ABSTRACT OF RESEARCH PLAN

INVESTIGATOR NAME/INSTITUTION	PROJECT TITLE
INSTITUTION: **Cooper University Hospital**	Adipose Derived Stem Cells In The Treatment Of Fractures With Bone Loss And Nonunions

Abstract of research plan: Please provide a 250 word abstract with 5 underlined phrases for project summary, to fit in the box below.

Avoid summaries of past accomplishments and the use of the first person. The abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application.

Abstract

A major challenge in orthopedic traumatology is the treatment of large segmental bone loss. Currently, none of the standard techniques for the reconstruction of large segmental bony defects are ideal. Bone grafting for a large segmental bony defect has a high rate of complications, failure, donor site morbidity and pain. Current techniques in tissue engineering based on the use of adipose derived stem cells (ASCs) provide opportunities for improving the healing of bone injuries. Several studies have documented the capacity of ASCs to induce both angiogenesis and osteogenesis and their use in bone regeneration shows marked potential. No studies have compared ASCs to BMPs alone or in combination in an attempt to heal bony defects or nonunions. ASCs are readily available, easy to obtain and can differentiate into bone and functional capillary networks and are not affected by age or disease. In addition, it has been shown that a critical component of successful tissue engineering outcomes is the distribution of cells within a three dimensional biomaterial scaffold which serves as a substrate for tissue regeneration. Accordingly, we have developed the *hypothesis* that freshly isolated ACS contained in a biomaterial scaffold can be used to tissue engineer large segmental bone regeneration, accelerate healing and decrease complications typically seen in orthopedic traumatology. Calibrated tibial fractures will be performed in rats followed by administration of OssimMend, OssiMend + hASCs, OssiMend + hASCs and hBMP and a proprietary scaffold + hASCs and BMP in 4 separate groups and evaluated at 2, 4 and 8 weeks for fracture recovery.

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A. SCIENTIFIC AIMS   (380 words)

Definition of the Research Question: One of the biggest challenges in orthopedic traumatology is the treatment of large segmental bone loss. Currently, none of the standard techniques for the reconstruction of large segmental bony defects are ideal. Bone grafting for a large segmental bony defect has a high rate of complications and failure. It also has donor site morbidity and pain. Distraction osteogenesis can create large amounts of well vascularized bone but can be technically demanding and time consuming. Pedicled and free bone graft require a high level of expertise and also result in significant donor defect and morbidity. Therefore the ideal treatment for large segmental bony defect would be a method that is not technically demanding, or time consuming and without further creation of a donor site defect. Tissue engineering, using multipotent adipose derived stem cells (ASCs) holds great promise for improved healing of bone injuries. Several studies have documented the capacity of ASCs to induce both angiogenesis and osteogenesis, and accordingly, their use in bone regeneration holds great promise.   [1-3]. However, no studies have compared ASCs to BMPs alone or in combination in an attempt to heal bony defects or nonunions. ASCs are readily available, easy to obtain and can differentiate into bone [2-4] and functional capillary networks [5] and are not affected by age or disease [6]. In addition, it has been shown that a critical component of successful tissue engineering outcomes is the distribution of cells within a three dimensional biomaterial scaffold which serves as a substrate for tissue regeneration [7-10]. Based on these observations, we have developed the *hypothesis* that freshly isolated ACS contained in a biomaterial scaffold can be used to tissue engineer large segmental bone regeneration, accelerate healing and decrease complications typically seen in orthopedic traumatology.

> **Specific Aim** - Freshly isolated human ASCs combined with osteoinductive BMP (OP-1) and a biomaterial scaffold will stimulate the regeneration of bone when applied to a rat model of non-union bone repair.

In this study rats will be subjected to non-union fracture of the femur, followed by the application of osteoconductive powder OssiMend (group 1), OssiMend containing hASCs (group 2), OssiMend containing both ASCs and hBMP (group 3), and a proprietary, injectable, thermogelling scaffold containing both ASCs and hBMP (group 4) and evaluated at 2, 4 and 8 weeks for fracture recovery.

B. BACKGROUND AND SIGNIFICANCE   (400 words)

Approximately 5-10% of all fractures fail to heal appropriately and result in a chronic non-union condition leaving patients in a compromised state with a marked reduction in quality of life. As there are no established regenerative methods for bone reconstruction that are standard of care at this time, traumatic bone regeneration for large segmental boney defects challenge the surgeon. One of the major components of bone healing is appropriate angiogenic activity which supplies a functional capillary network to the healing physiology of bone, and the lack of sufficient blood supply is thought to be a principle feature of non-union bone pathology [11-12]. Thus, collaboration between osteogenesis and neovascularization fails in these patients for reasons which have not been defined. Because ASCs have the potential to differentiate into bone, cartilage, fat, muscle and endothelial cells [13], it has been suggested that ASCs could be utilized to reconstruct defects in many tissues [13]. Recently, bone marrow mesenchymal stem cells (BMMSCs) have been used to reconstruct patients with bone defects [3]. However, BMMSC yield from bone marrow aspirates is low, is further reduced with both age and comorbidities [13-15], and marrow aspiration is quite invasive. Unlike BMMSCs, ASCs are easily harvested using standard liposuction techniques, their numbers are not reduced by aging or disease [6], and they share nearly all the phenotypic and regenerative features of BMMSCs [13]. Differentiation of ASCs to the osteogenic lineage is initiated in the presence of bone morphogenic protein (BMP) when added to ASCs in cell culture [16] or when directly added to freshly isolated ASCs prior to transplantation to a boney defect site [3, 17]. A feature of ASCs that we take advantage of is that they are immunoprivileged, which derives from their lack of cell surface MHC-II antigens [13]. Their immunoprivileged state allows us to focus our study on human ASCs (hASCs) in a relatively simple, inexpensive non-immunocompromised rat model of bone fracture healing. The use of hASCs in non-immunocompromised animal models has been successfully performed by others [11].

Hydrogels are three-dimensional water swollen networks [18]. Due to their lower critical solution temperature (LCST), at $32°C$, poly(N-isoproprylacrylamide) (PNIPAAm) gels form a miscible and injectable solution, whereas above the LCST, it forms a compact gel. Therefore, aqueous solutions of PNIPAAm can be implanted percutaneously and solidify *in situ* [19]. PNIPAAm has been shown to illicit little inflammatory response (like collagen) and supports growth of our ASCs [20].
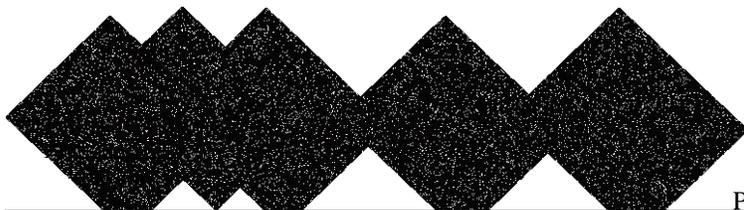

Fig. 5: Formation of EC-like tubes by CD31 ASCs isolated from human periumbilical fat and grown in ECGS for 7 days

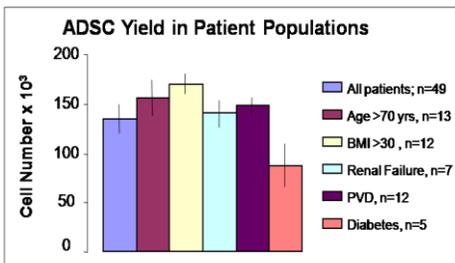## C. **PREVIOUS WORK DONE ON THE PROJECT**   (794 words)

Fig 1: ASDC cell number in patients with comorbidities. Neither age, BMI, renal failure nor per peripheral vascular disease affected the ASC cell yield. Only patients with diabetes had reduced cell yield, but still provide sufficient numbers for seeding.[6]
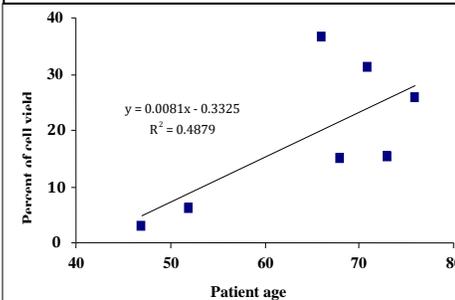

$$y = 0.0081x - 0.3325$$
$$R^2 = 0.4879$$

Fig 2: CD31 cell number yield in patients as a function of age. Note that, surprisingly, cell yield appears to increase with age.
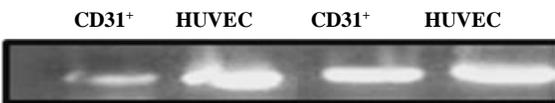

CD31^{+}   HUVEC   CD31^{+}   HUVEC

Fig. 3: Two human CD31^{+} cell lines (passage 2) naturally expressing eNOS. Human umbilical endothelial cells serve as positive controls. Similar results were obtained in canine CD31^{+} ASCs.
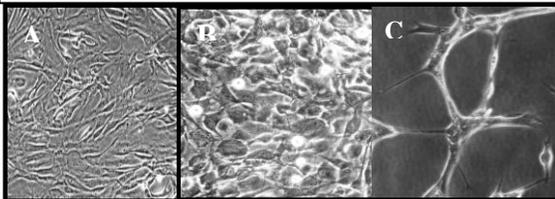

Fig.4: Differentiation of ASCs to an EC-like morphology when grown on fibronectin. A: On plastic, before fibronectin, B: cobblestone morphology and C: EC-like cords.

Stem cell availability One of the most important characteristics of a cell type used in tissue engineering is that it be readily and abundantly available in the specific patient population in which it is intended for use. In the case of bone regeneration, the cell must be obtainable in all age populations, including those with multiple risk factors such as obesity, vascular disease and diabetes. If a particular stem cell type is rare, ex vivo amplification becomes necessary before graft creation, increasing both time, complexity and expense in an already costly manufacturing process. Many of the original studies evaluating fat as a source of stem cells examined liposuction specimens obtained from young, healthy plastic surgery patients [21-22]. The applicability of such studies in the bone deformity population, more specifically those who are older, may be questionable because age and comorbidity adversely affect most stem cell populations [23-24].To address this issue, our group studied the availability of ASCs in 49 patients undergoing elective vascular surgical procedures [6]. After enzymatic dispersion, positive selection for ASC by absorbance to culture dish plastic for 1 week yields a homogenous population of ASCs [13]. Flow (FACS) sorting revealed these cells to be 98% positive for CD13, CD29, and CD90, cell surface antigens reported by others to be common to ASCs [21]. The number of stem cells per gram of adipose tissue harvested was analyzed with respect to patient age, gender, and body mass index, as well as the presence of diabetes, end-stage renal disease, and peripheral vascular disease. The major finding of this study was that ASC, unlike other stem cell populations [23], neither availability nor function were affected by advanced age, obesity, renal failure, or vascular disease [6] (Fig. 1). The presence of diabetes did appear to adversely affect the quantity of stem cells harvested, but not to a point where stem cell harvest would be considered impractical in this patient population. Interestingly, CD31^{+} cells (a "capillary endothelial precursor") isolated from the ASCs demonstrate a similar distribution across patient disease states, but unlike ASCs, their numbers actually increase with age (Fig 2). This feature, plus their pre-capillary endothelial phenotype make this cell type particularly attractive for angiogenesis in the context of bone regeneration. In sum, these findings suggest that ASCs are a practical source of adult, autologous mesenchymal stem cells that can be used for bone regeneration in clinical populations.

Osteogenic capacity Several laboratories including ours have demonstrated that hASCs can be differentiated to an osteoblast phenotype on incubation with osteogenic stimuli including hBMP (data not shown) confirming the clear ability of ASCs to differentiate to bone-like material [11].

Angiogenic capacity To date, investigators have not succeeded in differentiating ASCs to ECs. Accordingly, we have initiated efforts to find an ASC cell line with close similarity to ECs and which also naturally express eNOS. Using a FITC-labeled CD31+ antibody, we have isolated a CD31 expressing cell line from adipose tissue using FACS sorting. CD31 (platelet-endothelial cell adhesion molecule 1; i.e., PECAM) is a 130 kDa protein and is a member of the Ig gene superfamily that functions as a cell junctional molecule. It is expressed on the cell surface of hemopoietic and immune cells including endothelial cells, platelets, neutrophils, monocytes, megakaryocytes, natural killer cells and some T cells [25]. Approximately 5-40% of the cells (depending on donor age) contained in the stromal vascular fraction isolated from adipose tissue (Fig. 2) are CD31+. As shown in Figure 3, CD31+ cells naturally express eNOS RNA (and protein), and this expression maintains through multiple cell doublings. In addition, on fibronectin they acquire a cobblestone EC morphology (Fig. 4B), form EC-like cords (Fig. 4C), and undergo
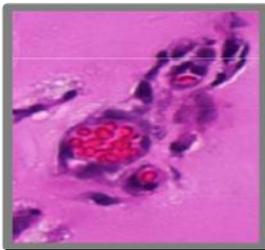
Fig. 6: Formation of functional capillary network in vivo with human CD31 cells implanted under the skin of the SCID mouse.

elongated EC-like tube formation in culture (Fig. 5), a precursor to capillary structures. CD31 cells are the only cells in the stromal vascular fraction that naturally posses these angiogenic features. Complementing this, they also display numerous markers of the capillary phenotype including CD34, vWF and uptake of acLDL (not shown). Lastly, our discovery that CD31 cells form EC-like chords on plastic led us to consider their potential for formation of 3 dimensional capillary networks in vivo. As illustrated in Figure 6, the effect of human CD31 expressing cells when grown in matrigel cultures followed by implantation under the skin of the wild-type mouse. In matrigel, they formed 3-dimensional networks that developed not only into capillaries in vivo, but also became functional as they clearly contain circulating mouse RBCs as shown.

It is important to point out that as written, this study can be made to meet both IRB and FDA regulations for autologous cell transplants; i.e., it can be performed intraoperatively (tissues do not leave the OR) and without cell manipulations. Accordingly, success in this study can result in taking this procedure quickly into clinical trials.
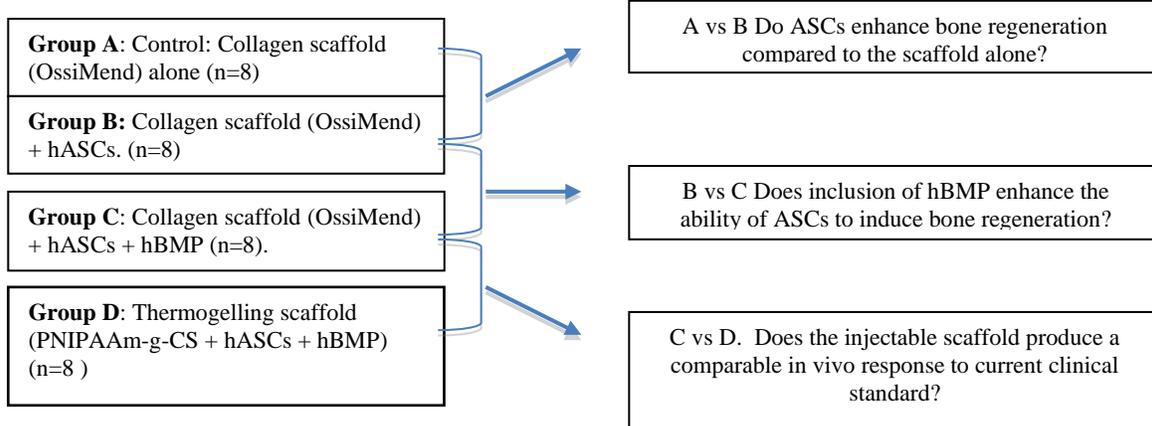
D. METHODS (1488 words)
D.!. - Vertebrate Animals
IACUC application pending

Overview: ASCs and hBMP will be studied to determine their relative contribution to bone regeneration in the context of non-union fracture healing. G*roup A*, the control group, will be treated with a current standard of care for this procedure; i.e., treatment with the collagenous (OssiMend) scaffold alone. Healing in this group will be compared against healing in *group B*, those animals treated with a combination of OssiMend to which hASCs have been added to determine the degree to which freshly isolated hASCs can differentiate into bone when combined with OssiMend. The role of BMP (*group C*) in inducing and/or accelerating hASC differentiation to bone will be examined by comparing the outcome of group C to that of group B. Lastly, hASCs and BMP will be combined with our proprietary thermogelling matrix (PNIPAAm-g-CS) *(group D)* to compare functionality of PNIPAAm-g-CS to OssiMend. The injectability of this matrix, ease of implantation, and ability to uniformly distribute cells and BMPs within it warrant its investigation. Outcomes will be defined by radiographic and histologic data while the mechanistic basis will be determined by vascularity achieved and specific cell signaling molecules associated with repair as determined by RNA (PCR) and protein (immunoblot) analyses.

Summary:

Group A: Control: Collagen scaffold (OssiMend) alone (n=8)	A vs B Do ASCs enhance bone regeneration compared to the scaffold alone?
Group B: Collagen scaffold (OssiMend) + hASCs. (n=8)	
Group C: Collagen scaffold (OssiMend) + hASCs + hBMP (n=8).	B vs C Does inclusion of hBMP enhance the ability of ASCs to induce bone regeneration?
Group D: Thermogelling scaffold (PNIPAAm-g-CS + hASCs + hBMP) (n=8)	C vs D. Does the injectable scaffold produce a comparable in vivo response to current clinical standard?

Fat harvest
Adipose tissue will be obtained by liposuction of subcutaneous periumbilical fat from patients undergoing cosmetic surgery. Consent to study will be obtained prior to surgery. 25-50 mL of tumescence solution (50 mL 1% lidocaine, 12 mEq sodium bicarbonate, and 1 mg epinephrine in 1 L normal saline) will be infiltrated into the liposuction site. Using a 3 mm Mercedez cannula, 50 cc of lipoaspirate will be obtained, placed on ice and transported to the laboratory for immediate processing.

Stem cell isolation
Stromal vascular cells containing the stem cells will be isolated as previously described by us [6, 26-28]. Briefly, the lipoaspirate will be will be washed with phosphate buffered saline (PBS), filtered through a 250 mm sieve, and incubated for 1 hour at 37°C with collagenase I solution (1 mg/mL + 4 mg/mL bovine serum albumin - Worthington). The slurry will be centrifuged for 10 min at 1000 rpm (1,500xg) followed by washing with 0.1% BSA (Sigma). The resultant pellet, termed the stromal vascular fraction (SVF), will be suspended in medium consisting of M199 + HEPES buffer at 37°C until its

application within 2 hours of its isolation.

Propriety scaffold preparation
Methacrylated chondroitin sulfate (mCS) will be prepared with methacrylic anhydride using a procedure developed by Bryant et. al. [29] and verified by ^{1}H NMR [30]. Redox polymerization of NIPAAm monomer [31] in the presence of methacrylated CS is then performed in a molar ratio of NIPAAm monomer units to mCS chains of 600:1. Reaction product will be freeze-dried and subsequently ground into powder before use. The powders will be re-dissolved in phosphate buffered saline (PBS, pH 7.4) to form flowable aqueous 5 wt% polymer solutions at room temperature. ASCs and BMP will also be incorporated into this solution and delivered to the bone defect locally upon gelation *in situ.*

Surgical Procedure
Adult male Sprague Dawley rats (Charles River) 220-320 gms will be used in this study. Animals and the study protocol will be approved by the local Institutional Animal Care and Use Committee. Anesthesia will be induced by intraperitoneal administration of a ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg) mixture. Non-healing femoral fractures will be induced in all animals by cauterizing the periosteum around the fracture site as described previously [32]. Gap formation size will be established at approximately 3 mm. Immediately after fracture induction, rats will receive local transplantation of either: A) the osteoconductive powder collagen scaffold (OssiMend) (*Group A*), B), hASCs (10^6 cells) suspended in 100 μl of PBS with OssiMend (*Group B*) and C), hASCs (10^6 cells) suspended in 100 μl of PBS with OssiMend containing osteoinductive BMP (OP-1) (*Group C*), D) 100 μl of PNIPAAm-g-CS in PBS, containing hASCs (10^6 cells) and BMP. The same amount of PBS with OssiMend (PBS group) applied to the contra-lateral side with an unfractured femur will be used as controls for histological and functional analyses, respectively. Rats will be euthanized with an overdose of ketamine and xylazine at the end of the study. The femurs will be directly frozen for subsequent biological and biomechanical analysis, whereas those for histological analysis will be embedded in OCT compound, snap frozen in liquid nitrogen and stored at −80°C.

Radiological Assessment
Rats will be fixed in supine position under anesthesia and radiographs of the fractured hindlimbs will be serially taken at week 0, 2, 4 and 8 post fracture. Fracture union will be identified by the presence of bridging callus on 2 cortices. Radiographs of each animal will be examined by three observers blinded to treatment. To evaluate the fracture healing process, relative callus areas around fracture sites in scanned radiographs at each time point will be quantified using the NIH Image software (National Institutes of Health, Bethesda, MD, USA).

Histological Assessments of Fracture Sites
Samples of the bridging callus will be sectioned at 6 μm thickness on slides followed by fixation with 4% paraformaldehyde at 4°C for 5 min. Histological evaluations will be performed with hematoxylin and eosin (HE) staining or toluidine blue staining to address the process of endochondral ossification on week 2, 4 and 8. The degree of fracture healing will be evaluated using the five-point scale (grade 0–5) proposed by Allen *et al [33].* Vascularity of soft tissue sections in peri-fracture sites will be evaluated by histochemical staining with FITC-conjugated CD31 (Vector Laboratories, CA, USA) to detect any human endothelial cells (capillaries). The number of capillaries will be counted under laser confocal fluorescent microscopy at four randomly selected fields in each section and averaged.

Laser Doppler Perfusion Imaging (LDPI)
LDPI system (Moor Instrument, DE, USA) will be used to measure serial blood flow in the hindlimbs over the healing course of 3 weeks post fracture according to the manufacturer's instructions. Rats will be fixed in the supine position under anesthesia. The femoral fracture site will be palpated under the skin and a small stainless steel marker will be pasted on the skin above the fracture site (on the contra-lateral intact limb, we will paste a marker on the center of the femoral bone). The steel marker will be scanned as a defect spot in the LDPI image and used as the center of the region of interest (ROI). The steel marker will be removed and scanned again. The ROI squares on the second scanned image will be compared to the first scanned image with the marker's defect spot. The blood flow recovery following fracture will be evaluated as the ratio of mean flux within the ROI in the fractured hind-limb to the same size ROI in the contra-lateral, intact hind-limb.

Biomechanical Analysis of Fracture Union
Four rats in each treatment group will be used for biomechanical evaluation at week 8 post fracture. Fractured femurs and the contra-lateral non-fractured femurs will be prepared and intramedullary fixation pins will be removed before the bending test. The standardized three-point bending test will be performed using load torsion and bending tester MZ-500S (Maruto Instrument, Tokyo, Japan). The bending force will be applied with cross-head at a speed of 2 □m/min until rupture occurs. The ultimate stress (*N*), the extrinsic stiffness (*N*/mm) and the failure energy (*N*/mm) will be interpreted and calculated from the load deflection curve. The relative ratio of the fractured femur to non-fractured femur will be calculated in each group and averaged.

RT-PCR and Quantitative Real-Time RT-PCR Analysis
The potential role of specific molecular markers of vascularization and osteogenesis will be assessed to shed light on the molecular mechanisms underlying bone healing in the three experimental groups. Granulation tissues and callus tissues surrounding the fracture gaps will be harvested at the end of the study. RNA will be isolated, first-strand cDNA synthesized and amplified by Taq DNA polymerase. PCR oligonucleotide primers will include: RT-PCR (h=human; r=rat): hCD31, hVEcad, hOC, hCol1, hGAPDH, rGAPDH. Real-time PCR: rVEGF, rANG-1, rBMP-2, rGAPDH, hCD31. RT-PCR and qPCR will be performed as described extensively by us [6, 27-28].

Immunobloting (western) analysis
Confirmation of activity of genes associated with bone recovery will be made by western blot analysis of the specific proteins synthesized using antibodies directed to these proteins. This will support the potential role of specific molecular markers of vascularization and osteogenesis identified by PCR analysis. Whole cell protein extracts and immunoblotting will be performed as described previously by us [6, 27].

Statistical analysis
ANOVA and two-tailed Student's *t*-test will be used throughout. Differences will be considered significant at *P* values of <0.05. Data will be expressed as mean ± standard deviation. We have performed a power analysis using preliminary data, or data generated in other systems (i.e., rat and human). These analyses indicate that in all studies using the in-vivo model, experiments must be performed in no less than 8 separate control and 8 separate test animals in each group.

D 2.. HUMAN SUBJECTS
IRB approved (IRB #: FWA 11-05EX, 5/8/11)

E. LITERATURE CITED

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SALARIES AND WAGES (List all personnel for whom money is requested)	% Of Time on this project	Requested from OTA Funds (Omit Cents)
Technician	25%	$ 11,975
	%	
	%	
	%	
Fringe Benefits _____% of Salaries and Wages Salaries and Wages plus Fringe Benefits	TOTAL	$11,975

PERMANENT EQUIPMENT (Justification to be appended)	
Subtotal	

CONSUMABLE SUPPLIES (Exclude animals and animal care)	
Surgical supplies	1,000
Antibodies	1,100
RT and qPCR supplies	1,160
Subtotal	3,260

ANIMALS AND ANIMAL CARE	
96 Sprague Dawley rats (@32.00/rat)	3,072
Animal housing (10/box at $2.23/box x 98 total per diem days)	1,967
Subtotal	5,039

ALL OTHER EXPENSES	
Travel 1 trip to national meeting for PI to present results	1,000
Equipment related costs	1,450
Institutional F&A	2.272
Subtotal	4,722

TOTAL DIRECT COSTS_$24,996_____