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RESEARCH ARTICLE

Responses of Blood Metabolites and Proteins to Different Vitamin A Levels in Korean Native Steers

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ABSTRACT

This study was conducted to determine the correlation between vitamin A restriction and serum metabolites as well as global proteins expression. Forty-eight animals were randomly divided into three groups and given different vitamin A supplementation: High (4.8 IU/g feed), Medium (2.43 IU/g feed) and Low (1.14 IU/g feed). Blood was collected individually via the external jugular veins. Results showed that the concentrations of Ca, total cholesterol, cortisol and cortisone, and the activity of 11beta-hydroxysteroid dehydrogenase (11-HSD) type 2 varied among the groups. An up-regulated (hibernation protein 25) and four down-regulated proteins (gelsolin a, alpha-1-microglobulin/bikunin precursor, transthyretin, and complement factor B) were identified in a proteomics study. These data demonstrated that vitamin A deficiency had considerable effects on the serum levels of several metabolites and global proteins expression and these metabolites and proteins may be used as physiological markers in the study of adipogenic differentiation prevention by vitamin A.

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INTRODUCTION

Vitamin A (retinol) and its active metabolite retinoic acid are essential for the development and function of many tissues (Beijer *et al.,* 2014). Previous studies suggested that the low serum Vitamin A level during the fattening stages III (21-23 month-old) may relate to producing high marbling score steers (Adachi *et al.,* 1999). In beef steers fed diets low in vitamin A, adipocyte differentiation and intramuscular fat deposition were increased without affecting subcutaneous adipocytes (Gorocica-Buenfil *et al.,* 2007). Moreover, slight changes in the fatty acid profile of subcutaneous adipose tissue were observed in vitamin A-restricted Angus steers, which suggested that vitamin A may affect the desaturase enzymatic activity (Gorocica-Buenfil *et al.,* 2008). In primary cultured stromal-vascular cells from bovine adipocyte, vitamin A showed a dose-dependent suppression on the adipose differentiation through completely blocking the stimulative action of thiazolidinedione (T-174) (Ohyama *et al.,* 1998). Moreover, vitamin A can suppress the adipogenesis of preadipocyte cell from porcine (Brandebourg and Hu, 2005) and ovine (Torii *et al.,* 1995). Currently, feeding a diet with appropriate vitamin A levels seemed to be a strategy for improving marbling scores on commercial Japanese beef farms (Kato *et al.,* 2011). However, little data is available on the mechanism underlying the prevention of adipogenic differentiation by vitamin A.

Generally, in the studies that aim to improve the level of intramuscular fat via vitamin A restriction of cattle, muscular tissues collected from animal carcass or biopsy are always indispensable. But none of these two sampling ways is ideal as the animal slaughter cost a lot while

biopsy lead to big stress to the animals. Therefore, alternative use of blood was explored in this study. We aimed to find some metabolites or proteins in the blood that have responses to the vitamin A restriction through proteomics and metabolomics studies.

MATERIALS AND METHODS

Experiment design, animals and sampling: Experiments conducted in this study were approved by the Animal Care and Use Committee of Pusan National University, Pusan, Korea. Forty-eight Korean native steers (14 months) were randomly divided into three groups that received three levels of vitamin A dietary supplementation (Table 1) according to a previous research (Gorocica-Buenfil *et al.,* 2007). Animals were housed in an environmentally controlled facility individually and were allowed ad libitum access to feed and water throughout the experimental period. Nine months later (23 months), blood samples were taken via the external jugular veins after the morning meal. Plasma or serum were recovered from the blood samples treated with or without heparin by centrifugation at 2,054×g at 4°C for 15 min, and stored at -80°C until required.

Analysis of vitamin A serum levels, metabolic profile test (MPT) and steroid concentration measurements: Vitamin A was extracted from serum (200 μL/sample) and dissolved in 95% methanol and subjected to High-performance liquid chromatography (HPLC) (Water 600 series; Waters Corporation, Milford, MA, USA) using a system equipped with a stainless steel Novapak C18 column (3.9 mm I.D x 150 mm; Waters Corporation) and a Water 486 tunable absorbance detector (Waters Corporation) (Siluk *et al.,* 2007). The mobile phase was 95% methanol with a flow rate of 0.8 mL/min. The concentration of vitamin A was calculated as the ratio of the individual sample area to that of a known standard (retinol, 95144, Sigma-Aldrich Corp.) after calibrating with the retinyl acetate internal standard.

The MPT was performed with a Toshiba Accute Biochemical Analyzer-TBA-40FR (Toshiba Medical Instruments, Otawara-shi, Tochigi-ken, Japan). Fifteen MPT criteria including total protein, aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyltranspepidase, blood urea nitrogen, calcium (Ca), inorganic phosphorus (IP), magnesium (Mg), high-density lipoproteins, low-density lipoproteins, total cholesterol, triglyceride, glucose, albumin, and creatinine were analyzed. All the reagents required for this procedure were purchased from Wako Pure Chemical Industries, Ltd. (Chuo-ku, Osaka, Japan).

Four concerned steroids including cortisol, cortisone, 11-dehydrocorticosterone and corticosterone were determined with an Agilent 6890 plus gas chromatograph interfaced with a single-quadrupole Agilent 5975 mass selective detector (Agilent Technologies; Palo Alto, CA, USA). Data were showed as the measured value for a pooled serum according to the levels of vitamin A dietary supplementation. In addition, the 11b-HSD type 2 activity was calculated as the ratio of 11-dehydrocorticosterone (metabolite) to corticosterone (precursor).

Two-dimension electrophoresis (2-DE): Aliquots of the pooled protein samples from animals in the high (H), medium (M), and low (L) groups were separated by the 2-DE technique. The detailed methods of ESI-Q-TOF/MS analysis and the identification of proteins were described in the previously published work (Wang *et al.,* 2013).

Statistical analysis: The data of vitamin A concentrations and metabolic profile test (MPT) are presented as Mean±SD (n=16) and analyzed using a one-way analysis of variance (one-way ANOVA, SPSS Inc., Chicago, IL, USA). In all cases, differences were considered significant if $P<0.05$.

RESULTS

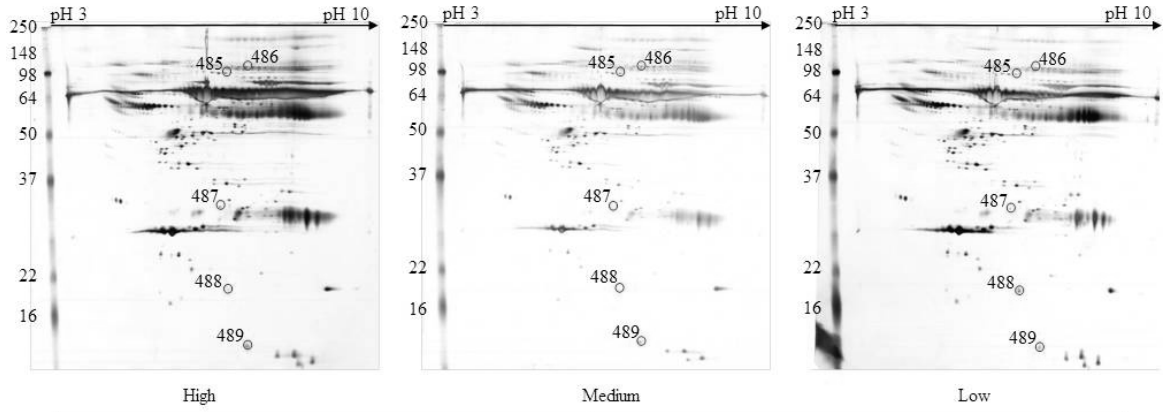
Vitamin A concentration in the Low group was significantly lower than those in the High or Medium groups ($P<0.05$). The concentrations of Ca in the Low group were significantly higher than that in the High group ($P<0.05$). The total cholesterol concentration in the Medium group was significantly higher than that in the High group ($P<0.05$). In addition, we did not observe any obvious differences in the other MPT parameters (Table 2). Among the steroids, the concentrations of cortisol and cortisone in the Low group were lower than those in the High group. Meanwhile the activity of 11-HSD type 2 in Low animals was lower than that in the High group too (Table 2). In the proteomics analysis, one up-regulated (hibernation protein 25) and four down-regulated proteins (gelsolin a, alpha-1-microglobulin/bikunin precursor, transthyretin, and complement factor B) were identified (Fig. 1; Table 3).

Table 1: Experiment design and the proximate (% dry matter) composition of the diet with vitamin A fed to 14 months old native Korean steers (n=16 in each group)

Parameters	Vitamin A levels		
	High (4.8 IU/g)	Medium (2.43 IU/g)	Low (1.14 IU/g)
Crude protein	11.50	11.50	11.50
Crude fat	4.20	4.20	4.20
Crude fiber	6.68	6.68	6.68
Calcium	0.70	0.70	0.70
Phosphorus	0.45	0.45	0.45
Total digestible nutrients	74.34	74.34	74.34
Vit. A (IU/g feed)	4.80	2.43	1.14
Dry matter	87.81	87.81	87.81
Moisture	12.23	12.23	12.23

DISCUSSION

Vitamin A concentrations detected in these groups were corresponded to the levels of vitamin A added to the different diets. A metabolic profile test (MPT) was initially designed as a presymptomatic diagnostic aid based on statistical analyses of blood chemistry to provide an early warning of certain types of metabolic imbalances. Recently, MPTs have also been used to measure physiological characteristics for selection programs designed to improve animal production traits (Kato *et al.,* 2011). In the current study, fifteen MPT criteria were analyzed in order to determine if any of these factors are correlated with vitamin A dietary restriction. The change pattern of Ca concentrations in these groups was in accordance with some previous findings showing that the



Up - regulated				Down - regulated			
No.	High	Medium	Low	No.	High	Medium	Low
488				485			
				486			
				487			
				489			

Fig. 1: Representative 2-DE images and spots corresponding to differently expressed proteins.

highest incidence of osteoporosis is found in populations with high vitamin A intake, excessive vitamin A may have toxic effects on bone mineral density (Boucher, 2003), and retinoic acid can inhibit osteoclast differentiation (Balkan *et al.,* 2011). These results demonstrated that dietary vitamin A intake may have a role in the maintaining Ca homeostasis in animals and humans. One previous report showed that when serum vitamin A concentration decrease from fattening stage 1 (less than 13 month of age, 86.8 IU/dL) to stage 4 (greater than 22 month of age, 38.9 IU/dL), the total serum cholesterol concentration increase from 125.9 to 149.3 mg/dL (Kato *et al.,* 2011).

About the concentrations of cortisol and cortisone, some different observations were reported in previous works. For instance, no consistent effect of vitamin A was found on serum cortisol concentrations in lambs (Bruns and Webb, 1990). To our knowledge few studies were focused on the responses of cortisol or cortisone or 11-HSD type 2 to vitamin A restriction in cattle. Therefore, much more works are still needed to support our finding.

HP-25 is a member of the hibernation-specific protein complex (HPc) that regulates hibernation in Siberian chipmunks (Kondo and Kondo, 1992). Originally, it was reported that the HP-25 gene is expressed in the liver of hibernating species such as chipmunks and ground squirrels, but not in a non-hibernating animals like tree squirrels (Kojima *et al.,* 2001). Recent research found that this hibernation-specific protein complex is also expressed in bovine liver and aortic endothelial cells, and it is delivered to the heart and kidneys via the blood stream (Fujita *et al.,* 2012). Results from the present study confirmed this finding. However, further studies on the correlation between vitamin A and HP-25 are still needed.

Gelsolin is an actin-binding protein that regulates the assembly and disassembly of actin filaments (Sun *et al.,* 1999). Gelsolin and another protein villin are two important Ca^{2+}-dependent actin regulatory proteins and both of them belong to the villin/gelsolin superfamily (Pestonjamasp *et al.,* 1997). Interestingly, it was thought that villin 2 may be associated with the induction of transdifferentiation and adipogenesis in bovine longissimus dorsi muscle (Jin *et al.,* 2012). However, whether gelsolin has a similar function in both transdifferentiation and adipogenesis are unknown.

Alpha-1-microglobulin/bikunin precursor (AMBP) is a lipocalin. This protein can be cleaved into three separate components including alpha-1-microglobulin, bikumin, and trypstain (Lindqvist, 1996). Alpha-1-microglobulin binds to and degrades heme, and acts as a radical scavenger as well as a reductase (Lindqvist, 1996). Bikunin inhibits serine proteases which are widely distributed in human tissues (Cui *et al.,* 1999). Finally, trypstain is a trypsin inhibitor. AMBP has been shown to interact with CD79A, a protein specifically expressed on the surface of B cells (Berggard *et al.,* 1997). It is unclear whether this interaction affects the immune modulation activity of vitamin A.

Transthyretin is a homotetramer with a dimer-of-dimers quaternary structure that has a molecular weight approximately 15.73 kDa (Hamilton and Benson, 2001). This protein gained its name from its ability to transport thyroxine and retinol in serum and cerebrospinal fluid (Monk *et al.,* 2013). During the transportation in the blood, vitamin A is first binds to retinol-binding protein (RBP) and then forms a complex with transthyretin (Hamilton and Benson, 2001). However, an excess of free transthyretin may inhibit vitamin A uptake through the RBP/transthyretin complex (Natarajan *et al.,* 1997). As expected, we found that the protein expression of transthyretin decreased as vitamin A dietary supplementation levels decreased in our study.

Table 2: Vitamin A concentrations, metabolic profile test (MPT) results, cortisol and cortisone levels, and 11beta-hydroxysteroid dehydrogenase type 2 (11b-HSD type 2) activities

Parameters	Vitamin A levels		
	High (4.8 IU/g)	High (4.8 IU/g)	High (4.8 IU/g)
Vitamin A (IU/dL)	136.38±9.93[b]	118.56±11.23[b]	81.06±8.36[a2]
Total protein (g/dL)	6.61±0.08	6.62±0.08	6.63±0.08
Aspartate transaminase (IU/L)	78.19±7.11	71.56±5.61	65.75±4.48
Alanine transaminase (IU/L)	19.56±0.64	18.69±0.62	18.31±0.62
γ-glutamyltranspepidase (IU/L)	28.88±1.67	31.56±2.59	26.63±1.97
Blood urea nitrogen (mg/dL)	12.60±0.42	12.73±0.41	12.40±0.70
Calcium (mg/dL)	10.29±0.09[a]	10.45±0.06[ab]	10.54±0.09[b]
Inorganic phosphorus (mg/dL)	9.58±0.14	9.79±0.22	9.53±0.14
Magnesium (mg/dL)	2.42±0.04	2.46±0.04	2.36±0.04
High-density lipoproteins (mg/dL)	152.71±4.94	161.86±4.40	158.21±3.08
Low-density lipoproteins (mg/dL)	58.25±8.05	58.88±6.67	62.50±6.75
Total cholesterol (mg/dL)	185.31±5.92[a]	207.06±7.56[b]	198.06±4.95[ab]
Triglyceride (mg/dL)	17.94±1.56	18.13±1.32	18.13±1.72
Glucose (mg/dL)	74.44±1.63	74.44±1.35	74.56±1.54
Albumin (g/dL)	3.73±0.04	3.76±0.04	3.70±0.03
Creatinine (mg/dL)	1.44±0.03	1.43±0.04	1.44±0.03
Cortisol (ng/mL)[1]	30.79	34.14	23.14
Cortisone (ng/mL)[1]	3.82	3.95	2.45
11b-HSD type 2 activity[1,2]	0.69	0.80	0.43

Values (mean±SD) in a row bearing different superscripts differ significantly (P<0.05).[1]Data were the measured value for a pooled serum according to the levels of vitamin A dietary supplementation; High (n=16, pooled), Medium (n=16, pooled) and Low (n=16, pooled); [2]11b-HSD type 2 activity = 11-dehydrocorticosterone (metabolite)/corticosterone (precursor).

Table 3: List of differently expressed proteins according to vitamin A supplementation levels

Protein No.	UniProtKB/ Swiss-Prot Entry	Protein Name	Theory Mol. Mass (kDa)/*PI*	Sequence Coverage (%)	Functions	Area of Spot		
						High[1]	Medium[1]	Low[1]
485	Q3SX14	Gelsolin a	80.73/5.54	22.54	Calcium regulation, Actin modulation	0.129	0.065	0.040
486	P81187	Complement factor B	85.37/7.87	16.82	Active in the complement alternate pathway	0.160	0.099	0.066
487	P00978	AMBP[2]	39.23/7.81	11.08	Protease inhibitor	0.090	0.028	0.035
488	Q2KIX7	HP-25[3]	22.56/6.96	17.92	Within the hibernation-associated family	0.050	0.008	0.125
489	O46375	Transthyretin	15.73/5.90	8.84	Thyroxine and retinol transporter	0.361	0.119	0.088

[1]Indicate the level of vitamin A dietary supplementation; [2]AMBP: alpha-1-microglobulin/bikunin precursor; [3]HP-25: hibernation protein 25.

Complement factor B circulates in the blood as a single chain polypeptide that forms the catalytic site of C3 convertase in the complement alternative pathway (Hourcade and Mitchell, 2011). Expression of complement factor B in retinal pigment epithelial cells can be up-regulated by amyloid-beta (Wang *et al.,* 2009). Variation in complement factor B gene expression is also associated with age-related macular degeneration, a common form of irreversible blindness (Gold *et al.,* 2006). However, the correlation between complement factor B and prevention of adipogenic differentiation by vitamin A still needs to be further explored.

Conclusion: These results suggested that vitamin A dietary restriction exerts considerable effects on the levels of several serum metabolites as well as the protein profiles. These findings may provide some theoretical basements for the studies about the improvement of intramuscular fat deposition via vitamin A restriction in Korean native steers.

Authors' contributions: HG conceived and designed the study. TW, KH, US, SH, MH and YC executed the experiment and analyzed the samples. TW analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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