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In vitro simulation of the equine hindgut as a tool to study the influence of phytosterol consumption on the excretion of anabolic–androgenic steroids in horses

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ABSTRACT

Traditionally, steroids other than testosterone are considered to be synthetic, anabolic steroids. Nevertheless, in stallions, it has been shown that β-Bol can originate from naturally present testosterone. Other precursors, including phytosterols from feed, have been put forward to explain the prevalence of low levels of steroids (including β-Bol and ADD) in urine of mares and geldings. However, the possible biotransformation and identification of the precursors has thus far not been investigated in horses. To study the possible endogenous digestive transformation, *in vitro* simulations of the horse hindgut were set up, using fecal inocula obtained from eight different horses. The functionality of the *in vitro* model was confirmed by monitoring the formation of short-chain fatty acids and the consumption of amino acids and carbohydrates throughout the digestion process. *In vitro* digestion samples were analyzed with a validated UHPLC–MS/MS method. The addition of β-Bol gave rise to the formation of ADD (androsta-1,4-diene-3,17-dione) or αT. Upon addition of ADD to the *in vitro* digestions, the transformation of ADD to β-Bol was observed and this for all eight horses' inocula, in line with previously obtained *in vivo* results, again confirming the functionality of the *in vitro* model. The transformation ratio proved to be inoculum and thus horse dependent. The addition of pure phytosterols (50% β-sitosterol) or phytosterol-rich herbal supplements on the other hand, did not induce the detection of β-Bol, only low concentrations of AED, a testosterone precursor, could be found (0.1 ng/mL). As such, the digestive transformation of ADD could be linked to the detection of β-Bol, and the consumption of phytosterols to low concentrations of AED, but there is no direct link between phytosterols and β-Bol.

1. Introduction

Doping is a hot topic in today's high-level horse sport industry, both olympic disciplines and racing, to prevent unfair advantages and to prevent horses from further damaging themselves by masking pain, or competing above their personal limits. Therefore, the presence of hundreds of forbidden substances such as narcotics, psychoactive drugs, corticosteroids and anabolic steroids is being controlled. Of the steroids the natural androgenic steroid testosterone is best known to the public. Closely related to testosterone (βT) in terms of chemical structure are, epitestosterone (αT), AED (androst-4-ene-3,17-dione), ADD (androsta-1,4-diene-3,17-dione) and β-Bol (androsta-1,4-diene-3-one-17β-ol or 1,2-dehydrotestosterone) (Fig. 1).

For a very long time boldenone was considered to be a synthetic hormone and zero-tolerance was maintained. As the number of boldenone-positive urine samples was increasing, the question arose whether this was due to illegal treatment of animals or if boldenone could possess an endogenous origin [1]. Indeed, boldenone has been shown to be naturally present in bovine urine and feces [1–3] whereas Pompa et al. (2006) described *de novo* synthesis of boldenone in cattle feces [4]. Moreover, β-Bol has been detected in urine from entire male horses [5,6]. According to these findings the IFHA (International Federation of Horseracing Authorities) and FEI (Fédération Equestre Internationale) set a threshold for boldenone at 15 ng free and conjugated boldenone per milliliter in urine from male horses (other than geldings) [7,8].

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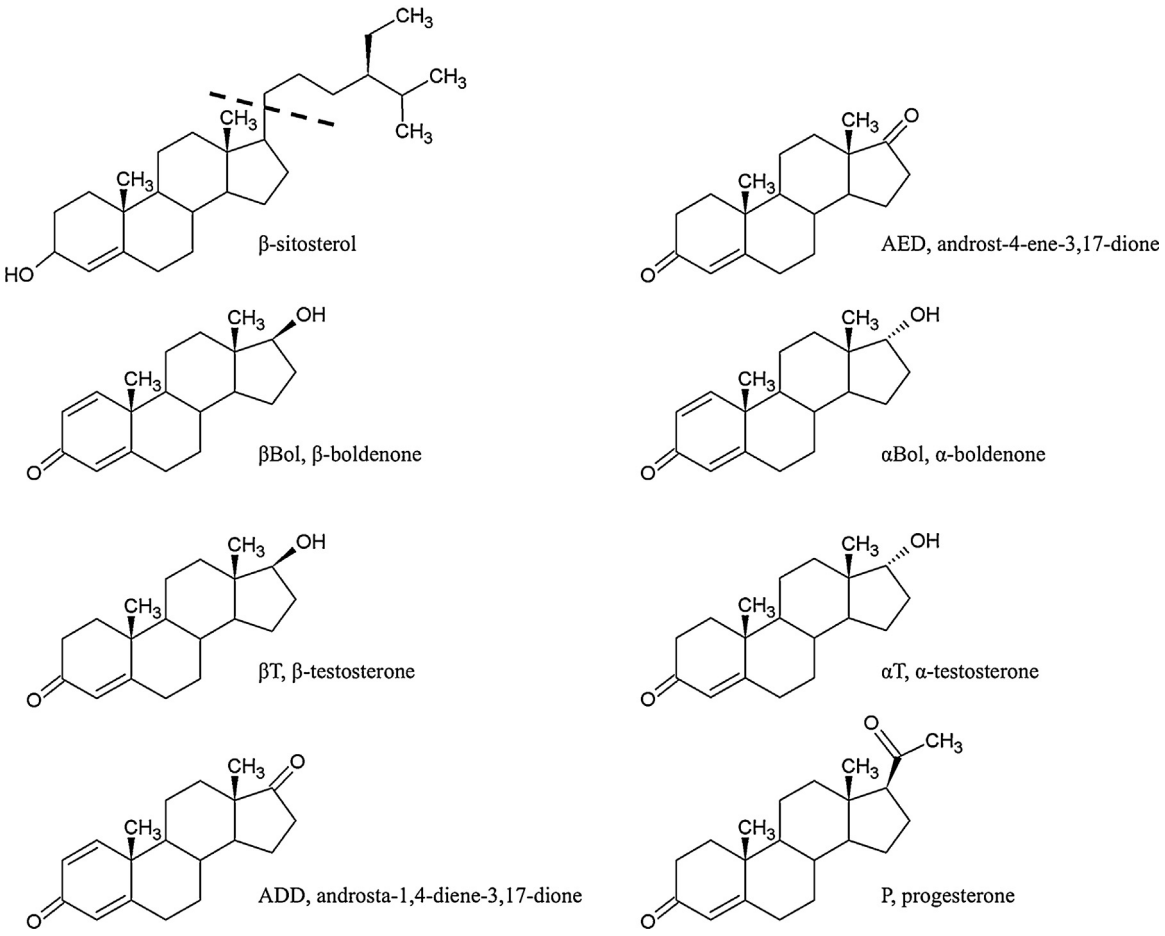

Fig. 1. Illustration of the closely related chemical structure of phytosterols and anabolic steroids. For the phytosterols, β-sitosterol, the most abundant phytosterol, is shown. The suggested microbial side chain cleavage is indicated with a dashed line (- - -). Campesterol and stigmasterol differ from β-sitosterol in the side chain double bond at C22 and the substituents at C24.

The presence of boldenone in urine from mares or geldings is however still prohibited.

Nevertheless, our previous research has demonstrated that AED and ADD, which are next to testosterone the main suspected precursors of boldenone [9], can be present in urine of both untreated geldings and mares at low parts-per-billion levels [10]. In the urine of one AED producing gelding even a low concentration (1.0 ng/mL) of boldenone was found. AED and ADD may originate from the microbial side chain cleavage of phytosterols (*e.g.* β-sitosterol) (Fig. 1).

Phytosterols are omnipresent in plants, regulating the fluidity of cell membranes and featuring in cellular differentiation and proliferation, just like cholesterol in animals [11,12]. The three major phytosterols are β-sitosterol, stigmasterol and campesterol (Fig. 1); they differ in the side chain double bond at C22 and the substituents at C24. All three types make good raw materials for the production of steroid hormones because of their typical A-ring molecular structure with a 3β-hydroxyl group and a 5,6-double bond. The C19-steroids, which include AED, ADD and testosterone, are the products of complete (microbial) side chain cleavage of phytosterols. This conversion of phytosterols to steroids has been frequently reported in different biological systems, mainly involving a variety of microorganisms [13] such as *Mycobacterium* sp. [14–16], *Arthrobacter* and *Nocardia* sp. [17]. In addition, a number of studies have been devoted to the ability of invertebrate organisms to convert phytosterols into anabolic steroids: maggots of *Lucilia Sericata* [9], Crustaceae [18] and zebra fish [19]. However, this biotransformation of phytosterols to steroid hormones has thus far not been demonstrated in horses. For other compounds, it has been proven that the oral uptake of feed contaminants can lead to the detection of these contaminants in the horse's urine. Selection of feed materials appears to be of great importance to prevent involuntary positive result to anti-doping tests [20].

To elucidate the endogenous origin of AED or ADD in horses and the possible transformation pathways to forbidden substances such as boldenone, a good experimental set-up was needed to study the horse's digestive metabolism. Horses are hindgut fermenters: the hindgut, caecum and colon, comprises roughly two thirds of the volume of the equine digestive tract [21]. As such, horses are especially adapted to grazing continually on marginal forages [22]. Complex plant material is fermented by microbes in the hindgut to short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which provide 60–70% of the daily energy needs of the horse [23].

In vitro incubation systems have been developed to simulate the gastrointestinal tract (GIT) of humans [24] and many different animals [25]. Such *in vitro* digestions were used to monitor digestion and passage rates [26] and transformations of different compounds *e.g.* phenolic compounds [27]. Generally, using an *in vitro* batch system enhances reproducibility; unlike when using an *in vivo* set-up, reaction parameters can be standardized. Though the use of these types of batch cultures has its limitations, *i.e.* absence of gastrointestinal absorption and lack of interaction with the host colonic mucosa, *in vivo* studies were not considered here due to lack of versatility in terms of mechanistic explorative potential as well as time consuming and costly nature [28]. Using