AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2020 – 2022

TITLE: : Increasing vitamin D levels to improve fertilization rates in cattle

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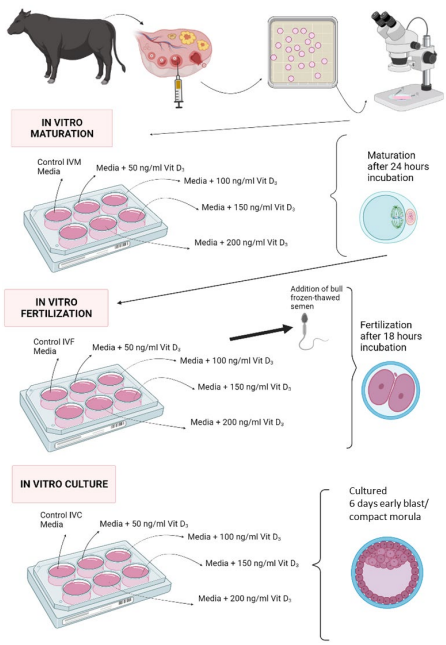
SUMMARY/ABSTRACT: These studies were designed to test if a simple, relatively inexpensive treatment was able to increase fertility in cattle during the breeding period. Based on current prices of injectable supplements this could be an additional $6-8 of cost during a synchronization protocol. However, compared to the cost of repeat breeding (semen alone $10-20+, depending on bull and type of semen), this is a very economical treatment. The in vivo study was matched with in vitro studies investigating the impact of vitamin D3 on ovarian function, something that remains under-appreciated in cattle species.

OBJECTIVES: The objectives of the proposed to study were to 1) Determine if there is a dose-response relationship between 1α,25-dihydroxyvitamin D/calcitriol levels and in vitro fertilization (IVF)/embryo development of bovine oocytes; 2) Perform acute supplementation of vitamin D3 to levels between 100 and 125 ng/ml to improve pregnancy rates during timed artificial insemination of indoor-housed dairy cattle; 3) To correlate vitamin D3 supplementation of mature cows with levels of calcitriol in large antral follicles.

PROCEDURES:

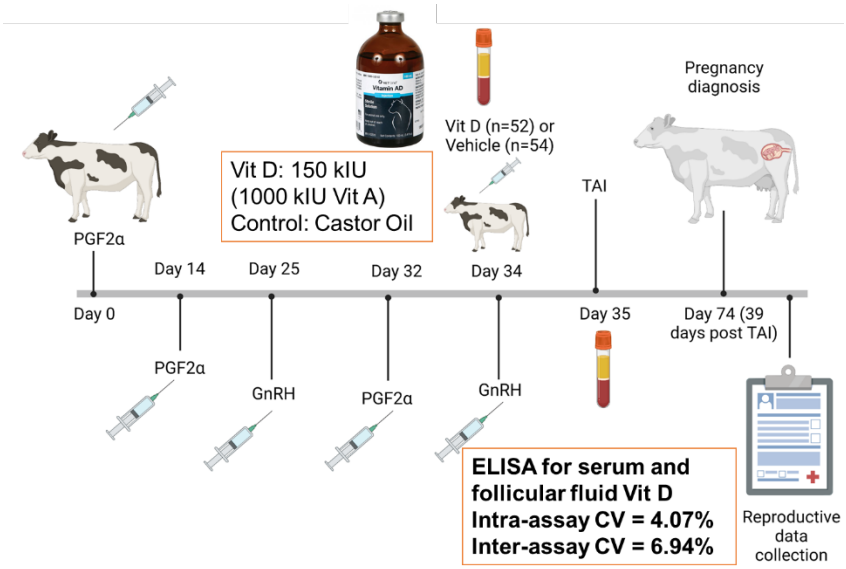


Objective 1) Bovine ovaries (n=130) were obtained in three rounds of ovary collection during October-December 2020 from a local abattoir from mixed breed female cattle and transported back to the PI's laboratory at Oregon State University on ice (<1 hour in transit). Antral follicles were manually aspirated to remove cumulus oocyte complexes (COCs). COCs were stimulated to mature the oocyte (in vitro maturation, IVM), fertilized using donated sperm (in vitro fertilization, IVF), and cultured to blastocyst stage in a commercial media (not containing sources of vitamin D or retinoids; n=25-38 COCs/group) in either baseline media (0 ng/ml calcitriol), 50 ng/ml calcitriol (Sigma Aldrich, USA), 100 ng/ml, 150 ng/ml and 200 ng/ml during the entire experimental culture period (Image, right). Images were captured following IVM, IVF and at the end of the culture period to determine number of embryos surviving each stage, as well as stage and quality of embryos at the end of

culture. All data were summarized into descrete variables by round of ovary collection and analyzed by non-parametric One-way ANOVA in SAS. Because commercial injectable supplements used in subsequent in cow studies contained vitamin A (beta-carotine), an additional cohort of COC's was exposed to the same calcitriol levels as well as a low, physiologically relevant level of all trans retinoic acid (5 nM).

Objective 2) To determine if increased supplementation of vitamin D3 during breeding increases fertility, studies were conducted on indoor housed dairy-breed cows at a large commercial dairy in the Willamette Valley. This herd was managed to consume a vitamin pre-mix by NRC requirements, and it was expected serum vitamin D3 levels would be between 60 and 80 ng/ml. We then designed our supplementation strategy to bring serum vitamin D3 levels up to 125-150 ng/ml [Nelson et al., 2016], only during the window of breeding, using a commercially available, FDA approved injectable (Vitamin AD, Vet One) administering 150 kIU Vitamin D/ 1000 kIU Vitamin A (n=52) or castor oil vehicle control (n=54). All cows were Holstein or Holstein crossbreds, and were on their 1st to 5th lacatation. Because of a delay in start of study due to COVID-19 protocols, this portion of the study began in April 2020, and was completed on June 2020.

These females were bred using a standard synchronization protocol, Presynch/Ovsynch, where females received 2 injections of prostaglandin F2α at day 0 and 14. Then 11-12 days later, females received an injection of gonadotropin releasing hormone. After 7 days, females again received prostaglandin F2α in the afternoon, followed by gonadotropin releasing hormone 2 days later in the afternoon. Females were bred by trained artificial insemination technicians at the Dairy 12 hours after the last injection (see timeline below). In this protocol, prostaglandin F2α is given to lyse any corpora lutea present on their ovaries, which would prevent the female from ovulating; gonadotropin releasing hormone (GnRH) is administered to first synchronize the follicle wave so an ovulatory follicle will be present on the ovary near the end of the protocol, and the second injection induces ovulation of that follicle. The female should be in heat (estrus) at time of artificial insemination (TAI), and ovulate shortly thereafter. This protocol allowed us to standardize our treatment scheme and reduce variability due to multiple breeding protocols. During their first synchronization cycle a single injection of either vehicle (castor oil) or Vitamin AD was administered 1 day prior to breeding (Timeline, right). Blood samples were taken at Day 34/35, just prior to vitamin AD or vehicle injection, and at time of insemination (TAI) to measure vitamin D3 levels by ELISA. Serum was also banked for later studies to



analyze other reproductive hormones. Vitamin AD injections were well-tolerated and no adverse effects on milk production and other health parameters were reported by staff at the dairy. Conception rates and subsequent days open were reported to research staff. All data were averaged into discrete variables by cow and analyzed by One-way ANOVA in SAS, using individual subjects as a covariate.

Objective 3) An additional study was performed in non-lactating dry cows to determine if vitamin D3 levels in serum adequately reflect the levels of vitamin D the COC is exposed to in the ovulating follicle. A total of 4 dairy-breed females (Jersey) managed as dry cows in sheltered housing at OSU Hogg Animal Metabolism Building were treated with the same Presynch/Ovsynch protocol described in the timeline above during 2 synchronization cycles, with 21 days in between synchronizations. The first cycle, females underwent blood sampling procedures and received an injection of vehicle (castor oil) in the AM the day of the second GnRH injection; 24 hours later these females underwent follicle aspiration and additional blood sampling (at same time of TAI in breeding protocol). In the subsequent synchronization cycle females received the vitamin AD injectable in place of vehicle. The follicular fluid and serum samples were analyzed for vitamin D3 levels using our same ELISA protocols. We had initially prepared for only 1 week inbetween treatment cycles, but the initial control cycle was completed in mid-March 2020, just prior to the COVID-19 lockdown. We received approval to begin the treated cycle in April, and were able to perform the final follicle aspirations in May 2020. Ratio between follicular fluid and serum levels of vitamin D was determined between control and treated cycles by individual cow.

SIGNIFICANT ACCOMPLISHMENTS: We determined there is a dose-response relationship between calcitrol exposure and embryogenesis in cattle. Of the doses investigated, 50 ng/ml showed the greatest benefits, with more advanced embryonic stage at the end of culture ($p<0.009$) and higher quality embryos ($p<0.03$) compared to non-exposed controls. Of note, addition of low levels of all trans retinoic acid (vitamin A) showed an additional benefit of increased fertilization rate ($p=0.02$) in the presence of 50 ng/ml calcitrol. In vivo studies demonstrated levels of vitamin D in serum are comparable to levels found in the follicular fluid of the ovulating follicle in both our control and treated cycles (ratio 1:1.3±0.28 and 1:1.21 ±0.28, respectively), suggesting any supplementation strategy would be successful in directly impacting the ovary . While we did not see a significant impact of a single dose of vitamin AD immediately prior to breeding (pregnancy rate, $p=0.14$; number of repeat inseminations $p=0.32$; abortion rate, $p=0.39$), it was apparent from the serum vitamin D levels while our treatment did increase serum vitamin D levels ($p=0.059$), we did have an unexpected discovery. The baseline levels of vitamin D in our indoor housed commercial dairy herd was much lower than expected (~33-37 ng/ml vs. expected 60-80 ng/ml), and was not different by month of study (treatment by month $p=0.31$). In contrast, in our dry cows in sheltered housing serum levels increased from ~30-45 ng/ml in March to a basline of 55-70 ng/ml in May. Our dry cows were consuming silage and were not supplemented with any oral premixes. This was an unexpected outcome and was reported to the commercial dairy.

BENEFITS & IMPACT: There appears to be a beneficial impact of supplementation of low levels of vitamin D during in vitro maturation, fertilization and early embryogenesis, and there may be a synergistic effect of combination with other fat soluble vitamins. While levels of vitamin D in serum are reflective of levels within the follicular fluid, short term treatments to elevate vitamin D levels may be less successful if cows have lower levels of vitamin D at start of treatment. Vitamin D premixes may not adequately elevate serum vitamin D as advertised. Additional studies are needed to evaluate a longer term supplementation strategy for this vitaimin in cattle.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM: Oregon Beef Council Grant to C. Bishop to study long term supplementation in beef cattle and impact on calves.

FUTURE FUNDING POSSIBILITIES: Application for continued support from Oregon Beef Council, Febuary 4, 2022. Planned proposal to USDA-NIFA AFRI Foundational and Applied Science Program, Priority Area Animal Health and Production and Animal Products, Program Area Priority Animal Reproduction. Submission date August 11, 2022.