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Metabolic Conversion of Zearalenone to α-Zearalenol by Goat Tissues

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ABSTRACT. Zearalenone (ZEA), an estrogenic mycotoxin produced by several *Fusarium* species, is converted into a more active metabolite, α-zearalenol (α-ZOL), and a less active metabolite, *β*-zearalenol (*β*-ZOL), by liver subcellular fractions, but evidence of this reaction in other tissues is limited. In order to clarify the role of various tissues in ZEA metabolism in ruminant, we investigated the *in vitro* metabolic conversion of ZEA by various tissues of adult male and female goats. The results indicate that in the liver, α-ZOL was a major metabolite in cytosolic fractions, whereas *β*-ZOL was a predominant metabolite in microsome fractions. Such a feature of ZEA metabolism was confirmed by the K_m and V_{max} values from an enzyme kinetics experiment. Post-mitochondrial fractions of the liver converted ZEA predominantly to α-ZOL, indicating that the goat liver may function as an activation organ rather than as an inactivation organ, for ZEA metabolism in goats. In most other tissues including rumen tissue, the activity converting ZEA to α-ZOL was higher than that to *β*-ZOL. The amount of α-ZOL formed by gastrointestinal tissues was 1/8–1/3 of that by the liver tissue in terms of the amount per mg protein, but the contribution of all gastrointestinal tissues to production of α-ZOL was estimated to be comparable to that of the liver because of the large mass of gastrointestinal tissues in ruminants. Overall the results show the importance of not only the liver tissue, but also other tissues, especially gastrointestinal ones, in the formation of a potent estrogenic metabolite, α-ZOL.
KEY WORDS: goat, *in vitro*, metabolism, tissue, zearalenone.

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Zearalenone (ZEA), 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-*β*-resorcyclic acid lactone, is an estrogenic mycotoxin produced by several *Fusarium* species and has been found to be a common and widespread contaminant in cereal grains and animal feedstuffs [23, 24, 29]. Various health problems associated with this mycotoxin have been documented in farm animals. The most important toxic effect of ZEA is its estrogenic effect, which has recently helped to identify ZEA and zearalenols as endocrine disrupters. ZEA can bind to estrogen receptors and cause diseases in the reproductive system, impaired fertility, and abnormal fetal development in farm animals [5, 15]. In addition to these estrogen-related effects, hepatic and renal lesions including hepatocarcinogenesis and nephropathy, and hematotoxicity in rodents [1, 17, 19], and the reduction of milk production in cows [28] have been observed. *In vitro* studies using cultured cells have shown cytotoxic effects of ZEA including DNA and protein syntheses [2, 14]. However, the health adverse effects of ZEA are unknown in goats.

Numerous studies have demonstrated that ZEA is metabolized in the livers of various animal species, including ruminants, the major metabolites being α-zearalenol (α-ZOL) and *β*-zearalenol (*β*-ZOL) (Fig. 1) [16, 20]. The reduction of ZEA has also been observed in human prostate glands [26], sow intestinal mucosa [21] and rumen fluid [13, 25], but little is known about ZEA metabolism in other organs. The estrogenic activity of α-ZOL is 3–100 fold higher than that of ZEA depending on the methods used to determine the activity, while the activity of *β*-ZOL is much lower than that of ZEA [4, 9, 11, 12, 18, 27]. Therefore, the organs capable of reducing ZEA to α-ZOL may contribute to the enhancement of the toxic effects of ZEA. However, ZEA metabolism in other organs remain unknown in any animals. Especially in ruminants, the mass of gastrointestinal tract tissues is enormous and therefore metabolism of ZEA in these tissues may play an important role as a whole, but little is known about it.

In order to clarify the metabolic conversion of ZEA in various tissues, we investigated ZEA metabolism by post-mitochondrial, microsome and cytosolic fractions of the liver, gastrointestinal tissues, kidneys, brain and lungs of adult male and female goats.

MATERIALS AND METHODS

Chemicals: Zearalenone (ZEA), α-zearalenol (α-ZOL), *β*-zearalenol (*β*-ZOL), α-zearalanol (α-ZAL), *β*-zearalanol (*β*-ZAL), zearalanone (ZAN), glucose-6-phosphate (G-6-P) disodium salt, glucose-6-phosphate (G-6-P) dehydrogenase, *β*-nicotinamide adenine dinucleotide phosphate reduced form (*β*-NADPH) and dithiothreitol (DTT) were purchased from Sigma Aldrich Chemicals (St. Louis, MO, U.S.A.). Ethylenediaminetetraacetic acid sodium salt (EDTA-2Na),

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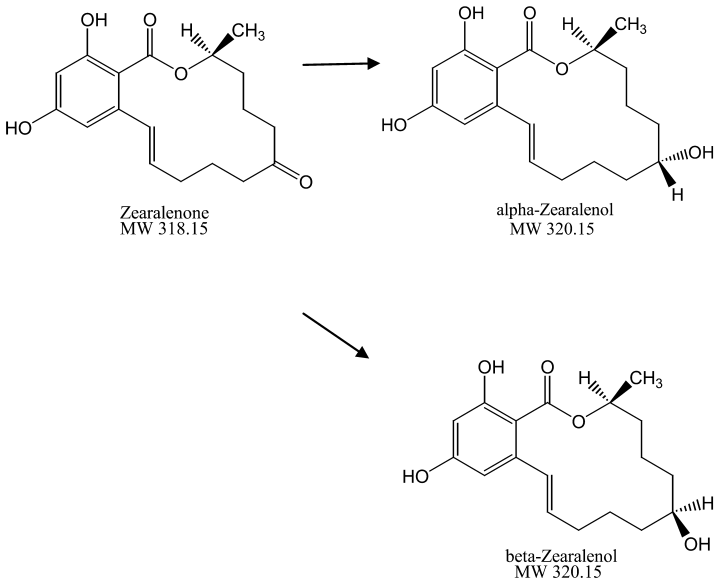

Fig. 1. Chemical structures of zearalenone, α-zearalenol and *β*-zearalenol.

chloroform, phosphoric acid, high performance liquid chromatograph (HPLC) grade methanol and acetonitrile were purchase from Wako Pure Chemicals (Osaka, Japan). Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad (Hercules, CA, U.S.A.). All other chemicals were of analytical grade and obtained from regular commercial sources.

Animals: Three female and three male adult Shiba goats, reared by the Research Unit for Animal Life Sciences, Animal Resource Science Center of the University of Tokyo, were used at their experimental ethics ages of 3 to 6 years.

Preparation of post-mitochondrial, microsome and cytosolic fractions: The goat liver, kidneys, lungs, brain, small intestines (jejunum and duodenum), rumen and abomasum were quickly removed under anaesthesia with intravenous injection of ketamine (30 mg/kg body weight). The rumen contents were taken from the rumen. The liver was perfused with ice-cold saline to remove excess blood. The organs were rapidly frozen in liquid nitrogen, and stored at –80°C until use. Microsomes and cytosolic fractions were prepared according to the method of Bammler *et al.* [3], except that a Teflon glass homogenizer instead of a blender was used for tissue homogenization [8], supernatants of centrifugation at 9,000 G of the tissue homogenates were used as post-mitochondrial fractions.

Determination of metabolic conversion of ZEA in tissue fractions: The reaction mixture (1 m*l*) containing the post-mitochondrial, microsome or cytosolic fraction, each equivalent to 1.0 mg protein, from various organs, 2.0 mM *β*-NADPH, 5.6 mM G-6-P and 0.5 unit of G-6-P dehydrogenase in potassium phosphate buffer solution (100 mM , pH 7.4) were pre-incubated in a shaking water bath for 5 min at 37°C. The reaction was started by adding 5 *μ*g of ZEA (5 *μ*g/10 *μl* methanol), and incubating for 30 min at 37°C. For kinetic studies, increasing concentrations of ZEA (final concentrations 7.5, 15, 30, 60, 120, 180 and 240 *μ*M in 10 *μl* methanol) were added to a 1 m*l* reaction mixture, and an incubation time of 10 min was used. Linear increases in the formation of ZEA metabolites were observed until 40 min of incubation in a preliminary experiment. The reaction was stopped by adding 20 *μl* of 1 M phosphoric acid and transferring the reaction mixture onto ice. ZEA and its metabolites were extracted twice with 2 m*l* ice-cold chloroform by vigorous vortex and centrifugation for 5 min at 3,500 rpm, and the combined chloroform layer was evaporated to dryness with a rotary evaporator. When the reaction mixtures of 1 m*l* were spiked with 2 *μ*g of different standards, the recovery rates were 91%, 90%, 86%, 82%, 86% and 80% for ZEA, α-ZOL, *β*-ZOL, α-ZAL, *β*-ZAL and ZAN, respectively.

Quantification of the ZEA metabolites by the HPLC method: The analysis of ZEA and its metabolites was performed by HPLC according to Fitzpatrick *et al.* [10] with minor modifications. Briefly, the sample was injected into a PEGASIL ODS column (4.6 × 250 mm, Senshu Scientific Co., Tokyo, Japan) equipped with a pre-column (4.6 × ~30 mm, Senshu Scientific Co., Tokyo, Japan). The mobile phase consisted of a mixture of water/ methanol/ acetonitrile (43:42:15, v/v/v), and the analyte was eluted at a flow-rate of 1 m*l*/min. The fluorescence detector was set at an excitation and emission wavelength of 280 and 390 nm, respectively. ZEA and its metabolites were quantified by comparing peak areas of samples to those of the standard solutions. Detection limits from the reaction mixture of 1 m*l* were 10 *n*g, 6.5 *n*g, 36.5 *n*g, 122 *n*g, 84 *n*g and 186.5 *n*g for ZEA, a-ZOL, *β*-ZOL, α-ZAL, *β*-ZAL and ZAN, respectively.

Protein assay: Protein concentrations of the microsomes

and cytosolic fractions from various organs were measured with a spectrophotometer (U-1500; Hitachi Co., Ltd., Tokyo, Japan) using Bio-Rad Protein Assay Dye Reagent Concentrate and albumin from bovine serum as the standard.

Statistical analysis: All results are presented as means ± SEM. Kinetic analyses were performed with non-linear regression analyses of the enzyme kinetic template of GraphPad prism version 4.00 for Windows (GraphPad Software, San Diego, CA, U.S.A.). Statistical differences were determined by Student's *t*-test. $P<0.05$ was considered significant.

RESULTS

Metabolic conversion of ZEA in microsomes and cytosolic fractions: Metabolic conversion of ZEA to zearalenols was found in various tissues from both sexes of adult goats (Fig. 2). In cytosolic fractions (Fig. 2A), most tissues showed activity to convert ZEA to α-ZOL and *β*-ZOL, but the lungs and kidneys did not produce any detectable amounts of *β*-ZOL formation. ZEA was converted more efficiently to α-ZOL than to *β*-ZOL in all tissues. In microsomal fractions (Fig. 2B), α-ZOL formation was detected in all tissues, whereas *β*-ZOL formation was detected only in the liver and lungs. ZEA was converted more efficiently to *β*-ZOL than to α-ZOL in the liver and lungs, but the reverse was the case in the other tissues. No sex-differences were observed in either the ZEA to α-ZOL or ZEA to *β*-ZOL conversion in any tissues. α-ZAL and *β*-ZAL were not detected in any fractions from any tissues.

Enzyme kinetics of ZEA biotransformation: To delineate more precisely the enzymatic activity converting ZEA focusing on comparative activity toward α-ZOL and *β*-ZOL, enzyme kinetics of the biotransformation of ZEA were measured using the microsomes and cytosolic fractions of the liver. Also, enzyme kinetics were measured for cytosolic fractions of the gastrointestinal tissues (rumen, abomasums, duodenum and jejunum). Data showed that the α-ZOL and *β*-ZOL formation increased with increases in the ZEA concentration (Fig. 3). The Michaelis-Menton constant (Km) and the maximum velocity (Vmax) values are shown in Table 1.

For *α*-ZOL formation (Fig. 3A, B), the largest value of V_{max} was found in liver cytosolic fractions (Table 1). The V_{max} values in the rumen, duodenum and jejunum were

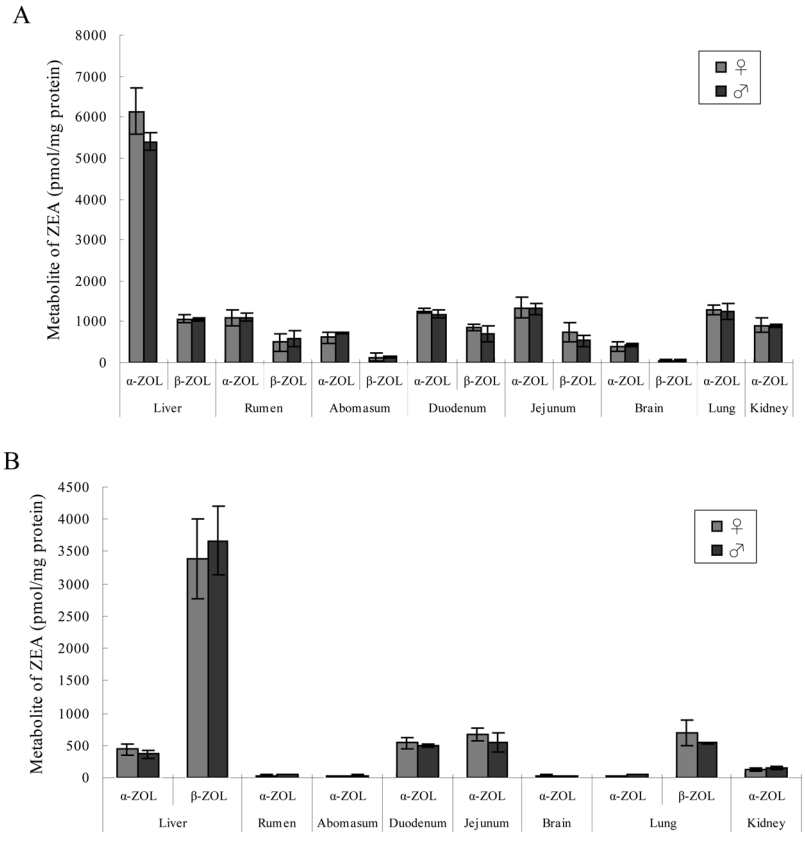

Fig. 2. Metabolic conversion of ZEA at a ZEA concentration of 5 *μ*g/m*l* in cytosolic fractions (A) and microsomal fractions (B) of various tissues from adult male and female goats. Values are indicated as mean ± SEM of metabolites for three goats. No significant differences were found between female and male goats.

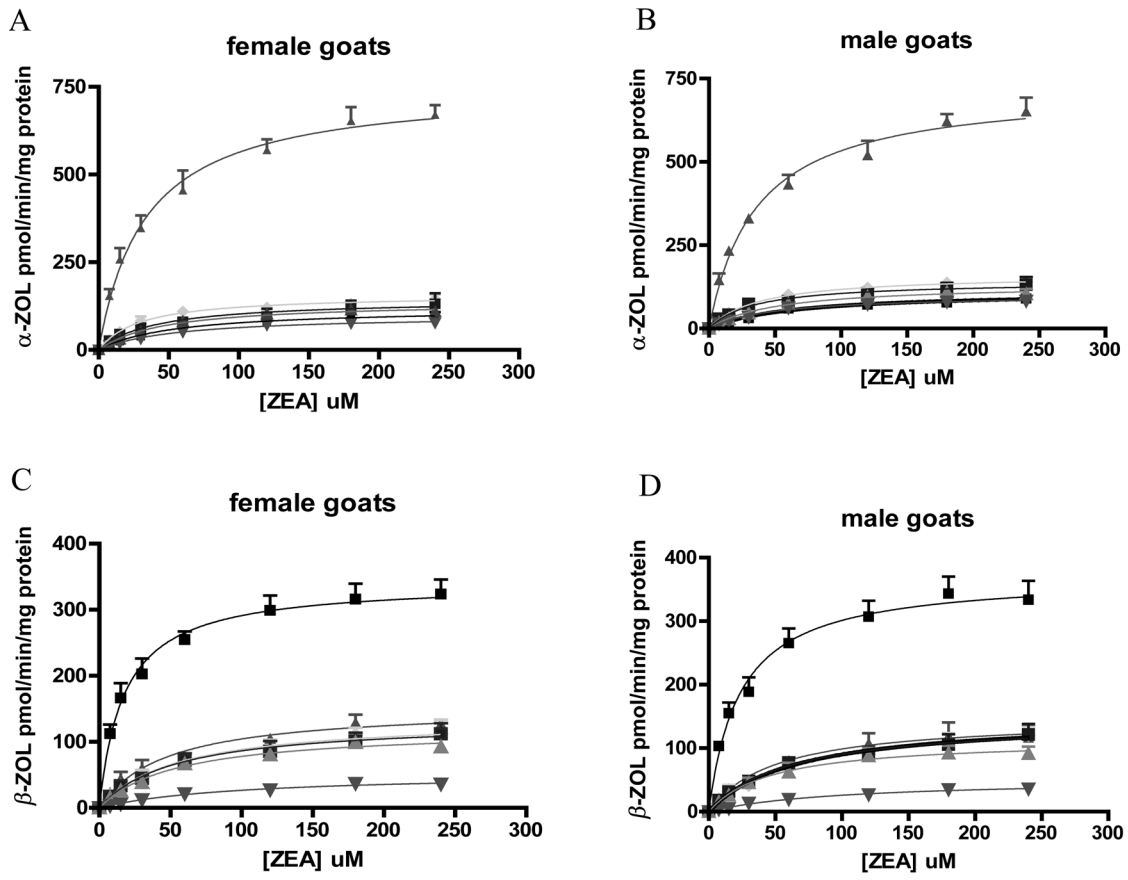

Fig. 3. Enzyme kinetics of ZEA biotransformation by microsomes of the liver (■) and cytosolic fractions of the liver (▲), rumen (▲), abomasum (▼) , duodenum (■) and jejunum (◆) from goats. A and B indicate the α-ZOL formation, and C and D indicate the *β*-ZOL formation. Values are indicated as mean ± SEM for three goats.

Table 1. Kinetic constants for metabolic conversion of ZEA to α-ZOL and *β*-ZOL by liver microsomes and liver, gastrointestinal tissues cytosolic fractions

		Liver microsome	Liver cytosol	Rumen cytosol	Abomasum cytosol	Duodenum cytosol	Jejunum cytosol
	α-ZOL						
Female	V_{max} (pmol/min/mg protein)	100.0 ± 2.0	751.8 ± 22.5	137.9 ± 5.5	70.1 ± 4.0	144.3 ± 9.0	157.9 ± 4.2
	K_m (μM)	49.2 ± 2.9	33.5 ± 3.4	46.7 ± 5.7	51.9 ± 8.5	40.8 ± 8.2	29.7 ± 2.8
Male	V_{max} (pmol/min/mg protein)	91.0 ± 5.5	723.6 ± 27.1	133.9 ± 5.8	75.91 ± 1.4	141.4 ± 7.8	162.0 ± 1.9
	K_m (μM)	45.4 ± 8.6	35.7 ± 4.5	51.8 ± 6.7	53.3 ± 2.8	34.5 ± 6.5	38.1 ± 1.5
	β-ZOL						
Female	V_{max} (pmol/min/mg protein)	341.7 ± 7.3	153.3 ± 7.7	119.9 ± 6.6	43.5 ± 3.1	131.9 ± 5.6	136.1 ± 7.1
	K_m (μM)	17.4 ± 1.6	46.9 ± 7.2	54.0 ± 8.8	81.3 ± 14.3	52.8 ± 6.6	54.1 ± 8.3
Male	V_{max} (pmol/min/mg protein)	371.3 ± 11.3	140.4 ± 7.6	114.6 ± 4.3	45.8 ± 3.1	124.4 ± 3.0	113.3 ± 4.5
	K_m (μM)	23.3 ± 2.7	41.9 ± 7.3	46.9 ± 5.4	81.2 ± 13.8	44.5 ± 3.4	45.6 ± 5.6

Values are indicated as mean ± SEM for three goats.

approximately the same, but they were relatively small in the abomasum. The liver microsomes showed relatively small V_{max} values. No large differences were noted in K_m values among tissues.

For *β*-ZOL formation (Fig. 3C, D), the smallest value of V_{max} was found in the abomasum cytosolic fractions (Table 1). The largest value of V_{max} was in the liver microsomes. No significant differences were noted in the V_{max} values among the cytosolic fractions of the liver, rumen, duodenum and jejunum. The K_m values in the abomasum tended to be larger than those in the other organs, the smallest value being found in the liver microsomes.

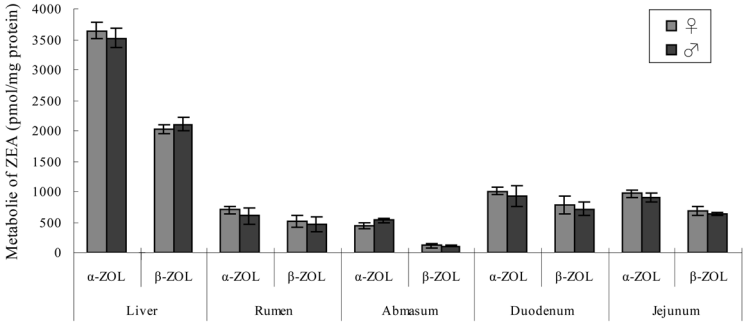

Fig. 4. Metabolic conversion of ZEA at a ZEA concentration of 5 μg/m*l* in post-mitochodrial fractions of the liver and gastrointestinal tissues from adult male and female goats. Values are indicated as mean ± SEM of metabolites for three goats. No significant differences were found between female and male goats.

Comparing the values of V_{max} and K_m, no significant differences were found between male and female goats.

Metabolic conversion of ZEA in post-mitochondrial fractions: Metabolic conversion of ZEA was also determined using post-mitochondrial fractions from the liver and gastrointestinal tissues (Fig. 4). Consistent with the results of the activity of the microsome and cytosolic fractions, the highest ZEA reducing activity was noted in the liver. α-ZOL formation was predominant in all tissues. The amount of α-ZOL formed by the gastrointestinal tissues was 1/8–1/3 of that by the liver.

DISCUSSION

The hepatic biotransformation of ZEA has been observed in various experimental and farm animals [20, 16]. The present study aimed to investigate metabolic conversion of ZEA in subcellular fractions of various tissues from adult goats. Previous studies by other researchers have demonstrated that ZEA is partially converted into α-ZOL and *β*-ZOL in the liver in animals. The rate of metabolic conversion of ZEA to α-ZOL and *β*-ZOL varies among animal species, which may account for the species differences in the sensitivity to ZEA [16]. The estrogenic activity of α-ZOL is 3–100 fold higher than that of ZEA, depending on the methods used to determine the activity, while the activity of *β*-ZOL is much lower than that of ZEA [4, 9, 11, 12, 18, 27]. Hence, the conversion of ZEA to α-ZOL is regarded as an activation process, whereas that to *β*-ZOL is an inactivation one.

This study showed that the conversion of ZEA is markedly different among tissues and between sub-cellular fractions, the liver activity being highest in the goat. In the liver, α-ZOL was the major metabolite in cytosolic fractions, whereas *β*-ZOL was the predominant metabolite in microsome fractions. In most other tissues, the activity converting ZEA to α-ZOL was higher than that to *β*-ZOL in both cytosol and microsome fractions, and only the lung microsomes possessed higher activities for converting ZEA to *β*-ZOL than those to α-ZOL. Such a feature of ZEA metabolism was confirmed by the K_m and V_{max} values calculated from the results of an enzyme kinetics experiment. Consistent with these findings using microsomes and cytosolic fractions, post-mitochondrial fractions of the liver were observed to convert ZEA predominantly to α-ZOL, indicating that the goat liver may function as an activation organ, rather than as an inactivation organ, for ZEA metabolism in goats. Also most extrahepatic tissues behaved in an activating rather than an inactivating manner, although this was not so clear in the liver.

Thus, the results indicate the importance of not only the liver, but also other tissues in the conversion of ZEA to the more active compound, α-ZOL. The amount of α-ZOL formed by the gastrointestinal tissues was 1/8–1/3 of that by the liver in terms of the amount per mg protein, but the contribution of the whole gastrointestinal tissue in production of α-ZOL is comparable to that of the liver because the tissue weight of the gastrointestinal tract is three to five times that of the liver in ruminants [6, 7]. Although no toxic effects of ZEA have been reported in the gastrointestinal tract, the findings of mRNA expression of estrogen receptors-α and -*β* in bovine gastrointestinal tissues [22] indicated the possible effects of ZEA and its metabolites on gastrointestinal tract function. α-ZOL formed from ZEA in the liver and the other organs may exert its effects not only at the site of formation, but also in all the target organs through the systemic circulation, increasing risks to animal health on the one hand, and to human health through ingestion of tissues containing these metabolites on the other.

Taken together, the present study firstly investigated the metabolic conversion of ZEA in various tissues in goats. The results demonstrate clearly that there is activity that converts ZEA to α-ZOL or *β*-ZOL in various tissues. The predominant formation of α-ZOL not only by the liver, but also by the other tissues, especially gastrointestinal tissues, may contribute to the enhancement of the adverse effects of ZEA in ruminants, resulting in increased risks to animal and human health.

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