RELATIONSHIP OF SINGLE NUCLEOTIDE POLYMORPHISM IN THE VITAMIN D-BINDING PROTEIN AND CALPASTATIN GENES WITH CALCIUM METABOLISM AND MEAT TENDERNESS FOR NELLORE CATTLE

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Abstract – Calpastatin (CAST) is a well-known inhibitor of the calpain enzymes, which affects meat tenderization. The 1,25-di-hydroxy-vitamin D_3 (1,25-OH_2-D_3) regulated by vitamin D-binding protein (DBP) have been related to plasma calcium, which may increase calpain activity and myofibrillar proteolysis. Hence, the aim of this work was to investigate a possible association of single nucleotide polymorphisms (SNPs) in the CAST and DBP genes with plasma calcium and meat tenderness in 86 Nellore cattle. The DBP SNP allele C was less frequent (3.5%) than allele T, while the CAST SNP alleles C and T had a similar frequency in evaluated animals. No association of DBP and CAST SNP with shear force was detected. For the DBP SNP, the genotypes CT had higher plasma calcium values than the genotypes TT. For the CAST SNP, the genotypes TT had the highest Myofibrillar Fragmentation Index (MFI) values and the genotypes CC had the lowest MFI values in the day 7 post-mortem (pm), while the heterozygous genotypes had MFI values similar to the CC and TT genotypes. Also, the cooking loss values for the steaks aged for 1 and 7 days pm were the highest for animals with genotypes CT. In conclusion, the DBP SNP influenced plasma calcium by possibly regulating 1,25-OH_2-D_3. The CAST SNP was associated with MFI values suggesting that CAST have a major role in the inhibition of the calpain enzymes.

Key Words – myofibrillar fragmentation, shear force, and SNP marker.

I. INTRODUCTION

Several works have related the meat toughness to a higher calpastatin (CAST) activity in *Bos indicus* cattle [1,2]. Association of single nucleotide polymorphisms (SNPs) in the CAST gene with shear force have been detected previously [3,4]. There is also evidence suggesting that other metabolic pathways could influence meat tenderness. Shear force values have varied in function of the plasma and muscle calcium [5,6], which may be regulated by the 1,25-di-hydroxy-vitamin D_3 (1,25-OH_2-D_3) [7,8]. Thus, the vitamin D-binding protein (DBP) gene may be considered a candidate gene for the calcium regulation and, subsequently, for the changes in meat tenderness. No work has investigated the relationship between SNP in the DBP gene and meat tenderness. Therefore, the aim of this work was to investigate a possible association of SNP in the CAST and DBP genes with plasma calcium and/or meat tenderness in Nellore cattle.

II. MATERIALS AND METHODS

A. Animals and sampling

Eighty-six intact and castrated Nellore beef cattle with average weight of 516 ± 39 kg and average age of 24 months were used. At slaughter, blood samples were collected during exsanguination of the animals and immediately centrifuged to obtain the plasma for the calcium analysis, while another whole blood sample was stored into a *vacutainer* tube with EDTA for the genotypes determination. Steaks from the *Longissimus lumborum* muscles were removed from the carcasses and aged for 1, 7, and 14 days post-mortem (pm) for Myofibrillar Fragmentation Index (MFI) and Warner-Bratzler shear force (WBSF) determinations.

B. DNA extraction and genotyping

Genomic DNA was extracted from blood using a salting out procedure described before [9]. Genotyping of one SNP marker for the DBP gene on 86 animals and one SNP marker for the CAST gene on 82 animals were carried out by Real-Time Polymerase Chain Reaction (RT-PCR). The pairs of oligonucleotide primers were designed and synthesized by the Applied Biosystems based on dbSNP [RefSNP: *rs136359868* (DBP gene: chromosome 6, exon 5, and C/T) and *rs135330728* (CAST gene: chromosome 7, exon 20, and C/T)] published on the National Center for Biotechnology Information (NCBI). The RT-PCR was conducted in 10 µL reaction volumes using 20 ng of genomic DNA, 0.25 µL Custom Taqman® SNP Genotyping Assays (Applied Biosystems), 5 µL Taqman® Universal PCR Master Mix (Applied Biosystems). Amplification conditions were 95ºC for 10 minutes followed by 50 cycles of 95ºC for 15 seconds, and an extension at 60ºC for 1.5 minutes.

C. Phenotypic traits

Plasma calcium was determined by an automated colorimetric method through the CA Arsezano Liquiform kit (Labtest Diagnóstica S/A, São Paulo, SP, Brazil) at the Diagnósticos Análises Clínicas laboratory.

MFI analysis was performed using 2 g of meat samples, which were homogenized with a MFI buffer (100 mM KCl, 20 mM KH_2PO_4, 20 mM K_2HPO_4, 1 mM EDTA, and 1 mM $MgCl_2$) for 30 s on 22,500 rpm using a Turratec TE 102 homogenizer, as described previously [10]. The protein concentration was determined in suspensions by the biuret method. In turn, the suspensions at a final concentration of 0.5 mg protein/mL were read at 540 nm using a spectrophotometer.

The WBSF determination was performed following previous recommendations [11]. Briefly, steaks with 2.54 cm of thickness were roasted on an oven until reaching internal temperature of 71°C. The steaks were cooled at room temperature and stored overnight at ±2°C. In the next day, cores were removed from each steak parallel to the direction of muscle fibers and analyzed using the Warner-Bratzler equipment. The results were expressed in kgf. Additionally, the weights of the steaks were recorded before and after the roasting to measure cooking losses (CL).

D. Statistical analysis

The plasma calcium, MFI, WBSF, and CL traits were evaluated with a mixed model that included fixed effects of sex condition, slaughter group, and genotype and random effects of sire and error. The age of the animals at the slaughter was included in model as a covariate. The allele substitution effects were estimated using the same model, but replacing the classification effect of genotypes by a linear regression on the number of favorable alleles (0 or 1 for the DBP SNP and 0, 1, or 2 for the CAST SNP). The data were analyzed by the PROC MIXED procedure of the SAS. When significant differences for *F* test were detected at 5%, the least squares means were separated using the *Tukey-Kramer* test.

III. RESULTS AND DISCUSSION

A. Genotypic and allelic frequencies

The genotypic and allelic frequencies for the SNP in the DBP and CAST genes are presented in Table 1. Among the animals genotyped for the *rs136359868* SNP (DBP gene), none had the genotype CC. On the other hand, all the genotypes were observed for the *rs135330728* SNP (CAST gene), where the heterozygous (CT) genotype was the most frequent. The minor allelic frequency (MAF) was observed in the DBP SNP for the allele C, while the frequency of both alleles C and T were similar in the CAST SNP.

Table 1 Genotypic and allelic frequencies of single nucleotide polymorphisms in the vitamin D-binding protein and calpastatin genes

Single Nucleotide Polymorphism	n	Genotypic Frequency (%)			Allelic Frequency (%)	
		CC	CT	TT	C	T
rs136359868[§]	86	-	7.0	93.0	3.5	96.5
rs109384915[£]	82	26.8	47.6	25.6	50.0	50.0

[§]Single nucleotide polymorphism in the vitamin D-binding protein (DBP) gene; [£]Single nucleotide polymorphism in the calpastatin (CAST) gene.

B. Genotype effects for the SNP in the DBP gene

The genotypes of the animals for the DBP SNP did not influence (*P>0.05*) the majority of the phenotypic traits (Table 2). A single exception was observed for the plasma calcium trait, which showed an effect of genotype and allele substitution (*P<0.05*).

Table 2 Influence of the single nucleotide polymorphism (rs136359868) in the vitamin D-binding protein (DBP) gene on the plasma calcium and meat tenderness

Trait	Genotype		*P*	Allele substitution effect	*P*
	CT	TT			
Ca_{plasma}	10.3 (0.18)[a]	9.9 (0.06)[b]	0.02	0.46 (0.189)	0.02
MFI_{day1}	51.1 (5.02)	56.2 (1.35)	0.33	–5.06 (5.212)	0.33
MFI_{day7}	78.6 (6.59)	77.9 (2.00)	0.91	0.79 (6.834)	0.91
MFI_{day14}	100.2 (6.84)	99.3 (1.85)	0.90	0.93 (7.105)	0.90
SF_{day1}	9.4 (0.82)	8.6 (0.22)	0.39	0.74 (0.852)	0.39
SF_{day7}	7.8 (0.59)	7.4 (0.16)	0.52	0.40 (0.608)	0.52
SF_{day14}	5.5 (0.56)	6.0 (0.15)	0.42	–0.47 (0.577)	0.42
CL_{day1}	21.7 (1.36)	21.7 (0.37)	0.96	0.07 (1.414)	0.96
CL_{day7}	25.7 (1.27)	23.9 (0.35)	0.18	1.79 (1.318)	0.18
CL_{day14}	23.3 (1.48)	23.0 (0.69)	0.83	0.32 (1.490)	0.83

Ca_{plasma} = plasma calcium (mg/mL); MFI_{day1} = Myofibrillar Fragmentation Index (MFI) at the day 1 post-mortem (pm); MFI_{day7} = MFI at the day 7 pm; MFI_{day14} = MFI at the day 14 pm; SF_{day1} = shear force (SF, kgf) at the day 1 pm; SF_{day7} = SF (kgf) at the day 7 pm; SF_{day14} = SF (kgf) at the day 14 pm; CL_{day1} = cooking loss (CL, %) at the day 1 pm; CL_{day7} = CL (%) at the day 7 pm; CL_{day14} = CL (%) at the day 14 pm; [a,b]Different lowercase letters between the genotypes differ significantly (*P<0.05*).

The substitution from allele T to allele C increased (*P<0.05*) the average plasma calcium in 0.46 mg/mL for each favorable allele. Although the genotypes CT had higher plasma calcium (*P<0.05*) than the genotypes TT, there was no improvement (*P>0.05*) for meat tenderness in genotypes CT. In this case, a higher 1,25-OH_2-D_3 concentration in animals with genotypes CT could explain the higher plasma calcium concentration through a direct effect. Quantification of the 1,25-OH_2-D_3 concentration in plasma and muscle are being conducted in our laboratory to check the possibility of a DBP SNP effect on the 1,25-OH_2-D_3 concentration. Because of the differences in plasma calcium would also be expected to find differences in MFI and WBSF values between the genotypes. However, the MFI and WBSF values may be affected by several other factors such as, for example, collagen content and solubility [12,13].

C. Genotype effects for the SNP in the CAST gene

The genotype of CAST was significant (*P<0.05*) for the MFI values at the day 7 pm and the CL values at the days 1 and 7 pm (Table 3). However, a significant additive effect (*P<0.05*) was only observed for the MFI values at the day 7 pm and for the CL values in the steaks aged for 1 day pm.

Table 3 Influence of the single nucleotide polymorphism (rs135330728) in the calpastatin (CAST) gene on the plasma calcium and meat tenderness

Trait	Genotype			*P*	Allele substitution effect	*P*
	CC	CT	TT			
Ca_{plasma}	9.8 (0.10)	9.9 (0.08)	10.0 (0.11)	0.79	0.05 (0.074)	0.49
MFI_{day1}	55.5 (2.66)	54.9 (1.97)	59.1 (2.76)	0.46	1.73 (1.959)	0.38
MFI_{day7}	72.7 (3.25)[b]	76.7 2.41)[ab]	85.0 (3.36)[a]	0.04	6.08 (2.383)	0.01
MFI_{day14}	97.2 (3.49)	98.8 (2.59)	103.9 (3.62)	0.39	3.35 (2.560)	0.19
SF_{day1}	8.6 (0.41)	8.7 (0.30)	8.7 (0.42)	0.99	0.05 (0.298)	0.87
SF_{day7}	7.5 (0.30)	7.5 (0.22)	7.1 (0.31)	0.45	–0.20 (0.223)	0.37
SF_{day14}	6.2 (0.27)	5.8 (0.20)	5.6 (0.28)	0.32	–0.30 (0.198)	0.13
CL_{day1}	20.1 (0.70)[y]	22.2 (0.52)[x]	22.2 (0.72)[xy]	0.05	1.05 (0.519)	0.05
CL_{day7}	23.3 (0.67)[b]	25.2 (0.51)[a]	23.2 (0.69)[b]	0.02	0.06 (0.500)	0.91
CL_{day14}	23.3 (0.95)	23.1 (0.79)	22.4 (0.94)	0.69	–0.44 (0.560)	0.43

Ca_{plasma} = plasma calcium (mg/mL); MFI_{day1} = Myofibrillar Fragmentation Index (MFI) at the day 1 post-mortem (pm); MFI_{day7} = MFI at the day 7 pm; MFI_{day14} = MFI at the day 14 pm; SF_{day1} = shear force (SF, kgf) at the day 1 pm; SF_{day7} = SF (kgf) at the day 7 pm; SF_{day14} = SF (kgf) at the day 14 pm; CL_{day1} = cooking loss (CL, %) at the day 1 pm; CL_{day7} = CL (%) at the day 7 pm; CL_{day14} = CL (%) at the day 14 pm; [a,b]Different lowercase letters between the genotypes differ significantly (*P<0.05*). [x,y]Different lowercase letters between the genotypes differ significantly (*P*=0.06).

A substitution from allele C to allele T increased (*P<0.05*) the average of the MFI values at 7 days pm and the CL values for the steaks aged for 1 day pm in 6.08 and 1.05%, respectively. The genotypes TT had the highest MFI values (*P<0.05*) and the genotypes CC had the lowest MFI values (*P<0.05*) in the day 7 pm, while the heterozygous genotypes had MFI values similar (*P>0.05*) to the genotypes CC and TT. A lower myofibrillar fragmentation for animals with genotypes CC could be attributed to a higher CAST activity, which inhibits calpain enzymes and decreases proteolysis pm [14]. The CAST activity is also being conducted in our laboratory to check if there is an association of CAST SNP with CAST activity, which can support the MFI results. Another report already showed association of CAST SNP with CAST activity [4].

Higher CL values for the steaks aged for 1 and 7 days pm found in animals with genotypes CT may suggest meat with lower water-holding capacity inherent to those animals, since steaks weight before the roasting and cooking time did not differ (*P>0.05*) among the genotypes (data not shown).

IV. CONCLUSION

In this preliminary study, the minor allele (C) of DBP SNP was only present in a small number of heterozygotes (MAF = 0.035). However, there did appear to be an effect on plasma calcium. This could be ascribed to a possible effect of DBP SNP on the 1,25-OH_2-D_3. A larger sample will be analyzed to confirm this effect. The CAST SNP was at a frequency of 0.5 and allele T was associated with high MFI values at 7 days pm and high CL values for the steaks aged for 1 day pm. The CAST SNP may have affected MFI, because CAST has a major role in the inhibition of the calpain enzymes.

ACKNOWLEDGEMENTS

The authors would like to thank the "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" for funding this work.

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