QUANTITATION OF OVINE PROTEIN GENE PRODUCT 9.5 (ovPGP 9.5) FROM HEAD-STUNNED DOHNE MERINO

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Abstract – The aim of the current study was to quantitate the expressions of the ovine protein gene product 9.5 (ovPGP 9.5) as a pain biomarker from head-stunned Dohne Merino at slaughter. Maxima SYBR Green/ROX qPCR Master reaction mix Green 1 and a LightCycler® carousel-based system of the Real-time PCR was used for the quantitation of the ovPGP 9.5 from the blood samples of the stunned sheep. The blood samples used for the analysis were collected after exsanguination from the castrates and ewes. Results showed that the application of 110volts across the head during stunning led to higher (27.2%) expressions of ovine ovPGP 9.5 mRNA by castrates against 22.7% from the ewes. The interactions between age and gender of the sheep were found to significantly ($p < 0.001$) contribute to the post-stunning secretion of ovPGP 9.5 mRNA and the expression of pain signals observed on the animals. It was observed that "head-only" stunning is not a zero pain-free approach and that castrates experienced more pains during stunning than the ewes. The quantitation of ovPGP 9.5 mRNA therefore suggested that it is a potential biomarker for detecting pain in head-stunned ovine species.

Key Words – Biomarker, Real time RT-PCR, Sheep stunning

- INTRODUCTION

Protein gene product 9.5 (PGP 9.5), also known as ubiquitin carboxyl-terminal hydrolase-1 (UCH-L1), is a 27-kDa protein originally isolated from whole brain extracts [1]. The activity of PGP 9.5 is so important to remove ubiquitin from partially degraded proteins and thus allowing the ubiquitin monomer to be recycled. Regulation of the ubiquitin pathway is very important as many physiological conditions are associated with defects in this pathway. Although PGP9.5 expression in normal tissues was originally felt to be strictly confined to neurons and neuroendocrine cells [2], evidence has not been provided hitherto that its expression could indicate pain when sheep are electrically stunned prior to exsanguination. In recent studies however, it has been shown that Dohne Merinos are one of the topmost rated sources of mutton, lambs and offal in Southern Africa [3,4]. Prior to its conversion to meat at the Halal abattoir, the slaughter of this dual purpose breed is usually preceded by "head-only" stunning [3]. This practice is actually supposed to exonerate the stunned animals from avoidable pains but observations at the abattoir have failed to confirm this supposition. Hence, the reason for the advocacy on the use of brain specific biomarkers for ascertaining whether electrical stunning is pain free or not [5]. Since PGP 9.5 is heavily expressed in neurons and the great abundance of this protein means that it is released from neurons in large amounts following injury or degeneration [6], thus the current study attempted its quantitation as a biomarker coding for pain when Dohne Merino sheep were electrically stunned at the abattoir.

- MATERIALS AND METHODS

Ethics Approval and Data Collection: The present study was conducted with the consent of the Research Ethics Committee of the University of Fort Hare, South Africa (UFH/UREC, 7-REC-270710-028). Data was generated at a high-throughput Halal abattoir where "head-only" electrical stunning was used. The average ages of the stunned Dohne Merino were 11 and 36 months for castrates and ewes, respectively. Prior to bleeding, the sheep were arranged

on a single line, held in a restraining conveyor, and were eventually stunned in the head by applying 110volts. During exsanguination, blood samples (5 to 10 ml) were collected from the jugular vein of each ewe (n=30) and castrate (n=30) into heparinised vacutainer tubes before the assay.

Total RNA Extraction and Primers Design*:* The
total mRNA was extracted rapidly from the blood samples using the Zymo Whole-Blood RNA MiniPrep™ kit (Zymo Research). This kit was chosen for having the ability to extract high quality RNA (A260/A280 >1.8, A260/A230 >1.8) suitable for all downstream RNA-based manipulations. Maxima SYBR Green/ROX qPCR Master Mix was optimized for its ability to produce sensitive and specific quantification of genomic, plasmid and cDNA templates. The Master Mix was used with the real-time thermal cyclers [LightCycler® 480 SYBR Green I Master (LightCycler® 480 instrument) and LightCycler® FastStart DNA MasterPLUS SYBR Green I (LightCycler® carousel instrument)] that were chemically modified by the addition of heat-labile blocking groups to amino acid residues. The design process for the primers did not follow a conventional route (Reference Patent Number: PA156691/P).

- RESULTS AND DISCUSSION

The results from this study showed that within the same nucleotide region of 24bp, the sequence for the forward primer (5'-TCCGGGTCTCATCTGTCTCCTCCT-3') and the reverse primer (3'-CGTCCATCTTCCAGTTGCTTGCCA-5') were in range of 9-42 and 231-208, respectively. The GC-content of 58.33% for the forward primer and 54.17% for the reverse primer were within the normal GC range of 45-60% portraying high annealing strength. The result of using SYBR Green Master mix in the temperature-dependent dissociation between DNA-strands revealed a typical primer-dimer formation. It is clear from the foregoing that at a higher inflection point (Tm), 50% of the primer was annealed. This result therefore indicated the stability of interaction between the primer-target gene and indicating a rise in absorbance intensity (hyperchromicity) that produced the desired single stranded PGP 9.5 mRNA amplicon.

The melting curve indicating positive ovPGP 9.5 mRNA expression (after stunning) was attained at 82.13^{o}C Tm (Figure 1).

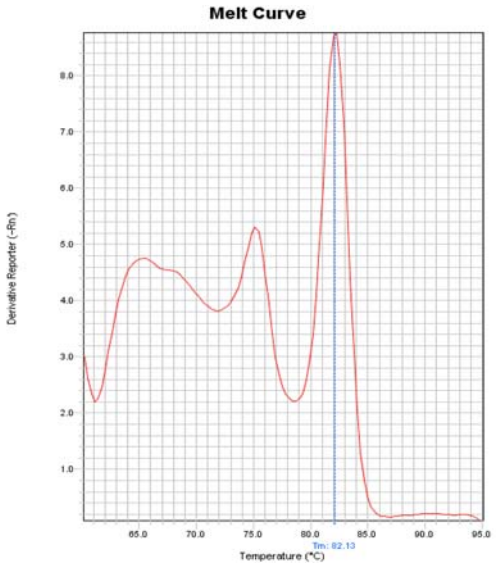

Figure 1 Melt curve from Dohne Merino sheep with positive post-stunning expressions of ovPGP 9.5

mRNA

The amplitude of the stunning voltage (101volts) and the flow of current during stunning resulted in the collapse of the animals in the range of 2-5seconds for most of them. The effects of electric insults on the animal produced higher expressions of ovPGP9.5 mRNA in the castrates. Results further showed that age and sex of the sheep significantly ($p < 0.001$) interacted to influence post-stunning expression of pain signals from castrates and ewes (Table 1). The effects of electric insults on the animal produced higher (27.2%) **)** expressions of ovPGP 9.5 mRNA in the castrates (Figure 2) which possibly suggests that the sensory receptors in the animal vary depending on their age and sex of the animal.

Table 1 Effects of age and gender on the expressions of PGP 9.5 mRNA by stunned Dohne Merino

Parametres	[1]Sig.
Sex	NS
Age	NS
Sex*Age	***

[1]Significant at $p < 0.001$; NS: Not significant $p > 0.05$.

The expression of ovPGP 9.5 mRNA in the current investigation has revealed that pain experienced by castrates and ewes might have been influenced by the differences in the pain receptivity. As earlier reported in literature[7], the amount of this protein product detected was directly linked with the melt peak resolution and the area under the curve of a melt peak. This findings is thus in agreement with earlier study [8] where the generation of a single product during a melt run suggested an association between a time-temperature binding pattern of the SYBR green 1 and the growth of the peak. Another study as well has associated the elevated UCH L1 mRNA expressions with abnormal blood-brain barrier function [9] while attributing neuronal vulnerability to neuronal injury caused by electrical or mechanical insults [10,11]. It could be argued therefore, that the release of electrical impulses in response to affective-motivational mechanism at the tonic phase triggered some nociceptions in the animal.

Thus, the transmission of these emotional signals possibly initiated sensory transduction by the receptor leading to the manifested as pain and the expression of ovPGP 9.5 mRNA in the sheep. Moreover, the present study has revealed a major weakness of “head-only” stunning with positive expressions of ovPGP 9.5 mRNA in about 50% of the Dohne Merino castrates and ewes. This further confirmed that the passage of current through the head sometimes may not produce a stunned state in the animal [12]. Prior to exsanguination, physical activities that might be resulting from ineffective stunning might be responsible for positive expressions of ovPGP 9.5 mRNA in the sheep especially the castrates.

Figure 2 Levels of PGP 9.5 mRNA expressions in castrates and ewes after “head-only” stunning

- CONCLUSION

The findings from this study have revealed high annealing strength since the GC-content for

the forward primer and the reverse primer were within the normal GC range of 45-60%. It has also shown that the bio-impedance of the castrates to electrical stunning is comparatively low compared to the ewes of the same species. The current investigation has given evidence that ovPGP 9.5 mRNA is a reliable biomarker for pain quantitation in ineffectively head-stunned sheep.

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