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RESEARCH ARTICLE

# Gene expression and enzyme function of two cytochrome P450 3A isoenzymes in rat and cattle precision cut liver slices

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**Abstract**

1. Precision-cut liver slices are one of the *in vitro* models used in studies concerning xenobiotic metabolism. Sparse information on this field is actually available for cattle and other veterinary species.
2. The aim of the current work was to study the effect of dexamethasone (DEX) on the gene expression and function of *CYP3A23* (in rat), *CYP3A28* (in cattle) and the transcriptional factors involved in their regulation.
3. DEX (at 100 μM) up-regulated *CYP3A23* mRNA (3.2-fold, $p = 0.028$) in rat liver slices after 12 h culture, whereas the gene expression profiles of transcriptional factors involved in *CYP3A* regulation were unaffected. A *CYP3A*-dependent enzyme activity (triacetyl-oleandomycin N-demethylase) increased 3.4-fold ($p < 0.05$) in rat liver slices cultured in the presence of DEX.
4. The protocol used for rat liver slices was used as reference to study the expression of a *CYP3A* isoenzyme in cattle liver slices. Oppositely, DEX did neither affect the gene expression profile of *CYP3A28* nor the *CYP3A* activity tested in cattle liver slices.
5. The data reported here are a further contribution to demonstrate the usefulness of liver slices as an *in vitro* tool for studies on the expression and function of xenobiotic metabolizing enzymes in cattle and in other ruminant species.

**Keywords**

Cattle, cytochrome P450, liver slices, rat, xenobiotic metabolizing enzymes

**History**

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## Introduction

The cytochrome P450 (CYP) enzyme system plays a crucial role in xenobiotic detoxification in mammalian species. Different CYP members belonging to families 1–4 convert a wide array of xenobiotic agents (e.g. drugs, feed additives, pesticides and pollutants) to products that can be readily excreted from the body via normal excretion routes. Most information on the expression and function of CYPs has been obtained from laboratory animals (e.g. rodent species) and humans. Although the characterization of CYPs in ruminants is still incomplete, recent investigations focused on the gene/protein expression of different members within this enzyme superfamily in these species (Fink-Gremmels, 2008; Ioannides, 2006). The molecular mechanisms involved in the pre-transcriptional regulation of different CYP isoenzymes in cattle also became a subject of interest (Giantin et al., 2008; Greger et al., 2006). Within the CYP enzyme system, the CYP3A subfamily is of particular relevance for the metabolism of most drugs clinically used in humans and in food-producing animals (Ioannides, 2006). Proteins cross-reacting with primary antibodies raised against rat, rabbit or human CYP3A isoenzymes have been identified in liver microsomes from cattle (Dacasto et al., 2005; Nebbia et al., 2003; Sivapathasundaram et al., 2001). Moreover, a complete phylogenetic analysis of the CYP3 family revealed that CYP3A28, CYP3A38 and CYP3A48 are the main CYP3A isoenzymes expressed in cattle (Zancanella et al., 2010). No orthology between cattle CYP3A isoenzymes and human CYP3A4/5 was found (Zancanella et al., 2010). In addition, the expression of CYP3A28 was completely characterised at post-transcriptional and post-translational levels in cattle liver (Cantiello et al., 2008; Giantin et al., 2008, 2010).

*In vitro* liver models represent a simpler system compared to the whole animal, allowing a step-by-step approach to investigate the fate of xenobiotics once inside the living organism. Therefore, these methods are useful to characterise the xenobiotic metabolite profiling and its effects on the expression and regulation of xenobiotic metabolizing enzymes (XMEs). Moreover, potentially harmful or beneficial drug–drug interactions may be identified by means of different *in vitro* methodologies. Subcellular fractions (i.e. S9 fractions, microsomes, cytosols), recombinant enzymes (e.g. Supersomes™), precision-cut liver slices, hepatocyte primary cultures and recombinant protein expression systems

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(or engineered cell lines) represent the *in vitro* liver models mostly used in studies concerning xenobiotic metabolism (Brandon et al., 2003; Ekins et al., 2000).

Precision-cut liver slices became popular since the introduction of Krumdieck™ and Brendel-Vitron™ tissue slicers and associated culture methods (Ekins, 1996; Fisher & Vickers, 2013; Ioannides, 2013; Maurel, 1996). Therefore, liver slices were used firstly for toxicology studies and then for xenobiotic metabolism (Ekins, 1996). One of the major advantages of liver slices compared with other *in vitro* models such as isolated or cultured hepatocytes is the maintenance of cellular diversity, thus being close to the physiological situation found *in vivo* (Maurel, 1996). Moreover, liver slices were used to investigate the inductive effects of model CYP inducers monitored at the mRNA/protein level or at the metabolic activity of different CYP isoforms (Ioannides, 2013).

Most of the published research in which xenobiotic metabolism processes were investigated using liver slices have been carried out with liver samples taken mostly from laboratory animals. Conversely, sparse information on this field of research is actually available for cattle and, to a wider scenario, for ruminants and other veterinary species. For instance, bovine liver slice preparations were used to study the metabolic pathways involved in the biotransformation of benzydamine (an indazole analgesic and antipyretic drug; Santi et al., 2002), testosterone (Wang et al., 2010) and the pro-hormone dehydroepiandrosterone (Rijk et al., 2012). In this context, and in view of the pivotal role of the CYP3A subfamily in xenobiotic metabolism, the work reported here was designed to gain further insight into the expression and function of two CYP3A isoenzymes in rat (CYP3A23) and cattle (CYP3A28) using liver slices as an *in vitro* model. The work also assessed the effect of dexamethasone (DEX), a known CYP3A inducer, on the gene expression of both isoenzymes and the transcriptional factors involved in their regulation.

## Materials and methods

### Chemicals

Dexamethasone sodium phosphate was purchased from Richmond Vet Pharma (Grand Bourg, Buenos Aires, Argentina). Insulin was purchased from Beta Laboratories (Buenos Aires, Argentina). Gentamicin was from Parafarm (Buenos Aires, Argentina). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Roche Applied Science (Buenos Aires, Argentina). Ascorbic acid was from Anedra (Buenos Aires, Argentina). D-Glucose and $HCO_3Na$ were purchased from Biopack (Buenos Aires, Argentina). Fungizone (amphotericin B) and all materials for the total RNA (tRNA) extraction, reverse transcription and quantitative PCR (e.g. RNA later, Trizol™ reagent, SYBR safe) were from Life Technologies (Carlsbad, CA). Salts (KCl, NaCl, $Cl_2Ca.H_2O$, $Cl_2Mg{\cdot}6H_2O$, $NaHCO_3$, $Na_2CO_3$, $Na_2HPO_4$, $NaH_2PO_4$, $K_2HPO_4$, $KH_2PO_4$ and $CH_3COONH_4$) were purchased from Baker Inc. (Phillipsburg, NJ). All other chemicals (e.g. Williams' Medium E, oleandomycin-triacetate, nicotinamide adenine dinucleotide phosphate, EDTA) were from Sigma-Aldrich Chemical Company (St. Louis, MO).

### Animals

Bovine liver samples from six (6) Aberdeen Angus/Hereford crossbreed steers (weighing 350 kg approximately) were obtained from a local slaughterhouse (Mirasur SA, Tandil, Argentina) located 16 km away from laboratory facilities. Immediately after the animal was sacrificed, the hepatic caudate process was excised, washed with ice-cold 1.15% KCl and immediately submerged in containers filled with ice-cold Krebs buffer (1 mM $PO_4H_2Na$; 2.5 mM $Cl_2Ca{\cdot}H_2O$; 4.7 mM KCl; 1.1 mM $Cl_2Mg{\cdot}6H_2O$; 0.004 mM EDTA; 11 mM glucose; 119 mM NaCl, 25 mM $CO_3Na_2$; 0.11 mM ascorbic acid). Containers were covered, chilled in ice and transported to the laboratory within 30–40 min for subsequent procedures.

Six (6) healthy adult male Wistar rats, weighing 280–310 g, were maintained under temperature controlled conditions (25 °C) and a normal photoperiod of 12 h of darkness and 12 h of light. Animal procedures and management protocols were carried out according to internationally accepted animal welfare guidelines (AVMA, 2007) and approved by the Animal Welfare Committee of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina (Internal Protocol: 12/2013; approval date: 30 October 2013). All animals were fasted overnight. Under anaesthesia with ketamine hydrochloride (100 mg/kg, intraperitoneal), the liver was rapidly excised, washed with ice-cold 1.15% KCl and immediately submerged in a container filled with ice-cold Krebs buffer and transported to laboratory facilities. After these procedures, animals were sacrificed by exsanguination.

### Preparation of liver slices

Cylindrical cores of liver tissue were produced by means of hand-held sharpened stainless steel tubes (Vitron Inc., Tucson AZ) of 8 mm (rat liver) or 10 mm (bovine liver) internal diameter. First, liver cores were placed in beakers containing oxygenated (95% $O_2$:5% $CO_2$) ice-cold Williams' Medium E (supplemented with 25 mM D-glucose, 50 μg/mL gentamicin, 1 μM insulin and 1.25 μg/mL amphotericin B). Then, liver cores were mounted on a Brendel/Vitron tissue slicer (Vitron Inc., Tucson, AZ) filled with oxygenated ice-cold Krebs buffer. Slices of $29.8 \pm 4.7$ mg (rat) and $31.9 \pm 2.5$ mg (cattle) wet weight and thickness of ~250 μm were produced by operating the slicer according to manufacturer's instructions. Immediately after preparation, slices were placed in a Petri dish containing ice-cold oxygenated culture medium. The most uniformly shaped slices were selected for incubation experiments.

### Culture of liver slices

Liver slices were floated onto type C titanium roller inserts (Vitron Inc., Tucson, AZ) and cultured in glass vials containing 1.7 mL of Williams' Medium E. Vials were placed into a dynamic organ culture incubator (Vitron Inc., Tucson, AZ), set at 37 °C and 2 rpm, and cultured under a humidified atmosphere of 95% $O_2$:5% $CO_2$. Slices were initially pre-incubated for 1 h ($t = 0$ h) in order to slough off any dead cells due to slicing. After that, a 12-h ($t = 12$ h) incubation period started by replacing the culture medium

with fresh oxygenated medium. An aliquot (87 µL) of DEX working solution (in water) was dissolved in the culture medium to reach a final concentration of 100 µM. Control vials had the same culture medium without DEX. Freshly cut liver slices and those harvested at $t = 0$ h and $t = 12$ h culture were snap-frozen in liquid $N_2$ and stored at −70 °C until analysis. The same procedure was applied to aliquots (1 mL) of culture medium taken at $t = 0$ h and $t = 12$ h.

### Preparation of tissue homogenates

Thawed slices were homogenised in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl. Homogenates were centrifuged at $10\,000 \times g$ (at 4 °C for 20 min) to obtain the post-mitochondrial fraction. Aliquots of this subcellular fraction were frozen in liquid $N_2$ and stored at −70 °C. Protein content was determined according to Lowry et al. (1951).

### Histopathology

Harvested slices were fixed in 10% buffered formalin and, after 24 h, dehydrated with ethanol and embedded in paraffin wax. Paraffin blocks were cross-sectioned (5 µm thickness) using a rotary microtome. Cross sections were rehydrated in distilled water, stained with haematoxylin and eosin (H/E) and examined by light microscopy (Leica Microsystems, Wetzlar, Germany).

### Biochemical determinations

Lactate dehydrogenase (LDH) leakage was assessed as a tissue viability parameter. This enzyme activity was measured both in the culture medium and diluted (1/20–1/50) post-mitochondrial fractions from liver slices following the method described by Moldeus et al. (1978). Briefly, sample aliquots were dissolved in a triethanolamine (TEA) buffer solution (0.5 M TEA, 0.1 M $Cl_2Mg$ $6H_2O$, 0.05 M EDTA sodium salt and 0.21 mM NADH). The reaction started with the addition of sodium pyruvate (1 mM final concentration) and the NADH oxidation was monitored at 340 nm for 5 min at 30 °C in a spectrophotometer (T80 + UV/Visible Spectrometer, PG Instruments Limited, Lutterworth, England). Percentages of LDH leakage were calculated as follows:

$$\text{LDH leakage}\ (\%) = \frac{\text{Activity in culture medium}}{\left(\begin{array}{c}\text{Activities in culture medium} + \text{Slice}\\ \text{post-mitochondrial fraction}\end{array}\right)} \times 100$$

where enzyme activities in culture medium or slice post-mitochondrial fractions are expressed as µmol of NADH oxidised per min µL multiplied by the total volume of culture medium in each culture vial (1700 µL) or the total volume of the post-mitochondrial fraction (usually between 1000 and 2000 µL).

The oxidative CYP-dependent N-demethylation activity toward triacetyl-oleandomycin (TAO), a CYP3A substrate, was measured in post-mitochondrial fractions prepared from control and DEX-exposed liver slices after 12 h culture. This enzyme activity was assayed using a NADPH-producing system (NADP + 0.32 mM, glucose-6-phosphate 6.4 mM, $MgCl_2$ 5 mM, EDTA 0.8 mM and 1.25 U of glucose-6-phosphate dehydrogenase in Tris-HCl 0.1 M, pH 7.4), 0.5 mg of protein and 0.3 mM substrate concentration (Nebbia et al., 2003). After a suitable incubation time, reactions were quenched with ice-cold trichloroacetic acid (10%, w/v) and the amount of the released formaldehyde was determined by fluorescence (excitation and emission wavelengths of 410 and 510 nm, respectively) in a spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu Corporation, Kyoto, Japan) on an aliquot of the clear supernatant with Nash's reagent as detailed by Werringloer (1978).

### Isolation of RNA and reverse transcription

Total RNA was isolated from about 2–3 snap-frozen liver slices, after 12 h culture either in the absence or in the presence of DEX, using Trizol™ according to manufacturer's protocols. To test the RNA for integrity and DNA contamination, the 28 S/18 S band intensity ratio was visualised for each extracted sample after 1.5% agarose gel electrophoresis using SYBR safe™ staining. Total RNA purity and concentration were determined in a spectrophotometer by recording the absorbance at 260 and 280 nm after a 1:500 sample dilution in RNase-free water. All extracted samples had 260/280 ratios (purity) higher than 1.8. The reverse transcription was performed with 2 µg of total RNA in a final volume of 20 µl, using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA) following the manufacturer's protocol. The reaction was performed in a thermocycler MyGene model MG96+ (LongGene Scientific Instruments Co. Ltd., Hangzhou, China) for 10 min at 25 °C, 120 min at 37 °C and, finally, 5 s at 85 °C for enzyme inactivation. The tRNA and cDNA were stored at −72 °C for future use.

### Quantitative real-time PCR (qPCR)

The NCBI (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) and Ensembl (Ensembl Genome Browser [http://www.ensembl.org/index.html]) databases were used for searching available rat gene sequences to design primers by means of Primer Express software (Applied Biosystems, Foster City, CA), taking into account the possible amplicon secondary structures. In all cases, the primer pairs were designed to fall on different exons or across exon–exon junctions avoiding possible amplification of contaminating genomic DNA; the amplicon specificity of the primer pair was tested by a Blast analysis against the genomic database. Reference and target genes from cattle were as previously published (Cantiello et al., 2009; Janovick-Guretzky et al., 2007). Primer sequences, amplicon sizes, GenBank/Ensembl accession numbers or bibliographic references for primer sets used for quantitative real time (qPCR) analysis are shown in Table 1. The selected reference genes were: *actin β* (*ACTB*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *ribosomal protein S9* (*RPS9*). Amplification efficiencies of reference genes (*ACTB* and *GAPDH* for rat; *GAPDH* and *RPS9* for cattle) were approximately equal to target genes and no differences in the expression of these genes were observed between control and DEX-exposed liver slices. The expression of rat *CYP3A23*, cattle *CYP3A28* and transcriptional

Table 1. Primer sequences, amplicon size, GenBank/Ensembl accession numbers or bibliographic references for primer sets used for quantitative real time PCR.

| Gen symbol | Primer sequence (5′→3′) | GC (%) | Amplicon Size (bp) | GenBank/Ensemblaccession N°/Reference |
|---|---|---|---|---|
| Rat | | | | |
| ACTB | F: ACCAGTTCGCCATGGATGAC | 55 | 57 | BC063166.1 |
| | R: TGCCGGAGCCGTTGTC | 69 | | |
| GAPDH | F: GGCAAGTTCAACGGCACAGT | 55 | 80 | ENSRNOT00000050443 |
| | R: CGCTCCTGGAAGATGGTGAT | 55 | | |
| CYP3A23 | F: GAGGCAAGAGAAAGGCAAACC | 52 | 60 | ENSRNOT00000075671 |
| | R: TCCATGCTGTAGGCACCAAA | 50 | | |
| PXR | F: CTAATGGCGGCTTCCAGAAG | 55 | 60 | ENSRNOT00000003934 |
| | R: TCAGCATGCAGTGGAATTTCA | 43 | | |
| RXRα | F: GGTGTCAGTACTGCCGATACCA | 55 | 57 | ENSRNOT00000012892 |
| | R: CGGCTTCCCGCTTCATG | 65 | | |
| GR | F: CCACAGCAGGGCCAACTT | 61 | 90 | ENSRNOT00000019409 |
| | R: GCTGGTCGACCTATTGAGGTTT | 50 | | |
| Cattle | | | | |
| GAPDH | R: TGGAAAGGCCATCACCATCT | 50 | 60 | Janovick-Guretzky et al. (2007) |
| | F: CCCACTTGATGTTGGCAG | 56 | | |
| RPS9 | R: CCTCGACCAAGAGCTGAAG | 58 | 62 | Janovick-Guretzky et al. (2007) |
| | F: CCTCCAGACCTCACGTTTGTTC | 55 | | |
| CYP3A28 | R: GCCAGAGCCCGAGGAGTT | 67 | 77 | Cantiello et al. (2009) |
| | F: GCAGGTAGACGTAAGGATTTATGCT | 44 | | |
| PXR | R: TGAAGGCCTACATCGAGTTCAAC | 48 | 68 | Cantiello et al. (2009) |
| | F: GGCCATGATCTTCAGGAACAA | 48 | | |
| RXRα | R: GCCTCAATGGTGTCCTCAAAG | 52 | 120 | Cantiello et al. (2009) |
| | F: AGCTGTACACCCCGTAGTGCTT | 55 | | |
| GR | R: AGCAGTGGAAGGACAGCACAA | 52 | 71 | Cantiello et al. (2009) |
| | F: TTCTTCGAATTTTATCAATGATACAATCAT | 23 | | |

ACTB: β-actin; bp: base pair; CYP3A23/CYP3A28: cytochrome P450 3A23 (rat) and 3A28 (cattle); F: forward; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GC: guanine/cytosine content; GR: glucocorticoid receptor; PXR: pregnane X receptor; RPS9: ribosomal protein S9; R: reverse; RXRα: retinoid X receptor alpha.



factors presumably involved in their regulation (*glucocorticoid receptor: GR; pregnane X receptor: PXR and retinoid X receptor alpha: RXRα*) was assessed in control and DEX-exposed liver slices from both species after 12 h culture.

The qPCR was carried out in an ABI Prism 7500 Real Time PCR System (Applied Biosystems, San Diego, CA). The reaction mixture included 10 µL of PCR SYBR Green Master Mix 2X (Applied Biosystems, San Diego, CA), 2 µL of each primer set, 1 µL of cDNA diluted 1:250 and 7 µL of water to obtain a final volume of 20 µL. The amplification reaction was performed in 96-well plates. The qPCR was carried out using the following thermal profile: 2 min at 50 °C followed by 10 min at 95 °C (Holding Stage), then 95 °C over 15 s followed by 1 min at 60 °C (40 cycles). Each primer set was assayed under these conditions in a 200–500 nM range to identify the concentration providing the highest sensitivity and specificity for each target sequence. No template controls were included for each primer pair and each qPCR reaction was carried out in duplicate. Gene-specific amplification was confirmed by a single peak in the melting-curve analysis and a single band on a 2% agarose gel stained with SYBR safe.

Calibration curves were performed after the amplification of decreasing amounts of a cDNA pool, making dilutions at 5-fold intervals, to evaluate the qPCR efficiency for a given gene in each tissue. Standard curves displayed slope values between −3.6 and −3.1 and $r^2$ higher than 0.980 as recommended in the ABI Prism 7500 Real Time PCR System guidelines (Applied Biosystems San Diego, CA). The amplification efficiency (%) for each primer pair was calculated from the expression $(10^{-1/\text{slope}} - 1) \times 100$, where slope is the mean of quantification cycles (Cq) against the logarithm of the cDNA dilution. Slope and $r^2$ values, qPCR efficiency and dynamic range for each target gene in each tissue are shown in Table 2. Selected reference genes showed linear dynamic ranges between 16.4 and 33.6, efficiencies between 95 and 100% and linear regression coefficients higher than 0.990.

Table 2. Validation parameters for the quantitative PCR assays of each target and reference gene studied in liver slices from rat and cattle.

| Target genes | Slope | Efficiency (%)[a] | Linear dynamic range[b] | $r^2$ |
|---|---|---|---|---|
| Rat | | | | |
| ACTB | −3.56 | 96 | 19.75–28.48 | 0.994 |
| GAPDH | −3.43 | 95 | 16.43–24.99 | 0.991 |
| CYP3A23 | −3.47 | 94 | 16.90–31.70 | 0.999 |
| PXR | −3.46 | 95 | 25.04–32.03 | 0.980 |
| RXRα | −3.38 | 98 | 25.70–32.40 | 0.980 |
| GR | −3.54 | 92 | 26.39–34.65 | 0.987 |
| Cattle | | | | |
| GAPDH | −3.25 | 100 | 19.70–31.90 | 0.998 |
| RPS9 | −3.32 | 100 | 21.60–33.60 | 0.996 |
| CYP3A28 | −3.47 | 94 | 21.50–34.30 | 0.980 |
| PXR | −3.62 | 88 | 21.82–32.55 | 0.994 |
| RXRα | −3.47 | 94 | 21.30–28.90 | 0.998 |
| GR | −3.50 | 93 | 22.27–34.30 | 0.986 |

ACTB: β-actin; bp: base pair; CYP3A 23/28: cytochrome P450 3A23 (rat) and 3A28 (cattle); GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GR: glucocorticoid receptor; PXR: pregnane X receptor; RPS9: ribosomalprotein S9; RXRα: retinoid X receptor alpha.

[a]Efficiency $(\%) = (10^{-1/\text{slope}} - 1) \times 100$.

[b]Range of Cq (cycle of quantification) obtained from target cDNA serial dilutions.

### Data and statistical analysis

Cq values were converted into RQ via the Δ-Cq method (Livak & Schmittgen, 2001), incorporating the calculated amplification efficiency for each primer pair. RQ values were calculated using the average Cq off all samples as a calibrator. The relative abundance of a specific mRNA (mean ± SEM) was normalised to the expression of reference genes and given as fold-change compared to control values. Metabolic activities (mean ± SD) were expressed in nmol of a metabolite formed per min per mg of microsomal protein (nmol/min mg). Statistical comparisons between the results obtained in both control and DEX-exposed liver slices were performed by unpaired Student's *t*-test with the Welch correction (Instat 3.0; Graph Pad Software Inc., San Diego, CA). $p$ Values lower than 0.05 were considered significant.

## Results

### Tissue viability

The microscopic examination of H/E stained cross-sections of liver slices from both rat and cattle is shown in Figure 1. Normal tissue architecture with easily visible nuclei was observed in liver slice specimens examined by light microscopy. Some vacuolization of the hepatocytes of the inner layers (rat liver slices) and several condensed nuclei (bovine liver slices) were the unique signals of cellular damage observed in all histological preparations after 12 h culture.

LDH activity was monitored in the culture medium and in post-mitochondrial fractions from liver slices after 1 h pre-culture period ($t = 0$) and following 12 h culture both in the absence (control) and in the presence of DEX (Figure 2). No changes in LDH enzyme activities were observed between $t = 0$ h and $t = 12$ h in the culture medium and in liver slices from rat. Conversely, LDH activity in bovine liver slices were lower ($p < 0.01$) at $t = 12$ h compared to $t = 0$ h, whereas a tendency to decrease along with the incubation time was also apparent in the culture medium. Higher percentages of LDH leakage were observed at $t = 0$ h and $t = 12$ h (control and DEX) in cattle compared to rat liver slice cultures (Figure 2, inserted table).

### Gene expression profiles and CYP3A-dependent enzyme activity

Table 3 shows the effect of DEX on mRNA relative abundances of rat *CYP3A23* and cattle *CYP3A28* and the most important transcriptional factors (*GR*, *PXR* and *RXRα*) presumably involved in CYP3A expression and regulation in the liver of both species. In rat liver slices, a 3.2-fold increase ($p = 0.028$) of *CYP3A23* gene expression was observed after 12 h culture in the presence of DEX. The presence of DEX did not affect the expression profiles of *GR*, *RXRα* and PXR, although the latter showed a tendency ($p = 0.058$) to be up-regulated by exposure to the glucocorticoid. On the other hand, DEX did not affect mRNA expression levels of cattle *CYP3A28* and all transcriptional factors analyzed.

Figure 3 shows TAO N-demethylase activities measured in post-mitochondrial fractions from both rat and cattle liver slices after 12 h culture. This enzyme activity increased 3.4-fold ($p < 0.05$) in rat liver slices cultured in the presence of DEX. Conversely, exposure to DEX did not affect this CYP3A-dependent enzyme activity in cattle liver slices.

## Discussion

Liver viability has a pivotal role in the outcome of studies on the expression and function of xenobiotic metabolizing enzymes carried out using tissue slicing methods. Liver tissue histopathology and LDH leakage are among different viability parameters that can be used for the assessment of slice quality (Fisher & Vickers, 2013). The ability to isolate total RNA for gene expression studies has been additionally used for this purpose (Vickers & Fisher, 2004). In the current work, microscopic observations of H/E-stained cross sections of liver slice specimens from both rat and cattle showed no signs of tissue necrosis after a 12-h incubation period. It has been shown that, under optimal culture conditions, tissue slices can

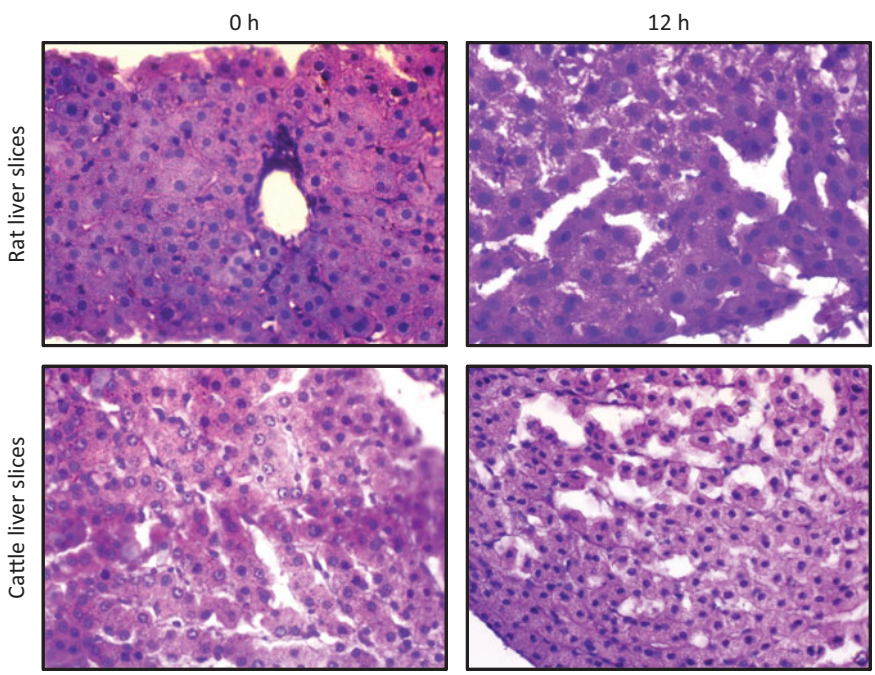


Figure 1. Histology of rat and cattle liver slice specimens at 0 h (left panel) and after 12 h in culture (right panel). Liver slice cross-sections were stained with Hematoxylin–Eosin and photomicrographs were taken using 400× magnification.

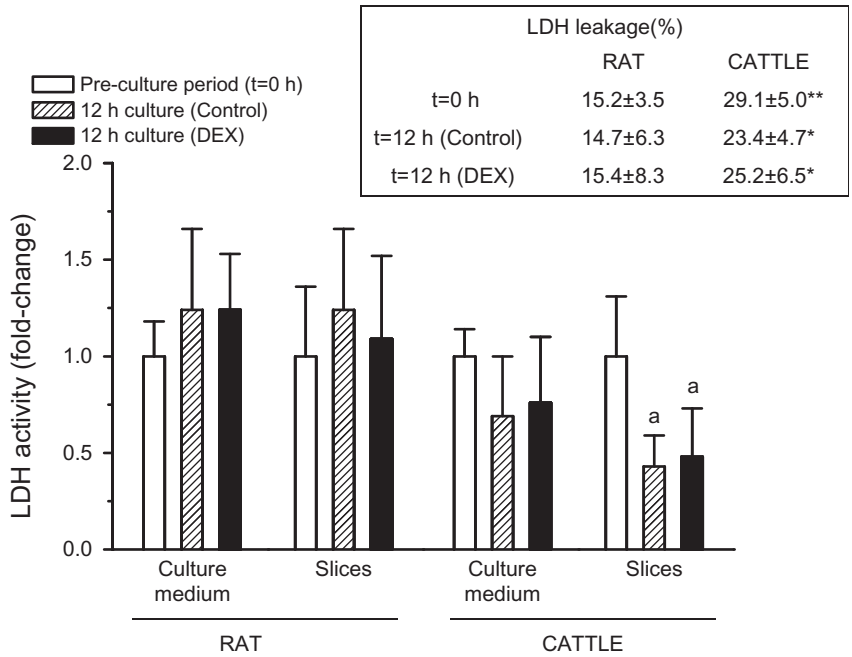


Figure 2. Lactate dehydrogenase (LDH) activities measured in the culture medium and liver slices (post-mitochondrial fractions) after a 1 h pre-culture period ($t = 0$ h) and 12 h culture either in the absence (control) or in the presence of 100 μM dexamethasone (DEX). Enzyme activities measured at 12 h are expressed as fold-change compared to those measured at $t = 0$ h. Mean (±SD) percentages of LDH leakage are shown in the inserted panel. (a) Significantly different ($p < 0.01$) compared to $t = 0$ h. LDH leakages observed in cattle liver slices were significantly different (*$p < 0.05$; **$p < 0.01$) compared to rat liver slices.

Table 3. Effect of dexamethasone (DEX) on the relative abundances of nuclear receptors (PXR, RXRα, GR), rat CYP3A23 and cattle CYP3A28 mRNAs in liver slices from both species.

| | Control | DEX | *p* |
|---|---|---|---|
| Rat | | | |
| PXR | 1.00 ± 0.23 | 1.85 ± 0.26 | 0.058 |
| RXRα | 1.00 ± 0.30 | 1.76 ± 0.49 | 0.160 |
| GR | 1.00 ± 0.24 | 1.12 ± 0.11 | 0.410 |
| CYP3A23 | 1.00 ± 0.21 | 3.19 ± 0.86 | 0.028* |
| Cattle | | | |
| PXR | 1.00 ± 0.16 | 0.92 ± 0.21 | 0.580 |
| RXRα | 1.00 ± 0.12 | 1.13 ± 0.33 | 0.950 |
| GR | 1.00 ± 0.15 | 1.22 ± 0.21 | 0.710 |
| CYP3A28 | 1.00 ± 0.11 | 1.19 ± 0.21 | 0.660 |

Liver slices from both species were incubated (12 h) without (control) or with DEX (100 μM). Data (mean ± SEM) are expressed as fold-change with respect to control liver slices. CYP3A23 and CYP3A28: cytochrome P450 3A23 (from rat) and 3A28 (from cattle); GR: glucocorticoid receptor; PXR: pregnane X receptor; RXRα: retinoid X receptor alpha. *Significantly different ($p < 0.05$) from its respective control value.

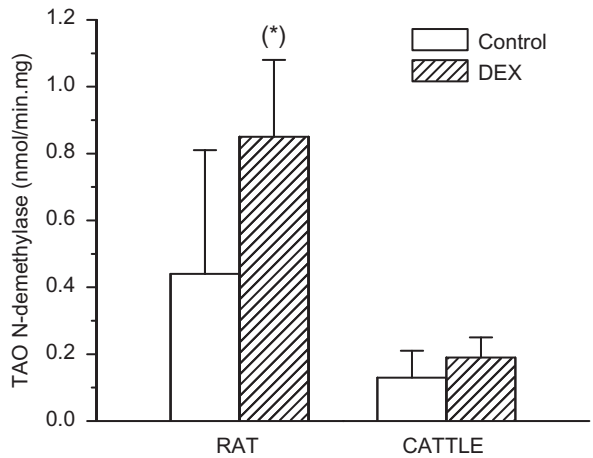


Figure 3. Triacetyl-oleandomycin (TAO) N-demethylase activity measured in post-mitochondrial fractions obtained from rat and cattle pooled liver slices after 12 h culture either in absence (control) or in the presence of dexamethasone (DEX). Liver slices were obtained from six (6) rats and six (6) steers. Homogenates for preparation of post-mitochondrial fractions were prepared with at least five (5) control or DEX-exposed liver slices from each animal. Data are expressed as mean ± SD. *Significantly different ($p < 0.05$) from control incubations.

maintain their normal architecture for up to 7 days (Fisher & Vickers, 2013). Moreover, electron microscopic observations denoted that nuclear and organelle integrity was maintained throughout extended culture periods (Vickers & Fisher, 2004). On the other hand, LDH leaking values observed in the current work correspond to medium-quality livers, considered suitable for studies on the expression and function of xenobiotic metabolizing enzymes (Fisher et al., 2001). Similar values of LDH leakage were reported previously after 12 h culture of liver slices from deer and cattle (Sivapathasundaram et al., 2004). However, higher percentages of LDH leakage were observed in liver slice cultures from cattle compared to rat. Such differences may be explained by different organ procurement procedures used for both species in the current work. The ischaemia time (the interval an organ remains at body temperature after sacrifice and then in a hypothermic preservation solution during transport) was longer when slices were produced from bovine liver instead of rat liver. Further to this observation, the data on LDH leakage, the absence of significant tissue damage and the ability to isolate total RNA for qPCR, may altogether indicate no or minimum detrimental effects on tissue viability during the 12-h culture period employed in the experimental assays described here.

A number of reports available in the literature described the DEX-mediated induction of CYP3A isoenzymes in rat and human liver. Even a single dose of DEX phosphate caused a temporal increase in a CYP3A-dependent activity (midazolam hydroxylation) in rat liver microsomes, reaching maximum values at 12 h after the glucocorticoid administration (Iwanaga et al., 2013). Studies performed with primary human hepatocyte cultures and HepG2 cells (human

hepatocellular liver carcinoma) showed that DEX produces a biphasic induction of human *CYP3A4* mRNA (Pascussi et al., 2001). These authors showed that DEX, at nanomolar concentrations, caused an induction of low amplitude (3 - to 4-fold), whereas an inductive response of higher magnitude (15- to 30-fold) was observed at micromolar concentrations (up to 100 μM); the latter are those that may be systemically achieved after bolus administration or under stress conditions in humans (Pascussi et al., 2001). The highest level of *CYP3A4* induction in human liver hepatocyte models was observed with 100 μM DEX (Pascussi et al., 2001), the concentration selected for the assays described herein. In this regard, this concentration is much higher than the highest level found in the systemic circulation of cows treated with a therapeutic dose of DEX by intravenous or intramuscular injections (Toutain et al., 1982).

Rat *CYP3A23* (formerly *CYP3A1*) is an inducible isoenzyme (Souček & Gut, 1992) and, for this reason, was selected for the current studies on CYP3A expression and function in rat liver slices. Moreover, an increased gene expression of *CYP3A23* and higher CYP3A-dependent enzyme activities were observed in male rat liver slices cultured during 6 and 24 h in the presence of 100 μM DEX (Glöckner et al., 2003). Compared to control liver slices, mRNA abundances were between 4-fold (6 h) and 14-fold (24 h) higher in the presence of DEX (Glöckner et al., 2003); the inductive effect was stronger (70-fold) following 3 days culture in the presence of the same concentration (Martignoni et al., 2004). Indeed, our results are in agreement with those previous observations on DEX-mediated induction of *CYP3A23* in rat liver slices. In fact, after 12 h culture, DEX caused a 3.2-fold increase on the gene expression of rat *CYP3A23* (Table 3), whereas the CYP3A-dependent enzyme activity tested (TAO N-demethylase) resulted 3.4-fold higher (Figure 3). The findings described here, together with previously published observations, indicate that rat liver slices may represent a reference model, useful for making comparisons on the expression and function of CYP3A isoenzymes in cattle.

The basic mechanism of glucocorticoid action involves an interaction with the GR followed by the regulation of glucocorticoid-responsive genes and, consequently, the expression and function of different proteins synthesised by target tissues (Yudt & Cidlowski, 2002). However, in the case of *CYP3A*, earlier studies showed that glucocorticoid compounds may utilize non-classical pathways involving the induction or the activation of other transcriptional factors such as *PXR* and the constitutive androstane receptor (*CAR*; Quatrochi & Guzelian, 2001). *PXR* has been implicated in two mechanisms involved in DEX-mediated induction of human *CYP3A4* and rat *CYP3A23* in primary hepatocyte cultures from both species (Pascussi et al., 2001; Quatrochi & Guzelian, 2001). These authors showed that low (nanomolar) concentrations of DEX modulate the induction of *PXR* through the classical *GR* pathway. Conversely, at micromolar concentrations, the inductive response is due to DEX binding and activation of *PXR*. Once in the nucleus, *PXR* binds to *RXRα*; the heterodimer formed (*PXR-RXRα*) can interact with specific response elements in *CYP3A4* or *CYP3A23* gene promoter regions (Pascussi et al., 2001; Quatrochi & Guzelian, 2001). Also, induction of *CYP3A* isoenzymes through activation of *PXR* by DEX has been observed in rat liver slices (Cui et al., 2005). In agreement with these previous observations, the micromolar DEX concentration used in the current assays did not modify the gene expression profiles of *PXR*, *GR* or *RXRα*. In conclusion, activation of *PXR* in the presence of high DEX concentrations, rather than increased gene expression profiles of related transcription factors, may be considered as a primary mechanism involved in *CYP3A23* induction in rat liver.

A remarkable species-specific induction profile for the CYP3A subfamily has been recognised. This fact precludes extrapolating data from rodents and other laboratory animals (Quatrochi & Guzelian, 2001). It is therefore not surprising that, unlike in rat liver slices, the present work shows that DEX neither affected the gene expression profile of *CYP3A28* nor the *CYP3A* activity tested in cattle liver slices after 12 h culture. Previous investigations showed contradictory results on the expression levels of different CYP3A isoenzymes in the liver of DEX-treated cattle. A number of factors affecting the expression and function of xenobiotic metabolizing enzymes (i.e. administered dose, treatment schedule, diet, sex, age of animals) may have accounted for such observations, which complicates the comparison of the data obtained in different experimental assays. For instance, neonatal calves receiving sub-therapeutic doses of DEX (30 μg/kg b.w., twice daily during 4 days) had ∼40% lower mRNA abundances of both *CAR* and *PXR* compared to untreated animals, whereas *CYP3A* mRNA levels remained unaffected in treated animals (Greger & Blum, 2007). Similarly, decreased hepatic *CYP3A28* mRNA, apoprotein content and CYP3A-dependent catalytic activities were observed when DEX sodium phosphate was orally administered (as a growth promoter) to veal calves for 23 days (Cantiello et al., 2009). Conversely, up-regulation of *CYP3A28*, *CAR* and *RXRα* mRNAs in cattle liver was observed after the oral administration of a sub-therapeutic dose of DEX for 50 days, while *CYP3A28* and *RXRα* mRNAs increased when DEX was administered by the oral route for 43 days in combination with 3 intramuscular doses of 17β-oestradiol at 15-day intervals (Giantin et al., 2010). The *in vitro* model used in the current research did not reproduce any of the observed *in vivo* effects of DEX on the expression of *CYP3A28* transcription factors in cattle liver. Undoubtedly, a further optimization of tissue slicing methodologies is needed for the improvement of studies on the expression and function of xenobiotic metabolizing enzymes in cattle liver slices. In this regard, longer culture periods or incubations in the presence of different concentrations of DEX (or other xenobiotics) may be necessary.

## Conclusion

In conclusion, a micromolar DEX concentration up-regulated *CYP3A23* mRNA in rat liver slices after 12 h culture, whereas the gene expression profiles of transcriptional factors involved in *CYP3A* regulation were unaffected. The *CYP3A23* induction was accompanied with an increased CYP3A-dependent enzyme activity. The protocol used for rat liver slices was used as a reference to study the expression of *CYP3A28* in cattle liver slices. Oppositely to the observations in rat liver slices, DEX neither affected the gene expression profile of

*CYP3A28* nor the CYP3A activity tested in cattle liver slices. Also, the glucocorticoid compound did not affect the expression of transcriptional factors presumably involved in CYP3A regulation. To the best of our knowledge, the current work is the first attempt to study the modulation of a xenobiotic metabolizing enzyme employing cattle liver slices. Although a further improvement is needed, this *in vitro* model may be useful for studies on the expression and function of xenobiotic metabolizing enzymes in cattle and in other ruminant species.

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## Declaration of interest

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