Effect of Zilpaterol and Ractopamine on biometric parameters and muscle fiber thickness in Pelibuey lambs

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Scientific article
Received: April 25, 2021
Accepted: January 19, 2022


ABSTRACT. The objective of this study is to evaluate the effect of β-agonists (BA), Zilpaterol Hydrochloride (ZH), and Ractopamine Hydrochloride (RH) on the biometric parameters and the muscle fiber thickness in Pelibuey lambs (n = 6). The control group was fed a standard diet (16% protein content and 12 MJ per day); treatment groups received a standard diet supplemented with ZH or RH for 37 days and was assessed presence of BA in samples of muscle tissues, and urine. The effect of Daily Weight Gain (DWG) and Food Conversion Rate (FCR) on muscle fiber thickness was determined by Scanning Electron Microscopy (SEM) and Digital Image Analysis (DIA). Results showed an increase of 30% in DWG in the ZH group, compared with RH. While FCR was lower in ZH (3.83) than in RH (4.85). In both BA treatments, the withdrawal time was sufficient to minimize drug concentration in meat. In contrast with the control group, muscle fiber thickness increased in BA treated groups (ZH = 69.2%, and RH = 15.6%). In conclusion, ZH displayed a higher carcass yield in Pelibuey lambs.

Key words: β-adrenergic, digital image analysis, meat, Pelibuey sheep.

RESUMEN. El objetivo de este estudio fue evaluar el efecto de los β-agonistas (BA), Clorhidrato de Zilpaterol (ZH) y el Clorhidrato de Ractopamina (RH) sobre los parámetros biométricos y el grosor de la fibra muscular en corderos Pelibuey (n = 6). El grupo control recibió una dieta estándar (DS) (16% de contenido de proteína y 12 MJ por día), mientras que los grupos de tratamiento recibieron DS+ZH o DS+RH durante 37 días. Se evaluó la presencia de BA en tejido muscular y orina, determinó el aumento de peso diario (DWG), la tasa de conversión de alimentos (FCR) sobre el grosor de la fibra muscular, mediante microscopía electrónica de barrido (SEM) y análisis de imagen digital (DIA). Los resultados mostraron 30% más en DWG en el grupo ZH, que en RH. Mientras que la FCR fue menor en ZH (3.83) que en RH (4.85). El tiempo de supresión aplicado fue suficiente para que los grupos ZH y RH no mostraran residuos. En contraste con el grupo de control, el grosor de las fibras musculares aumentó en los grupos tratados con BA (ZH = 69.2% y RH = 15.6%).

Palabras clave: β-adrenérgico, Análisis Digital de imágenes, carne, Borrego pelibuey.
INTRODUCTION

Meat is one of the most nutritious foods due to its supply of high biological value proteins, lipids, B-complex vitamins, and minerals like iron, zinc, and phosphorus (McAfee et al. 2010). In the last decades, the increase of the worldwide population has generated a higher demand for meat products (fresh, refrigerated, frozen, or processed) despite their high economic cost. The most consumed meat products, in descending order, are beef, lamb, mutton, poultry and pork (Boler et al. 2012, Sans and Combris 2015). To meet the consumers’ demand, countries such as Mexico, Brazil, and the United States of America (USA) have approved the use of growth promoters in livestock to increase carcass yield (Giannetti et al. 2016, Parr et al. 2016, Chu et al. 2017). β-agonists (BA) are highly utilized in the meat industry, including Zilpaterol Hydrochloride (CA) and Ractopamine Hydrochloride (RH) however, Clenbuterol Hydrochloride (CC) remains unregulated by food safety authorities (Mondragón et al. 2010, Montes-Nino et al. 2017, Ma et al. 2018).

Due to their anabolic effects, BA at high doses can increase lean muscle mass in animals (Whipple et al. 1990, Domínguez-Vara et al. 2009). These compounds are analogs of adrenaline and noradrenaline, derived from phenylethanolamines, thus differ in structure from the endogenous catecholamines in the aromatic ring and the amino group (Giannetti et al. 2016, Parr et al. 2016). These present powerful pharmacological activity in humans acting as bronchodilators and tocolytic agents (Al-Majed et al. 2017), but when administered to livestock species, they promote growth and weight gain (Parr et al. 2016). β-2 adrenergic receptor agonists such as clenbuterol and cimaterol, produce the greatest effect on muscle growth (Parr et al. 2016); RH binds to both β-1 and β-2 adrenergic receptors and produces similar results (Rikard-Bell et al. 2009). Zilpaterol or terbutaline also improve the growth rate and the feed efficiency of lambs under thermo-neutral conditions (Avendaño-Reyes et al. 2011, Avendaño-Reyes et al. 2018). Few reports exist on the effects of BA on muscle fibers and the effectiveness of withdrawal time in sheep (Hernández-Martínez et al. 2014, Costa-Lima et al. 2015, Dávila-Ramírez et al. 2015). The objective of this study is to evaluate the effect of Zilpaterol hydrochloride (ZH), and Ractopamine hydrochloride (RH) on biometric parameters and muscle fiber thickness in Pelibuey lambs.

MATERIALS AND METHODS

Experimental Animal Testing

Eighteen Pelibuey male lambs aged 2.5 months and weighing 18 ± 1 kg were acquired from Rodeo Ranch in Jalapa municipality, state of Tabasco, in south-eastern Mexico. Animals were dewormed with closantel 10% (Digavet, Belgium), 1 mL oral albendazole per 10 kg of body weight (Pfizer, USA) and treated with an intramuscular injection of vitamins A, D and E (2 mL 10 kg⁻¹ of bodyweight, Pfizer, USA) following the technical specifications of the products and per accordance with the UK Animals (Scientific

Lambs were fed with a standard diet (SDiet) -12 MJ per day with 16% protein- formulated with milled corn, whole wheat, soy paste, grass, coconut paste, molasses, and a supplement of minerals and vitamins (AFRC 1993). Water was provided ad libitum through 5 L dispensers. After being weighed, animals were randomly divided into three groups of six lambs each, according to the BA they received: SDiet or negative control, ZH group = SDiet+ZH (Zilmax, Merck Sharp & Dohme Corp, USA), and RH group = SDiet+RH (Lapi-Racto. REG. SAGARPA Q-20183-158, Lapisa). Dosage of growth promoters was given in accordance to the NOM-EM-015-Z00-2002 (SAGARPA 2002) and the technical specifications of the product (C115 g ZH, and 125 g RH). The experiment lasted 40 days: growth promoters were supplied daily for 37 days plus the withdrawal period of 72 h. All feeding was withdrawn 12 h before slaughter. The response variables were final live weight gain (FW), average daily weight gain (ADG), feed conversion ratio (FCR), and feed consumption for 40 days. In addition, urine samples were taken from each animal (days 0, 10, 20, 30, and 40) and placed in sterile assay tubes, frozen immediately by using liquid nitrogen and stored at -20 °C until analysis.

All lambs were slaughtered following the Mexican Official Standard on animal welfare, NOM-033-ZOO-1995 (SAGARPA 2014). For each animal, 350 g of different cuts from the hindquarter were stored at -20 °C until analysis.

**Extraction of BA from muscle samples**

To extract the BA, 100 g of muscle was blended with 40 mL of acetonitrile and 20 mL of isopropanol during 30 s. NaCl (1.2 g) was added to the blend, mixed on vortex for 2 min, supplemented with 4 g of Na₂SO₄ and 0.5 g of MgSO₄ and mixed again for 2 min (Water, 2012). Lastly, the samples were centrifuged (Hermle Model Z323K, Germany) at 1960 g for 10 min. The supernatant was dried using a rotary evaporator (Büchi R-215, Labortechnik, Switzerland). The pellet was dissolved in 1 mL of 0.1% formic acid, sonicated for 2 min, passed through cartridges (Oasis MCX 3 cc Vac Cartridge, 60 mg Sorbent per cartridge, 30 µm, Waters, USA) and filtered (Membrane Filter, 0.45 µm and 0.22 µm pore size, Merck Millipore, Ireland) (Water 2012).

**Determination of BA in urine and meat samples**

Identification and quantification of growth promoters (BA) were determined through HPLC using a five-point calibration curve of the standards in the following concentrations for each group: ZH (0.47, 0.23, 0.11, 0.05, 0.02 g mL⁻¹ diluted in 50:50 methanol: water), and RH (0.66, 0.33, 0.16, 0.08, 0.04 g mL⁻¹ diluted in 50:50 methanol: water) (Sumano et al. 2002). After extraction, samples were analyzed according to the USDA (2012). Urine samples were injected directly after filtration (0.45 µm). The standards were filtered (0.45µm) and injected into the HPLC (HPLC 1200 (Agilent Technologies, Germany), at 4°C, in a C-18 column (ZORBAX Eclipse column, XDB 80Å C18, 4.6 x 150 mm, 5 µm, Agilent, USA), with a flow of 20 µL min⁻¹ at 210 nm to 280 nm wavelengths, using as mobile phase (A) water and 1% formic acid, and as a gradient (B) acetonitrile and 1% formic acid (Waters 2012).

**Light microscopy**

Micrographs of the meat samples from each treatment group were taken through optical microscopy. Samples were cut in 5 mm sections and placed in 35 mL of fixative FAA (30 mL of 40% formaldehyde, 150 mL of ethanol, 15 mL of acetic acid, and 105 mL of distilled water) for 72 h. Afterwards, they were washed and dehydrated with water: ethanol (3:1, 1:1, 1:3) and absolute ethanol, for 40 min. Samples were placed in xylol: ethanol (3:1,1:1,1:3) until absolute xylol was reached, during 20 min; then embedded in paraffin: xylol, set in a microscope slide, and stained with haematoxylin-eosin. Longitudinal and transverse sections were observed under LEYCA RAM optical microscope at 60X, 150X, and 300X (Arzate-Vázquez et al. 2011).
Scanning Electron Microscopy (SEM)

Using a scalpel blade no. 10, muscle tissue cuts were made to obtain longitudinal and transversal sections (1.0 x 1.0 x 0.5 cm). These were fixed in 4% formaldehyde and 25% glutaraldehyde, later washed, and dehydrated with water: ethanol (25:100). Finally, the sample sections were dried until critical point in an SPI-Module Sputter (SPI-supplies, West Chester, PA), carbon coating equipment at 34-35 °C and 1200 psi, and covered with gold for 40 s (Estrada-Solis et al. 2016). Micrographs were taken at 5 kV through scanning electron microscope (JSM-7800F, JEOL, Japan).

Digital Image Analysis (DIA)

Ten micrographs were analyzed with ImageJ software (1.52q, National Institutes of Health, USA) to determine muscle thickness. The scale was set to 2.55 pixels/µm based on SEM imaging (5 kV, 200X magnification) previously obtained, and the images threshold was automatically adjusted by the software (approximately 100). Average muscle fiber thickness was determined from a minimum of 20 muscle fibers per micrograph using ImageJ software measuring tool.

Statistical analysis

The results of biometric parameters and muscle fiber thickness were analyzed with an ANOVA test using Sigma Plot 12.5 software (Systat Software, USA) and reported as average ± standard error. The contrast of means was performed through the Tukey Test, assuming a significance level of 0.05.

RESULTS

In vivo assessment of β-agonists in lambs

Table 1 shows a greater DWG in the animals fed with BA (93 and 49% for ZH and RH, respectively), compared with the control group, during the 37 days of BA treatment. DWG pairwise comparisons between all three groups were statistically significant (p ≤ 0.05). FCR was lower in ZH in contrast to RH and the control group (p ≤ 0.05). FE was higher in the ZH group (26.00%) than in the RH group (20.63%), (p ≤ 0.05).

Kinetics and urinary excretion of β-agonists

Firstly, to detect BA on muscle samples, the UV spectrum of each standard was obtained using a UV detector of 190-380 nm; maximum wavelengths for standard samples were: ZH = 210 nm, 8.9 min retention time and RH = 280 nm, 12.49 min retention time.

Secondly, the concentration of BA excreted in urine was evaluated. Higher urinary excretion of BA occurred in the first 10 days of treatment (Table 2). At day 40, the excreted amount of BA was minimal (ZH = 0.14 mg mL⁻¹ and RH = 0.0039 mg mL⁻¹), indicating that a pre-slaughter withdrawal period of 72 h allows proper elimination of ZH and RH.

Determination of β-agonist in meat samples

Analysis of meat samples through HPLC reported the absence of ZH and RH residues, probably due to their urinary excretion and the withdrawal period (72 h) before slaughter.

Optical microscopy of lamb samples

Optical microscopy allowed to characterize different cellular structures of lamb samples - to assess the effect of BA on their lipid content. Optical micrographs of the transverse sections of the lamb samples reflect the intensity of lipolysis in the BA treatment groups, compared to the control group (Figure 1).

SEM of lamb samples

Figure 2 displays morphological aspects of lamb meat samples in the treated groups. Elongated muscle fibers surrounded by dense connective tissue (epimysium) were identified in groups ZH and RH (1). While the control group, showed contracted fibers with a corrugated appearance (2). this effect may indicate stress on the lambs during slaughter. Abundant connective tissue was detected in ZH treatment samples (3).

Muscle thickness

200 muscle fibers were measured with DIA.
Table 1. Biometric parameters of lambs fed with a control diet and BA-supplemented diet over a 40-day experiment.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Control</th>
<th>ZH</th>
<th>RH</th>
</tr>
</thead>
<tbody>
<tr>
<td>IW (kg)</td>
<td>18.03 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.94 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.01 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FW (kg)</td>
<td>25.3 ± 0.84&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.94 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.01 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWG (g)</td>
<td>181.66 ± 19.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>350.00 ± 18.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>270.00 ± 20.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FCR</td>
<td>7.83 ± 0.66&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.83 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.85 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FE%</td>
<td>13.63 ± 1.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.25 ± 1.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.63 ± 0.79&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

IW = Initial weight, FW = Final weight, DWG = Daily weight gain, FCR = Feed Conversion Ratio, FE = Food efficiency, ZH = Zilpaterol Hydrochloride, RH = Ractopamin Hydrochloride. Results correspond to the mean of n = 9 ± SE. Different letters in the same row indicate statistically significant differences (Tukey Test, p < 0.05).

Table 2. Kinetics of BA present in the urine of lambs treated with two growth promoters.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>ZH (µg/mL)</th>
<th>RH (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.86 ± 0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.06 ± 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.81 ± 0.005&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.88 ± 0.005&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.09 ± 0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.38 ± 0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.14 ± 0.005&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0039 ± 0.009&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

ZH = zilpaterol hydrochloride, RH = ractopamin hydrochloride. Results correspond to the mean of n = 9 ± SE. Different small letters in the same row indicate statistically significant differences (Tukey Test, p < 0.05) between treatments. Different capital letters in the same column indicate statistically significant differences (Tukey Test, p < 0.05).

Figure 1. Kinetics of BA present in the urine of lambs treated with two growth promoters.

software using the longitudinal-section micrographs obtained by SEM. Results show that muscle fiber thickness may be proportional to drug bioavailability. This seems to be correlated with the observations under optical microscopy (Figure 1) in which RH treatment might increase fat content in lamb samples. A deeper study is currently in progress to assess differences in fatty acids content of the samples in the BA-treated groups.

**DISCUSSION**

**In vivo assessment of β-agonists in lambs**

Positive effects in DWG were observed from day 13 of BA supplementation due to their capacity to stimulate muscle hypertrophy (Mersmann 1998) through inhibiting proteolysis and lipogenesis, stimulating protein synthesis, and redirecting energetic substrates towards muscle accretion (Mills 2002, Chu *et al*. 2017, Claffey *et al*. 2018). In the
present study, DWG was higher than reported by López-Carlos et al. (2010) who fed sheep a diet of 28% alfalfa and 47.65% yellow corn, which supplied 22 and 9% of raw protein into the diet, respectively. Differences in weight gain can be caused by multiple factors, for instance, the quality and amount of dietary protein, duration of treatment, sex, and hygiene (Parr et al. 2016, Tang et al. 2016, Rivera-Villegas et al. 2019). DWG reflects lean muscle mass gain, because BA act on the calpain-calpastatin complex, diminishing protein degradation (Dávila-Ramirez et al. 2015, González-Ronquillo and Angeles-Hernández 2017, Smith et al. 2019). These results are consistent with Resendiz et al. (2013), which declared that a high FE is an indicator of the efficiency and profitability of productive systems.

**Kinetics of β-agonists excretion in urine**

According to Waters (2012) and Morales-Trejo et al. (2013), selective analyses can reliably identify growth promoters in biological matrices of ruminants such as liver, meat, and urine. BA absorption depends on the administration pathway; drug levels in plasma and blood are higher after oral dosing than intramuscular administration. After reaching effective concentrations in muscle, BA are excreted into the urine (Meza-Márquez et al. 2012). Similarly to present findings, Van Hoof et al. (2005) reported urinary excretion of RH and ZH in calves; the maximum elimination rate (12.6 µg mL⁻¹) of both compounds occurred after the first 5 days of treatment. For this reason, urine is a reliable biological matrix to detect and quantify the elimination rate of BA (Van Hoof et al. 2005).

In all species, BA are mostly excreted through urine (50-58%), but a smaller portion are eliminated through feces (5-30%), and milk (0.9-3%) as non-metabolized products (Ma et al. 2018). Sumano et al. (2002) indicated that BA are bio transformed and rapidly inactivated by Catechol-O-Methyltransferase (COMT) enzyme, which methylates the hydroxyl groups on the aromatic ring. RH is metabolized by hepatic glucuronidation; hence, it is not a substrate for COMT.

**Determination of β-agonists in meat samples**

Lamb samples at day 40 were BA-free, after the withdrawal period recommended in the technical specifications for using ZH and RH. Mondragón et al. (2010), López-Carlos et al. (2011) and Avendaño-Reyes et al. (2018), agree that BA are fully eliminated from meat samples after 72 h.

**Optical microscopy of lamb samples**

Gianetti et al. (2016) indicated that the decrease of lipids or lipolysis in sheep-produced by lipases that hydrolyze triacylglycerols to glycerol and free fatty acids-facilitates glycolysis, oxidation, and the tricarboxylic acid cycle, which can derive in the synthesis of proteins. Vahedi et al. (2014) manifested...
that the addition of BA to the diet of ruminants increases muscle mass and decreases adipose tissue (depending on the cut area).

**SEM of lamb samples**

Meat has a complex structural organization, and its components impact its quality (Lu et al. 2000). Moreover, meat morphological structure indicates the post-mortem treatment to which the animal was subjected, this may modify quality attributes, such as tenderness (Li et al. 1999). Romero-Peñuela et al. (2011) stated that stress can only be measured by biomarkers or cortisol levels. The present study’s findings revealed that in ZH groups the sarcoplasm was more compressed (F), compared with RH and control, which showed open basal cisterns that function as calcium storage. After the death of the animal, calcium is released to activate endogenous enzymes such as calpains and catechins that improve meat tenderness (Rubio-Lozano et al. 2011).

**Muscle fiber thickness**

The greater muscle fiber thickness was observed in the ZH group, which may be due to the bioavailability of the treatments. RH bioavailability ranges between 45-50% in ruminants, and 88% in pigs; while ZH bioavailability is 80% in ruminants (Sumano et al. 2002, Estrada-Solis et al. 2016). Results suggested that muscle fiber thickness is correlated with the yield in weight gain, as reported by Chulayo and Muchenje (2016), who described that the use of growth promoters in ruminants improved protein synthesis, hence, greater muscle thickness. As observed in Table 3, the percentage of increase in fiber thickness was higher after ZH treatment (69.2%) than after RH intervention (15.6%) compared to control. The reason beneath this significant difference is directly related to the increase that RH produced in FW respect to the control, this is, RH increased 14.7% the weight of the sheep, but such increase seems to be explained not only by the increase in muscle fiber thickness because the end percent increase of this parameter was the lowest after RH treatment.

### Table 3. Statistical difference found in muscle fibre thickness and DWG between treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean muscle thickness (µm)</th>
<th>increase of thickness (%)</th>
<th>increase of FW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.3 ± 0.997</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ZH</td>
<td>24.2 ± 1.104*</td>
<td>69.2 ± 7.6</td>
<td>26.2 ± 0.51</td>
</tr>
<tr>
<td>RH</td>
<td>16.54 ± 0.522c</td>
<td>15.6 ± 3.6</td>
<td>14.7 ± 0.27</td>
</tr>
</tbody>
</table>

DWG = Daily weight gain, Control, ZH = Zilpaterol Hydrochloride, RH = Ractopamin Hydrochloride groups. Results correspond to the mean of n = 9 ± SE. Different letters in the same column indicate statistically significant differences (Tukey, p < 0.05).

Since the same diet was provided to all lambs, differences in muscle fiber thickness and weight gain depended only on the type of BA that was supplied; like the results of Giannetti et al. (2016) and Rivera-Villegas et al. (2019) on using ZH in cattle to promote weight gain. Furthermore, RH treatment increased muscle mass and fat simultaneously, probably due to the rapid metabolism of this BA (Sumano et al. 2002).

**CONCLUSIONS**

Utilizing BA at the dosage recommended by the manufacturers ensures the obtention of BA-free meat products, as they are efficiently excreted through urine. When comparing the treatment groups, ZH gave better results in weight gain and muscle fiber thickness. The BA, and particularly ZH, proving that it is more apt to achieve better performance lamb carcass yield. Using SEM and DIA, the effect that BAs have on the thickness of the muscle fiber was evidenced, showing that these techniques can be a tool in the evaluation of the quality of the meat if what you are looking for is to confirm its organic quality.

**ACKNOWLEDGEMENTS**

Authors thank the financial support provided by SIP Project 20196662, and CONACyT through a Ph.D. scholarship (Exp. 451044).
LITERATURE CITED


