

# Early Inflammation Disorder in Neonatal Calves

## A.S. Leaflet R2969

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### Summary and Implications

In the present study, 30 Holstein calves were acquired at birth and were randomly subjected to one of six treatments. One treatment group was colostrum deprived (CD) and five other treatment groups were fed colostrum replacer (CR). Of the five CR groups, one group was not further supplemented (CR), one received vitamin A (CR-A), one received vitamin D (CR-D), one received vitamin E (CR-E), and one received vitamins A, D, and E (CR-ADE). Additionally, all calves were inoculated with *Mycobacterium avium*, subsp. *paratuberculosis* on d 1 and d 3 of age. One d after birth, CD calves exhibited lower IgG1, haptoglobin, and serum amyloid A concentrations in serum compared with the other five CR groups. These findings point to an endogenous early inflammation disorder in calves that are not fed colostrum.

### Introduction

The passive benefits of colostrum intake by neonatal calves are well described and are primarily attributed to acquisition of exogenous immunoglobulin, but recently interest has been generated regarding effects of bioactive compounds in colostrum on endogenous functions. Endogenous immune capabilities are especially important during early life when microbial colonization of the intestine takes place. Colostrum contains fat soluble vitamins that have been implicated in intestinal barrier and immune response functions. The aim of this study was to assess the effects of colostrum supplementation and additional supplemental vitamins A, D, and E on intestinal health in neonatal calves.

### Materials and Methods

#### Animal Procedures

All procedures were approved by the animal care and use committee of the National Animal Disease Center, ARS, USDA (Ames, IA 50010). Thirty Holstein calves were obtained from two dairy farms in central Iowa and were randomly assigned to one of six treatment groups, with 5 calves per treatment, in a 14 d feeding trial. One treatment group was colostrum deprived (CD), and the other five treatment groups received 375 g of colostrum replacer (Milk Products, Chilton, WI 53014) reconstituted in 1.9 L water at approximately 40 degrees C. The colostrum replacer contained 150 g bovine globulin protein from fractionated

colostral whey. Calves in the CD group were fed 1.9 L of pasteurized whole milk (Iowa State University Dairy Farm, Ames, IA 50014) brought to approximately 40 degrees C as a control. All calves received first feeding within 3 h of birth.

All calves received one of six vitamin or control treatments at the time of first feeding. Calves in the CD group were injected with 3 mL of placebo carrier solution. Similarly, one group fed colostrum replacer (CR) was also injected with 3 mL of placebo carrier solution. The other four treatment groups received colostrum replacer as described and then were injected with various vitamin solutions (Stuart Products, Bedford, TX) in a 3 mL volume. The CR-A group received 150,000 I.U. of retinyl palmitate, the CR-D group received 150,000 I.U. of cholecalciferol, the CR-E group received 1,500 I.U. of d- $\alpha$ -tocopherol, and the CR-ADE group received all three compounds in the concentrations described.

Calves were transported within 12 h of birth to experimental indoor housing at the National Animal Disease Center. Upon arrival, calves were placed on a twice daily feeding schedule. At each feeding, calves were fed 2.7 L of pasteurized whole milk. Once per day, calves received additional vitamin supplementation according to treatment group in 1 mL volume, consisting of placebo, 25,000 I.U. retinyl palmitate, 5,000 I.U. cholecalciferol, 500 I.U. d- $\alpha$ -tocopherol, or the triad combination. All calves were offered milk via bottle and nipple, but calves were not allowed to refuse dietary milk; refusals of greater than 0.1 L were administered to the calf via esophageal tube. Day 1 of the trial was marked by the first morning feeding in the trial location. All calves were inoculated via dietary milk with  $10^8$  cfu of *Mycobacterium avium*, subsp. *paratuberculosis*, which is the causative agent of Johne's disease, during the morning feedings of d 1 and d 3. Calves were fed according to schedule for 14 d.

#### Sample collection and analysis

Whole blood was collected via jugular venipuncture from all animals on d 0 within four hours of birth and again on d 1 at 12 to 24 h post-partum, on d 7, and on d 14 after a 12 h fast. Blood was allowed to clot at room temperature for 1 h, and then was centrifuged at 2500 RPM for 30 min for collection of serum. Haptoglobin was quantified using a bovine-specific haptoglobin ELISA (Immunology Consultants Laboratory Inc., Newberg OR). Serum amyloid A (SAA) was quantified using the PHASE<sup>TM</sup> RANGE Multispecies SAA ELISA kit (Tridelta Development Ltd., Maynooth Co. Kildare, Ireland). Immunoglobulin G1 concentration was quantified by capture ELISA using sheep anti-Bovine IgG1 antibody (Bethyl Laboratories, Inc., Montgomery, TX 77356) and sheep anti-Bovine IgG1-HRP

conjugate (Bethyl Laboratories, Inc.) for catalysis of trimethyl blue (TMB) colorimetric reaction.

Retinol and  $\alpha$ -tocopherol were quantified by reverse phase high performance liquid chromatography (RP-HPLC) using a SUPELCOSIL™ LC-18DB HPLC column (5  $\mu$ m particle size, dimensions 25 cm x 4.6 mm, Sigma-Aldrich) after extraction from serum using hexane as a solvent using retinyl acetate (125 ng suspended in 200  $\mu$ L ethyl alcohol) as the internal standard.

Total 25-(OH)-Vitamin D<sub>2</sub> and D<sub>3</sub> equivalents were quantified by competitive radioimmunoassay after extraction using acetonitrile. Radioiodinated 25-(OH)-vitamin D (Diasorin) was used as an internal standard (10,000 cpm in 25  $\mu$ L ethanol with addition of 0.01 M phosphate). Gamma radiation of samples was counted using a gamma-radiation well-type counting system (Packard Cobra II gamma counter, GMI, Ramsey, MN 55303). Concentration of 25-(OH)-vitamin D equivalents for each sample was determined directly by the counting system.

### *Statistical Analysis*

Data for vitamin concentrations in serum were analyzed by repeated measures ANOVA using mixed procedures of SAS (version 9.3, SAS Institute Inc., Cary, NC). Calf served as the experimental unit. The model included fixed effects of treatment and sample day; calf was included in the model as a random effect. Contrast statements were used to separate least square means of calves supplemented with each respective vitamin from calves not supplemented with the vitamin.

Data for acute phase proteins were initially analyzed by repeated measures ANOVA as described, but disparity among standard error values for individual treatment means rendered the model invalid, so d 1 values for acute phase proteins were analyzed as a unique response variable using contrast statements in mixed procedures of SAS. Contrast statements were used as described to separate least square means on the basis of vitamin supplementation. An additional contrast statement was used to separate least square means of the CD group and the five CR groups. Data for IgG1 at d 1 were analyzed similarly, using only a contrast statement to separate means on the basis of colostrum supplementation.

## **Results and Discussion**

### *Treatment verification*

To determine success of colostrum and vitamin treatments, IgG1, retinol, 25-(OH)-cholecalciferol, and tocopherol were quantified in serum. Values are reported as mean  $\pm$  pooled standard error. Calves supplemented with colostrum at first feeding (CR, CR-A, CR-D, CR-E, CR-ADE) all exhibited greater concentrations of IgG1 in serum by d 1 of age (Figure 1). The statistical contrast of d 1 IgG1 values of CR groups against the CD groups produced a *P* value of <0.0001. Treatment group averages for IgG1 in serum at d 1 did not exceed 6.0 mg/mL for any group.

Calves supplemented with retinyl acetate individually (CR-A) and as part of the triad vitamin treatment (CR-ADE) exhibited greater overall concentrations of retinol in serum when contrasted against treatment groups not supplemented with retinyl acetate (*P* < 0.05). Figure 2 shows mean concentrations of retinol in serum of all treatment groups at all of the time points tested. Mean concentration of retinol in serum did not differ between the CR-A and CR-ADE groups at any of the time points tested.

Calves supplemented with cholecalciferol individually (CR-D) and as part of the triad vitamin treatment (CR-ADE) exhibited greater overall concentrations of 25-(OH)-vitamin D equivalents when statistically contrasted against calves that did not receive the cholecalciferol supplement (*P* < 0.0001). Figure 3 shows mean concentrations of 25-(OH)-vitamin D equivalents in serum of all treatment groups at all of the time points tested. Additionally, CR-ADE calves exhibited greater 25-(OH)-vitamin D in serum at d 7 than did CR-D calves (*P* < 0.0001), but the difference was not observed at d 14. Concentration of 25-(OH)-vitamin D in serum of calves not supplemented with the compound declined below 20.0 ng/mL by 14 d of age, indicative of severe vitamin D deficiency.

Calves supplemented with d- $\alpha$ -tocopherol individually (CR-E) and as part of the triad treatment (CR-ADE) also exhibited greater overall concentrations of  $\alpha$ -tocopherol in serum throughout the study period (*P* < 0.0001). Figure 4 shows mean concentrations of  $\alpha$ -tocopherol in serum of all treatment groups at all of the time points tested. Mean concentration of  $\alpha$ -tocopherol did not differ between CR-E and CR-ADE calves at any of the time point tested.

### *Acute Phase Inflammation*

Acute phase inflammatory proteins haptoglobin and serum amyloid A (SAA) were quantified in serum of calves at d 0, 1, 7, and 14 to test the hypothesis that colostrum and vitamin supplementation of various forms would affect initial immune responses to first microbial encounter after birth and also later responses indicative of the degree of homeostasis maintained with intestinal and environmental microbiota. Panel A in Figures 5 and 6 show mean  $\pm$  pooled standard error concentrations of haptoglobin and serum amyloid A, respectively, in serum of all treatment groups at all four of the time points analyzed by repeated measures ANOVA. The repeated measures ANOVA revealed no overall effect of treatment, but a significant effect of day (*P* < 0.05), on stimulation of haptoglobin expression; the same outcome was observed for SAA (*P* < 0.0001). Furthermore, standard errors of individual treatment means for d 1 haptoglobin values were orders of magnitude smaller than standard errors of treatment means for d 7 and d 14 values (data not shown). The results are consistent with our working hypothesis that immediate post-natal immune responses that stimulate inflammation may be of a different nature than the responses that have been characterized for traditional pathogen challenge. Thus, we

determined that the repeated measures ANOVA was not robust to early postnatal (d 1) values for the acute phase response, so d 1 values were analyzed as a unique response variable. The fixed effect of treatment in the model tended to be significant ( $P < 0.1$ ) for both haptoglobin and SAA and therefore warranted further statistical contrasts according to colostrum and vitamin treatments.

Colostrum deprivation (CD) was contrasted against the other five CR treatments for effect on concentrations of haptoglobin and serum amyloid A in serum of calves at d 1. Panel B of Figures 5 and 6 shows mean concentrations  $\pm$  pooled standard error of the contrast for haptoglobin and serum amyloid A. Calves supplemented with colostrum replacer as part of experimental treatment exhibited approximately 6-fold greater concentrations of haptoglobin at d 1 ( $P < 0.01$ ) and 2-fold greater concentrations of serum amyloid A ( $P < 0.05$ ) than did CD calves. Calves supplemented with the various vitamins were contrasted against the unsupplemented treatment groups as previously described, but no significant differences were detected. Thus, differences in acute phase protein expression at d 1 are independent of vitamin supplementation.

Every previously characterized aspect of colostrum supplementation is an asset to the newborn calf. Therefore,

induction of haptoglobin and serum amyloid at d 1 of age in calves fed colostrum should be viewed as a positive event and indicates that inflammation is a normal and necessary adjustment to the ex-utero environment. Additionally, these results indicate that failure to express acute phase inflammatory proteins is associated with humoral immunodeficiency as a result of colostrum deprivation. Thus, we identify the endogenous condition of early inflammation disorder separately from failure of passive acquisition of humoral immunity and we characterize it as independent of vitamin supplementation.

### **Acknowledgements**

The authors recognize the contributions of National Animal Disease Center animal caretaker staff and Iowa State University dairy farm employees, especially Brian Conrad, Ben Samek, and Hannah Schroeder. We also extend thanks to laboratory technicians Margaret Walker, Tracy Porter, and Derrel Hoy for assistance with assays, and to Dave Wood of Animix, Inc. and Dave Cook of Milk Products, Inc. for contributions to experimental design and colostrum replacer product used in the study.

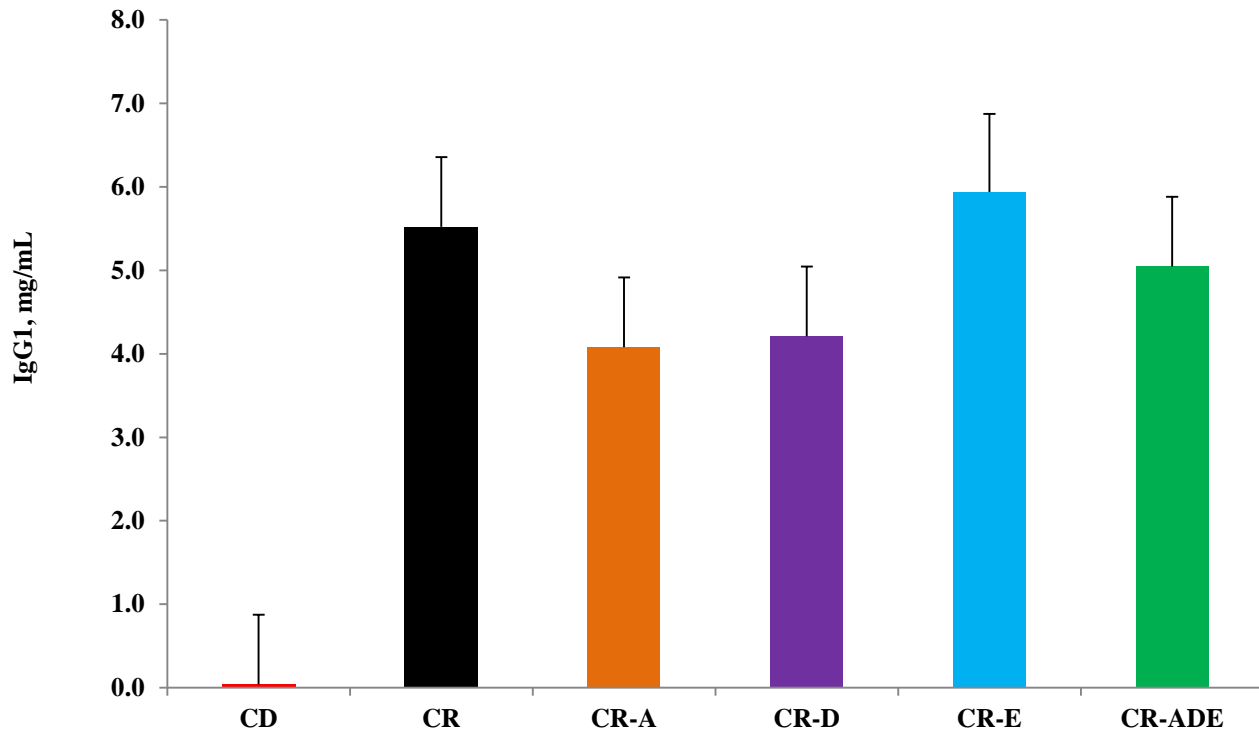


Figure 1. Mean concentrations of IgG1 in serum of calves 12-24 h post-partum.

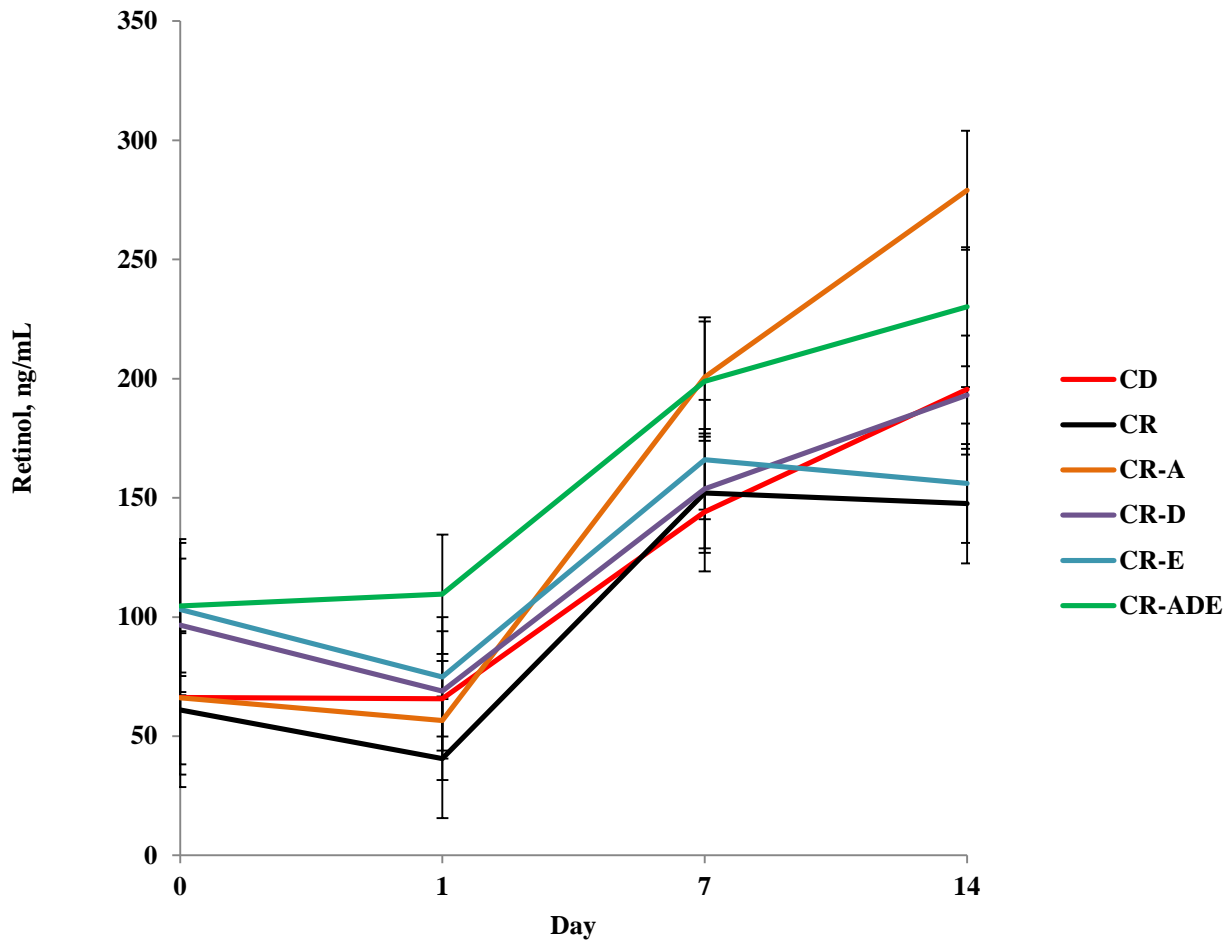


Figure 2. Mean concentrations of retinol in serum of calves.

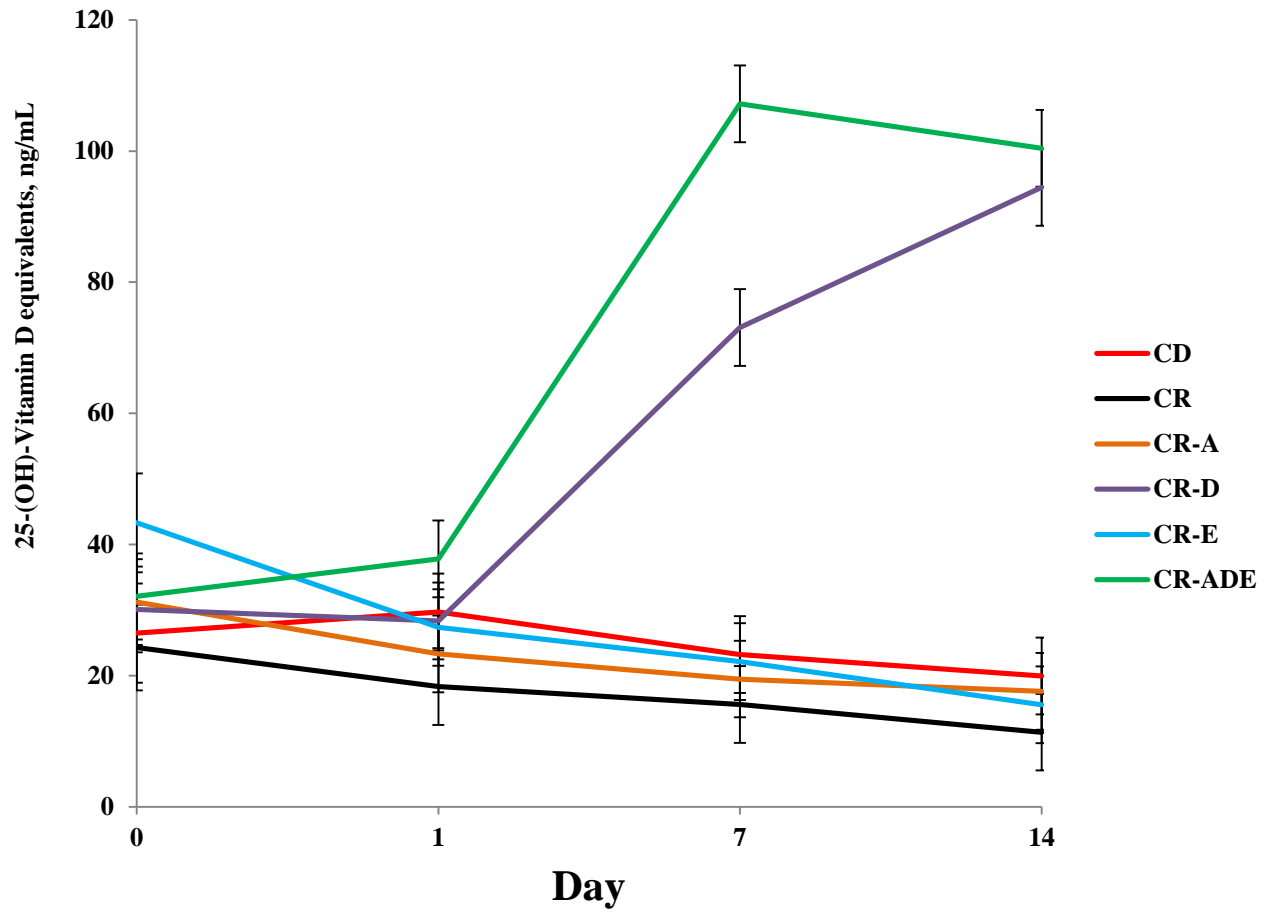


Figure 3. Mean concentrations of 25-(OH)-vitamin D equivalents in serum of calves.

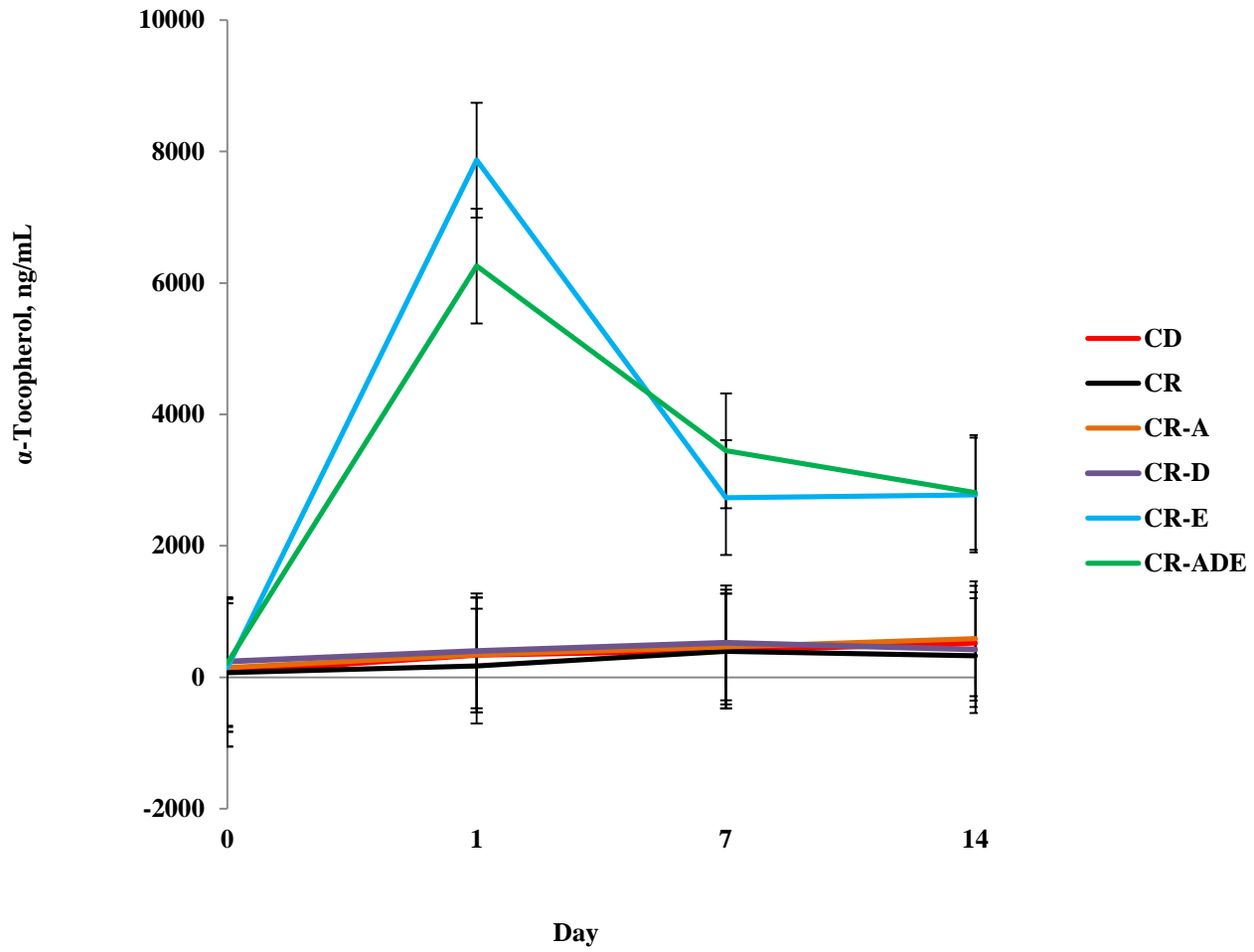
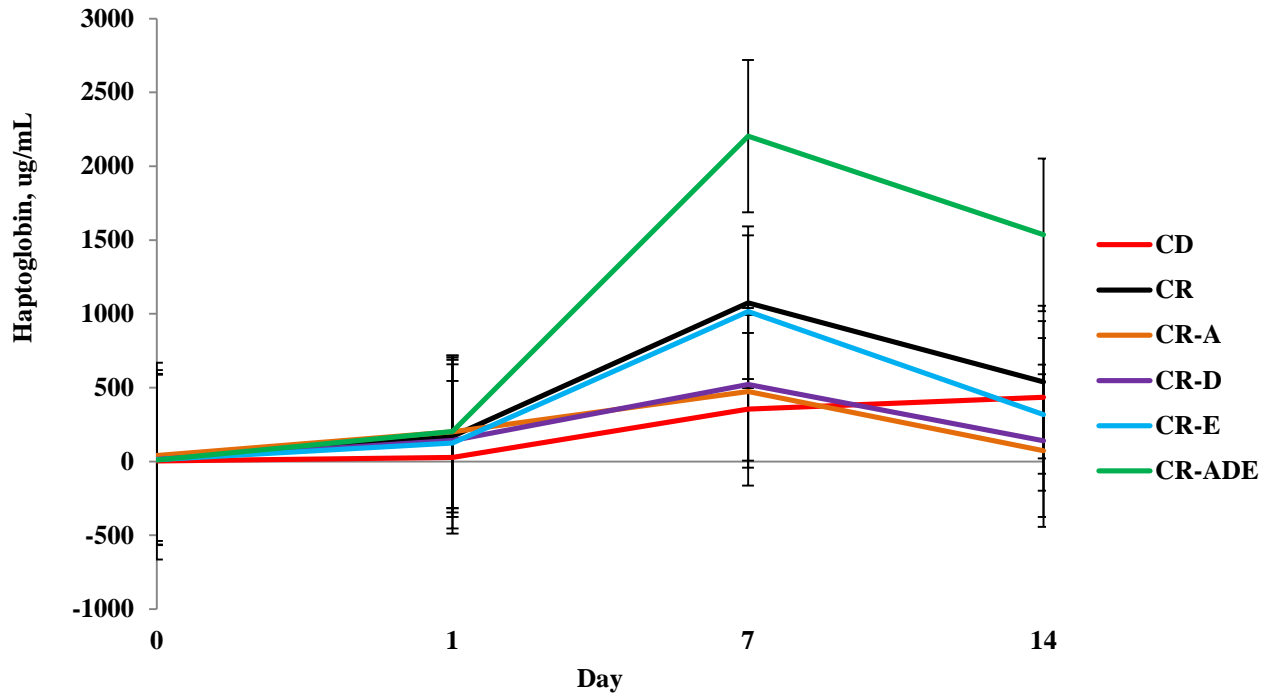


Figure 4. Mean concentrations of  $\alpha$ -tocopherol in serum of calves.

a.



b.

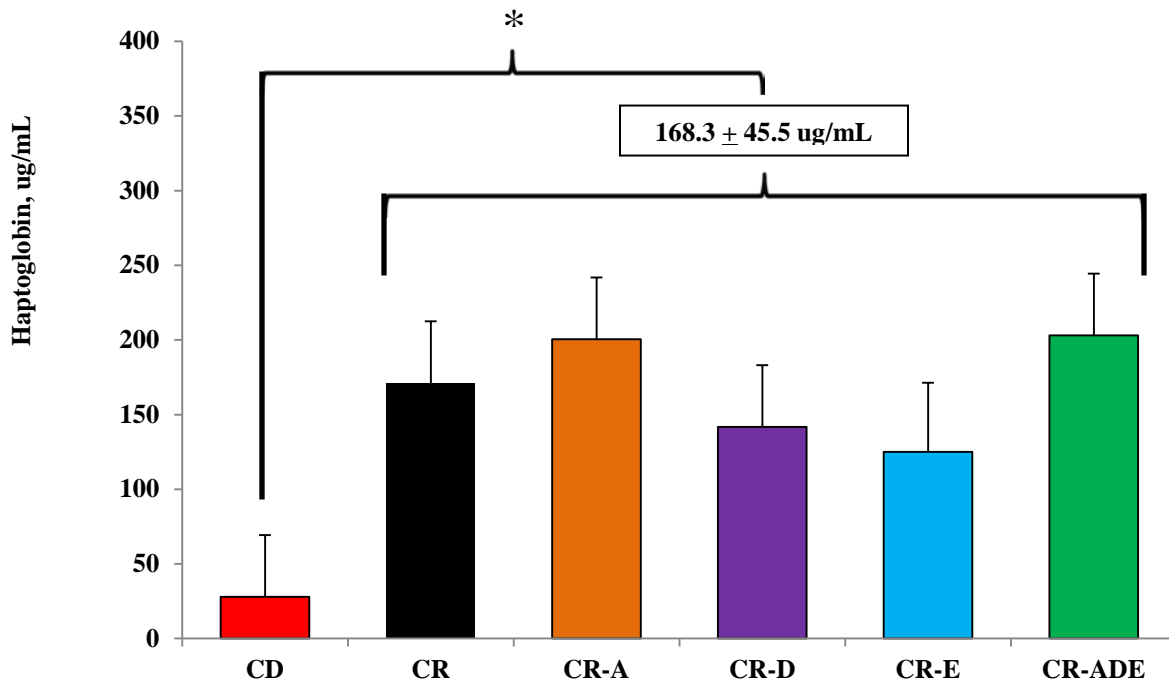
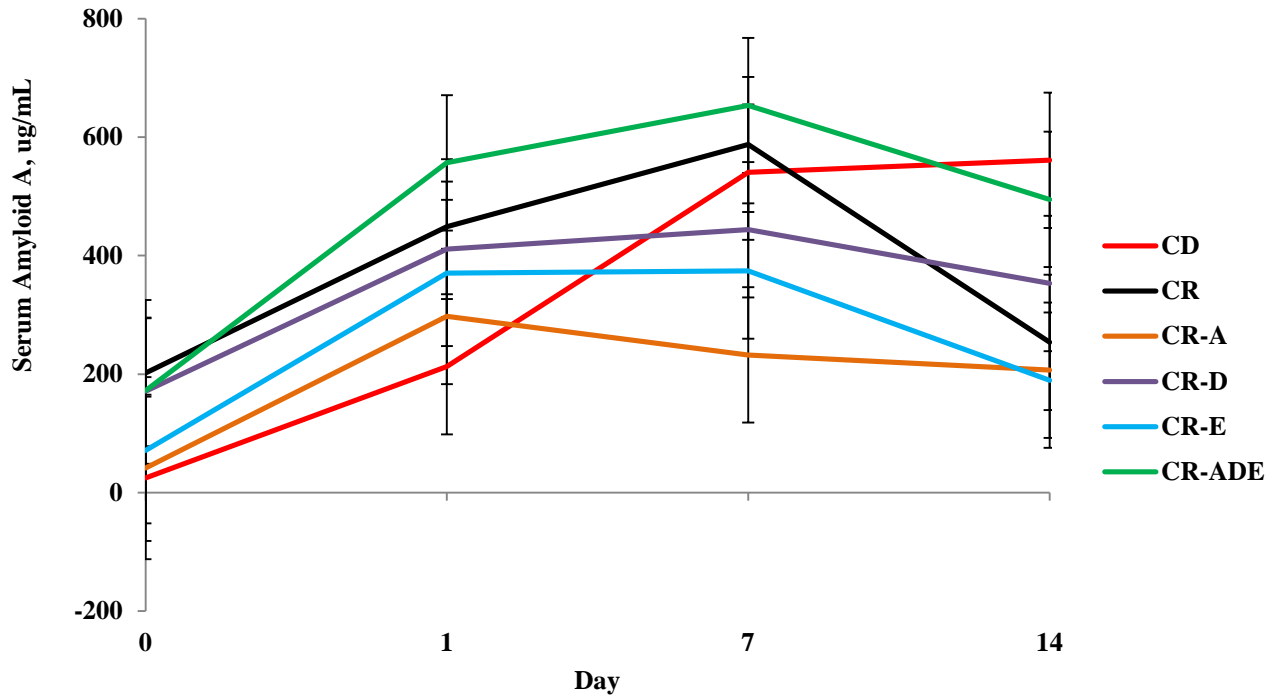


Figure 5. Concentrations of haptoglobin in serum of calves a) for all time points analyzed by repeated measures ANOVA and b) for d 1 values analyzed by statistical contrast. \* $P < 0.01$ .



a.



b.

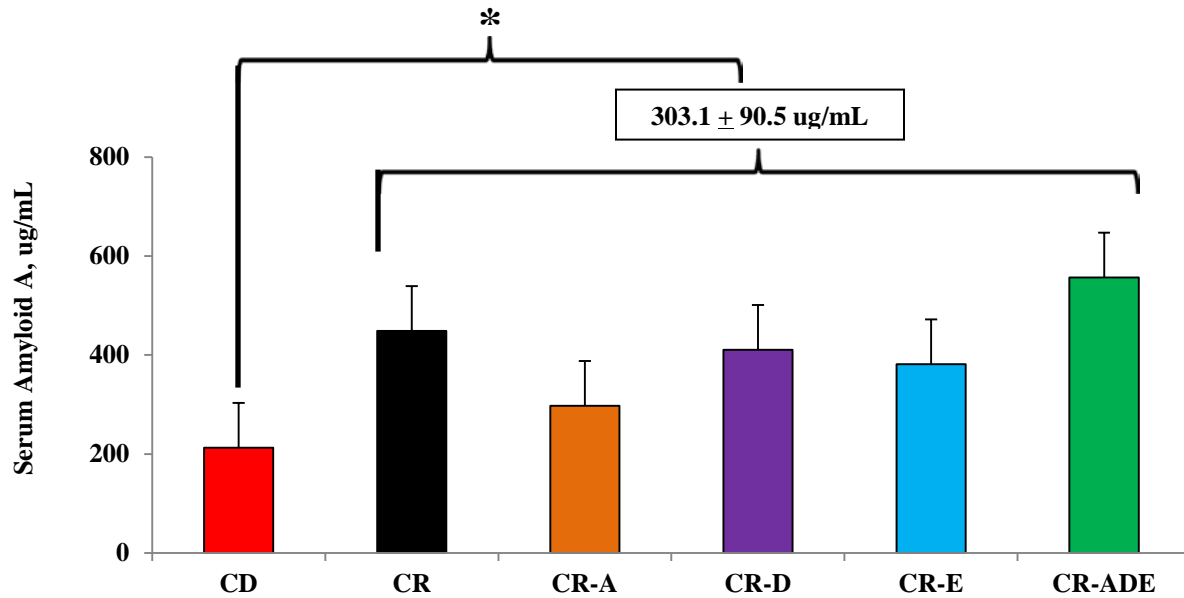


Figure 6. Concentrations of serum amyloid A in serum of calves a) for all time points analyzed by repeated measures ANOVA and b) for d 1 values analyzed by statistical contrast. \* $P < 0.05$ .