AWARD NUMBER: W81XWH-14-1-0164

TITLE: Development of Nanomedicines for Treatment of Posttraumatic Osteoarthritis

PRINCIPAL INVESTIGATOR: Dong Wang

CONTRACTING ORGANIZATION: University of Nebraska
Omaha, NE 68198

REPORT DATE: March 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2016	Final	5 June 2014 – 4 Dec 2015

4. TITLE AND SUBTITLE

Development of Nanomedicines for Treatment of Posttraumatic Osteoarthritis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0164

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dong Wang, P. Edward Purdue, Steven R. Goldring

E-Mail: dwang@unmc.edu, purduee@hss.edu, goldrings@hss.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nebraska Medical Center
987835 Nebraska Medical Center
Omaha NE 68198-6810

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

For this project, *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates (including dexamethasone and BMS-345541 conjugates) were synthesized and labeled with near-infrared dye IRDye CW 800, Alexa Fluor 488 or Alexa Fluor 647. Mice with surgical destabilization of the medial meniscus (DMM) were established as an osteoarthritis model for our evaluation. P-IRDye and P-Alexa were given to the DMM mice via intraarticular injection. Optical imaging demonstrated that the DMM joints retained the injected P-IRDye. Fluorescence microscopy studies, in line with the *in vivo* imaging results, confirmed that P-Alexa was retained within the joint space and was primarily localized to synovial lining cells and articular chondrocytes. To further confirm the ability of chondrocytes to retain the copolymer conjugates, mouse femoral head explants were cultured in presence of P-Alexa. Imaging confirmed internalization of the conjugate by chondrocytes. Cell culture studies demonstrated that P-Alexa was sequestered within an endosomal/lysosomal compartment. Cell culture studies showed that P-BMS, a NF-κB inhibitor copolymer conjugate, inhibited LPS induction of pro-inflammatory cytokine expression and provided sustained inhibition of osteoclast formation.

15. SUBJECT TERMS
nanomedicine, PTOA, DMM, osteoarthritis, prodrug, glucocorticoid, dexamethasone, HPMA copolymer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. **Introduction…….1**
2. **Keywords…….….1**
3. **Accomplishments……..……...1**
4. **Impact……...……5**
5. **Changes/Problems...….……5**
6. **Products…….…….…..…….5**
7. **Participants & Other Collaborating Organizations…….……5**
8. **Special Reporting Requirements……7**
9. **Appendices……7**

1. **INTRODUCTION:**

The current proposal aims to design nanomedicines to selectively target cells within the osteoarthritic joint, and to exploit this to enhance the delivery, retention, and efficacy of therapeutic agents. Osteoarthritis and post-traumatic osteoarthritis (OA/PTOA) are the leading cause of disability and loss of work capacity in the adult population, yet there remains an unmet clinical need for effective therapies to prevent disease progression. The proposed studies are designed to investigate novel nanomedicine mechanisms of pharmacotherapy for OA, using a combination of in vitro and in vivo studies.

2. **KEYWORDS:**

Nanomedicine, PTOA, DMM, osteoarthritis, prodrug, glucocorticoid, dexamethasone, HPMA copolymer

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

- Task 1. Identify patterns of cellular uptake and retention of nanomedicines in the knee joints of mice with OA induced by destabilization of the medial meniscus (DMM). Months 1-12.

- Task 2. Optimize structural parameters for delivery and sustained therapeutic efficacy of Dexamethasone and BMS 345541 prodrugs. Months 13-18.

What was accomplished under these goals?

- Synthesis and characterization of HPMA copolymers. Months 1-2.

To synthesize IRDye and Alexa-labeled HPMA copolymers, *N*-(2-Hydroxypropyl)methacrylamide (HPMA), *N*-(3-aminopropyl)methacrylamide (APMA), 2,2'-azobisisobutyronitrile (AIBN) and *S,S'*-bis(α, α'-dimethyl-α"-acetic acid) trithiocarbonate were dissolved in methanol, transferred into an ampule, purged with argon for 5 min and then flame-sealed. It was maintained at 45 °C for 48 hr for polymerization, after which the polymer product was purified by re-precipitation and LH-20 column. The content of the primary amine in the copolymer was determined by ninhydrin assay. The polymer precursors were then incubated together with NHS esters of IRDye or Alexa (in the presence of DIPEA) at room temperature overnight to introduce the fluorescent labels. The labeled copolymers were further purified by LH-20 column and their molecular weight (MW) and polydispersity (PDI) were determined by FPLC using HPMA homopolymer calibration. The content of labels were determined by UV/Vis spectrophotometry.

HPMA copolymers	M_w (kDa)	PDI	Dex content (μg/mg)	Alexa content (μmol·g^{-1})	IR-Dye content (μmol·g^{-1})
1	16.0	1.35	120.31 ± 10.69	10.89 ± 0.15 (488)	-
2	16.0	1.35	120.31 ± 10.69	-	6.20 ± 0.14
3	27.5	1.40	117.79 ± 4.41	13.82 ± 0.27 (488)	-
4	27.5	1.40	117.79 ± 4.41	-	6.08 ± 0.31
5	35.4	1.49	125.02 ± 12.90	10.25 ± 0.20 (488)	-
6	35.4	1.49	125.02 ± 12.90	-	7.89 ± 0.04

7	45.3	1.34	123.75 ± 7.22	12.63 ± 1.28 (488)	-
8	45.3	1.34	123.75 ± 7.22	-	7.55 ±0.14
9	45.6	1.18	-	-	4.10 ±0.06
10	45.5	1.22	-	13.03 ± 0.37 (647)	-

To conjugate N-(2-aminoethyl)-1,8-dimethylimidazo[1,2-a] quinoxalin-4-amine (BMS-345541) to HPMA copolymer, the primary amine in BMS-345541 was used to conjugate with Gly-Phe-Leu-Gly. Using AIBN as the initiator and S,S'-bis (α,α'-dimethyl-α''-acetic acid)-trithiocarbonate as the RAFT agent, MA-Gly-Phe-Leu-Gly-BMS was copolymerized with HPMA. After purification with LH-20 column and dialysis, the FPLC analysis revealed that the apparent weight average molecular weight (M_w) of the copolymer (P-BMS) was 3.41×10^4 kDa with a PDI of 1.39. The BMS content in P-BMS was 9.20 % measured by UV spectrometer.

- Surgical induction of DMM in mice. Months 1-4.

DMM surgery was performed on a total of 15 mice. In each mouse, the right knee was subjected to transection of the medial tibial ligament, and the left knee was unoperated (control).

- Intraarticular injection of HPMA copolymers, optical imaging to define patterns of retention within the joints. Months 3-8.

Five mice with DMM surgery were administered a mixture of 0.5mg P-IRDye + 0.5mg P-Alexa in a total volume of 5 microliters of PBS into each knee by intraarticular injection at three weeks post surgery. Successful introduction of the HPMA copolymers into the joint space was confirmed by optical imaging 2 hours after injection. This showed strong IRDye signals in the injected knees. Two additional mice were injected with PBS (controls). An additional 5 mice and 3 controls were treated with HPMA copolymers in the same way at 4 weeks post surgery. All mice were optically imaged over 2 weeks. This analysis showed that the intraarticularly injected HPMA copolymers were retained within the joint space over the time course, with signal still present at 2 weeks after injection (Figure 1). Both operated and unoperated knees showed retention of HPMA copolymers over 2 weeks, although some variations in the intensity of the relative signals were noted.

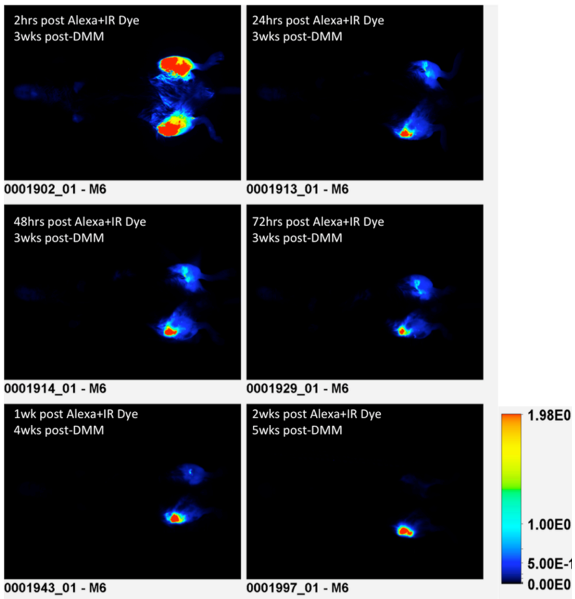

Figure 1. Optical imaging of mouse knee joints post-intraarticular injection of P-IRDye

- Recovery of joint tissues and cells, establishment of disease progression and cellular localization using faxitron, u-CT, histological and immunohistological analyses, flow cytometry. Months 8-16.

Following optical imaging at 2 weeks after intra-articular injection of the HPMA copolymers, the mice were sacrificed and the knee joints were fixed with paraformaldehyde, decalcified with EDTA, paraffin-embedded, and sectioned. Fluorescence microscopy has been performed on sections from knee joints

(both with and without DMM surgery) two weeks after intraarticular injection of HPMA copolymers. These studies have revealed that, in line with the in vivo imaging results, copolymer is retained over this time period within the joint space. Furthermore, the HPMA copolymers within the knee joint are primarily localized to cells, including cells at locations consistent with synoviocytes and articular chondrocytes (Figure 2).

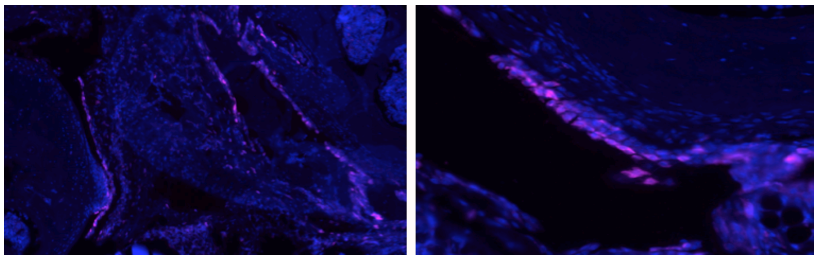

Figure 2. Fluorescence microscopy images demonstrating localization of HPMA copolymers to cells adjacent to the joint space 2 weeks after intraarticular injection (5 weeks following DMM surgery). Several distinct cellular compartments are labeled, consistent with uptake into synoviocytes and articular chondrocytes.

To further confirm this finding, we performed a femoral head explant study:

Mouse femoral heads were harvested from 5- to 6-day-old wild-type mice and cultured in complete medium for 24h. At 24h after harvesting, medium was changed for fresh complete medium for additional 96 h. At day 5, medium was changed for complete medium containing vehicle (PBS/BSA) or IL-1beta (1ng/ml) for additional 24h. At 24h after stimulation with IL-1beta, the cultures were treated with vehicle (control) or P-Dex-Alexa. Samples were processed for imaging at 24, 48 and 72 hours after addition of the copolymer. As shown in Figure 3, the "histological" sections of explants fixed in PFA and paraffin-embedded showed positive signal in P-Dex-Alexa groups (with high background in the PBS control groups), which validate the finding our in vivo data with the fluorescence microscopy.

- Treatment of cultured cells with P-Alexa-647 and P-BMS *in vitro.* Analysis of uptake using flow cytometry and fluorescence microscopy. Evaluation of the effects of copolymers on generation of inflammatory and catabolic mediators in cell cultured in vitro. <u>Months 14-18</u>

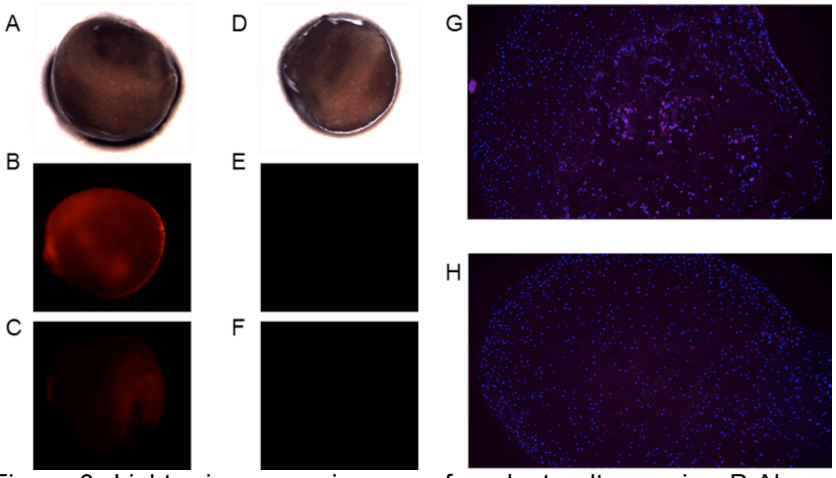

Figure 3. Light microscope images of explant culture using P-Alexa-647 (A) or vehicle-treated (D) control explant cultures at 72 hours after P-Alexa-647 addition (IL1beta-treated group not shown). Fluorescence images of samples after PBS washes and before PFA fixation are shown in (B) and (E) for P-Alexa-647 and Control groups, respectively. Representative fluorescence images of samples after PFA fixation and washes are shown in (C) and (F) for P-Alexa-647 and control groups, respectively. PFA-fixed, paraffin-embedded sections are shown in (G) and (H) for P-Alexa-647 and control groups, respectively.

In addition to the explant study, we have initiated cell culture studies to characterize uptake of P-Alexa-647 and signaling effects of HPMA copolymer BMS-345541 conjugate (P-BMS) in cultured bone marrow derived macrophages (BMM) and chondrocytes. Uptake of P-Alexa-647 into BMM was evaluated by fluorescence microscopy and flow cytometry, with both

assays revealing uniform uptake of the copolymer by the cells after a 24 h incubation (Figure 4). The copolymer displayed a speckled appearance, consistent with the previously demonstrated ability of HPMA copolymer to be sequestered within the lysosomal/endosomal compartment.

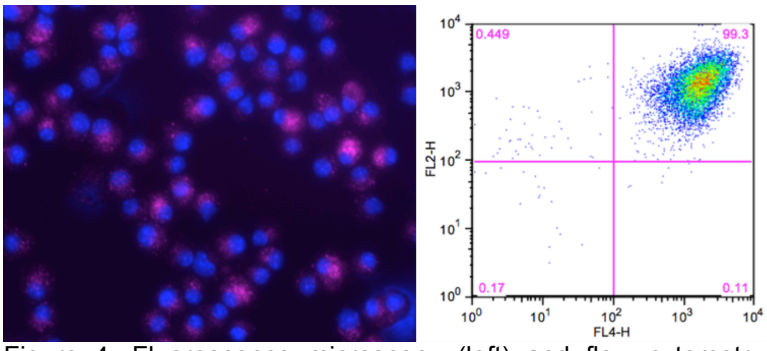

Figure 4. Fluorescence microscopy (left) and flow cytometry (right). Essentially all the cells showed a positive fluorscence signal, and were double-positive for the myeloid marker cd11b (FL4-H) and P-Alexa-647 (FL2-H)

To study the ability of P-BMS to provide sustained protection from inflammatory mediators, BMM were cultured with P-BMS for 24 h (to allow uptake and sequestration), then cultured in normal medium for 96 h and challanged with LPS. Under these conditions, the P-BMS inhibited LPS induction of pro-inflammatory cytokine expression, as assessed by real-time quantitative RT-PCR. In an additional study we evaluated the differential effects of P-BMS on formation of osteoclasts (OC), myeloid lineage cells responsible for pathologic bone destruction in a variety of inflammatory conditions. We found that P-BMS inhibited OC formation (an NF-κB dependent pathway) when present throughout an 11-day culture with the OC-inducing factors receptor activator of NF-κB ligand (RANKL) and monocyte colony stimulating factor (MCSF). However, removal of the BMS-345541 after the first 24 h resulted in loss of its' capacity to inhibit OC formation. In contrast, the P-BMS inhibited OC formation not only when present throughout the entire culture period, but also when administered only for the initial 24 h (Figure 5).

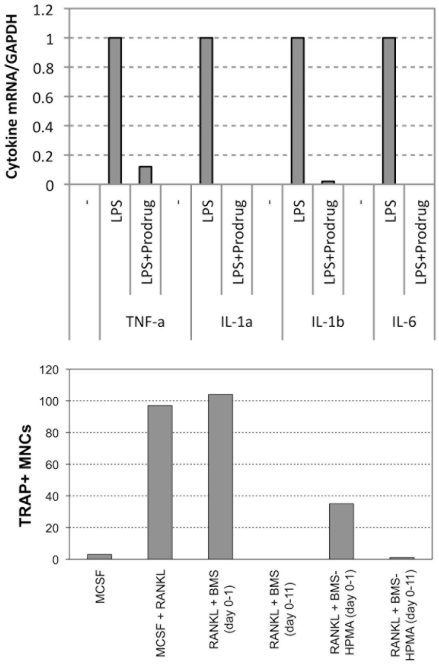

Figure 5. Upper panel: Human monocytes were pulsed with P-BMS (equivalent to a 20μM dose of BMS-345541) for 24 h, then cultured in drug-free medium for 96 h and challenged for 6 h with 4 pg/mL LPS. Cytokine gene expression was assessed by qPCR. Lower panel: P-BMS inhibits osteoclast (OC) formation (induced by MCSF and RANKL) when present throughout the culture period, but not when present for only the first day. BMS-345541 prodrug, however, inhibits OC formation completely when present throughout the culture period, but also when only present for the first day.

What opportunities for training and professional development have the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Although the funding period has been completed, we are currently finalizing the data collection and planning to develop future proposals around the findings collected during this funding period. These

studies will focus upon detailed characterization of the cell types that internalize P-Alexa-647 after injection, and quantitation of the kinetics of uptake and retention. The ex vivo/in vitro studies will model the ability of HPMA copolymers containing BMS-345541 to provide protracted protection of chondrocytes and macrophages.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Our preliminary data obtained from this project confirms that locally injected polymeric prodrug can be sequestered and processed by the cellular components, (including synovial cells and chondrocytes) in the joints of DMM mouse model of OA. This result sets the stage for the future development of novel intraarticular therapies for improved treatment of OA.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. **CHANGES/PROBLEMS:**

Actual or anticipated problems or delays and actions or plans to resolve them

The in vivo imaging of all the mice has been completed, and all the tissues have been processed for subsequent histological, fluorescent microscopic and immunocytochemical analysis. Several of these samples have been analyzed by histological staining and fluorescence microscopy, demonstrating localization and retention of P-Alexa-647 in cell populations in the synovial lining and articular cartilage. Additional samples are currently being processed to complete the data collection from these experiments, and will be completed within the next months. The flow cytometry approaches have been delayed due to limitations of the amount of retrieved tissue to work with, and to allow us to focus upon the histological analysis, which should provide comprehensive data upon the cellular localization of the injected HPMA copolymer. To establish uptake parameters into the articular chondrocytes, we decided to add an explant culture component (see above), which has allowed us to demonstrate that copolymer uptake into these cells can be recapitulated ex vivo. This has delayed the chondrocyte monolayer cultures, which are now in process. The macrophage cultures have been performed to demonstrate robust uptake of copolymer and ability to achieve sustained activation of P-BMS. These cells are major components of the synovial lining of joints and represent therapeutic targets for treatment intervention.

6. **PRODUCTS:**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Dong Wang
Project Role:	Principle investigator
Person month worked:	1.2
Contribution to Project:	Dr. Wang is responsible for overseeing the entire project, especially the synthesis and characterization of the various HPMA copolymers, and HPMA copolymer-drug conjugates used in the study.
Funding Support:	N/A

Name:	P. Edward Purdue
Project Role:	Principle investigator at the subcontract site (HSS)
Person month worked:	1.2
Contribution to Project:	Dr. Purdue directed and helped oversee experiments involving the murine OA model, which were established within Dr. Mary Goldring's Research Laboratory, and the processing of tissue for histological analysis. He also performed the BMM cell culture experiments.
Funding Support:	N/A

Name:	Mary B. Goldring
Project Role:	Co-investigator at the subcontract site (HSS)
Person month worked:	0.6
Contribution to Project:	Dr. M. Goldring participated in experimental design and troubleshooting, analysis of data and interpretation of results.
Funding Support:	N/A

Name:	Steven R. Goldring
Project Role:	Co-investigator at the subcontract site (HSS)
Person month worked:	1.2
Contribution to Project:	Dr. S. Goldring participated in experimental design and troubleshooting, analysis of data and interpretation of results.
Funding Support:	N/A

Name:	Aaron Daluiski
Project Role:	Co-investigator at the subcontract site (HSS)
Person month worked:	0.6
Contribution to Project:	Dr. Daluiski is the Co-I at HSS. He assisted in carrying out the mouse OA model and evaluating the outcomes of the surgeries.
Funding Support:	N/A

Name:	Jianbo Wu
Project Role:	Postdoc with Dr. Wang
Person month worked:	3
Contribution to Project:	Dr. Wu focused on the synthesis and characterization of polymer prodrugs.
Funding Support:	N/A

Name:	Kirstey Culley
Project Role:	Postdoc with Dr. M. Goldring
Person month worked:	1.8
Contribution to Project:	Dr. Culley established DMM model and performed the intraarticular injections on the mice and tissue retrieval, and the explant study.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

New funding

Grant Title:	Identification of novel pathways of osteoclast activation and deregulated bone remodeling in inflammatory arthritis and bone
Funding Agency:	Boehringer Ingelheim Pharmaceuticals
Duration:	11/1/2015-10/31/2017
PI:	Steven R. Goldring. Co-I: P. Edward Purdue

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: None