AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
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TITLE: Is Periparturient Immunosuppression in Beef Cows Attenuated by Feeding Selenium Fertilized Alfalfa Hay in the Third Trimester?

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SUMMARY:

Our goal is to prevent diseases in beef cattle by optimizing immune function with supranutritional selenium (Se) biofortification. Minimizing cattle losses from pests and diseases depends on maximizing the protective capacity of the immune system responsible for containment and mitigation of parasites, bacteria, and viruses. During the periparturient period, beef cows experience a state of natural immunosuppression, which increases susceptibility to diseases. Clinical and subclinical diseases result in decreased milk production as well as higher morbidity and mortality. We will determine if and how supranutritional Se supplementation during the dry period alters immune responses and disease incidence.

Selenium has been recognized for years as an essential trace element for animals. The Northwest region is among those with the lowest amounts of Se in soils and plants. In general, the majority of livestock raised in low-Se regions do not receive sufficient dietary Se for optimum health. Severe *Se deficiency* in ruminants results in nutritional myodegeneration known as "white muscle disease", whereas insufficient Se intake has been implicated as the cause of a group of Se-responsive disorders including unthriftiness, reduced weight gain, and immunosuppression.

Livestock can be supplemented with Se by various means, including licks, drenching with Se compounds (e.g., selenite/selenate or Se yeast), intraruminal boluses, selenite injections or selenate depot injections, adding Na-selenite to the drinking water, and various methods of pasture and soil applications (Se fertilization). The most promising Se supplementation method is Se fertilization, as it increases Se concentrations in plants, and in animals consuming Se-biofortified forages and hay. Nitrogenous fertilizers, widely hailed as one of the most important advances in agricultural technology, increase biomass but dilute essential minerals like Se, emphasizing the need for Se amendments. Se-fertilization has been used in several countries including Finland, Denmark, New Zealand, and the United Kingdom to increase Se concentrations in the food chain. In Oregon, the Department of Agriculture does not control the use of Se as a plant fertilizer, making it possible to produce Se-biofortified forages.

The goal of enhancing immunity is to increase disease resistance. All components of the immune system, e.g. antibody titers to vaccination, lymphocyte proliferation, and ability of neutrophils to destroy phagocytized bacteria are affected by Se. In general, *Se deficiency* decreases neutrophil function, antibody production, and proliferation of T and B lymphocytes in response to mitogens, resulting in increased morbidity and mortality. Supplemental Se can reverse those effects. Selenium supplementation of *Se-deficient* animals results in fewer disease events, including metritis, mastitis, laminitis and pinkeye. It is unknown whether consumption of Se above currently recommended levels (*supranutritional Se*) enhances immunity and improves host resistance in *Se-replete* livestock exposed to new pests or diseases. Our goal is to determine whether supranutritional Se biofortification of *Se-replete* animals enhances immunity and prevents, contains, or improves recovery from pests and diseases of livestock.

OBJECTIVES:

1) To determine if feeding pregnant beef cows Se-fortified alfalfa hay in the last 8 wk of pregnancy enhances innate immunity at parturition based on serum concentrations of inflammation biomarkers: non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), haptoglobin, serum amyloid A, and albumin.

2) To determine if feeding pregnant beef cows Se-fortified alfalfa hay in the last 8 wk of pregnancy enhances innate immunity at parturition based on the expression levels of Se-responsive genes and functional measures of phagocytosis.

PROCEDURES:

The study design consisted of three groups of beef cows that were housed in the Hogg Animal Metabolism Barn on campus during the last 8 wk of pregnancy. Because pen is the experimental unit in animal nutrition studies, we put 5 cows per pen and had 3 pen replicates for each of three Se-treatment groups (total of 45 cows). Group 1 (control): Three pens of cows (n=15) were fed non-Se fortified alfalfa hay as a major portion of the ration plus a mineral supplement containing 120 mg/kg Se (US FDA regulations) from sodium selenite. Group 2 (M-Se): Three pens of cows (n=15) were fed alfalfa hay harvested from a field fertilized with a moderate level of Se (M-Se) and a mineral supplement without added Se. Group 3 (H-Se): Three pens of cows (n=15) were fed alfalfa hay harvested from fields fertilized with a high level of Se (H-Se) and fed mineral supplement without added Se. Cows were maintained on their respective diets for 8 wk prior to calving.

For the Se-enriched alfalfa forage, sodium selenate was mixed with water and sprayed onto the soil surface of an alfalfa field after the second cutting of hay. Two application rates of selenate were used for the M-Se and H-Se forage. In our pilot study we showed that fertilizing with 45.0 (M-Se) or 89.9 (H-Se) g Se/ha resulted in corresponding increases in alfalfa hay Se content of 1.55 and 3.26 mg Se/kg dry matter, respectively. Hay was harvested from the respective field plots, and then sampled for Se content. A Penn State forage sampler was used to take 25 cores from random bales in each alfalfa hay source (control, M-Se, and H-Se) for Se analysis at Utah Veterinary Diagnostic Laboratory, Logan.

Whole blood samples were collected from all cows prior to feeding Se-fortified alfalfa hay and at parturition to measure blood-Se concentrations, which were determined by Utah Veterinary Diagnostic Laboratory using an ICP-MS method. Serum was also collected at the same time points to detect subclinical diseases by measuring serum concentrations of inflammation biomarkers: non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), haptoglobin, serum amyloid A, and albumin as indicators.

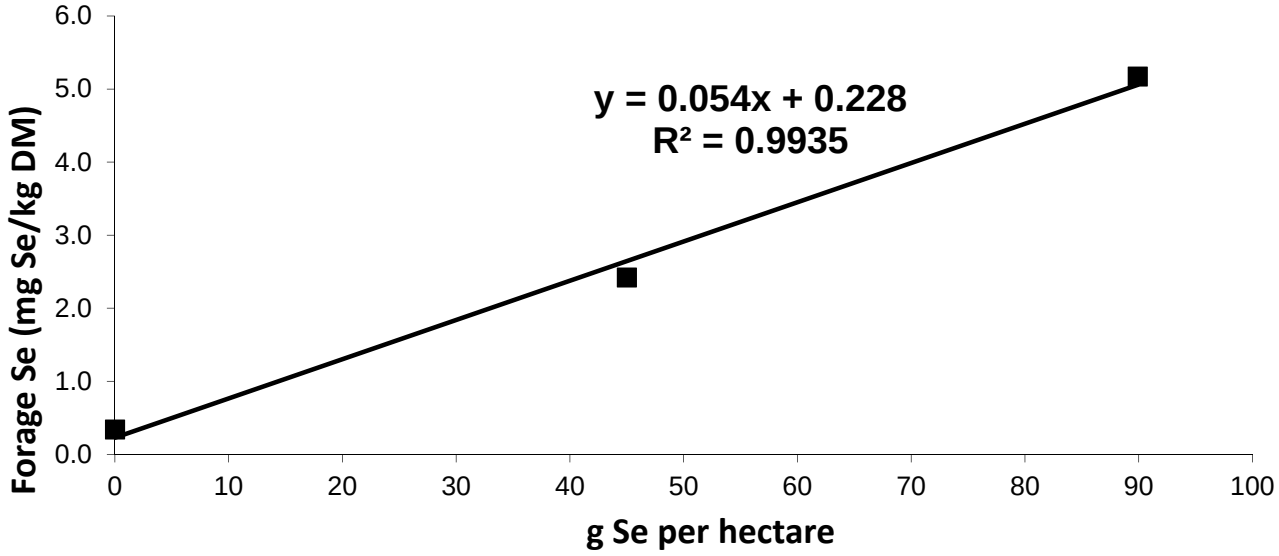
Macrophages and neutrophils are the cells primarily responsible for carrying out innate immune effector functions at sites of infection via phagocytosis. We assessed phagocytosis by incubating blood leukocytes with fluorescently labeled latex beads and measuring bead uptake by flow cytometry. Correlating the expression levels of Se-responsive genes (e.g., selenoprotein S) with the functional measures of phagocytosis will allow us to address our hypothesis that Se enhances innate immune functions. Complement proteins present in blood can directly kill bacteria, which were measured by isolating plasma from animals and mixing serial dilutions of the plasma with laboratory strains of *E. coli* bacteria. Bacterial outgrowth was monitored by counting colonies 24 h after plating bacteria. We compared complement-mediated bacterial killing in plasma of cows fed Se-biofortified forage vs. control diet to determine if supranutritional Se enhances the innate immune responses associated with complement-mediated bacterial killing. Activated macrophages generate large amounts of nitric oxide (NO) to damage pathogens, which was measured by a colorimetric method when culturing macrophages in the presence of activators. In contrast to activated macrophages, regulatory macrophages convert arginine, the precursor for NO production, to urea and, thus, prevent NO release. Urea was also measured by a colorimetric method. We compared the ratio of NO to urea production in macrophages isolated from blood of cows fed Se-biofortified forage versus control diet to determine if supranutritional Se induces a shift towards NO production, which is indicative of activated macrophages and an enhanced innate immune response.

Objective one is accomplished by determining serum concentrations of indicators of subclinical diseases at parturition. We hypothesize that supranutritional Se-supplementation provided by feeding Se fertilized alfalfa hay during the dry period will increase blood-Se concentrations and decrease morbidity (e.g., mastitis, metritis, retained placenta) in Se-supplemented cows compared with control cows. Objective two will be accomplished by measuring expression levels of Se-responsive genes and isolating cells and measuring phagocytic ability. This will allow us to address our hypothesis that Se enhances innate immune functions. We hypothesize that innate immunity will be enhanced in cows fed Se-fortified alfalfa hay compared with control cows.

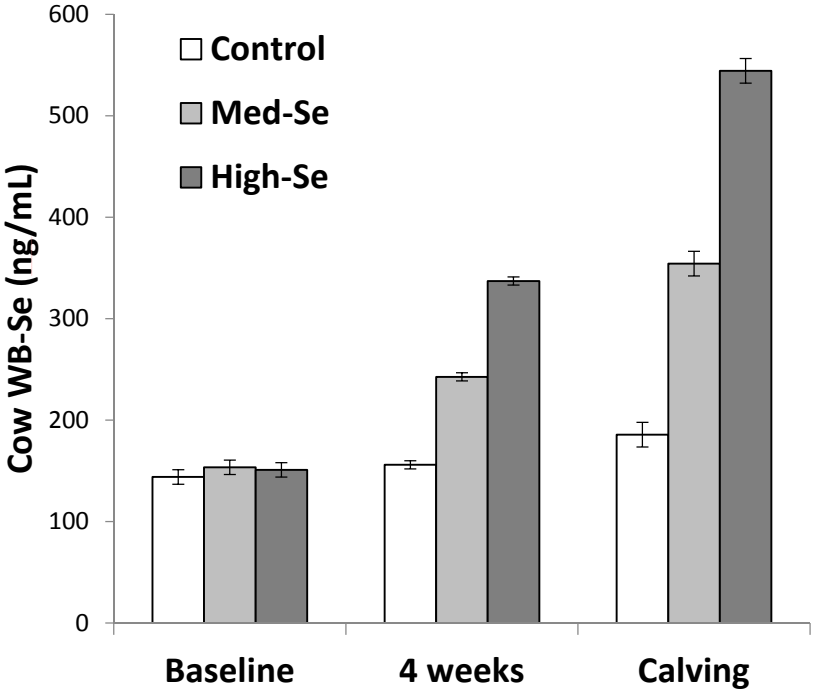
Statistical analyses: Differences in variables measured pre and post Se supplementation will be evaluated using an ANOVA method and Statistical Analysis Software (SAS). Significance will be accepted at $P \leq 0.05$.

SIGNIFICANT ACCOMPLISHMENTS:

Sodium selenate mixed with water was sprayed onto the soil surface of an alfalfa field after the second cutting of hay. Fertilizing fields with increasing amounts of Na selenate increased the Se-concentration of third cutting alfalfa hay from 0.36 mg Se/kg DM (non-fertilized control) to 2.42 and 5.17 mg Se/kg DM for Na selenate application rates of 45.0 and 89.9 g Se/ha, respectively. Fertilizing fields with increasing amounts of Na selenate increased the Se-concentration of third cutting alfalfa hay from 0.34 mg Se/kg DM (non-fertilized control) to 2.42 and 5.17 mg Se/kg DM for Na selenate application rates of 45.0 and 89.9 g Se/ha, respectively. The relationship between amount of Se applied by fertilization (g Se/ha) and observed forage Se concentration (mg Se/kg DM) was y = 0.054 (± 0.002) x + 0.228 (± 0.095), $r_{Pearson} = 0.99$. Calculated Se intake from dietary sources was 5.3, 27.6, and 57.5 mg Se/head/d for cows consuming alfalfa hay with Se concentrations of 0.34 to 2.42 and 5.17 mg Se/kg DM, respectively. Cows receiving non-fertilized control hay received an additional 3 mg Se/head/d from the mineral supplement containing Na-selenite.

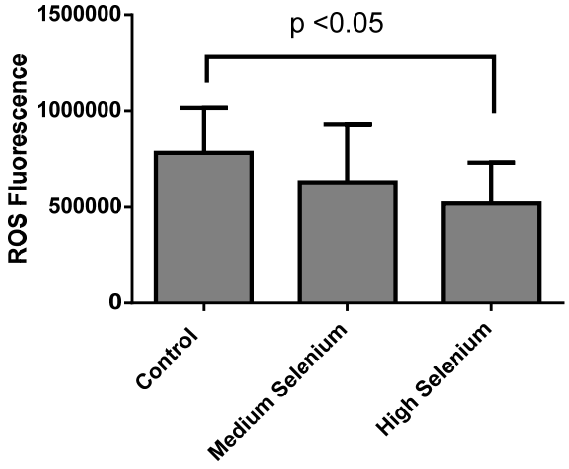


Whole blood and serum samples from cows were collected and processed for further analysis. Prior to agronomic biofortification, cows had whole blood-Se concentrations of 151 ± 4 ng/mL (mean ± SEM; range, 106-243 ng/mL), which were within the reference interval of adult cows (120-300 ng/mL). Whole blood-Se concentrations increased after feeding Se-fertilized alfalfa hay for 4 weeks depending upon the Se-application rate (0, 45.0, or 89.9 g Se/ha) to 156 ± 4, 243 ± 4, and 337 ± 4 ng/mL, respectively, and to 186 ± 13, 354 ± 12, and 544 ± 12 ng/mL at parturition (both $P_{Linear} < 0.001$). Whole blood-Se concentrations were similar after 8 weeks of consuming Se-fertilized alfalfa hay with the lower Se-application rate (45.0 g Se/ha) compared with 4 weeks of consuming Se-fertilized alfalfa hay with the higher Se-application rate (89.9 g Se/ha; $P = 0.48$).

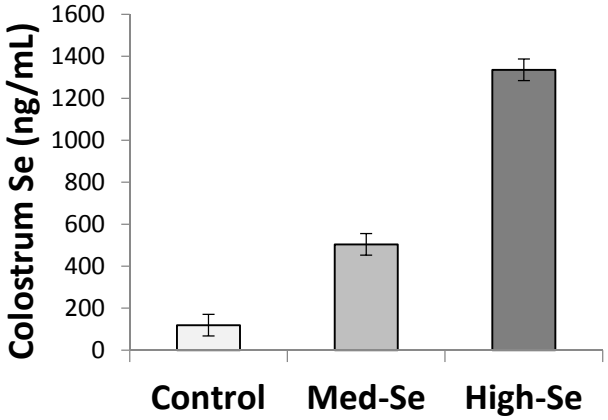


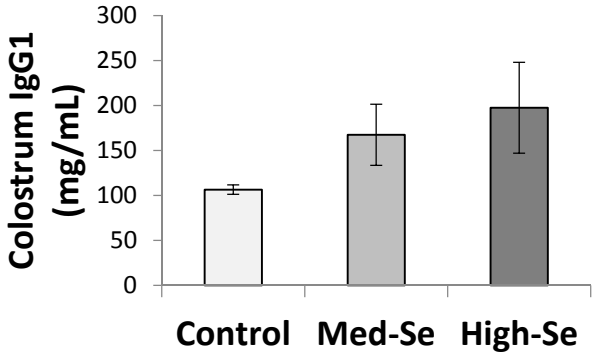
White blood cells, such as neutrophils and monocytes are a first line of defense against infectious microbes. Upon stimulation, they generate huge amounts of reactive oxygen species (ROS), in a process termed oxidative burst. The ROS can either have direct anti-microbial effects, or can be converted into more potent microbicides, such as nitric oxide (NO) and hypochlorite. These chemicals can also damage healthy tissues and induce inflammation, so their production needs to be tightly controlled. To determine if feeding Se-fortified hay altered respiratory burst in granulocytic cells (neutrophils), we treated isolated cells with 1 μM phorbol 12-myristate 13-acetate (PMA), a mitogen known to induce immune cell activation, for ten minutes at 37°C. Following activation, the dye 2′, 7′-dichlorofluorescein diacetate (DHR) was added to cells at a final concentration of 5 μM, and cells were incubated for 15 minutes at room temperature. DHR is oxidized by hydrogen peroxide generated during oxidative burst to create a fluorescent dye. The fluorescence of each cell in a sample was measured using flow cytometry and the mean fluorescence intensity (MFI) of 10,000 cells was recorded. The average MFI of three technical replicates was used as a final measurement and the background fluorescence (determined by measuring cells exposed to DHR but not PMA) was subtracted. Our data indicate that cows fed Se-fortified hay had lower ROS production compared to cows fed a control diet. Whereas these data may at first seem contradictory to our hypothesis that Se supplementation enhances immune responses, it is important to note what a healthy immune response entails. ROS generation leads to an inflammatory response that damages tissues, which would further activate the immune response. The resulting immunopathology can, in some instances, be more damaging then the microbial infection that triggered the response. Therefore, it is beneficial to limit immune pathology. Perhaps the diminished ROS generation that results from feeding Se-fortified hay dampens tissue responses and prevents excessive damage to host tissue while still limiting the microbial infection.

ROS production in granulocytes from cows fed increasing levels of Se-biofortified hay.



We have submitted one paper to *Journal of Animal Science* (currently under review) looking at the effects of feeding Se-biofortified hay to beef cows during the last 8 weeks of gestation on passive transfer of antibodies to their calves. Colostrum and whole blood were collected from cows at calving, and whole blood was collected from calves within 2 hours of calving, and at 12, 24, 36, and 48 hours of age. Concentrations of IgG1 and J-5 *Escherichia coli* antibody in cow colostrum and calf serum were quantified using ELISA procedures. Supranutritional Se supplementation of beef cows during the last 8 weeks of pregnancy using Se-biofortified alfalfa hay resulted in increased, in a linear dose- and time-dependent fashion, whole blood-Se concentrations in cows and their newborn calves (both $P_{Linear} < 0.001$). In addition, supranutritional Se supplementation increased in a linear dose-dependent fashion colostral Se concentrations ($P_{Linear} < 0.001$), and colostral IgG1 concentrations ($P = 0.04$), but had no effect on serum IgG1 concentrations in calves or colostral and calf serum J-5 *Escherichia coli* antibody concentrations in the first 48 hours of age. Thus, we suggest that feeding Se-biofortified alfalfa hay promotes the accumulation of Se and antibodies in colostrum, but a physiologic limitation of gut cells to absorb additional antibodies may have limited us to observe short-term differences in serum antibody concentrations in calves.





Additional publications are in preparation. In addition, we will use these results as pilot data to submit future grants for larger funding amounts from the USDA to advance our overall goal of improving the production and health of beef cattle in Oregon by increasing the practice of Se fertilization.

BENEFITS & IMPACT:
Results of this study will be used as evidence for hay producers in Oregon to adopt the practice of Se-fertilization of forages to provide an enhanced quality of hay, which will then be used to benefit performance and health of beef cattle. This is an innovative and economically viable way of supplementing Se to cattle in our Se-deficient state that we hope will be adopted by hay producers and cattle producers. These results will be shared via peer-reviewed publication, at state meetings, e.g., the Oregon Cattlemen's Association annual meeting, and through extension work and publications.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:
We received funding from the Oregon Beef Council to answer the question: Does feeding selenium fertilized alfalfa hay for eight weeks decrease gastrointestinal parasite load in weaned beef calves?

FUTURE FUNDING POSSIBILITIES:
We have two pending grant proposals: 1) USDA FY16 Animal Health and Disease Program: Feeding cows and calves Se-biofortified hay: Effects on health and disease, and 2) Agricultural Research Foundation: How is selenium (Se) utilized by forage plants after Se is applied to soils as a fertilizer amendment?