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# MODE OF ACTION AND METABOLIC PATHWAYS OF CLEMBUTEROL IN BOVINE

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**Abstract:** Clenbuterol considered as a powerful broncho-vase-dilator, used to control respiratory diseases, both in animals and in humans; Clb is a ß$_2$-adrenergic agonist (ß$_2$-AA), which causes an increase in muscle mass in birds, pigs, cattle and sheep, however, the metabolic routes to date are not well elucidated; The objective of this study was to determine the metabolites that arise with the intake of clenbuterol and its possible derivatives that are stored in the liver, pancreas, adrenal gland, muscles and genital organs, mainly in cattle. Fat content is dramatically reduced when clenbuterol is used as an anabolic, meaning anabolic, when Clb is administered orally or intramuscularly above the therapeutic dose, between 5 and 10 times its therapeutic concentration (0.8µg/ body weight). twice daily). For this study, 17 different metabolites were measured to determine which metabolic parameters are disturbed, preventing homeostasis at the level of the different organs in which it intervenes. The study showed that there are several metabolites that are altered by clenbuterol, among these we have: glucose, triglycerides, alkaline phosphatase, gamma glutamyl transferase, transaminases (ALT/ GPT y AST/GOT), lactic dehydrogenase, prostatic and non-prostatic acid phosphatase, cholesterol, and calcium. This indicates that it has an effect on altering the metabolism of several metabolic pathways, contributing to its lipolytic and antilipogenic activity and induces nitrogen retention, increases glycolysis, lactate production and oxygen consumption, increases levels of glucose, which varies according to the time of treatment, since at the level of the pancreas insulin decreases and adipocytes are less sensitive to this hormone, there is an increase in the use of energy, bringing with it an increase in body temperature-thermogenesis.
**Keywords:** Bioavailability, biotransformation, metabolic profile, metabolic pathways, **β**-adrenergic agonist (**β**AA).

## INTRODUCTION

The use of ß-agonists, mainly clenbuterol (Clb), zilpaterol and ractopamine, are producing an increase in intoxications in humans and animals, mainly due to the excessive use of these components that are used as food additives (Sumano et al., 2002, Caicedo et al., 2009, Caicedo et al., 2021). On the other hand, other components have also been developed that improve the body quality of animals such as: antibiotics, prebiotics, enzymes, antimicrobials, immune system modifiers, metabolic modifiers or anabolic agents. In recent years, the use of β2-adrenergic agonists (ß-AA) in economically important animals has increased; Consider that the excessive use of this drug such as clenbuterol (Clb) has had and continues to have a very significant impact at the human and animal level (increase in intoxications and animal welfare, both are altered, respectively). Its use increases meat production in the short term, in three months the live weight of the animal can be increased by 50-80% (Caicedo et al., 2021), since they retain nitrogenous compounds, increasing muscle mass (Smith, 1998). βAR agonists increase lipid-degrading metabolism in adipocytes in vitro and in vivo. In mammalian tissues, three distinct subtypes of βARs currently exist: β1 (β1AR), β2 (β2AR), and β3 (β3AR). Individual tissues (eg liver, heart) have different proportions of subtypes, this will depend on the vertebrate species, and it varies between different species. Consequently, certain βAR agonists are expected to have different effects in the same tissue in different species due to the different distribution of βAR subtypes and/ or amino acid sequence (Mersmann, 2002); For this reason, it can be added that the effects produced by a βAR agonist (or antagonist) in

adipose tissue in vivo depend not only on the species and distribution of the adipocyte βAR subtype, but also on the pharmacokinetics and pharmacodynamics of the adipocyte. compound in that species, including blood flow to the tissue, and the compound's multiple metabolic and endocrine effects on other tissues in the body (Mersmann, 2002).

These β-AA drugs are also chemical agents that act specifically at the level of cellular adrenergic receptors (β-AR), metabolizing nutrients and energy, increasing the metabolism of proteins and fats, modifying the permeability of the cell membrane, increased lipolysis, and glycogenolysis (Meyer and Rinke,1991), (**figure 1**).

In addition, they bring with them an increase in the formation of muscle mass, and this is due to the fact that the OH group that other ß-AA possess, in the case of Clb, is replaced by a halogen called chlorine (Cl), this Cl ion prevents the biotransformation by COMT (catechol-O-methyl-transferase) enzymes at the tissue level and liver biotransformation slows down (Courtheyn et al., 1996), this chlorine ion in clenbuterol makes it more fat-soluble than its analogs (zilpaterol, salbutamol and ractopamine) and as a result tends to diffuse deeper into animal tissues and fat (Martin 1971, Ruffolo, 1991, Waldeck and Widmark, 1995). **Figure 2**.

The application of ß-AA to mammals amplifies weight gain, this is possibly due to the increase in the amount of tRNA (transfer ribonucleic acid) for several skeletal muscle proteins, in this case, after treatment with ß-AA mRNA for myosin light chain (Smith et al., 1998), mRNA for a-actin (Helferich et

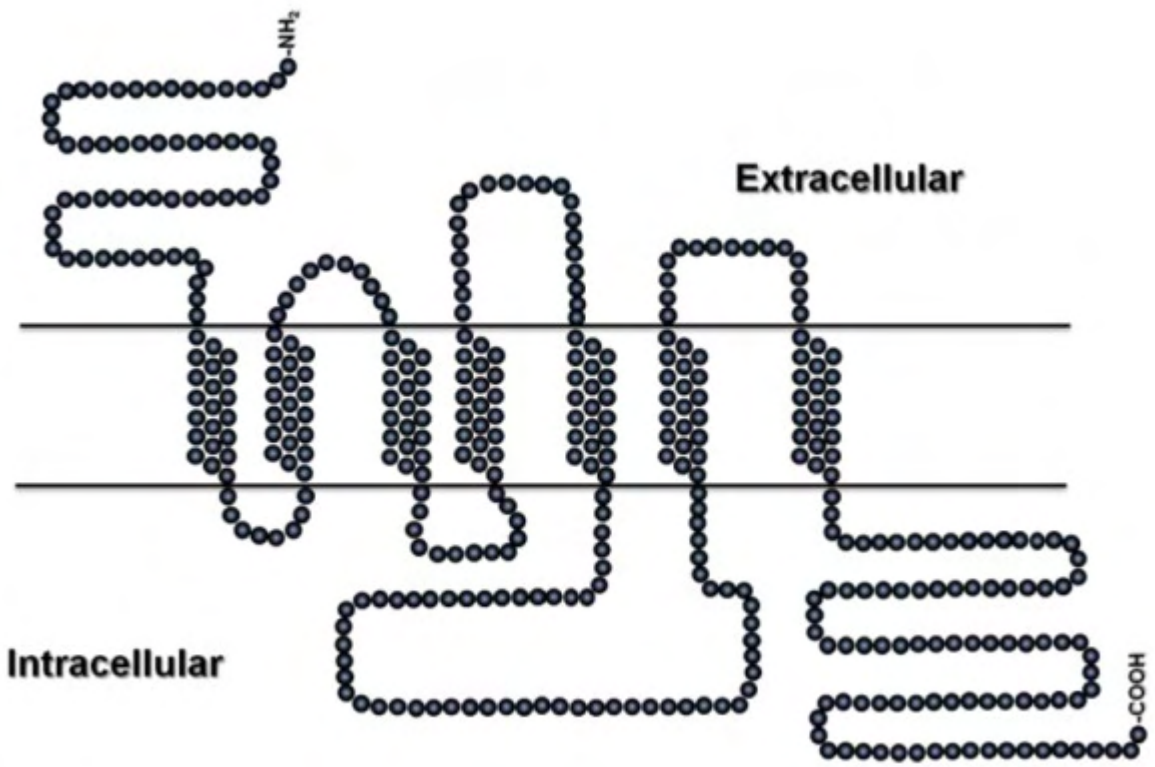


Figure 1: All β-ARs contain seven transmembrane hydrophobic domains that allow this component, like Clb, its effect to be much longer, mainly in adipocyte tissues. According to Johnson et al., 2014, (Asian Australasian J. Anim. Sci., Vol.27, No. 5: 757-766.

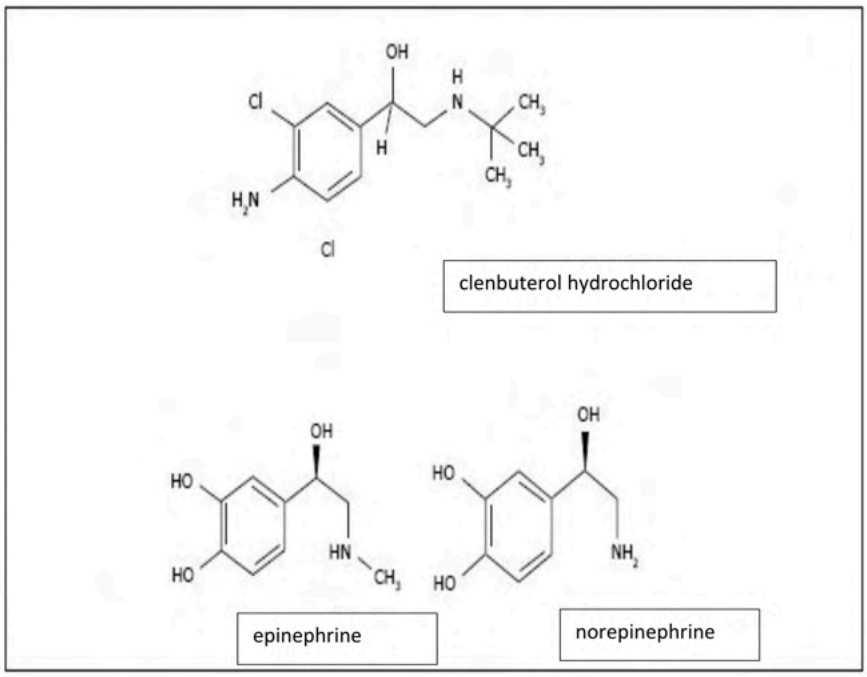


Figure 2: Comparison of the chemical structure of three components called phenethanolamines, two produced physiologically by the body: epinephrine and norepinephrine, and a synthetic component called clenbuterol, which is characterized by having two chlorine-Cl atoms in its structure, which gives it a specific action. prolonged. (Taken from Valladares et al., 2015).

al., 1990), and the protease inhibitor calpain-calpastin (Higgins et al., 1988) are increased. ). ß-AAs can increase blood flow in certain regions of the body, it allows the process of skeletal muscle hypertrophy by containing greater amounts of substrate and energy sources for protein synthesis. Theoretically, the use of these substances presents a series of advantages related, not only to the improvement of productivity, but also to the quality of the meat, since meat from animals treated with ß-AA present a higher percentage of lean tissue (Beermann, 1993; Waldeck and Widmark 1995 and Mersmann, 1998). However, the increased use of ß-AA is related to the increase in intoxications in humans, according to Kuri, et al (2007); The therapeutic dose (TD) is considered to be 0.8 µg/kg of body weight twice a day. The maximum duration of treatment in non-lactating cattle allowed is 10 days orally or intravenously. The illegal use of Clb and analogs in cattle is any dose that exceeds the therapeutic dose (Sauer et al, 1995). Based on the above, it indicates that until now the metabolic routes (biotransformation and bioavailability) of degradation of this food additive are unknown, therefore, the objective of this study was to determine the possible metabolic routes of Clb in cattle.

## MATERIALS AND METHODS

**Animals**: 2567 bovines (Bos taurus X Bos indicus) were used, 95% were male and 5% female, coming from different zoogeographic zones of the country and from different municipal slaughterhouses as well as from private farms. All farms under study were georeferenced with a GPS. The ages of the animals fluctuated from 22-38 months, the predominant breeds crossed, Angus X Brahman = Brangus, Angus, Zebu, Holstein, Charolais and Creoles.

**Sampling:** Vacuum test tubes were used, the first tube without anticoagulant, to obtain blood serum to determine the metabolic profile and hormonal profile of steroids mainly (progesterone and 17ß-estradiol) and another tube with EDTA, to perform blood smears. and thus determine the blood count (the differential count of leukocytes and measurement of hemoglobin). The blood without EDTA was centrifuged at 2,500 rpm/10min, the serum obtained was separated in eppendorf tubes and frozen at -20ºC for subsequent analysis of blood metabolites. Kit-Bio-System-USA was used, 18 metabolites were measured, such as: macrominerals: calcium, phosphorus, liver enzymes: Gamma-glutamyl transferase (γGT), lactic dehydrogenase (L-DH), alkaline phosphatase (AP); transaminases such as: alanine amino-transferase (ALT/GPT) and aspartate amino-transferase (AST/GOT), metabolites such as: albumin, direct and total Bilirubin, total cholesterol, Glucose, total proteins, Urea/BUN, prostate enzymes: acid phosphatase total (FAt), non-prostatic and prostatic; measurements were made in a spectrophotometer (Spectronic 20). For the determination of clenbuterol, the RIDASCREEN kit, Clenbuterol Fast (R-Biopharm AG, Darmstadt, Germany) was used to measure the concentrations of steroids: progesterone (P4) and 17β-estradiol (E2), the immunodiagnosis technique of ELISA (Enzyme-linked immunosorbent assay), diagnostic kits were used, USA, they were measured in an ELISA reader (Stat Fax-2100, Microplate Reader).

**Statistical Analysis:** the data obtained underwent an analysis of variance (ANOVA), with the statistical program Stat-2 (Olivares 1984) and to determine the significance between averages, the Duncan New Multiple Range Test was used.

## RESULTS

The results showed that Clb, (β2-AA) alters muscle growth and composition, apparently decreasing muscle growth when lipogenesis decreases, stimulates lipolysis; the increase in muscle mass is associated with the proliferation of satellite cells, stimulating myofibrils in protein synthesis and suppression of myofibril degradation in protein degradation. The sampled animals increased their body weight between 50 to 70% of their initial weight, in a time of 90 to 112 days of treatment, the daily feeding with Clb was twice a day, at a concentration between 5 to 10 times the dose therapeutic (0.8µg/Kg-body weight), bone at anabolic dose.

Regarding the metabolic profile, high concentrations of transaminases were obtained: the AST/GOT values of clinically healthy animals (ACS) were 533.9±0.26 U/L compared to animals treated with Clb, whose detected value was 264.7±0.22 U. /L, there was a significant decrease $p<0.01$, which indicates that there is an enormous physiological change at the liver level, at the level of the liver parenchyma, possibly degradation of the liver parenchyma without tissue destruction, since these enzymes are intracellular, the same occurred with ALT/GPT, whose value in animals not treated with Clb was 345.5±0.60 U/L compared to cattle treated with Clb, values of 277.9±0.50 U/L were detected, there is also a significant decrease in p<0, 05; ; on the other hand, the hepatic enzyme Gamma-glutamyl transferase (γGT), significantly very low values ($p<0.01$) were detected with reference to ACS animals, between 16.7±0.34 and 26.5±0.30 U/L, respectively, the Clb It is also capable of disguising certain liver pathologies, a work already presented in 2011, it is very likely that these animals already have certain pathologies at the liver and kidney level and that due to the time of the damage, there is still no hepatobiliary and kidney damage,

although it is not known. has determined for this very particular case alkaline phosphatase. Other metabolites such as glucose, their detected values were 115.8±0.31 mg/dL for ACS and in treated animals they had values of 195.4±0.34 mg/dL. They are low, due to the high metabolic demand that this Clb requires of the different structures, including the liver., pancreas, thyroid glands and adrenal glands, since, initially, these metabolic parameters are very high, as the hours and days pass after the intake of this food additive, this activity decreases, but certain components from the degradation of Clb accumulate, including part of the Clb that is not fully degraded, indicating that the bioavailability is sufficient to continue to accumulate and continue the degradation of lipids, with accumulation of nitrogen, through the adipocytes, these as an energy reserve of the organism and when degraded they release acids fatty acids and triglycerides into the bloodstream. The degradation of adipocytes leads to increased lipolysis, decreased lipogenesis and increased thermogenesis, while in the muscle, accumulation occurs at an overdose of Clb, which makes it an anabolic for the animal, biotransformation at the muscle entails an increase in muscle fibers, it is due to the fact that there is an increase in protein synthesis, a decrease in protein degradation, an increase in glycolysis, in the production of lactate and in the use of oxygen. In the pancreas there is a decrease in the production of insulin and an increase in glucagon, producing diabetes in the animal; An increase in glycogenolysis and gluconeogenesis occurs in the liver, producing liver damage in the animal such as cirrhosis and fibrosis, which was detected in 62.3% of the animals studied, as well as the presence of cysts in the liver.

Prostatic acid phosphatase of ACS animals, its detected value was 3.87±0.30 U/L, compared to animals treated with Clb, whose concentration was detected at 11.3±1.2 U/L.

Additionally, cholesterol concentrations for this study decreased from 264.5±0.33 mg/dL to 207.8±0.48 mg/dL, this decrease is significant at $p<0.01$. Cholesterol tends to fall when there is thyroid dysfunction induced by excess food additives, since it is known that the thyroid gland stimulates the elimination of cholesterol by direct secretion in bile and bile acids and also stimulates its synthesis by controlling the functional level of cholesterol. hepatocyte, where approximately 90% of endogenous cholesterol is formed and this can clarify the variability of changes in serum concentration in pathologies such as hyperthyroidism and hypothyroidism in cattle treated with this βAA, for a period of time beyond what is regulated, since many producers subject their animals to more than 100 days of treatment and to concentrations higher than the anabolic dose, between 12 and 15% more.

As for Urea/Bun, it constitutes one of the most abundant non-protein nitrogenous components in the ruminant organism, it is the main nitrogenous waste product of protein catabolism and is only synthesized in the liver, the values obtained for this study ranged between 30.4±0.23 mg/dL in ACS and 95.8±0.28 mg/dL in treated animals. These last values greatly increased and significant ($p<0.01$). showing renal deficiency, either due to increased blood pressure at the level of the juxtaglomerular apparatus.

It is also worth mentioning that the remaining measured metabolites did not show significant changes for this reason, in this study we will not mention it, but we do want to emphasize that all the 17 measured metabolites showed changes according to the ACS values.

The levels of Clb detected in this study fluctuated between 245±34.5 -1623±146.6 ng/kg, these are values of concentrations of Clb

in bovine animals ready to be distributed to supermarkets and grocery stores and then be consumed by the population, the which, do not coincide with the values accepted by the FAO/WHO-(Codex Alimentary), whose values must not exceed 125.0 ng/kg, for human consumption.

When the values of steroid hormones linked to reproductive capacity in females were detected, estradiol (E2) values were detected between 152.1±8.91 to 1152.3±89.74 pg/ml, and progesterone (P4) values fluctuated between 248.51±12.51 at 852.42±90.2 pg/ml, these high values of estrogen and progesterone were obtained from females with very prolonged postpartum anestrus greater than 120 days (postpartum).

## DISCUSSION

For this study it must be considered that one of the most important characteristics in terms of the structre of Clb is that it has a halogen in its chemical structure which is the chlorine ion, this chlorine ion (Cl), allows slowing down the metabolic activity of Clb Therefore, its effect is much longer than other ß-AA agonists and its total excretion is also more delayed (Martin 1971, Ruffolo, 1991, Waldeck and Widmark, 1995). In addition, the effect that Clb has on the activities of the reproductive system in females and males is not very well elucidated, because in animals with a high intake of Clb, reproductive activity decreases (Caicedo et al., 2010), for On the other hand, there is an increase in prostatic acid phosphatase, possibly due to the thermogenic effect of Clb, what this caloric phenomenon does is alter the sperm morphology (Paz-Calderón et al., 2011). In addition, we can consider that ß-AAs stimulate the adrenal gland by producing glucocorticoids and corticosteroids (dexamethasone and betamethasone); the effect of Clb at the level of this adrenal gland is not yet clear. It is probable that in females it stimulates the production of steroids (P4 and E2) at the level of the ovaries, such as progesterone and estradiol, and in males it increases the levels of testosterone (T) and prostatic acid phosphatase, however, the probable damage that it can cause is unknown. produce clenbuterol in the reproductive organs (Caicedo et al., 2021), because these steroids are produced in them, it is likely that the Clb in the adrenal gland increases glucocorticoids and mineralocorticoids, which can also increase steroids (estrogens and progestins) through this organ (Caicedo et al., 2021).

It can be considered that Clb produces an increase in the activity of the nervous system, which leads to loss of appetite, which may be due to the feeling of discomfort of the animal or to glycogenolytic and lipolytic activity, blocking the appetite centers through overload signals from chemostatic receptors (Caicedo et al., 2009; Saavedra et al., 2019). Being able to cross the blood-brain barrier, it is possible that the reduction in food consumption can be attributed to excessive stimulation of ß2-adrenergic receptors (β-AR) in the central nervous system. The growth-promoting effects exerted by Clb are strongly mediated by direct stimulation of β2-adrenergic receptors (β2-AR) (Helferich et al., 1990, Ni et al., 2010), located in muscle tissue and also, indirectly due to variations in plasma concentrations of catabolic or anabolic hormones (Higgins et al., 1988), such as glucocorticoids, growth hormone (GH) or insulin. If hormones can alter the response of adipose tissue to endogenous catecholamines, they can also affect the response of skeletal muscles to exogenous β2 agonists (Sumano et al., 2002). The study confirms that clenbuterol, therefore, modifies the muscle composition of the animals, since in animals treated with β2-AA, an increase in protein deposition

(15%) and a decrease in fat (18%) (Lueso and Gómez, 1990).

Muscle growth, in response to treatment with β2-AA, is a hypertrophy of striated skeletal muscle tissue, which is demonstrated by the studies carried out by Beerman et al., (1986) in rats and by Martin et al., (1990) in cows. The effects of β2-AAs on the endocrine system are largely due to the release of other hormones (Caicedo et al., 2009, Saavedra et al., 2019).

Among the actions of catecholamines are the inhibition of insulin secretion, the increase of glucagon and the stimulation of the release of adrenocorticotropic hormone (ACTH), somatotropic hormone (STH) and gonadotropins such as: FSH and LH, (Beermann et al., 1987). However, there is a surprising lack of information on the effects of Clb on the adrenal gland, which is even more surprising, considering that there are β-adrenergic receptors (β-AR) in this gland, that the adrenal medulla is one of the tissues that synthesize and secrete the natural catecholamines adrenaline and noradrenaline and that the adrenal gland synthesizes and secretes glucocorticoids, finally, it must be considered that the direct involvement of this gland in the mechanisms of adaptation of the organism to stress, both in the short term as long term.

The effects of β-AAs on fat metabolism are very difficult to define, however, they act indirectly on fat deposition, by increasing the metabolic rate and energy expenditure of treated animals and reflecting with thermogenesis, part of the ingested energy prevents the formation of fat and on the other hand the direct effect is in the increase of the levels of cyclic AMP in the adipose tissue, ATP is transformed into cyclic AMP that activates certain proteins such as protein kinases that by phosphorylation stimulate an intracellular lipase that converts triglycerides into fatty acids and glycerol. This mechanism increases lipolysis and decreases lipogenesis. Likewise, this mechanism will depend on the animal species in which we are treating, which is why the increase in the administered concentration and the time in which the animals are subjected to this β-AA plays a role. a very important role in the effect in the short and medium term, for its administration. Based on previous studies and the data obtained in this study, the following can be added: The administration of Clb, at an anabolic dose, causes an alteration in the functionality of the hypothalamic-pituitary-adrenal-gonadal-hepatic axis in cattle, which in some cases is reversible, after a period of withdrawal or quarantine. In addition, that the routes of Clb in cattle, which leads to the formation of: 4-nitro-clenbuterol, hydroxylamine of clenbuterol, 4-amino sulfonic acid of clenbuterol, alcohol-hydroxyamino 2,5-dichloro-a-benzyl and acid 4-amino-3,5-dichloromandelic, all these components are highly toxic to animal physiology according to Fiems (1987), Zalko 1997 a and b; Caicedo et al., 2011 and 2021 (**Figure 3**).

Add to this that the maximal response of β-AA-clenbuterol is affected by the dose administered and the duration of a sustained dose (Ricks et al., 1984). This has been well documented and that the β-AR mediated increase in cAMP is temporary and that continued activation of the receptor by β-AA ligand is necessary to maintain cAMP levels to sustain the response. Exposure to a constant dose of βAA to the receptor will eventually cause acute desensitization or inactivation of receptor-mediated signaling. Phosphorylation of both protein kinase A and β-AR kinases are the major steps in initiating the signaling cascade that follows cAMP activation by β-ARs (Hausdorff et al., 1990). Acute desensitization can be prevented to some extent by increasing the dose and boosting the signal. Long-term

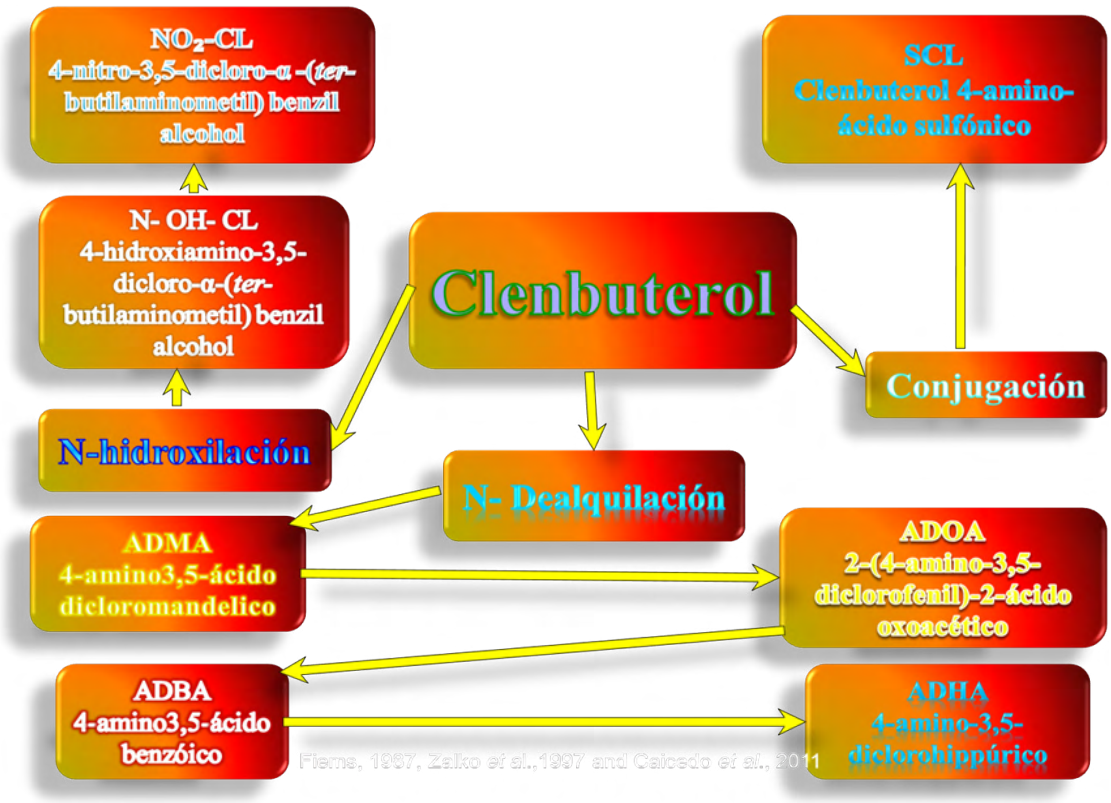


Figure 3: Bioavailability and Biotransformation of Clenbuterol in bovines and possible metabolic routes of Clenbuterol in the different bovine structures, according to Fiems (1987), Zalko 1997 a and b and Caicedo et al., 2011 and 2021.

(chronic) exposure to high doses of β-AA leads to internalization or loss of the cell surface receptor and decreased abundance of β-AR mRNA (Hausdorff et al., 1990). These alterations appear to be irreversible at least in the short-term feeding period.

β-Adrenergic agonists are analogs of the catecholamines epinephrine and norepinephrine. They may function through specific beta-adrenoceptors (βARs) on the surface of adipocytes and skeletal muscle cells. It is proven that βAA. Clenbuterol promotes growth, likewise it is elucidated to some extent that these drugs Clb and zilpaterol reduce total carcass fat and increase total carcass protein in four species: cattle, sheep, domestic fowl and rats. Many β-adrenergic agonists reduce carcass lipids by stimulating lipolysis and blocking lipogenesis in adipose tissue.

## CONCLUSIONS

β-AA-Clenbuterol consistently improves beef cattle performance and increases muscle growth when mixed with finishing rations.

Changes in mRNA abundance of multiple genes associated with myogenic differentiation may indicate an important effect of $\beta_2$AA on the proliferation, differentiation, and/or recruitment of satellite cells into muscle fibers to promote muscle hypertrophy.

The physiological activity of Clb depends on the inherent activity of the receptor and its absorption, rate of metabolism, elimination and distribution in the target tissue.

Note that cattle receiving β-AA-clenbuterol tend to have very low marbling scores, less backfat, and higher beef toughness.

The elevation of the types of glycolytic fibers with the treatment with β-AA-clenbuterol is the main responsible for the increase in muscle hypertrophy, however, it correlates negatively with the amount of adipose tissue, both intramuscular and intermuscular.


## THANKS

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We clarify that this work does not have any type of particular interest, it only wants to demonstrate that much work is needed to increase the quality of life of the animal and its welfare to ensure the production of safe food in our region and improve the environment. ambient.

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