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Characterization of the TRPC3 Gene in Myotonic Goats: Further Insight Into *Myotonia congenita* and Muscular Dystrophy

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Abstract

Myotonia congenita (Mc) is a muscle disorder seen in both Myotonic goats and humans, caused by mutations in the chloride ion channel gene (CLCN1). Calcium signaling has been coupled to the function of the CLCN1, but this interaction is not well understood, as individuals with Mc do not experience muscular dystrophy (MD). Over expression of the Transient Receptor Cation Channel 3 (*TRPC3*), a protein responsible for calcium influx and muscle contraction causes an elevation in calcium which results in a phenotype of MD. Evaluation of the *TRPC3* gene in Myotonic goats has not been conducted. Therefore the objective of this experiment was to evaluate gene expression of the *TRPC3* gene in Myotonic vs. Spanish goats (control). Total RNA was isolated from whole blood samples. Cross species primers were designed from the human, bovine, and mouse *TRPC3* cDNA alignments. The goat partial *TRPC3* gene showed 98%, 92% and 91%, and 100%, 98%, and 98% nucleotide and amino acid sequence homology to the bovine, human and mouse *TRPC3* genes respectively. Quantitative Real Time PCR showed that gene expression of *TRPC3* was 77% higher ($P<0.05$) in Myotonic than Non-Myotonic (Spanish) goats. Male Myotonic goats expressed 67% higher levels ($P<0.05$) of TRPC3 than females. The *TRPC3* gene expression was 90% higher ($P<0.05$) in Myotonic goats older than 4 years of age. These data indicate that the *TRPC3* gene is a potential biomarker to further study *Myotonia congenita* in Myotonic goats and the interrelationship of the mechanism of calcium signaling in human Mc and MD.

Keywords: TRPC3, gene expression, *Myotonia congenita* Goat

1. Introduction

1.1 Significance of the Problem

The Myotonic goat (“falling goats”, “stiff legged goats”, epileptic goats, or fainting goats), was first known as the oldest recognized animal model for inherited muscular dystrophy (MD) (Bryant, 1979), since they exhibit an inherited disorder called *Myotonia congenita* (Mc). Myotonia is characteristic of hyperexcitability of the muscle cell membrane. Humans with this disorder often have prolonged muscle contractions and are unable to relax certain muscles after use (Curran & Keating, 1994; George, Crackower, Abdalla, Hudson, & Ebers, 1993; Koch et al., 1992). Myotonic disorders are classified either non-dystrophic or dystrophic myotonias. The non-dystrophic myotonias thus far have been shown to only involve the muscle system, whereas the dystrophic myotonias exhibit multisystem involvement concurrent with additional muscle weakness. Genes involved in Mc are the muscle voltage-gated sodium and chloride channel genes SCN4A ("Dinucleotide repeat polymorphisms at the SCN4A locus suggest allelic heterogeneity of hyperkalemic periodic paralysis and paramyotonia congenita," 1992; Du et al., 2012; Ruscák, 1997; Shirakawa, Sakai, Kitagawa, Hori, & Hirose, 2002) and CLCN1 (Bernard, Poulin, Puymirat, Sternberg, & Shevell, 2008; Grunnet et al., 2003; Wijnberg et al., 2012; Zhang, Bendahhou, Sanguinetti, & Ptácek, 2000), the Myotonic dystrophy protein kinase (DMPK) gene (Meola & Cardani, 2014), and the CCHC-type zinc finger, nucleic acid binding protein gene CNBP (Meola, 2013; Sun et al., 2011). In most forms of MD, membrane damage occurs after prolonged muscle contraction (Blake, Weir, Newey, & Davies, 2002; Petrof, Shrager, Stedman, Kelly, & Sweeney, 1993), which causes a delayed relaxation of the muscles (Beck, Fahlke, & George, 1996). Like humans (Curran & Keating, 1994; Skov, de Paoli, Lausten, Nielsen, & Pedersen, 2014), Mc in goats is attributed to a mutation in a chloride ion gene (clcn1) (Beck et al., 1996; Bryant & Conte-Camerino, 1991). Positively charged sodium ions signal the brain's message for the muscle cells to contract, while negatively charged chloride ions, tell the muscle cells to

relax. *Myotonia congenita* results from an abnormal channel of chloride ions, which throws this relationship out of equilibrium. The muscle cells end up with more than enough sodium but not enough chloride, which causes abnormal repetitive electrical signals from the brain resulting in stiffness. Like the Myotonic goat, humans with Mc, do not exhibit obvious muscle wasting as seen with MD, and in most cases live a normal life span. This has led to a transition from viewing the Myotonic goat as an identical model to study human MD, to a renewed interest in what causes non-muscle wasting in this form of Mc. However, though there is a Myotonic phenotype, conflicting studies have shown that on one hand, Mc is not related to MD, as the characteristic muscle wasting does not occur, and on the other hand, loss of CLCN1 function is due to binding of the clc-1 mRNA to elevated levels of a CUG splicing regulator protein in skeletal muscle of individuals with Type 1 MD (Charlet-B et al., 2002). Other studies have found that mutations in both the CLCN1 and CNBP coexist in individuals with MD (Sun et al., 2011), indicating that there is a need for further understanding of the mechanism of Mc. Studies suggested that decreased calcium release or increased uptake may protect Myotonic goat muscle from destructive changes of calcium overload, which has been proposed to be a common factor for dystrophic change (Atkinson, Swift, & Lequire, 1981; Millay et al., 2009; Skov et al., 2014; Skov, Riisager, Fraser, Nielsen, & Pedersen, 2013; Swift, Atkinson, & LeQuire, 1979)**.** It has been shown that disruption in calcium influx causes malfunction in muscle contraction (Allen, Gervasio, Yeung, & Whitehead, 2010). Furthermore, calcium alone is enough to induce dystrophy in skeletal muscle in mice (Millay et al., 2009). The Transient Receptor Potential Canonical proteins, particularly 3 (*TRPC3)* is involved in calcium cycling (Gonzalez-Cobos & Trebak, 2010) and muscle contraction (Ambudkar, 2009; Gailly, 2012; Tsvilovskyy et al., 2009). Over expression of TRPC3, coupled with an increase in calcium influx results in a phenotype of MD nearly identical to that observed in disease animal models lacking the cytoskeletal protein dystrophin (Millay et al., 2009)**.** It was shown that inhibition of TRPC channels in mice considerably reduced calcium influx and dystrophic characteristics associated with a mutation in the dystrophin gene (mdx) (Vandebrouck, Martin, Colson-Van Schoor, Debaix, & Gailly, 2002; Whitehead, Yeung, & Allen, 2006). Experiments have shown that mouse mdx muscles are susceptible to stretch-induced damage caused by a series of stretched contractions resulting in a prolonged increase in resting intracellular calcium concentration. This is caused by calcium entry through stretch-activated channels via TRPC genes. This causes opening of the stretched channels thereby allowing calcium entry. The end result is significant muscle damage and consequently MD (Allen et al., 2010). The fact that TRPC3 is directly implicated in MD, and Mc is somewhat interconnected with calcium signaling, warrants further study.

1.2 Justification

The TRPC channels and muscular disease has resurrected interest in calcium signaling mechanisms relative to Mc (Beech, 2005; Guibert, Ducret, & Savineau, 2011; Nilius & Owsianik, 2011; Nilius, Voets, & Peters, 2005). We have isolated a partial sequence of the *TRPC3* gene in Myotonic goats (Accession: HQ847409). Gene expression of *TRPC3* in Myotonic goats has not been evaluated. Therefore this study was conducted to first isolate a partial sequence of the goat TRPC3 gene and evaluate gene expression of the *TRPC3* in Myotonic goats using quantitative Real Time PCR.

2. Method

2.1 Animals and Experimental Design

Animals used for the study were housed at Virginia State University Randolph farm in accordance with animal care and use guidelines. A total of 20 Spanish and Myotonic goats (5 males, 5 non- pregnant females of each breed), grazing pasture and supplemented with hay, cracked corn and ground soybean meal were selected. Spanish goats were used to represent the non-Myotonic or non-Mc goats in the study.

2.2 Blood Collection and Serum Preparation

Goats were adequately restrained and blood collected via jugular venipuncture. In brief, the vein was identified by palpation and visual inspection. The area was swabbed with 70% alcohol. Gentle pressure was applied at the thoracic inlet to produce distension of the vein. Blood samples (3 ml) were collected in vials without coagulant using 16-20G needles. Blood samples were subsequently stored at -80^{o}C for later molecular analysis.

2.3 Total RNA Isolation from Goat Blood

Total RNA was isolated from selected goat whole blood samples previously stored at -80°C using the modified RNA extraction protocol (Gauthier, Madison, & Michel, 1997). Approximately 500 µl of each sample was mixed with 3 ml of 1X Dulbecco's phosphate buffered saline (PBS) (Invitrogen; Grand Island, NY 14072) and centrifuged at 5,000 rpm for 20 minutes at 4°C in a Heraeus MegaFuge 16R centrifuge (ThermoFisher-Scientific). After discarding the supernatant, the addition of PBS and centrifugation was repeated until cells were clean. Once the cells were sufficiently washed, 1 ml of guanidinium thiocyanate solution (4M GTC, 25 mM Sodium citrate pH

7.0 and N-0.5% lauroylsarcosine, Sigma-Aldrich) was added and the cells were resuspended by pipetting. Under the fume hood, 10 microliters of β-mercaptoethanol (Omni-Pur), 2M sodium acetate (pH4) at one-tenth of the volume and 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Fisher Scientific; Fair Lawn, New Jersey 0740) were added and mixed before placing on ice for 15 minutes. The tubes were then centrifuged for 15 minutes at 4°C at 5,000 rpm. After centrifugation, the aqueous layer was removed and placed in a sterile tube. The samples were placed on ice for 10 minutes, 2 volumes of 95% ethanol added, and centrifuged for 15 minutes at 5,000 rpm, allowing RNA to precipitate. Two additional washes with 15 minutes of centrifugation at 5,000 rpm were completed using 200 µl of 70% ethanol before allowing samples to air dry. Samples were then resuspended in Diethylpyrocarbonate (DEPC) treated water and stored at -80°C. A NanoDrop 2000 Spectrophotometer (ThermoScientific) was used to measure RNA concentration and purity.

2.4 Isolation of Goat TRPC3

A comprehensive screening of the GenBank nucleotide databases was performed. The *TRPC3* sequences (bovine, human, mouse) were retrieved from the GenBank and sequence alignments generated using CLC Main WorkbenchBioinformatics software (clcbio.com). Oligonucleotide primers were designed from conserved regions of mRNA of the bovine human, and mouse *TRPC3* nucleotide sequence alignments. Primers and target regions used for isolation of the goat *TRPC3* gene are given in Table 1. The RT-PCR was conducted using the recommended protocol of the Verso 1-step RT-PCR kit (Thermo Scientific). Modified thermocycling conditions for 40 cycles were as follows: 50^{o}C 15 minutes, 95^{o}C, 2 minutes (initial denaturation), 95^{o}C, 30 secs, 55^{o}C, 1 minute, 72^{o}C, 1 minute repeated 39 times and a final extension at 72^{o}C for 5 minutes. The target *TRPC3* cDNA was visualized by 1.5% agarose gel electrophoresis and a UGenius UV gel documentation system (SynGene, Fredericksburg, MD) equipped with a high resolution CCD camera.

Table 1. Primers and Target Regions used for Isolation of the goat TRPC3 gene from goat whole blood

Accession No.	Primer name	Conserved Primer sequence	Target Region	Fragment Length (bp)
NM001104960	Forward	AGGATGACAGTGATGTAGA	2033-2057	213
	Reverse	ACCTGGACTTTGAGTTAC	2228-2245 (Rev-comp)	
NM001130698			2326-2538	
NM019510			2407-2619	

Table showing a summary of the GenBank Accession numbers of the TRPC3 genes used for nucleotide alignments and design of oligonucleotide primers from conserved regions among the species (*Bos taurus, Homo sapiens, Mus musculus*). The CLC Main Workbench Bioinformatics software was used to generate primers.

2.5 Nucleotide Sequencing of Goat TRPC3 cDNA

For *TRPC3* nucleotide sequencing, the cDNA (213bp) products were cut out and purified from agarose gels (Qiagen and Bio-Rad). Samples were sent for sequencing at GeneWiz (South Plainfield, New Jersey). Raw nucleotide sequences were analyzed using sequence analysis software (NCBI-BLAST, CLC Main Workbench). Subsequently, quantitative Real Time PCR (qRT-PCR) analysis was conducted to determine gene expression of goat *TRPC3*.

2.6 Measurement of Goat TRPC3 Gene Expression

Gene expression of *TRPC3* in whole blood was measured using (qRT-PCR). The analytical parameters were as previously published (Corley & Ward, 2013). In brief, the analysis was conducted using the iScript One Step RT-PCR kit with SYBR Green (BioRad). For each reaction 100ng of total RNA was used. The Actin gene was used as an internal standard for relative quantitative analysis of *TRPC3* gene expression.

2.5 Statistical Analysis

All data were analyzed using the General Linear Model procedure of SAS. Means were considered significant at the 5% level of probability using Duncan's Multiple Range Test.

3. Results and Discussion

3.1 Isolation and Analysis of Goat TRPC3

This study aimed to accomplish two objectives. Since the TRPC3 gene had not yet been identified in goats, our first aim was to isolate a partial sequence of this gene in the goats. After RNA isolation from goat blood, RT-PCR was performed. Agarose gel electrophoresis was performed to visualize the goat *TRPC3* cDNA. The cross species

primers designed to target the Spanish and Myotonic goat *TRPC3* genes successfully amplified the expected 213 bp fragments (Figure 1). The goat *TRPC3* cDNA was sequenced and compared to the bovine, human, and mouse *TRPC3* genes to identify sequence similarities. The BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990) of the goat *TRPC3* cDNA showed 98% and 92% and 91% sequence homology to the bovine and human and mouse *TRPC3* genes respectively (Figure 2). The BLASTp (Altschul et al., 1997) of the goat *TRPC3* amino acid alignment of the translated 213 bp nucleotide sequence was compared to the bovine, human, and mouse TRPC3 showing 100%, 98% and 98% homology respectively (Figure 3).

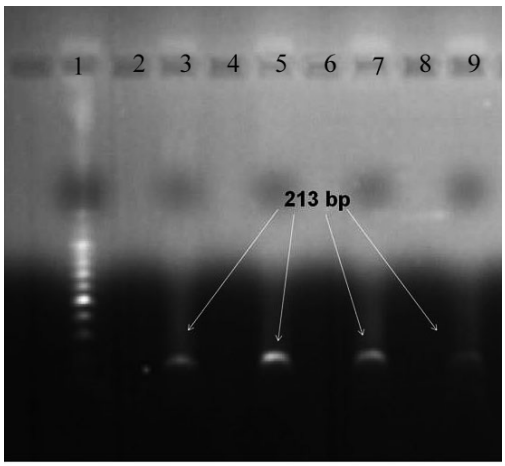


Figure1. Agarose Gel Electrophoresis of Goat TRPC3 cDNA after Reverse Transcriptase PCR

Gel electrophoresis of Goat TRPC3 cDNA. The gel is 1.5% agarose with 0.05mg/ml ethidium bromide.

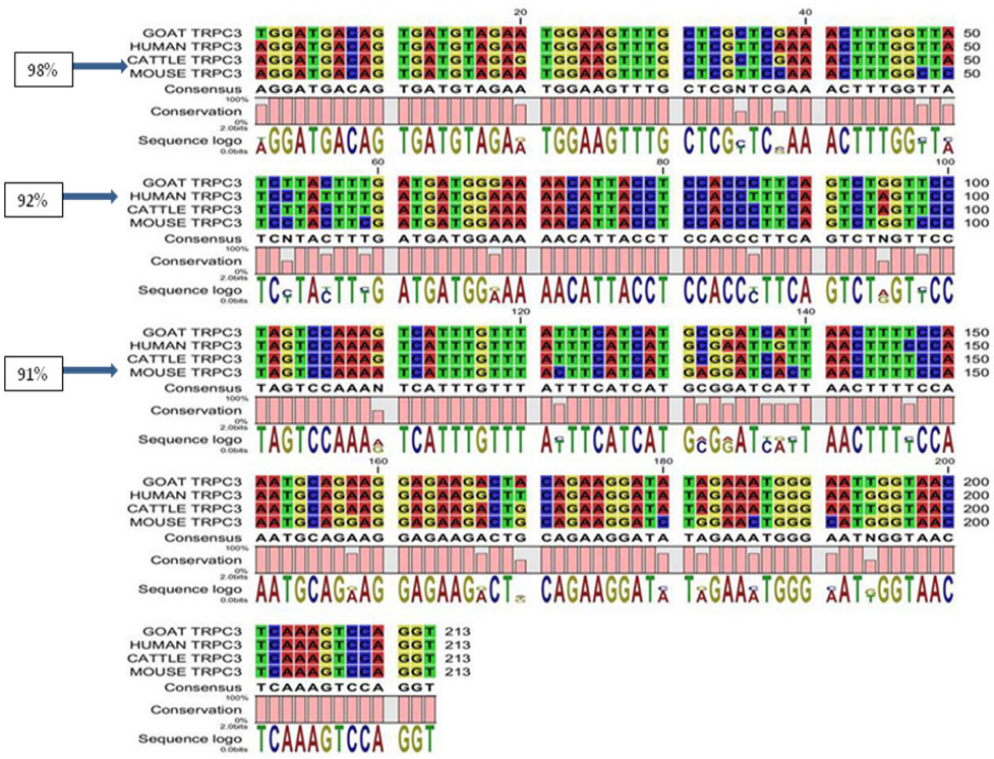


Figure 2. Nucleotide Sequence Alignment Showing Homology of Goat, Human, Bovine, and Mouse TRPC3 Genes

Nucleotide sequence alignment of partial goat TRPC 3 gene is shown. Alignment is shown to validate the conserved regions used to design the cross species oligonucleotide primers. Generated using CLC Main.

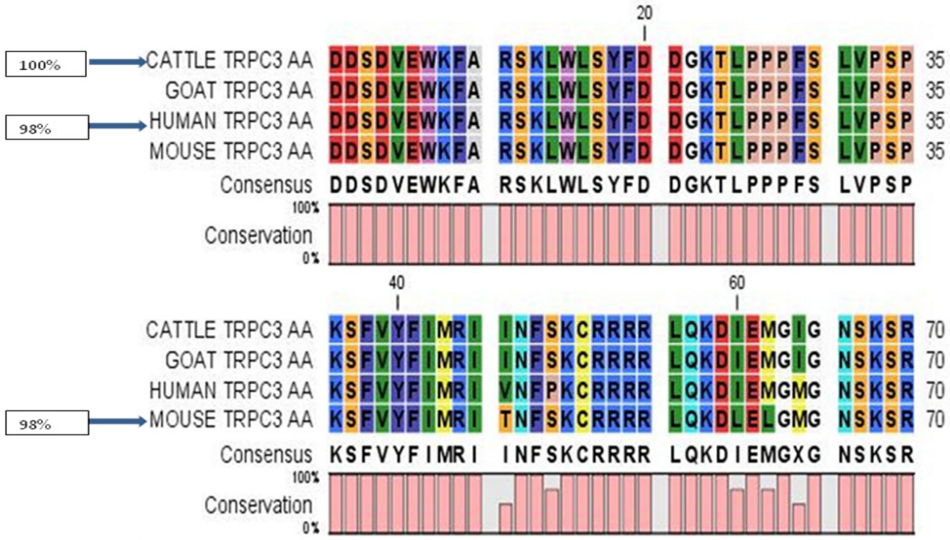
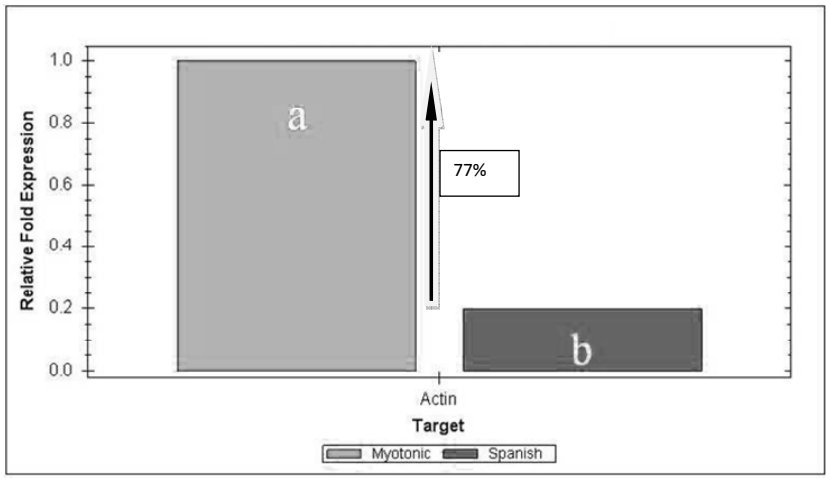


Figure 3. Amino Acid Alignment of Translated Goat, Human, Bovine, and Mouse TRPC3

Amino acid translated sequence alignment of partial goat TRPC3 gene is shown. Alignment is shown to validate the conserved regions used to design the cross species oligonucleotide primers. Degeneracy lends to a greater amino acid sequence homology of the partial TRPC3 genes. Generated w CLC Main Workbench Bioinformatics software Percentages represents the degree of homology among partial amino acid sequences of the TRPC3 gene.

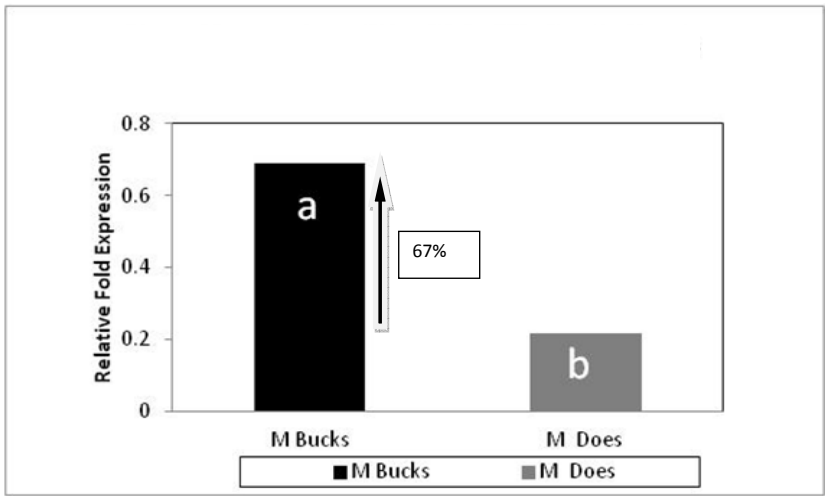
3.2 TRPC3 Gene Expression

Our second aim was to measure gene expression of goat TRPC3 after nucleotide sequence verification of the target gene. Spanish goats (non-Myotonic) versus Myotonic goats were evaluated. Quantitative Real Time PCR successfully amplified the *TRPC3* gene in Spanish and Myotonic goats. The *TRPC3* gene was expressed 77% higher ($P<0.05$) in Myotonic goats when compared to non-Myotonic (Spanish) goats (Figure 4). This observation seems to be in line with previous studies in mice which demonstrated that an over expression of TRPC3 was correlated with characteristics of MD (Millay et al., 2009). Though Myotonic goats do not exhibit obvious muscle wasting (Bryant, 1979), there seems to be some involvement of the calcium signaling mechanism in goats that exhibit Mc as opposed to those that don't, as indicated by the TRPC3 gene expression data of this study. Myotonic males had 67% higher ($P<0.05$) gene expression of *TRPC3* than females (Figure 5). The Myotonic goats used in this study were sexually mature non-pregnant females and intact males. It has been shown that Mc is more severe in males than females, (Burge, Hanna, & Schorge, 2013), studies have shown that progesterone and testosterone inhibit CLCN1 channels containing the mutation F297S associated with dominantly inherited Mc, but is a non-genomic effect (Fialho, Kullmann, Hanna, & Schorge, 2008). Higher gene expression of the TRPC3 gene in males versus females in this study coincides with the severity of Mc in males with non dystrophic Mc. This further broadens the scope of insight into the mechanism of calcium signaling and muscle excitation or contraction in Mc. We evaluated gene expression of TRPC3 within two age groups of Myotonic goats; those younger and older than 4 yrs of age. These goats were exhibiting Mc, which is usually first manifested between 20 days to 6 months of age (Bryant, 1979). The *TRPC3* gene expression was 90% higher ($P<0.05$) in Myotonic goats older than 4 yrs old (Figure 6), indicating some form of progression of increased expression of TPRC3 over time. This could be indicative of the calcium cycling homeostasis depreciating with age in goats with Mc. It has already been documented that a high influx of calcium is enough to cause the onset of MD in mice (Millay et al., 2009), though Myotonic goats' Mc has been widely accepted as only the nondystrophic form. Molecular genetic studies have documented a coexistence of both CLCN1 mutations (characteristic of non dystrophic Mc), and CNBP (characteristic of MD) (Sun et al., 2011). Therefore further studies need to be conducted to determine the relationship between TRPC3, CLCN1 and CNBP in Myotonic goats. Such studies can then open the door for further study on the molecular characterization of these genes (TRPC3, CLCN1 and CNBP), and how they relate to Mc and MD. Older non-Myotonic goats (Spanish) also expressed higher levels ($P<0.05$) of TRPC3, but trended lower than that of Myotonic goats (Figure 6).



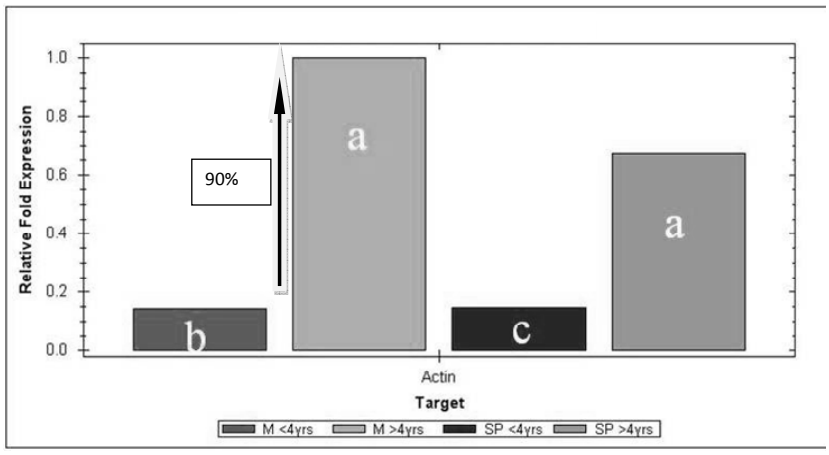
a, b means with different superscripts differ at P<0.05

Figure 4. Gene Expression of *TRPC3* in Myotonic and Non-Myotonic (Spanish) Goats



a, b means with different superscripts differ at P<0.05, n=10

Figure 5. Gender Effect on Gene Expression of *TRPC3* in Myotonic Goats



a, b means with different superscripts differ at P<0.05

Figure 6. Age Effect on Gene Expression of *TRPC3* in Myotonic Goats

4. Conclusion

The results of this study indicated that the cross species oligonucleotide primers designed from the conserved regions of the *TRPC3* genes used to amplify the goat *TRPC3* can be used on a blood sample derived from the cattle and mouse, but most importantly the human. It is clear that though TRPC3 is involved in MD, it may also be interrelated with the nondystrophic Mc in Myotonic goats. These data will serve as a basis for further study on calcium signaling in Mc in humans and other species, and also contribute to the beginning stages of the study of the goat *TRPC3* gene as a genetic marker for Mc. The Myotonic goat may then serve as a model not only for Mc, but a potential model to study other calcium signaling related neuromuscular disorders.

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