INTRODUCTION
The healing time and the extent of repair following ischemic necrosis of the femoral head (INFH) vary considerably depending on the age of onset. In patients near skeletal maturity and adults, the healing time is prolonged and the extent of repair is decreased compared to children with INFH. Even in childhood, the age of onset affects the healing time and the outcome. We hypothesize that revascularization and repair of the femoral head following ischemic injury is impaired as a function of age, and that impaired revascularization is due to an age-dependent reduction in the expression of a key angiogenic factor, VEGF and its transcription factor, HIF-1α. To test this hypothesis we developed an epiphyseal cartilage explant model and studied VEGF and HIF-1α expression in the explants from 2 age groups of animals following hypoxia induction. Epiphyseal cartilage was studied since it is the only viable tissue remaining on the femoral head following total head infarction and it has been shown previously to upregulate VEGF expression in response to ischemic necrosis.

METHOD
The Local Animal Care and Use Committee approved the study. Two age groups of pigs were obtained; 4 young pigs (4 wks old, 5-8kg) and 4 older pigs (24 wks old, 50-60kg). Epiphyseal cartilage overlying the bony epiphysis of the femoral heads were collected and cut into 5mm length x 5mm width x 1mm thick explants. The explants were incubated in DMEM without serum for 30 minutes followed by placement in a hypoxia chamber (1% O2, 5% CO2 and 94% N2) for up to 24 hrs. Control samples were incubated in a normoxic condition (5% CO2, 95% Air). The explants were analyzed at 2, 4, 12, and 24 hrs.

Confirmation of hypoxia induction was performed using Hypoxyprobe-1 (Hypoxyprobe kit, Chemicon International). Cell viability testing was performed using calcein and propidium iodide staining. Ribonuclease Protection Assay (RPA) was performed using a customized multiprobe template for porcine VEGF and HIF-1α. Western blot analysis for VEGF and HIF-1α were performed using A-20 (Santa Cruz) and NB 100-123 (Novus) antibodies, respectively. Optical density (OD) analyses of RPA and Western blots were performed using a Scion Image software. Samples from each animal were repeated twice and results were found to be consistent.

Results
Confirmation of Hypoxia:
Brown staining of cells in the explants, which are due to formation of adducts under low oxygen tension (pO2<10 mmHg), was observed only in the cartilage explants subjected to hypoxia. Presence of brown staining was observed as early as 2 hrs after hypoxia and it increased progressively over time (Fig 1).

Cell viability testing: At 2hrs of hypoxia, >90% of cells in the explants had calcein staining and only few cells had propidium iodide staining, indicating that most cells were viable. By 24hrs, 60% of cells remained viable. (Fig 2)

RPA: VEGF expression in the explants from the older animals was reduced at 2 hrs and to a greater extent than those from the younger animals at all time points (Fig 3A, 3B).

Fig 1: Hypoxyprobe-1 staining: Positive staining was observed only in the explants subjected to hypoxia induction.

Fig 2: Cell viability test: After being subjected to hypoxia for 24 hrs, majority of the cells were Calcein positive indicating that they were viable.

Western blot analysis: No difference in VEGF protein expression was observed between the explants subjected to hypoxia and controls for both young and old animals at all time points (data not shown). Moreover, there was a lower rate of increase of HIF-1α expression in the explants from the older animals compared to those from the young animal at 24 hours of hypoxia (Fig 4A, 4B). Moreover, there was a lower rate of increase of HIF-1α expression in the explants from the older animals compared to the young animals. All results had been normalized over SP1 expression (internal control).

DISCUSSION
Age of onset is one of the most important factors that determine the duration of healing and the clinical outcome following INFH. Current study was performed to investigate a clinically relevant question of why the healing time and repair response is impaired in the older individuals that suffer INFH. Our results support the hypothesis that there is an age dependent reduction of VEGF and HIF-1α expression following hypoxic injury. In response to hypoxia, the absolute quantity and the rate of increase of VEGF mRNA and HIF-1α protein expressions were reduced in the explants from the older animals compared to the young animals. Interestingly, the explants from the older animals had decreased baseline expression of VEGF and HIF-1α compared to the younger animals. We postulate that an increase in VEGF expression following hypoxia was not observed on the western blot because of the short study period (24 hrs), limited by cell viability in the explant system, and because western blot analysis is less sensitive than RPA. We believe that no increase in HIF-1α mRNA expression was observed following hypoxia since it is expressed constitutively and the regulation of HIF-1α has been shown to be at the post-translational level. In summary, our results provide a new insight into a clinical problem where revascularization and repair are impaired in older individuals following INFH.

REFERENCE:
1. Kim et al. JBMR 19:2041, 2004

Founded by OREF and Shriners Hospitals for Children