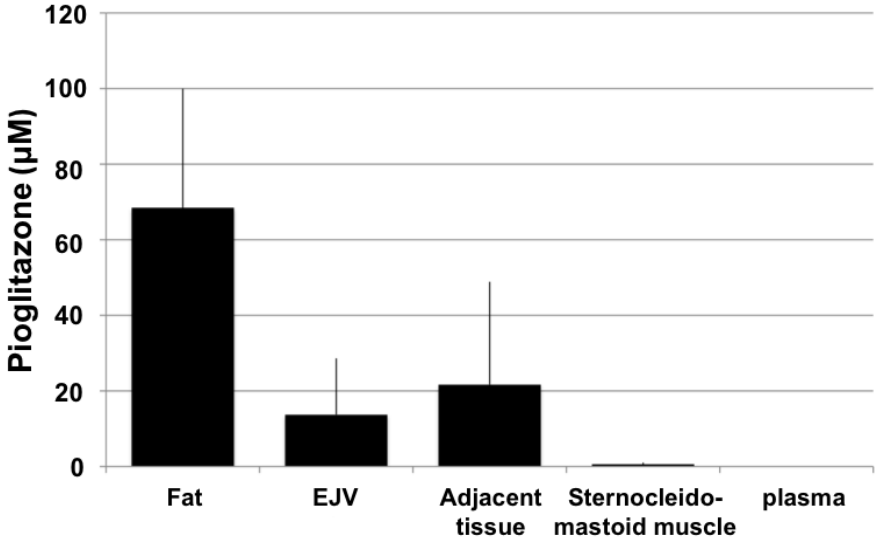


Figure 8. **Pioglitazone (PGZ) concentrations in tissue after fat/PGZ transplant perivascular to external jugular vein.** Average PGZ concentrations in the fat depots (fat), external jugular veins (EJV), adjacent tissues, and sternocleidomastoid muscles collected from three pigs one week after transplantation of 10 g of autologous porcine subcutaneous fat with PGZ (200 µM initial concentration). No drug was detected in plasma.

MRI of the fat/PGZ depots after transplantation. Animals underwent MR imaging to monitor fat/PGZ depot location one week after transplant. The fat transplants remained near the perivascular region of the external jugular vein (**Figure 9**). For better visualization of the fat transplants, the image fusion function of the DICOM imager was used to highlight the fat signal in blue, as shown in **Supplementary Figure 3**.

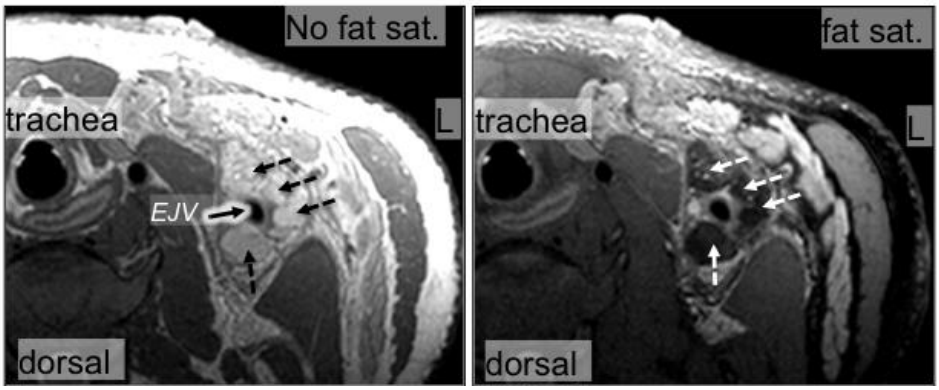


Figure 9. **Examples of MR imaging of the autologous fat transplants in swine.** Swine underwent MRI one week after autologous fat transplant. The images show a cross-section of the animal, with the dorsal side of the animal at the bottom, and focuses on the left (L) side, where the fat transplants were placed. Flowing blood appears black in black-blood images, thus the external jugular vein (EJV) lumen is black. Black-blood MR images were obtained without (No fat sat.) (left image) or with fat signal suppression (fat sat.) (right image). Fat appears white when fat signal suppression is not used and it appears hypointense (gray) when fat signal suppression is used. Lymph tissue appears white in both "No fat sat." and "fat sat." images. The "No fat sat." and the "fat sat." images were obtained in immediate succession in each

animal. The fat transplant is indicated in each animal in the "No fat sat." images by black dashed arrows, and is indicated in the "fat sat." images by white dashed arrows.

Adiponectin protein in vein adjacent to the fat/PGZ transplant. External jugular vein were explanted at 2 weeks, with the transplanted fat attached, from swine treated with fat alone or fat mixed with PGZ (200 μM), then immunostained with anti-adiponectin antibody. As shown in **Figure 10**, the external jugular vein and perivascular fat had greater adiponectin immunoreactivity in the animal exposed to the fat/PGZ transplant compared to the vein exposed to fat transplant alone. The endothelial layer (indicated by arrows) was highly immunostained in the fat/PGZ transplanted vessel.

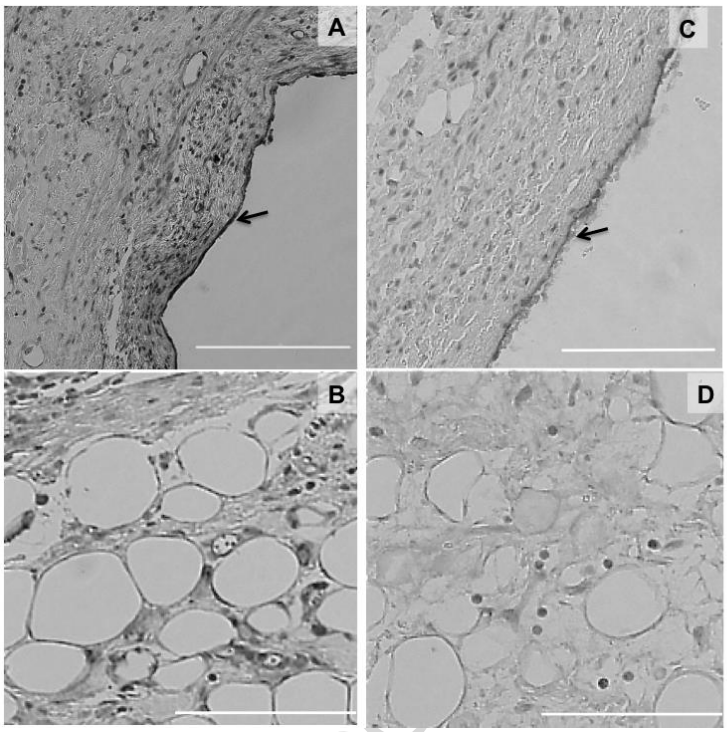


Figure 10. **Effect of autologous subcutaneous fat transplant with or without PGZ on adiponectin expression in the simple transplant model.** Fat with or without PGZ (200 μM) was placed perivascular to the left and right external jugular vein, respectively, in a pig. The veins were explanted at 2 weeks post-transplantation. The tissue was immunostained with anti-adiponectin antibody. Images A and B are from the vein exposed to the fat transplant with PGZ. Images C and D are from the vein exposed to the fat transplant without PGZ. Images A and B are of the vein wall and B and D are of the adipose tissue perivascular to the vessel wall. Dark gray indicates positive antibody binding. The length bar indicates 200 μm.

Fat/PGZ depot effects on hyperplasia in a porcine arteriovenous graft model. Autologous fat was mixed with PGZ (6000 μM) and the depots were placed perivascular to the vein-graft anastomosis region. Control animals received arteriovenous grafts but no fat/PGZ depots. Serum chemistries and

hematology parameters in blood collected at surgery and at four weeks are shown in **Table 1**. No statistically significant differences in blood chemistries or hematology parameters were observed between treated and control animals at four weeks. All tested values were within normal range for swine and similar to values observed in our previous studies[12, 15].

Table 1. Preoperative and four week serum chemistries and hematology.

	Glucose (mg/dL)	Insulin (mg/dL)	Creatinine (mg/dL)	WBC (10^3/mL)	ALT (IU/L)	AST (IU/L)	BUN (mg/dL)	Bilirubin (mg/dL)
Baseline								
Control	126.0 ± 40.4	9.4 ± 1.8	0.9 ± 0.1[c]	15.6 ± 3.3	20.4 ± 2.6[a]	14.2 ± 3.9	7.4 ± 2.1	0.1 ± 0.0
Treated	96.5 ± 34.1	7.0 ± 1.5	1.0 ± 0.2[d]	17.5 ± 4.2	24.4 ± 6.2[b]	20.3 ± 6.7	7.9 ± 1.1	0.2 ± 0.1
4 weeks								
Control	159.0 ± 44.1	12.0 ± 2.7	1.1 ± 0.2	12.8 ± 2.2[e]	28.6 ± 3.4	20.4 ± 2.6	7.2 ± 1.6	0.1 ± 0.0
Treated	131.5 ± 3 7.5	11.6 ± 6.0	1.2 ± 0.2	13.3 ± 1.6	28.8 ± 4.2	23.4 ± 6.2	8.5 ± 1.3	0.1 ± 0.0

[a] p<0.002, control baseline vs. control four-week
[b] p<0.020, treated baseline vs. treated four-week
[c] p<0.040, control baseline vs. treated four-week
[d] p<0.029, treated baseline vs. treated four-week
[e] p<0.033, treated baseline vs. treated four-week

At four weeks, the graft and attached veins were explanted, formalin-fixed and paraffin embedded for histomorphometry. The fat/PGZ depots were well tolerated with no overt swelling or infections. As shown in **Figure 11**, pigs treated with fat/PGZ depots had similar graft lumen areas as compared to control untreated pigs.

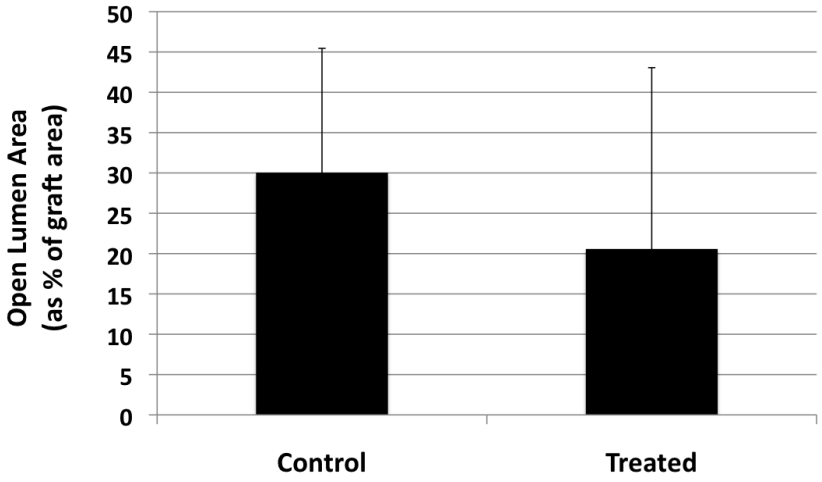


Figure 11. **Open lumen area at the vein-graft anastomosis region as a percentage of total graft lumen area.** There was no statistically significant difference in open lumen area in the synthetic graft between control pigs (n=5) and pigs treated with fat/PGZ depots (n=9) (p=0.367).

DISCUSSION

This study was performed to determine if autologous fat could be repurposed as a biocompatible drug depot for the sustained delivery of ROS or PGZ, and serve as a biofactory for the production of the vasoprotective protein adiponectin. We further examined if fat laden with either ROS or PGZ could decrease the development of intimal hyperplasia at the anastomosis of vein and graft in a porcine model of synthetic arteriovenous grafts. The powder form of ROS and PGZ was bioactive within fat, as adiponectin production was increased to extents that were similar to that reported when PGZ was dissolved in a solvent and introduced into the culture media of adipose tissue explants [39].

We also provide *in vitro* and *in vivo* data demonstrating the sustained release of PGZ or ROS from fat explants when the drug is incorporated into the fat in powdered form. Powdered drug was used to avoid possible deleterious effects of solvent [40]. ROS, which is more hydrophilic than PGZ (calculated Log P 2.95, aqueous solubility of 0.038 mg/mL vs. Log P 3.33, aqueous solubility 0.004 mg/mL, respectively),[16] was released to media more rapidly than PGZ. The concentrations of drug observed in the fat/drug-conditioned culture media were within range of the mean maximal concentrations observed in serum in healthy volunteers administered a 45 mg dose of PGZ (~3.9 µM) [40] and within range of concentrations used in *in vitro* studies when the drug is dissolved in solvent for the purpose of adiponectin induction[39, 41]. Thus, our *in vitro* studies supported the notion that clinically relevant localized levels of drug could be achieved with this approach.

When concentrations of PGZ or ROS were serially diluted in the fat explant cultures, lower levels of drug were detected in the conditioned media, as expected. However, the concentrations of drug in the media from the explants did not mirror the proportional decreases in concentration of drug initially incorporated into the fat. For example, only 2-4-fold greater concentrations of PGZ were detected in the media collected from the cultures incorporated with 600 µM PGZ compared to the

media from explants incorporated with 60 µM, when a 10-fold increase would be expected for first-order drug release. The aqueous solubility of PGZ is 0.004 mg/mL (~12 µM); thus the media was close to saturation with concentrations greater than 10 µM. It is conceivable that the drugs were metabolized within the adipose explant, but this possibility was not investigated. The kinetics of the ROS or PGZ release from fat requires more detailed study, but an important factor to be considered is that vascular, fibrotic and lipid components of adipose tissues are highly variable among individuals, which could alter drug release profiles.

The release of MCP-1 from fat explants was decreased in the presence of ROS or PGZ, indicating an inhibitory effect of the drug on fat inflammation, similar to that reported in tissue culture studies using these drugs dissolved in solvent and in *in vivo* studies after oral administration [42-45]. The induction of TNFα from LPS-induced monocytes was inhibited to a similar degree by conditioned media from either the fat/drug or fat explant cultures without drug, suggesting adiponectin was the more important factor than the drug with our approach. A trend toward greater inhibition of PDGF-stimulation of smooth muscle cell proliferation was observed in cultures exposed to conditioned media from fat/drug cultures compared to fat alone. However, studies utilizing adiponectin-blocking antibodies are needed to define the precise roles of adiponectin and ROS and PGZ.

The *in vivo* efficacy studies did not decrease hyperplasia development at the venous anastomosis of the synthetic graft. The studies however showed that the fat/PGZ depots were well tolerated and that clinically relevant levels of drug could be achieved in the target blood vessel wall. Although PGZ was detected in tissue adjacent to the depot, levels were below detection in peripheral blood suggesting the fat/PGZ depot resulted in minimal systemic exposure to drug while providing levels of drug in the target tissue that are typically therapeutic.

The location of the fat/PGZ depots after one week was monitored *in vivo* by MRI where the majority of the fat signal was detected perivascular to the target vessel. At time of explantation, the fat was observed around the location of original placement. This indicates that the fat depot stays at the site of application, which is an important attribute for an effective drug delivery depot.

Immunostaining for adiponectin, performed on vein and fat depot explanted from the animal model revealed enhanced immunoreactivity for adiponectin in the vessels exposed to fat/PGZ transplants compared to vessels exposed to fat alone. These data suggest that the PGZ enhanced the adiponectin levels in the tissues adjacent to the depot *in vivo*. In particular, the endothelium had very high immunoreactivity for the anti-adiponectin antibody even though this tissue type does not synthesize adiponectin. This suggests that the adiponectin released from the fat/PGZ depot was able to migrate from the fat depot through the vascular wall to bind to the endothelium. Studies by others have shown that PGZ increases plasma adiponectin levels by increasing translation of the adiponectin gene in the adipocyte, and not by an increase in message transcription [46, 47]. Adiponectin exists as an ~30 kD adiponectin monomer that is further assembled into complexes of increasing molecular weights, and with a variety of post-translational modifications[48]. The different multimers trigger overlapping and distinct signaling pathways and have different binding affinities to the three known adiponectin receptors. Thus the fat/PGZ or fat/ROS transplant likely provides the different native adiponectin complexes with all the natural secondary modifications that are difficult to replicate in recombinant adiponectin products that might be delivered by a polymer depot.

A number of factors contribute to intimal hyperplasia development in arteriovenous grafts, including i) surgical trauma to the vessel wall, ii) the introduction of foreign material in the form of synthetic graft and/or sutures, and iii) the introduction of aberrant blood flow shear stresses. These factors induce inflammation, cell proliferation and damage to the endothelium. The anti-diabetic ROS and PGZ inhibit oxidative stress [49-53] and smooth muscle cell proliferation [54, 55], promote vasodilation [56, 57], and induce adiponectin, suggesting they would be beneficial in this setting. However, in this model of very robust hyperplasia development, the delivery of PGZ and adiponectin to the vein-graft anastomosis did not decrease stenosis. We have shown here that the fat/PGZ depots were well tolerated, provided prolonged delivery of drug to adjacent tissue without appreciable systemic exposures, induced adiponectin, and decreased inflammation and cell proliferation and induced adiponectin. Thus this novel approach may still be an effective treatment to inhibit stenosis

and improve maturation rates of native arteriovenous hemodialysis fistulas that lack the presence of synthetic, pro-inflammatory graft material. Further the approach may be useful in other settings of pathological cell proliferation or inflammation, but this remains to be determined.

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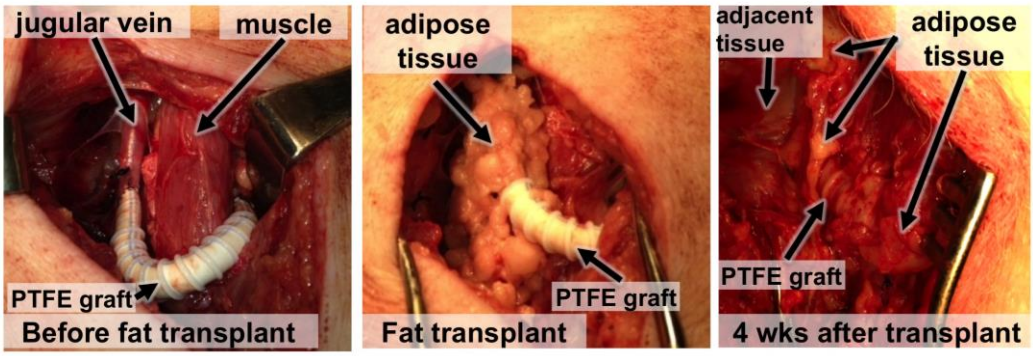
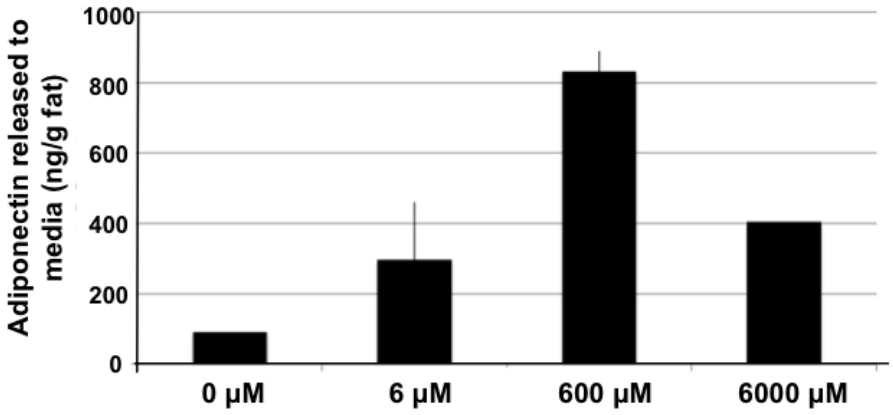
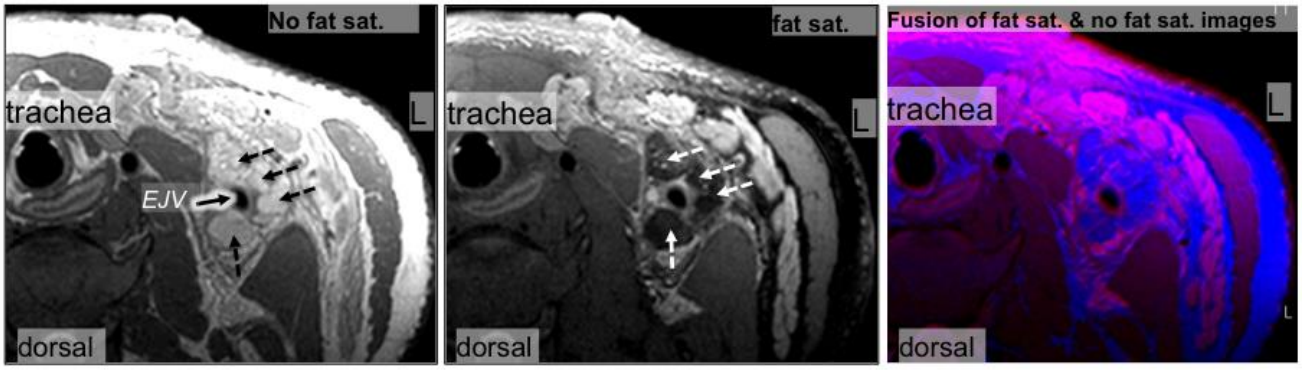
Supplemental Figures



Supp. Figure 1. Placement of fat/PGZ depot at anastomosis of synthetic graft and jugular vein. A synthetic polytetrafluoroethylene graft (PTFE) was surgically placed between the carotid artery and ipsilateral external jugular vein. Fat collected from the dorsal region of the neck was minced and mixed with PGZ (6000 μM) then placed perivascular to the vein-graft anastomosis region. The wound was sutured closed and at 4 weeks graft and attached vessels were explanted for histomorphometry. Adipose tissue was often observed attached to both the vein and the graft material.



Supp. Figure 2. **Release of adiponectin from cultured human subcutaneous fat explants.** Freshly isolated human subcutaneous fat explants from 3 subjects were mixed with powdered ROS to achieve initial concentrations of 6 to 6000 μM or with no ROS, then cultured. The culture media was removed and replaced at 2 and 5 days. Shown is the concentration of adiponectin detected in the media removed at day 5. The media from the 60 μM concentration was lost due to technical difficulties.



Supp. Figure 3. **Examples of MR imaging of autologous fat transplants in swine.** MR imaging was performed one week after autologous fat transplant perivascular to the left external jugular vein. The images show a cross-section of the animal, with the dorsal side at the bottom, and focuses on the left side, where the fat transplants were placed. Flowing blood appears black in black-blood images, thus the vessel lumens are black. Black-blood MR images were obtained without (No fat sat.) (image in first column) or with fat signal suppression (fat sat.) (image in second column). Fat appears white when fat signal suppression is not used and it appears hypointense (gray) when fat signal suppression is used. Lymph tissue appears white in both "No fat sat." and "fat sat." images. The last column is an image created by fusing the "No fat sat." into the "fat sat" image wherein the fat is assigned a blue color. The "No fat sat." and the "fat sat." images were obtained in immediate succession in each animal. The fat transplant is indicated in the "No fat sat." images by black dashed arrows, and is indicated in the "fat sat." images by white dashed arrows.

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