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RESEARCH ARTICLE

Assessment of liver slices for research on metabolic drug–drug interactions in cattle

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Abstract

1. Precision-cut liver slices (PCLS) from food-producing animals have not been extensively used to study xenobiotic metabolism, and thus information on this field of research is sparse.
2. The aims of the present work were to further validate the technique of production and culture of bovine PCLS and to characterize the metabolic interaction between the anthelmintic albendazole (ABZ) and the flavin-monooxygenase (FMO) inhibitor methimazole (MTZ).
3. Nine steers were used as donors. PCLS were produced and incubated under two methods: a dynamic organ culture (DOC) incubator and a well-plate (WP) system.
4. Tissue viability, assessed through both structural and functional markers, was preserved throughout 12 h of incubation. ABZ was metabolized to its (+) and (-) albendazole sulfoxide stereoisomers (ABZSO) in bovine PCLS. The interaction between ABZ and MTZ resulted in a reduction ($p < 0.001$) in the rates of appearance of (+) ABZSO. Conversely, in presence of MTZ, the rates of appearance of (−) ABZSO increased under both systems ($p < 0.05$).
5. Both culture systems were suitable for assessing the interaction between ABZ and MTZ.
6. Overall, the results presented herein show that PCLS are a useful and reliable tool for short-term studies on metabolic drug–drug interactions in the bovine species.

Keywords

Albendazole, cattle, metabolism, precision-cut liver slices, viability

History

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Introduction

The liver plays a major role in metabolism of both endogenous and exogenous compounds (Kanter et al., 2002). A wide array of *in vitro* methods has been developed in order to mimic structural and functional features within this organ. Such systems, including subcellular fractions (e.g. microsomal and cytosolic fractions), recombinant enzymes, primary hepatocytes, precision-cut slices, and engineered cell lines, are routinely used to evaluate specific aspects of metabolism, including drug–drug (D–D) interactions, gene regulation, toxicity, and stress response (Asha et al., 2010; Brandon et al., 2003; Lake & Price, 2013). However, insights provided by such *in vitro* models are not readily extrapolated to the living organism. As a general rule, the simpler the system in terms of inherent structure, the worse a predictive model it becomes (Brandon et al., 2003).

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Precision-cut liver slices (PCLS) are tissue explants produced by semi or fully automated methods (de Graaf et al., 2010). As cell lines and primary tissue cultures, PCLS can be maintained *in vitro* for a finite period under optimal conditions, which depending on the species amounts to around 5 days (Asha et al., 2010; Fisher et al., 2013). Originally used in physiology and toxicology studies (Bach et al., 1996; Lerche-Langrand & Toutain, 2000; Worboys et al., 1997), PCLS have since shown clear advantages in other research areas, including the evaluation of metabolic and excretory pathways and D–D interactions in different species (de Graaf et al., 2007; De Kanter et al., 2002; Ioannides, 2012; van de Kerkhof et al., 2008). In contrast to other *in vitro* models, the original scaffolding system, cell populations and relationships are maintained in PCLS, thus closely mimicking the cellular and humoral interactions within the intact organ (de Graaf et al., 2010; Elferink et al., 2011; Olinga et al., 2001). For human and rat PCLS, a better *in vitro–in vivo* correlation has been shown compared with other *in vitro* methods, including microsomal fractions and primary hepatocytes (de Graaf et al., 2007).

A crucial aspect of PCLS preparation and culture is viability (Godoy et al., 2013). Viability can be assessed by a

variety of methods, including the leakage of liver enzymes (e.g. aspartate aminotransferase or lactate dehydrogenase), ATP content, intracellular potassium (K_i^+) content, reduced (GSH) to oxidized (GSSG) glutathione ratio, and histomorphological evaluation (de Graaf et al., 2003; Fisher et al., 2002). The latter is considered a highly sensitive and reliable viability marker, bearing in turn a good correlation with both ATP and K_i^+ levels (Godoy et al., 2013). The use of two markers of tissue structural integrity and an additional parameter, such as metabolism of a model compound, to assess functional integrity of PCLS, is recommended (de Graaf et al., 2010).

A variety of culture systems for both short- and long-term incubation of PCLS have been developed in order to preserve tissue viability and allow for different experimental approaches. Such systems include dynamic organ culture (DOC) incubators, 6- or 12- well-plate (WP) systems and, more recently, perifusion systems, which rely on biochips equipped with microfluidics-based circulation (van Midwoud et al., 2010; van Midwoud et al., 2011). The DOC incubators and the WP systems remain the most widely used techniques for long- and short-term studies, respectively.

Metabolic interactions with xenobiotic metabolizing enzymes may drastically affect the disposition kinetics of different drugs used in animal production, which will have a relevant impact on the pattern of drug/metabolite residues in edible tissues, a major concern for public health and consumer safety. Therefore, the assessment of reliable bioassays to investigate D–D metabolic interactions in target species is a major concern for veterinary pharmaco-toxicologists. Understanding these mechanisms could lead to better treatment protocols based on a rational understanding of species-specific characteristics. This knowledge also has a direct impact on the handling of drug residues in livestock. Most of the published research on tissue slicing methodologies has been carried out with liver samples from humans and laboratory animals. To the best of our knowledge, liver slices from ruminants and other food-producing animals have not been extensively used to study metabolism of exogenous compounds, and thus information on this field of research is sparse. Therefore, in view of the potential usefulness of bovine liver slices as an *in vitro* tool for the evaluation of metabolic/excretory pathways involved in the elimination of xenobiotics, the aims of the present work were to further validate the technique of PCLS in this species, and to compare slice structural and functional integrity under both the WP system and the DOC incubator. Three markers of tissue structural integrity, namely histomorphological evaluation, K_i^+ content, and GSH/GSSG measurements were assessed. Metabolism of the anthelmintic albendazole (ABZ) by cattle PCLS was evaluated as a marker of functional integrity. Furthermore, the metabolic interaction between this anthelmintic and methimazole (MTZ), a well-known inhibitor of ABZ metabolism, was evaluated. This interaction, which has been extensively characterized in ruminants, was assayed in order to establish the usefulness of PCLS for the assessment of metabolic D–D interactions in cattle.

Materials and methods

Chemicals

Bovine insulin was purchased from Beta Laboratories (Buenos Aires, Argentina). Gentamicin was obtained from Parafarm (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 O-pthalaldehyde were from Thermo Fisher Scientific (Carlsbad, CA). Salts (KCl, NaCl, $Cl_2Ca{\cdot}2H_2O$, $Cl_2Mg{\cdot}6H_2O$, $NaHCO_3$, Na_2CO_3, Na_2HPO_4, $NaH_2PO_4{\cdot}H_2O$, K_2HPO_4, KH_2PO_4, CH_3COONH_4 and NaOH), and acetonitrile (ACN) were purchased from Baker Inc. (Phillipsburg, NJ). Reference standards of ABZ, albendazole sulfoxide (ABZSO) and albendazole sulfone ($ABZSO_2$), Williams' Medium E (WME), ampicillin, EDTA, N-ethylmaleimide, $HClO_4$, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

Animals

Bovine liver samples from nine Aberdeen Angus/Hereford crossbreed steers with an approximate weight of 350 kg were obtained from a local slaughterhouse (Mirasur SA, Tandil, Argentina) located 16 km away from the laboratory facilities. Immediately following sacrifice, a sample from the caudate lobe was obtained. Each sample was rinsed with ice-cold 1.15% KCl and immersed in ice-cold Krebs buffer (1mM $NaH_2PO_4{\cdot}H_2O$; 2.5mM $Cl_2Ca{\cdot}2H_2O$; 4.7mM KCl; 1.1mM $Cl_2Mg{\cdot}6H_2O$; 0.004mM EDTA; 11mM glucose; 119mM NaCl, 25mM Na_2CO_3; 0.11 mM ascorbic acid). Containers were covered, chilled in ice, and transported to the laboratory within 30–40 min for subsequent procedures.

Preparation of PCLS

PCLS were obtained as previously described (Maté et al., 2015). Briefly, cylindrical cores with a diameter of 10 mm were produced by means of a hand-held sharpened stainless steel punch (Vitron Inc., Tucson, AZ). The liver cores thus obtained were placed in aerated (95% O_2:5% CO_2) ice-cold WME supplemented with 25 mM D-glucose, 50 mg/mL gentamicin, 1 mM insulin, and 1.25 mg/mL amphotericin B. The cores were then mounted on a Brendel/Vitron tissue slicer (Vitron Inc., Tucson, AZ) filled with oxygenated ice-cold Krebs buffer. Precision-cut slices with a thickness of 0.25 mm and a wet weight of 19.6 ± 4.8 mg were produced. Immediately after preparation, slices were transferred into a Petri dish containing ice-cold oxygenated WME.

Culture of liver slices

Dynamic organ culture (DOC) incubator

Liver slices from six animals were placed in type C titanium roller inserts (Vitron Inc., Tucson, AZ) and cultured in glass vials containing 1.7 mL of WME (one slice per vial). Vials were placed into a DOC 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.

Well-plate (WP) system

Liver slices from three animals were transferred to 12-well culture plates (Corning Incorporated, Corning, NY) with 1.3 mL of WME (one slice per plate) inside a container with a humidified atmosphere of 95% O_2:5% CO_2. The container was placed on an orbital shaker (Ferca, Buenos Aires, Argentina) set at 60 rpm and maintained at 37 °C.

PCLS experiments

In both the DOC incubator and the WP system, slices were initially preincubated for 1 h ($t = -1$ h) in order to remove cell debris due to the slicing procedure. Following the preincubation period, the medium was completely replaced with fresh oxygenated WME in both culture systems. After 0, 6, and 12 h slices were harvested, snap-frozen in liquid N_2 and stored at −70 °C until analysis. Slices sampled for intracellular K^+ measurement were additionally washed using NaCl 0.9% prior to snap freezing. Samples for histological evaluation were stored in 10% buffered formalin at room temperature.

Histopathological evaluation

Histological analysis was carried out to evaluate the structural integrity of incubated PCLS. Slices were harvested at 0, 6, and 12 h and immediately fixed in 10% buffered formalin for histopathological evaluation. After 24 h, the slices were dehydrated with ethanol and embedded in paraffin. Paraffin blocks were sectioned using a rotary microtome to obtain 5 μm thick cross-sections. The sections were then rehydrated in distilled water and stained with hematoxylin and eosin (HE). The histomorphological evaluation was carried out by a trained veterinary pathologist. The pathologist was blind to the identity of each sample. Three morphological indicators, i.e. hepatocyte loss, hydropic degeneration and hepatocyte fragmentation, were evaluated. For each individual indicator, the degree of severity was scored using a subjective scale comprising degrees 0 (absence of histological damage), 1 (mild), 2 (moderate), and 3 (severe damage).

Preparation of tissue homogenates and biochemical determinations

Five individual PCLS were pooled for homogenization; at least 2 (DOC incubator) or 3 pools (WP system) per animal were prepared for each incubation time. For determination of glutathione content, thawed slices were homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl and 18 μM butylated hydroxytoluene. Homogenates were centrifuged at 800 g for 10 min at 4 °C. Aliquots (200 μL) of supernatant were frozen in liquid N_2 and stored at −70 °C. Protein content was determined according to Lowry et al. (1951), using bovine serum albumin as standard. Absolute and relative amounts of reduced (GSH) and oxidized (GSSG) glutathione were assessed following a modified procedure from that described by Hissin & Hilf (1976). Briefly, sample aliquots (200 μL) of post-mitochondrial fractions were diluted (1/3 v/v) with 5% trichloroacetic acid (containing 5 mM EDTA) and centrifuged at 10 000 g for 25 min at 4 °C. Fractions for GSSG measurement were further processed by the addition of N-ethylmaleimide 0.04 M to prevent oxidation of GSH to GSSG, followed by the addition of NaOH 0.1 M. The fluorescent reagent O-pthalaldehyde 0.1% was then added to all of the samples, and GSH and GSSG contents were measured by means of a RF-5301 PC spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan). Excitation and emission wavelengths were 350 and 412 nm, respectively. A calibration curve was built using known GSH and GSSG concentrations in the range of 0.5–10 nmol/mL. Results are expressed as pmol of GSH or GSSG per mg of slice.

For determination of K_i^+ content, thawed slices were homogenized in 1 mL distilled water by means of an ultrasonic cell disruptor (XL 2000-010, Misonix, Farmingdale, NY). Protein content was then determined as previously mentioned, and 20 μL of 70% $HClO_4$ was added in order to precipitate proteins. The homogenate was then centrifuged at 10 000 g for 5 min at room temperature. The supernatant was then collected and stored at −70 °C. Intracellular potassium (K_i^+) content was determined following the method described by Fisher et al. (2002). Samples were assayed using an atomic absorption spectrophotometer (GBC Scientific Equipment, Braeside, Australia). A calibration curve was built using known K^+ concentrations between 10.2 and 30.7 nmol/mL. Results are expressed as nmol of K_i^+ per mg of slice.

ABZ metabolism

ABZ was selected as a model compound to evaluate the metabolic ability of bovine PCLS by the amount of its main metabolite, namely ABZ sulfoxide (ABZSO), present after12-h incubation with ABZ in both the DOC incubator and the WP system. Following the preincubation period ($t = -1$ h), the medium in both the DOC incubator and the WP system was completely replaced with fresh oxygenated WME containing either 50 μM ABZ or 50 μM ABZ 50 μM plus 100 μM MTZ. At least 2 (DOC incubator) or 3 slices (WP system) per animal were incubated for each condition. Samples (150 μL) of the culture medium were taken at 2-h intervals, followed by reposition with drug-free WME. At the end of the incubation period, the slices were harvested, snap-frozen in liquid N_2, and stored at −20 °C until analysis by high performance liquid chromatography (HPLC).

Chromatographic analysis

About 20 μL of 0.25 mM OBZ (internal standard solution in methanol) was added to culture medium samples. Samples were then mixed with 100 μL of ACN, shaken and centrifuged at 9000 g for 15 min at 10 °C. For drug extraction from tissue samples, 10 μL of the internal standard solution was added to thawed slice specimens. Samples were briefly stored at 4 °C for 15 min and then homogenized manually. Homogenates were centrifuged at 9000 g for 15 min at 10 °C. Supernatants were evaporated to dryness in a Savant Automatic SpeedVac® System (Thermo Savant, NY). The dry residues were resuspended in the mobile phase (300 μL) and injected into the HPLC system.

Samples were analyzed for ABZ, ABZSO, and $ABZSO_2$. For both medium and tissue samples, a volume of 50 μL was injected into a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a Kromasil C18

reverse-phase column (250 × 4.6 mm, particle size 5 µm, Eka Chemicals, Marietta, GA) and a UV detector (SPD-10 A UV detector, Shimadzu Kyoto, Japan) reading at 292 nm. Chromatographic procedures, including method validation, were undertaken (with slight modifications) following the methodologies described by Virkel et al. (2014). For each sample, the peak fraction corresponding to ABZSO was collected in order to obtain the relative proportions of each enantiomer following previously described procedures (Delatour et al., 1991; Virkel et al., 2014).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The total amounts of ABZSO enantiomers in medium and tissue samples are expressed as nmol. Rates of appearance are expressed as pmol per mg of tissue per h [pmol/(mg.h)]. Ratios between (+) and (-) stereoisomers represent the mean value of the ratio between the total amount of each enantiomer measured in each individual assay. Linearity was determined by linear regression analysis. Biochemical measurements of GSH, GSSG, and K_i^+ across incubation times were compared by means of one-way ANOVA or non-parametric Kruskal–Wallis test depending on whether the SDs were similar or not between time groups. Comparisons between culture methods (DOC incubator and WP system) were performed using two-tailed Student's *t* test with Welch correction for unequal variances among groups. The same statistical test was employed for comparisons among ABZ incubations in absence and presence of the metabolic inhibitor MTZ. Statistical analysis was performed using Instat 3.0 (Graph Pad Software Inc., San Diego, CA). The Shapiro–Wilk method was used as a normality test (R software, version 3.2.2). Differences were considered statistically significant for *p* values below 0.05.

Results

Histomorphological evaluation

The results of the comparative analysis of HE-stained sections of bovine PCLS incubated under either the DOC incubator or the WP system are shown in Table 1 and Figure 1. The mean value of two (DOC incubator) or three (WP system) individual slices was considered as the overall score for each time point, as illustrated in Table 1. Histomorphological evaluation showed no overall differences in terms of structural integrity. Among the selected morphological indicators, hydropic degeneration was the only observed alteration. Mild degrees (score = 1) of hydropic degeneration were observed in all samples at 6 h of incubation under both systems (Figure 1), except for one slice incubated under the DOC system, which exhibited a moderate degree (score = 2). At 12 h of incubation, all the slices incubated under the DOC incubator and one of the slices incubated under the WP system showed a moderate degree of hydropic degeneration.

Table 1. Histological evaluation observed in hepatic slices incubated either in a DOC incubator or in a well-plate system (WP).

Incubation time	Hydropic degeneration	Hepatocyte fragmentation	Hepatocyte loss
DOC			
0 h	0	0	0
6 h	1.5	0	0
12 h	2	0	0
WP			
0 h	0	0	0
6 h	1	0	0
12 h	1.33	0	0

Each value represents the mean of at least two observations. Microscopic evaluation: severity was scored between 0 and 3, with 0 indicating no microscopic lesions and values between 1 and 3 indicating increasingly severe microscopic lesions.

Biochemical determinations

GSH levels in PCLS after 0, 6, and 12 h incubation are shown in Figure 2. The mean contents at $t = 0$ were 0.077 ± 0.003 and 0.072 ± 0.003 pmol/mg of slice for the DOC incubator and the WP, respectively, with no significant differences between both systems. GSH content decreased over time. For the DOC incubator, mean levels dropped significantly (21%, $p < 0.01$) following 12 h incubation when compared with $t = 0$. For the WP system, GSH content at 12 h was significantly lower (17%, $p < 0.01$) than GSH content at $t = 0$ and $t = 6$. GSH/GSSG ratios were calculated for each time point, with no significant differences being observed along the whole incubation period of 12 h. Under the DOC incubator, GSH/GSSG ratios were 1.2 ± 0.5, 1.5 ± 0.2, and 1.2 ± 0.6 following 0, 6, and 12 h incubation, respectively. Under the WP system, GSH/GSSG ratios were 1.2 ± 0.5, 1.4 ± 0.1, and 1.1 ± 0.6 following 0, 6, and 12 h incubation, respectively.

K_i^+ levels after 0, 6, and 12 h incubation are also shown in Figure 2. At $t = 0$, mean K_i^+ content for the DOC incubator and the WP system was 21.1 ± 3.2 nmol/mg of slice and 27.3 ± 3.0 nmol/mg of slice, respectively. No significant differences were observed between both culture systems. Additionally, K_i^+ levels did not differ significantly along the 12-h incubation period within each culture system.

ABZ metabolism

ABZ was metabolized to ABZSO in bovine PCLS. Figure 3 shows the accumulation and the rates of appearance of total ABZSO in the culture medium under each incubation system. Mean accumulation rates exhibited a lineal tendency up to 12 h, with a 1.8-fold higher ($p = 0.001$) accumulation rate of ABZSO under the WP system compared with the DOC incubator. Likewise, the total amounts of ABZSO recovered from the culture medium at 12 h were higher ($p < 0.001$) for the WP system (1240 ± 94 pmol/mg of slice) compared with the DOC incubator (660 ± 53 pmol/mg of tissue). Trace amounts of $ABZSO_2$ were recovered from the culture medium following incubations carried out with the DOC incubator; the mean rate of $ABZSO_2$ accumulation was 4 ± 1 pmol/(mg.h). Conversely, no sulfonation was detected in the WP system after 12 h.

Table 2 shows the rates of appearance of ABZSO enantiomers in the culture medium after incubation of PCLS with ABZ, either in absence (control incubations) or

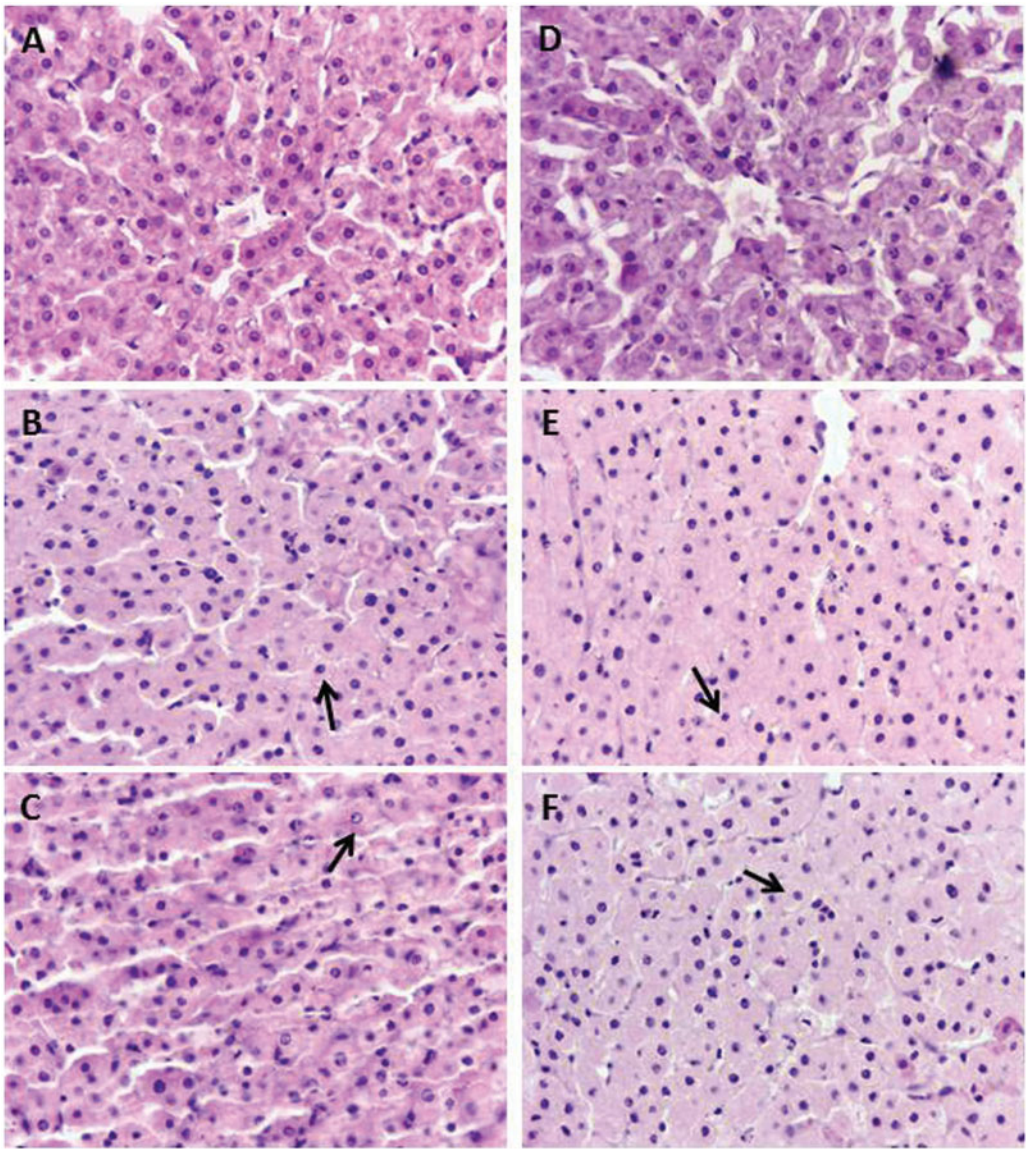

Figure 1. Histopathology of bovine PCLS cultured either in a DOC incubator or in well plates (WP). Liver slice cross-sections were stained with hematoxylin and eosin and photomicrographs were taken using 400× magnification. A and D: Fresh hepatic tissue; B: DOC incubator, 6 h incubation; C: DOC incubator, 12 h incubation; E: WP, 6 h incubation; F: WP, 12 h incubation. PCLS obtained at 6 and 12 h incubation exhibit mild hydropic degeneration (arrows).

in presence of MTZ. When ABZ was incubated alone, (+) ABZSO prevailed in the medium under both culture systems in control incubations, with significant higher ($p<0.001$) rates of appearance compared to those observed for (−) ABZSO. The interaction between ABZ and MTZ resulted in a net reduction ($p<0.01$) in the rates of appearance of (+) ABZSO, which were 80% and 94% lower in the DOC incubator and the WP system, respectively. Conversely, in presence of MTZ the rates of appearance of (−) ABZSO increased 4-fold and 2-fold in the DOC incubator and the WP system, respectively ($p<0.05$). Only in control incubations, the rate of appearance of (+) ABZSO was 2-fold higher ($p<0.001$) under the WP system compared to that observed in the DOC incubator. The amounts of both (+) and (−) ABZSO enantiomers were further quantified in culture medium and tissue samples at 6 h incubation of PCLS with ABZ, either in absence or in presence of MTZ (Figure 4). In presence of MTZ, the amount of (+) ABZSO decreased significantly ($p<0.001$) in the culture medium under both incubation systems, whereas a slight increase ($p<0.001$) of this enantiomer was detected in the tissue only in WP. In the WP system, a slight decrease ($p<0.05$) in the amount of (−) ABZSO was observed in the culture medium when ABZ was incubated in presence of MTZ. Conversely, higher ($p<0.01$) amounts of (−) ABZSO were detected within the liver tissue under both culture systems when ABZ was incubated in presence of the metabolic inhibitor. In accordance with these observations, in control incubations performed under both the DOC incubator and the WP system, the (+) ABZSO/(−) ABZSO enantiomeric ratios in the culture medium were significantly higher ($p<0.001$) than those observed within the liver tissue (Figure 4, inserted tables). When ABZ was incubated in presence of MTZ, the (+) ABZSO/(−) ABZSO enantiomeric ratios in tissue decreased significantly (DOC incubator: 37%, $p<0.001$; WP system: 20%, $p<0.05$) compared to those observed in control incubations. In the

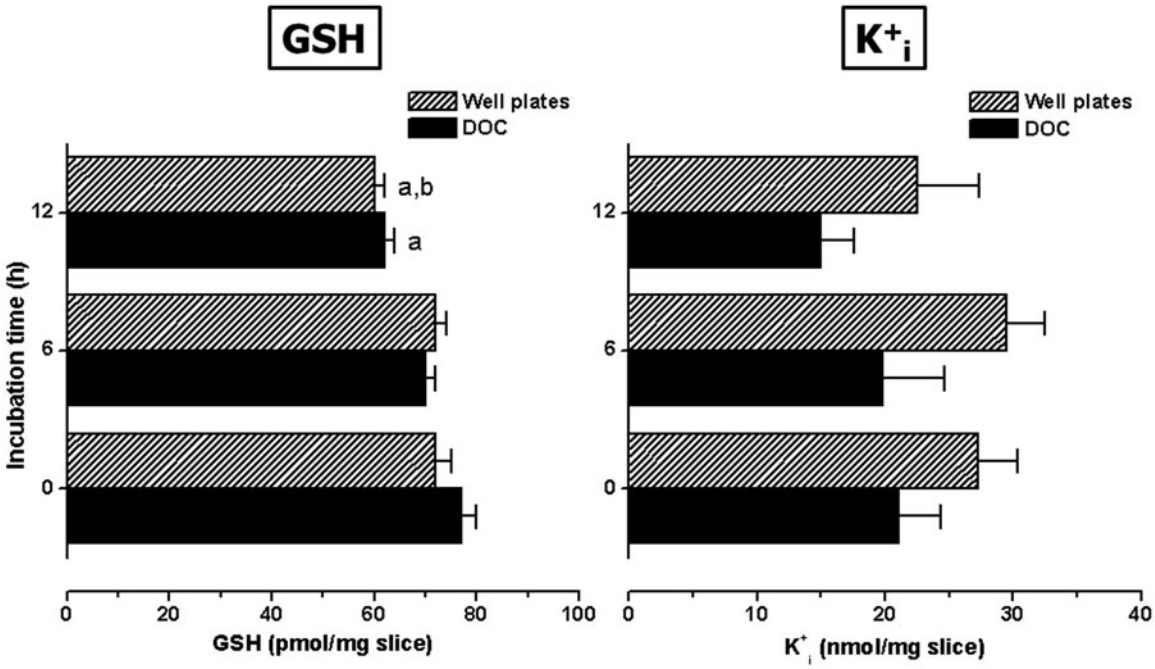

Figure 2. Intracellular reduced glutathione (GSH) (left panel) and K_i^+ contents (right panel) in 0, 6, and 12-h cultured PCLS, incubated either in a DOC system or in well plates (WP). Values (mean ± SEM) are expressed as pmol of GSH and nmol of K_i^+ per mg of slice. Significantly different from 0 h: $^a p < 0.01$. Significantly different from 6 h: $^b p < 0.01$.

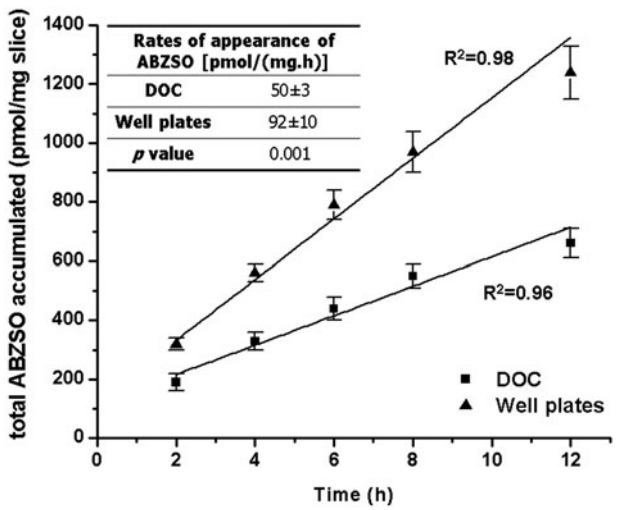

Figure 3. Accumulation of albendazole sulfoxide (ABZSO) in the culture medium after incubation of bovine PCLS in presence of the anthelmintic albendazole, either in a DOC system or in well plates. The inserted table shows the rates of appearance of ABZSO in both incubation systems. R^2: Coefficients of determination for each linear regression. Data (mean ± SEM) were obtained from 6 (DOC) or 9 (WP) individual assays and are the amounts (pmol) of ABZSO in the culture medium corrected by slice weight (mg).

Table 2. Rates of appearance (pmol/(mg.h) of albendazole sulfoxide (ABZSO) enantiomers in the culture medium after the incubation of cattle liver slices with the anthelmintic albendazole (ABZ) in the absence and in presence of the flavin-monooxygenase inhibitor methimazole (MTZ). Incubation assays were performed either in a DOC incubator or in well plates (WP).

	Rates of appearance of ABZSO enantiomers [pmol/(mg.h)]			
	DOC incubator		WP	
	ABZ control	ABZ + MTZ	ABZ control	ABZ + MTZ
(+) ABZSO	$45 \pm 2^{\S}$	$9 \pm 2^{£,a}$	$88 \pm 10^{\S}$	$5 \pm 2^{£,a}$
(−) ABZSO	5 ± 1	20 ± 4^{b}	6 ± 1	12 ± 3^{b}

Data are the mean (±SEM) of the rates of appearance obtained in 6 (DOC incubator) or 9 (WP) individual assays.
Significantly different from the control:
$^a p < 0.001$,
$^b p < 0.05$.
Significantly different from (−) ABZSO:
$^{\S} p < 0.001$,
$^{£} p < 0.05$.

culture medium, the (+) ABZSO/(−) ABZSO enantiomeric ratios were also significantly lower (DOC incubator: 83%, $p < 0.001$); WP system: 92%, $p < 0.001$) in presence of the metabolic inhibitor.

Discussion

The study of xenobiotic metabolism and disposition depends to a large extent on the use of *in vitro* methods (Brouwer et al., 2013; Kanter et al., 2002). Compared with *in vivo* studies, such methods are usually simpler to run, allow for more controlled experimental settings, and are often the only available empirical means due to ethical or practical constraints (Kanter et al., 2002). The study of species-specific metabolism and response to xenobiotic exposure requires the use of consistent and robust methodologies. However, in both human and veterinary medicine, interspecific differences prevent extrapolation of data and call for the replacement of laboratory animals with valid *in vitro* or *ex vivo* alternatives based on the particular species under study. In this context, validation of methodologies such as PCLS for metabolism studies in species of veterinary interest is of primary

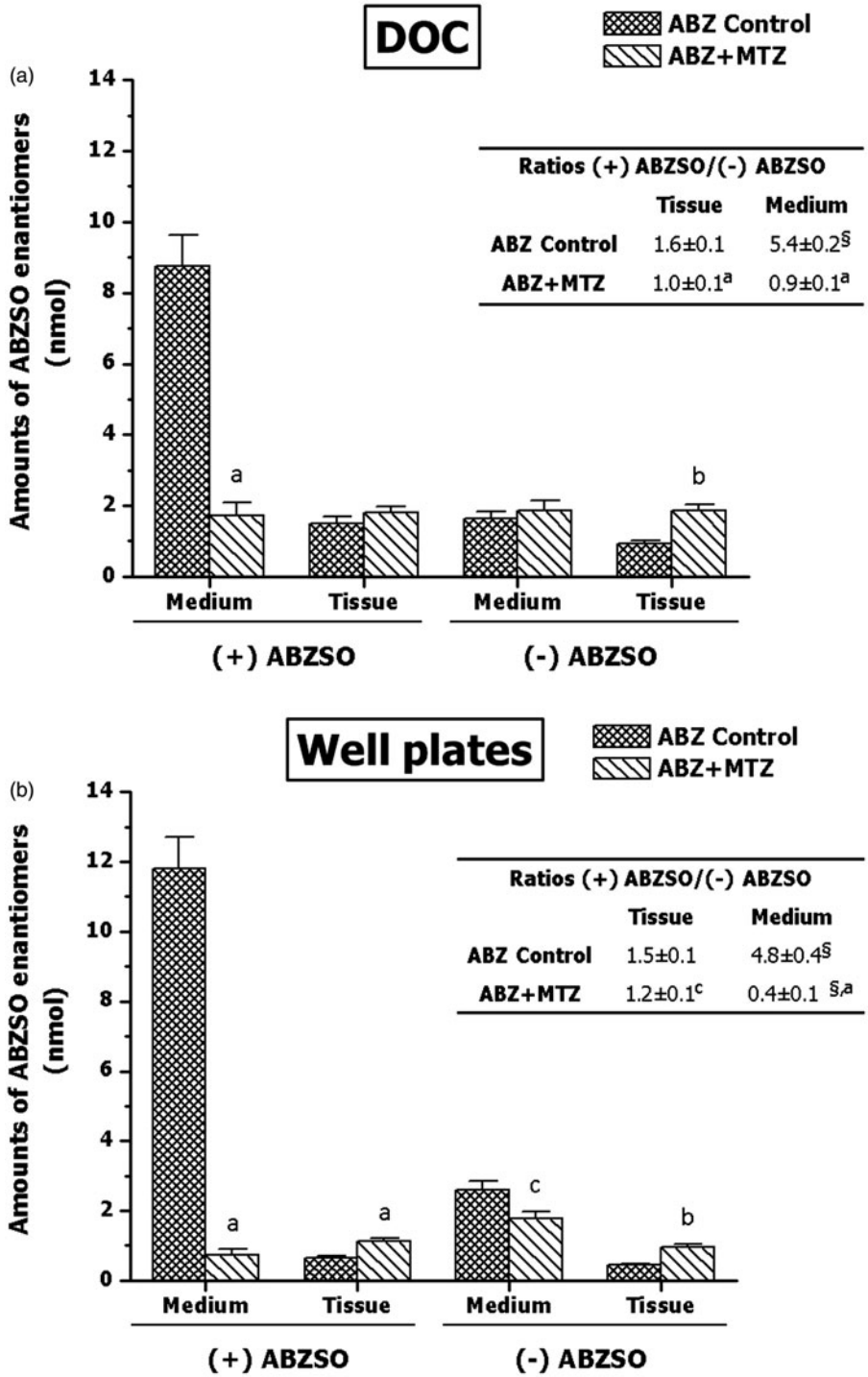

Figure 4. Amounts of albendazole sulfoxide (ABZSO) enantiomers in tissue and culture medium at 6 h of incubation of bovine PCLS with the anthelmintic albendazole (ABZ) in absence and in presence of the flavin-monooxygenase inhibitor methimazole (MTZ). Incubation assays were performed either in a DOC incubator (a) or in well plates (b). The inserted tables show the enantiomeric ratios in tissue and culture medium. Data are the mean (±SEM) of the amounts/enantiomeric ratios found in 6 (DOC) or 9 (well plates) slices and their respective culture mediums. Significantly different from the tissue (control assays): $^{\S}p<0.001$. Significantly different from the control: $^{a}p<0.001$, $^{b}p<0.01$, $^{c}p<0.01$.

importance, increasing the likelihood of detecting species-specific metabolic responses to drug use and drug combinations.

In both humans and laboratory animals, PCLS have proved to be a reliable methodology, exhibiting good correlation with both *in vivo* studies and validated *in vitro* methods, including microsomal fractions. Viability, though, is a crucial factor in determining the quality of data obtained by means of this *in vitro* method. The maintenance of an adequate electrochemical gradient, enzyme function, gene transcription, regulatory pathways and the overall cellular structural and functional integrity determines the outcome of assays performed using PCLS (Fisher et al., 2013), and the resulting conclusions contributing to scientific advancement. In the current work, both structural and functional markers were assessed to evaluate bovine PCLS integrity. Although a variety of such markers have been validated and correlated in human and laboratory animals, no such studies have been performed in the bovine species. In a study with bovine PCLS, Wang et al. (2010) compared ATP content with histomorphological evaluation of slices incubated for 6 h. The authors found a strong correlation between both parameters. This approach was later simplified by Rijk et al. (2012), who relied solely on ATP content to assess viability during 24-h incubation of bovine PCLS. In both cases, the WP system was used.

Results from the evaluation of the selected viability parameters showed satisfactory culture conditions for bovine PCLS during 12 h under both incubation systems. Of the parameters described in the literature, histomorphological evaluation is routinely used to assess structural integrity of PCLS (de Graaf et al., 2010). At 0, 6, and 12 of incubation, no differences between systems in terms of structural integrity were observed in bovine PCLS. HE-stained sections exhibited preserved tissue architecture and staining pattern (see Figure 1). Hydropic degeneration is considered an indicator of mild reversible alteration of the tissue parenchyma (Acorda et al., 1995). Although severe hydropic degeneration can be associated with shortened tissue life span (D'Alessandro et al., 1991), mild degeneration is a normal finding in tissue explants, and its relevance in short-term incubation can be overlooked. No hepatocyte fragmentation or hepatocyte loss was observed.

K_i^+ content is considered a reliable indicator of membrane integrity (Cervenková et al., 2001; Nave et al., 2006). K_i^+ levels bear a strong correlation with ATP levels, and thus with energetic balance, as described by Godoy et al. (2013). According to previous reports, K_i^+ values tend to increase during the 1-h equilibration period and the first 2 h of incubation, after which they stabilize and maintain approximately constant levels unless loss of structural integrity takes place (Price et al., 1998). A similar pattern of K_i^+ levels was observed in the present work, with values in the range of 15–29 nmol of K_i^+ per mg of slice (see Figure 2). These values are in line with previous reports in PCLS from humans and rats (Fisher et al., 2001; Olinga et al., 1997).

Intracellular contents of GSH and GSSG are accepted indicators of oxidative stress and, therefore, of cellular viability (de Graaf et al., 2007). In the current study, GSH levels decreased steadily over the 12-h incubation period. Compared with $t = 0$, the GSH content dropped roughly by 21 and 17% for the DOC incubator and the WP system, respectively. In cases where the decrease in GSH content is concurrent with an increase in GSSG levels, it is assumed that loss of antioxidant ability has led to oxidative stress (Tafazoli et al., 2005). In the current study, GSSG levels did not increase, whereas GSH/GSSG ratios remained constant. Based on these observations, it is unlikely that cellular integrity was negatively affected by oxidative stress throughout the 12-h incubation period.

The hepatic metabolism of the model compound ABZ has been extensively evaluated under a variety of *in vitro* methods and *in vivo* animal models. ABZ undergoes a two-step sequential S-oxidation to its sulfoxide (ABZSO) and sulfone ($ABZSO_2$) metabolites. Two enzymatic complexes, flavin-monooxygenase (FMO) and cytochrome P450 (CYP), are responsible for ABZ S-oxidation in ruminants (Capece et al., 2009). Both complexes display asymmetrical enantioselectivity, resulting in the production of differential quantities of (+) and (−) stereoisomers (Testa & Mayer, 1988). In the bovine species, the CYP system is responsible for ABZ oxidation to (−) ABZSO, whereas FMO activity accounts for roughly 94% of total (+) ABZSO (Virkel et al., 2004). Overall, the (+) ABZSO enantiomer has been found as the predominant stereoisomer in both microsomal fractions (Virkel et al., 2004) and plasma from ABZ-treated animals (Delatour et al., 1991). As expected, the same situation was observed in bovine PCLS incubated with this anthelmintic molecule. Thus, in control incubations with ABZ alone, the rates of appearance of (+) ABZSO in the culture medium were higher compared to those observed for its antipode (see Table 2).

In ruminants, the existence of metabolic interactions between the antithyroid molecule MTZ and the anthelmintic ABZ has been previously shown *in vitro* (i.e. liver microsomes) and *in vivo* (Lanusse et al., 1993; Virkel et al., 2004). MTZ is a well-known inhibitor of the FMO complex and therefore of ABZ metabolism to ABZSO, in particular to the (+) enantiomer (Virkel et al., 2004). In sheep liver microsomes, for instance, exposure to roughly 25 μM of MTZ was enough to decrease (+) ABZSO production by 50%, whereas more than 1000 μM were necessary to produce a matching effect on (−) ABZSO production (Virkel et al., 2014). The current study demonstrates that such interactions also occur in bovine PCLS. A net decline in the rate of appearance of (+) ABZSO (between 80 and 94% depending on the culture system) was observed when bovine PCLS were incubated with ABZ in presence of MTZ (see Table 2). Higher concentrations of ABZ would therefore be available for the CYP-dependent production of (-) ABZSO. Consequently, the accumulation rates of this enantiomer increased (between 4-fold and 2-fold in the DOC incubator and the WP system, respectively) when ABZ was incubated in presence of the FMO inhibitor. Therefore, the alternative pathway catalyzed by the CYP complex, which accounts for a negligible participation in total ABZSO production in the absence of inhibition, is enhanced. The increased appearance of (−) ABZSO, distinctly observed under this *in vitro* model, reflects the shift from the FMO-dependent to the CYP-mediated S-oxidation after co-incubation of ABZ and MTZ.

The relative amounts of each ABZSO enantiomer were measured at 6 h incubation in both the culture medium and liver tissue. Interestingly, enantiomer measurements within the PCLS and in the WME exhibited a pronounced asymmetry (see Figure 4). In control incubations, the relationship (+) ABZSO/(-) ABZSO in the culture medium exhibited a marked asymmetrical accumulation in favor of (+) ABZSO, which was less evident within the liver tissue (see Figure 4, inserted tables). In presence of the metabolic inhibitor, the amount of (+) ABZSO decreased in the culture medium but not within the liver tissue. Enantiomeric ratios changed in presence of MTZ, being lower in both the culture medium and the slice. However, the magnitude of the reduction of the ratio (+) ABZSO/(−) ABZSO was markedly higher in the culture medium compared to the liver tissue. In absence of further evidence, no explanation as to the cause of this situation can be put forward. However, one possible mechanism is an active secretion process of (+) ABZSO by the hepatocyte. This could in turn be explained by a differential affinity of both enantiomers for one or more membrane efflux proteins. In this regard, the *in vitro* transport of ABZSO by the breast cancer resistance protein (BCRP) has been clearly shown in cultured cells (Merino et al., 2005; Muenster et al., 2008). Thus, the lower production of (+) ABZSO within the hepatocytes is reflected by a reduced efflux of this enantiomer to the culture medium. Enantioselectivity in the activity of

membrane transporters has been demonstrated by means of both *in vitro* studies and animal models (Zhou et al., 2014).

Since the development of the technique, several incubation methods for PCLS have been developed. In a comparative study involving a variety of alternatives including the DOC incubator and WP system, Olinga et al. (1997) found no significant differences among methods, whereas Hashemi et al. (1999) reported better overall results with the WP system. More recently, Fisher et al. (2013) have suggested the use of DOC systems in order to ensure adequate oxygenation of the PCLS. The assays were performed on either laboratory animal or human PCLS, and such comparative studies on food-producing animals are currently lacking. The DOC incubator and the WP system display some practical differences. The design of the WP system allows for a large number of samples to be incubated. Additionally, it permits a simpler collection method that optimizes sampling time and reduces exposure to uncontrolled conditions during the sampling procedure. Under the DOC system, PCLS are placed on a titanium screen. Fisher et al. (2013) suggest that this screen may act as a scaffold for tissue repair and regeneration, thus allowing for culture up to 7 d for human PCLS (Fisher et al., 2013; Vickers et al., 2011). However, organ remodeling as evidenced by an increased collagen deposit and enzyme downregulation is evident under prolonged culture, and might affect the interpretation of metabolic data. In particular, the rate of CYP-mediated metabolism decreases, as shown by de Graaf et al. (2007). The descent is attributed to lack of positive stimuli to the synthesis of enzyme *de novo* (Cervenková et al., 2001). In the current study, no advantage of the DOC incubator compared with the WP system was observed in terms of structural integrity during a 12-h incubation period. However, the higher rates of appearance of ABZSO using WP may indicate an advantage of this culture system for short-term studies on metabolic D–D interactions in cattle.

Conclusions

The present study involves a comparative analysis of the structural and functional integrity of bovine PCLS under two culture methods: the DOC incubator and the WP system. Bovine PCLS were obtained by a standardized, repeatable methodology that ensures defined and homogeneous weight, thickness, and culture conditions. Under the described conditions, the DOC incubator and the WP system are comparable in terms of preserved viability of bovine PCLS as shown by structural indicators, and can therefore be used as a reliable approach to evaluate organ-specific metabolism in the bovine species. In practical terms, however, the WP system allows for more versatile sampling procedures while enabling more samples to be incubated. A well-described metabolic D–D interaction between ABZ and MTZ was observed as a consequence of MTZ-mediated inhibition of the FMO complex, resulting in a reduced rate of appearance of (+) ABZSO. Furthermore, secretion of ABZSO from tissue to the culture medium appears to be enantioselective, warranting further research. In the light of these findings, PCLS may be considered as a useful tool to obtain relevant information on D–D interactions in cattle.

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Declaration of interest

The authors have no conflicts of interest to declare.

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