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Nanofibrous antibiotic-eluting matrices: Biocompatibility studies in a rat model

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Acknowledgments: M.C. Bottino acknowledges funding from the National Institutes of Health (NIH)/National Institute of Dental and Craniofacial Research (NIDCR) (grants DE023552 and DEDE026578).

Abstract: This study evaluated the biocompatibility of degradable polydioxanone (PDS) electrospun drug delivery systems (hereafter referred as matrices) containing metronidazole (MET) or ciprofloxacin (CIP) after subcutaneous implantation in rats. 60 adult male rats were randomized into 6 groups: SHAM (sham surgery); PDS (antibiotic-free matrix); 1MET (one 25 wt.% MET matrix); 1CIP (one 25 wt.% CIP matrix); 2MET (two 25 wt.% MET matrices); and 2CIP (two 25 wt.% CIP matrices). At 3 and 30 days, animals were assessed for inflammatory cell response (ICR), collagen fibers degradation, and oxidative profile (reactive oxygen species, ROS; lipid peroxidation, LP; and protein carbonyl, PC). At 3 days, percentages of no/discrete ICR were 100, 93.3, 86.7, 76.7, 50, and 66.6 for SHAM, PDS, 1MET, 1CIP, 2MET, and 2CIP, respectively. At 30 days, percentages of no/discrete ICR were 100% for SHAM, PDS, 1MET and 1CIP and 93.3% for 2MET and 2CIP. Between 3 and 30 days, SHAM, 1CIP, and 2CIP produced collagen, while 1MET and 2MET were unchanged. At 30 days, the collagen fiber means percentages for SHAM, PDS, 1MET, 1CIP, 2MET, and 2CIP were 63.7, 60.7, 56.6, 62.6, 51.8, 61.7, respectively. Antibiotic-eluting matrices showed similar or better oxidative behavior when compared to PDS, except for CIP-eluting matrices, which showed higher levels of PC compared to SHAM or PDS at 30 days. Collectively, our findings indicate that antibiotic-eluting matrices may be an attractive biocompatible drug delivery system to fight periodontopathogens.

Keywords: Drug delivery; Biomaterials; Electrospinning; Nanofibers; Periodontitis

Running Head: Biocompatibility of Drug Delivery Systems

INTRODUCTION

Periodontitis is an inflammatory disorder that leads to the destruction of the teeth supporting tissues, ultimately resulting in tooth loss. According to data from the World Health Organization (WHO), 10.8% of the adult populations worldwide have severe periodontitis.[1] Moreover, severe periodontitis is the sixth most prevalent condition in the world.[2] Over the last three decades, several regenerative-based strategies aimed at periodontal tissue reconstruction have been proposed[3-6] and yet the ideal biomaterial and/or scaffold is still to be found.[7]

Barrier membranes have been employed in the so-called guided tissue/bone regeneration (GTR/GBR) approaches.[8,9] Nevertheless, current membranes, do not possess the structural integrity required to resist compressive forces to maintain the space necessary for periodontal regeneration.[10,11] Conventional, non-resorbable membranes have the disadvantage of necessitating a second surgery, with the associated additional discomfort and economic burden. Polyester-based membranes (e.g., polydioxanone, PDS) are biodegradable, allow for tissue integration, and are easier to handle when compared to non-resorbable counterparts. In terms of periodontal defect disinfection prior to tissue regeneration, biodegradable nanofibrous antibiotic-eluting delivery systems have the potential to endow a bacteria-free microenvironment, reducing risk of GTR/GBR failure due to infection,[12] while minimizing adverse effects associated with the use of systemic antibiotics.[13]

Numerous authors have explored the electrospinning technique to generate nanofibrous matrices for drug delivery.[10,14-18] Bottino and colleagues[19,20] presented mechanically strong biodegradable PDS-based antibiotic-eluting matrices, which demonstrated: (i) adequate mechanical properties to sustain handling and maintain the overall structure during regenerative procedures; (ii) an inhibitory effect on the biofilm of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* with no detrimental effects on commensal bacteria biofilm (e.g., *Lactobacillus casei)*, and (iii) controlled release of an adequate drug dose over 7 days. Moreover, 25 wt.% MET matrices and its CIP counterparts released only 12% and 40%, respectively, of the antibiotic during this period, demonstrating the ability to sustain a controlled release for a longer time period. Taken together, the ability to inhibit periodontal pathogens enhances the clinical potential of the proposed nanofibrous antibiotic-eluting matrices as a drug delivery system for applications in periodontics. Thus, the purpose of this investigation was to determine the inflammatory response of biodegradable PDS-based electrospun antibiotic-eluting matrices containing MET or CIP using a rat subcutaneous implantation model. Initial (3 days) and late (30 days) inflammatory responses were assessed based on degree of local inflammatory cell response, tissue oxidative status, and collagen degradation.

MATERIAL AND METHODS

Synthesis of electrospun drug delivery systems

Polydioxanone monofilament surgical sutures (PDS II®, Ethicon, Somerville, NJ, USA) were cut into pieces and placed into glass vials containing dichloromethane (Sigma Aldrich, St. Louis, MO, USA) to remove the violet dye.[19] Next, undyed PDS was then dissolved in

1,1,1,3,3,3-hexafluoro-2-propanol (Sigma Aldrich) at a 10 wt.% concentration. Metronidazole (Sigma Aldrich) with a molecular weight (M_w) of 171.15 g/mol, or ciprofloxacin (Sigma Aldrich) with M_w of 331.346 g/mol, were directly added at 25 wt.% concentration (respective to the total polymer weight), and mixed together under vigorous stirring.[19,20]

Pure (i.e., antibiotic-free) PDS or antibiotic-containing PDS solutions were individually loaded into plastic syringes fitted with a 27-gauge stainless steel needle and electrospun under optimized parameters (2 mL/h, 18-cm distance, and 15 kV). The fibers were collected at room temperature (RT) on an aluminum-foil covered rotating mandrel. The electrospun matrices were dried in vacuum desiccators at RT for at least 48 h to remove any residual solvent.[19]

Experimental design

The experimental protocol (#4141250315) followed the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and was approved by the Committee on Animal Research of the Institution (Federal University of Santa Maria, Santa Maria, RS, Brazil).

Sixty adult male rats (*Rattus norvegicus, albinus*, Wistar) 90-days old (250-300 g) were housed at a controlled temperature (23±1°C), relative humidity (60%) with noise control (maximum 85 decibels) and a standard light-dark cycle (12 h). The animals were fed with regular rat pellets and distilled water *ad libitum* and were acclimatized to the laboratory environment for a period of 2 weeks before the experimental procedures.

The animals were allocated into 2 groups (30 per group) to evaluate the initial (3 days) and late (30 days) inflammatory response to matrix subcutaneous implantation. Computer software (Random Allocation Software, version 1.0, May 2004) was used to randomly

allocate the animals into the experimental groups (Table 1). One study team member (RM) performed the randomization process. The surgical procedures were performed by one operator (PCP) in randomized sequence order. The operator made subcutaneous pockets and was then informed whether or not the animals received the nanofibrous matrix. All matrices presented the same coloration and thus avoided any potential bias.

The animals were anesthetized with intramuscular ketamine (70 mg/kg) and xylazine (6 mg/kg). The back of each rat was shaved and scrubbed with chlorhexidine (2%) and each rat was draped for sterile surgery. One incision (3 cm) was performed through the skin on the right side of the midline. For animals in the 2MET and 2CIP groups, one incision (3 cm) on each side of the midline with at least a 2-cm separating distance was made. For each incision, a pouch was created subcutaneously (3-4 cm^2) using blunt dissection. One matrix (2×2 cm^2) was implanted into each subcutaneous pouch, except for the SHAM animals. The skin incisions were subsequently stitched with non-resorbable 6.0 sutures (Ethicon) and adhesive strips (Leukostrip, Smith & Nephew Ltda., São Paulo, SP, Brazil).

At 3 and 30 days, the animals were anesthetized with an inhalation of isoflurane (3%) (Isoflurano, Instituto BioChimico Indústria Farmacêutica Limitada, Itatiaia, RJ, Brazil) in an oxygen carrier. Blood was collected by cardiocentesis (22G needle and a 5-ml syringe) in EDTA anticoagulant tubes. Euthanasia was performed by total exsanguination and the matrices were explanted together with the surrounding peri-implantation tissue to analysis. The specimens were sectioned in 2 equal segments. One segment was used to analyze the tissue oxidative status and the other segment was fixed in 4% buffered formalin (72 h) prior to histological analysis (Figure 1).

Histological analysis

The segment used to provide histological analysis was cut into two identical pieces.[21] These tissue segments contained the peripheral and center region (Figure 1) of the implantation bed. Further processing involved dehydration of the biopsies and paraffin embedding. Accordingly, from each animal, 2 consecutive 5-µm serial sections were cut from each segment (peripheral region and center of the biopsies). Hematoxylin and eosin (H&E) staining was used for the first section, and Masson's trichrome (MT) (Sigma-Aldrich) and Picrosirius Red (PRed, Picrosirius Red Staining, Easy Path®, São Paulo, SP, Brazil) for the second and third sections, respectively.

The H&E sections were used to evaluate for the presence and degree of inflammatory cell response to the matrices. Three fields of view (100×) from the peripheral and central sections were observed and scored from verbal description using a 4-point scale[22] considering polymorphonuclear leukocytes/mononuclear cells: 0=absent (no inflammatory cells); 1=mild or discrete (few inflammatory cells); 2=moderate (several inflammatory cells and increased reaction zone); and 3=intense or severe (increased reaction zone, occasional foci of neutrophil granulocytes and/or lymphocytes).[23] This evaluation was performed by one calibrated examiner (JM) via double measurements of 10 specimens using a 1-week interval. The Kappa value was 0.95.

The MT stained sections were digitized at 200× magnification using optical microscopy (Axiovision; Carl Zeiss MicroImaging, Jena, Germany). From the left margin of the tissue sample, fields of view were numbered consecutively towards the right margin. On average, 14 fields were displayed. Of these, fields 7, 8, and 9 were selected to determine the percentage of

area occupied by collagen fibers from the peripheral and central region, totaling 6 fields of evaluation per rat. The image of the fields was obtained using image-processing software (Zen lite 2012 blue edition, Carl Zeiss MicroImaging). The images were transferred to ImageJ software (v1.50i, National Institutes of Health, Bethesda, MD, USA), binarized and the percentage of area filled by collagen fibers was calculated.[24] Descriptive analysis of collagen fiber type was performed in the same fields (7, 8 and 9) by polarized microscopy (Leica, model DM2000, Wetzlar, Germany) at 400× using sections dyed with PRed. The Figure 2 show the position of the analysis fields where the percentage of collagen fibers was performed.

All histometric analyses were performed by examiners (PCP and JM) who were blinded to the experimental groups.

ROS generation with DCH

The ROS levels were quantified using the oxidant-sensing fluorescent probe, 2,7-dichlorofluorescein diacetate (DCHF-DA).[25] Oxidation (DCHF-DA) to fluorescent dichlorofluorescein (DCF) ratio was determined at 488 nm for excitation and 525 nm for emission. Tissue samples were added to a medium containing Tris—HCl buffer (0.01 mM, pH 7.4) and DCHF-DA was added to the solution for 1h until fluorescence measurements were obtained. DCF-RS levels were corrected by protein content[26] and expressed as a percentage of values from control.

Oxidative cellular damage by protein carbonyl (PC) determination

Protein carbonyl (PC) determination is a type of protein oxidation that can be promoted by ROS, referring to a process that forms reactive ketones or aldehydes that can be reacted by 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. PC level was measured according to a previously established protocol[27] with minor modifications. In brief, aliquots of tissue samples homogenized in Tris—HCl buffer (10 mM, pH 7.4) were mixed with 0.2 ml of DNPH (10 mM). After 1 h of incubation at RT in the dark, 0.5 ml of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, 3% SDS), 2 ml of heptane (99.5%) and 2 ml of ethanol (99.8%) were added sequentially, mixed for 40 s and centrifuged for 15 min. Thereafter, the protein isolated from the interface was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in denaturing buffer. Each sample was measured at 370 nm against the corresponding HCl sample (blank), and total carbonylation was calculated using a molar extinction coefficient of 22 000 M^{-1} cm^{-1}.[28] The results were expressed as nmol carbonyl/g $tissue^{-1}$.

Oxidative cellular damage by lipidic peroxidation (LP) estimation

Lipidic peroxidation (LP), or the reaction of oxygen with unsaturated lipids, occurs when reactive oxygen species attack polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids and the end-products of such LP reactions are hazardous to cell viability. One product of LP is malondialdehyde (MDA), which presents a facile reaction with thiobarbituric acid (TBA), yielding an intensely colored fluorescent chromogen. LP was measured by the reaction of MDA with TBA.[29] A pink chromogen was generated and spectrophotometrically measured at 532 nm. The TBARS levels (substances that react with TBA) were expressed as nmol of

MDA g tissue.

Statistical analysis

The rat was the unit analysis. The degree of inflammatory cell response data were showed as median, minimum and maximum values. Distribution frequency of each score of degree of inflammatory cell response was calculated per rat (considering 6 evaluation fields). Then, the mean percentage of absent/mild (score of 0 and 1) and moderate/severe (score of 2 and 3) was calculated. The data of collagen fibers' percentage, ROS, PC, and LP levels were presented as mean and standard deviation. Normality of the data was analyzed using the Shapiro-Wilk test. The intergroup analyses were performed with Two-way ANOVA and post hoc Duncan' test. Differences were considered significant when $p<0.05$. The program *Statistics 8.0* was used for all analyses.

RESULTS

No animal was lost during the experimental period, and no adverse effects were observed.

Degree of inflammatory cell response

The median (minimum and maximum) of the inflammation scores are presented in Table 2. At 3 days, the implantation of 2 MET matrices revealed higher median values. This finding agrees with the analyses of the mean percentage of fields presenting no or discrete inflammatory infiltration (score of 0 and 1), for which 2MET showed the lowest percentage 50%, thus being the only group that presented statistical difference from SHAM ($p=0.041$). In the other groups, the mean percentage of fields presenting no or discrete inflammatory infiltration were of 100%, 93.3%, 86.7%, 73.2%, and 66.6% for SHAM, PDS, 1MET, 1CIP, and 2CIP, respectively (Figure 3A), with no statistical difference between groups. At 30 days,

1MET, 2MET and 2CIP groups still had some area with moderate inflammation (Table 2). No statistically difference was observed between groups. Nearly all the groups presented 100% of no/discrete cellular infiltration, except 1MET, 2MET and 2CIP, which showed 96.6%, 90% and 90% of fields in this condition, respectively (Figure 3B). At both evaluation times (3 and 30 days), no rat showed any field with severe inflammation. Figure 4 and Figure 5 show representative H&E micrographs after 3 days at different magnifications.

Collagen fibers

Table 3 shows the mean percentage of collagen fibers in the groups at 3 and 30 days. At 3 days, the PDS matrices seemed to stimulate collagen production. 2MET, 2CIP and 1CIP showed lower statistically percentage of collagen fibers than PDS. All groups were statistically similar to the SHAM. The reduction percentage of collagen fibers when compared to SHAM, was 14.5%, 9.1% and 7.7% for 1CIP, 2CIP, and 2MET, respectively. Interestingly, between 3 and 30 days, the SHAM, 1CIP and 2CIP groups produced collagen, while the 1MET and 2MET maintained the same percentage of collagen fibers seen at 3 days. The 2MET group demonstrated a reduction of approximately 18%, in the collagen fiber percentage at 30 days when compared to the SHAM. The other groups (PDS, 1MET, 1CIP, 2CIP) were statistically similar to the SHAM. Most of the fibers were type I, dense, and thick. Few type III fibers, interlaced with type I fibers, were noted.

Oxidative status profile

The oxidative status data are shown in Figure 6. At 3 days, the PDS matrices significantly increased the ROS levels ($p<0.01$) and cellular damage from LP ($p<0.01$) and PC ($p=0.012$)

when compared to the SHAM. At 30 days, PDS and SHAM showed similar oxidative profile. At 3 days, the cellular damage from LP was in ascending order: SHAM=2MET<1MET=2CIP<1CIP<PDS ($p<0.05$). At 30 days, the antibiotic-eluting matrices presented lower LP levels when compared to the PDS or SHAM ($p<0.05$). Concerning PC levels at 3 days, CIP-eluting matrices showed similar behavior to the SHAM. The 2CIP was the only that presented PC levels lower statistically than PDS ($p=0.02$). At 30 days, all groups were statistically similar to the SHAM, with exception of 1CIP ($p=0.025$). In general, antibiotic-eluting matrices displayed a protective effect in terms of oxidative cellular damage with regard to LP analyses, but not in terms of PC when compared to antibiotic-free PDS matrices.

DISCUSSION

The aim of this study was to investigate the biocompatibility of PDS-based antibiotic-eluting matrices in a rat subcutaneous implantation model. Tissue response was evaluated based on the degree of inflammatory cellular response, percentage collagen fibers by histological analysis, and oxidative profile by biochemical analysis. Alterations during early inflammatory response (3 days) associated with the matrices without antibiotics were observed when compared to the SHAM. However, at 30 days, these alterations were nullified. Regarding the antibiotic-eluting matrices, the alterations were more prominent at 3 days for all outcomes. At 30 days, 1MET, 2MET and 2CIP did not reach the same inflammatory parameters as observed in PDS or SHAM groups, which showed 100% of no/discrete inflammatory cell response. Moreover, MET-eluting matrices impaired collagen fibers formation between 3 and

30 days.

A higher degree of inflammatory cellular response was observed when two incisions were performed (on each side of the midline) and two antibiotic-eluting matrices were implanted per animal. In the 2MET and 2CIP animals, one antibiotic matrix was implanted on each side of the midline, but the histological analysis was carried out from only one tissue side. Then, it is possible that the drug released from matrix localized on the left side reached the opposite side by tissue diffusion and/or bioavailability. Worth mentioning, tissue diffusion and bioavailability of a drug depends on pH, local vascularization, presence of a bacterial enzyme, and drug physical and chemical properties, such as size and molecular weight, charge, dissociation constants, liposolubility, and partition coefficient.[30,31] Drugs administered subcutaneously can reach systemic circulation either by uptake via blood capillaries or indirectly by lymphatic vessels,[32] and also can exhibit diffusion and advection into extracellular matrix before uptake by the lymphatic capillaries draining the site.[31] Another plausible explanation for these differences is the fact that, regardless of the antibiotic-eluting matrix implantation, two incisions were performed, promoting higher tissue injury, and thus a greater inflammatory cell response at both implantation sites.

Oxygen reactive species (ROS) are produced in the mitochondria during respiratory chain electron transport, where they are rapidly transformed into more inactive species by the antioxidant defenses. However, increased production of ROS during the inflammatory process cannot be counteracted by a concomitant increase in antioxidant defenses, thus leading to cellular damage that can be measured as protein carbonylation and lipidic peroxidation. At the sites of inflammation, there is a considerable overproduction of various oxygen reactive

species by activated neutrophils and macrophages. At 3 days, 2 MET was the only group that simultaneously produced higher cellular damage from oxidative mechanisms (PC and LP) when compared to the SHAM. In general, antibiotic-eluting matrices demonstrated similar or lower oxidative damage when compared to the antibiotic-free PDS-based counterparts. At 30 days, antibiotic-eluting matrices presented similar behavior to the PDS or SHAM groups.

With respect to collagen fibers, only MET-eluting matrices were associated with impaired collagen fibers' formation during the experimental period, since the collagen lost during the initial inflammatory response was not regenerated. However, one should interpret these findings with caution, since 1MET showed an 11% reduction relative to SHAM; and a 6.7% reduction relative to PDS at 30 days. Since PDS is an FDA-approved bioresorbable synthetic polymer, such impairment (6.7%) should not be considered clinically significant. Therefore, although quantitative data obtained from pre-clinical studies cannot be applied directly to humans, one could anticipate a fairly similar behavior.

Although it was not the focus of the present study, our data show a discrete presence of multinucleated giant cells, which are characteristic of foreign body reaction, at 3 and 30 days for PDS, 1MET, 2MET, 1CIP and 2CIP (data not shown). No difference was verified among the groups. The discrete presence of this cellular type at 30 days might indicate that the biomaterial was not yet completely degraded.

In the present study, antibiotic-eluting PDS-based matrices were evaluated using a subcutaneous implantation model in the rat. The proposed matrices have been designed to be used to treat periodontal infection and/or to perform a barrier function as required in GTR/GBR regenerative approaches. Worth mentioning, the overall tissue response upon

matrices' implantation was investigated in the absence of a microbial challenge. Thus, it is possible that in the presence of bacterial infection, the antibiotic-eluting nanofibrous matrices could work reducing the inflammatory alterations when compared to the antibiotic-free PDS matrices; therefore, this issue should be considered in future studies.

CONCLUSIONS

Collectively, the findings of this study demonstrate that antibiotic-eluting biodegradable polymeric matrices did not result in significant damage to tissue healing beyond that obtained from surgical handling (SHAM) or the use of antibiotic-free (PDS only) matrices. Considering the previously established antimicrobial properties of the polymer-based matrices evaluated herein, the results from this biocompatibility study suggest that our antibiotic-eluting matrices may offer an attractive drug delivery system for treating periodontal infection. Further *in vivo* studies assessing not only bacterial reduction but also the overall tissue response within a more clinically relevant scenario (i.e., presence of infection) are warranted to confirm the clinical potential of the proposed antibiotic-eluting drug delivery systems.

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LIST OF TABLES

TABLE 1. Experimental Groups Description.

Experimental groups		# of animals to evaluate initial inflammatory response: 3 days	# of animals to evaluate late inflammatory response: 30 days
SHAM	One subcutaneous pocket (no matrix)	5	5
1PDS	One PDS matrix	5	5
1MET	One 25 wt.% MET PDS matrix per rat	5	5
1CIP	One 25 wt.% CIP PDS matrix per rat	5	5
2MET	Two 25 wt.% MET PDS matrices per rat	5	5
2CIP	Two 25 wt.% CIP PDS matrices per rat	5	5

PDS: polydioxanone, CIP: ciprofloxacin, MET: metronidazole.

TABLE 2. Median Inflammation Scores (Minimum and Maximum) at 3 and 30 Days.

	SHAM	PDS	1MET	2MET	1CIP	2CIP
3 days	1 (0-1)	1 (1-2)	1 (0-2)	1.5 (0-2)	1 (1-2)	1 (1-2)
30 days	0 (0-1)	0 (0-1)	0 (0-2)	1 (0-2)	1 (0-1)	1 (0-2)

Score system from verbal description using a 4-point scale considering polymorphonuclear leukocytes/mononuclear cells: 0=absent (no inflammatory cells); 1=mild or discrete (few inflammatory cells); 2=moderate (several inflammatory cells and increased reaction zone); and 3=intense or severe (increased reaction zone, occasional foci of neutrophil granulocytes and/or lymphocytes). Sections were viewed at 100× and graded by a blinded and calibrated examiner. Five animals per group and time point were used.

TABLE 3. Mean Percentage of Collagen Fibers (standard deviation) at 3 and 30 Days.

	SHAM	PDS	1MET	2MET	1CIP	2CIP
3 days	56.8 (5.0)	66.9 (4.8)	58.7 (7.4)	52.4 (7.0)*	48.6 (3.4)*	51.6 (2.6)*
30 days	63.7 (7.0)	60.7 (4.9)	56.6 (4.2)	51.8 (3.0)#	62.6 (3.4)	61.7 (3.6)

One-way ANOVA and Tukey's post hoc test ($p<0.05$). Five animals per group and time point were used.

At 3 days: No statistical difference was observed among antibiotics groups. All groups were statistically

similar to the SHAM. Groups with 2 antibiotics matrices (2MET, 2CIP) and the 1CIP group presented statistically lower percentage of collagen fibers than PDS (*).

At 30 days: No statistical difference was observed among antibiotics groups. Only 2MET group presented statistically lower collagen fiber percentage when compared to the SHAM (#). None of the groups showed difference when compared to the PDS.

*: represents statistically significant difference compared to PDS at 3 days.

#: represents statistically significant difference compared to SHAM at 30 days.