3. Risk assessment for human embryonic development of triclabendazole residues in milk and cheese in the diet of a rural population in Cajamarca (Peru): A preliminary approach

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Abstract. Triclabendazole (TCBZ) is a veterinary drug used against Fasciola hepatica in cattle. The Cajamarca Valley in Peru is an endemic area of fascioliasis with a high infection rate in animals producing milk for human consumption. The administration of TCBZ during the lactating period can lead to TCBZ derivative residues in milk and cheese entering the human food chain. Milk-derivatives from treated animals have been found
positive for TCBZ metabolites. One of these metabolites, triclabendazole sulfoxide (TCBZSO), is embryolethal during early developmental stages in vitro in mouse and zebrafish. In this study, we have calculated the estimated daily intake (EDI) of TCBZSO due to milk and cheese consumption among a rural population in Cajamarca in order to evaluate the associated risk for human embryonic development. Although the expected maximum plasma concentration of TCBZSO after a worst-case scenario simulation would be below the reported lowest observed adverse effect concentration (LOAEC) for embryolethality in vitro (10 µM), several limitations on the available information for exposure, bioavailability and interspecies differences still impede the accomplishment of an accurate risk assessment.

Introduction

Triclabendazole (TCBZ) is a benzimidazolic compound used in veterinary medicine to treat fascioliasis. This parasitic disease, induced by the liver trematode *Fasciola hepatica* (see life cycle in Fig.1 [1]) is endemic in the Cajamarca Valley in Peru, where more than 78% of livestock are infected [2]. Treating the infected animals is a necessity in order to reduce the number of hosts and thus to reduce the disease impact in humans, but also to avoid production losses in cattle industry by means of reductions in animal weight gain, fertility and milk yield [3-5]. TCBZ is the drug of choice to treat fascioliasis in animals because it has high activity against adult and juvenile flukes [6-8] and because it can be administered as a single treatment at the start of the dry-off period, when no milk is being produced for human consumption, and thus avoid TCBZ residues in milk [9].

However, in fascioliasis endemic areas like Cajamarca, TCBZ treatment is administered three to four times a year, even during the period when cows produce milk for human consumption [10]. This use during the lactating period can lead to TCBZ derivative residues entering the human food chain as several studies have shown the presence of TCBZ residues in milk and its transference to dairy products [9].

Taking that into consideration, a maximum residue limit (MRL) of 10 µg/kg of TCBZ in milk has been fixed by the European Union [11], and also an acceptable daily intake (ADI) of TCBZ of 0 to 3 µg/kg body weight (b.w). [12]. However, neither MRL has been established for TCBZ derivatives in milk, nor for TCBZ or TCBZ derivatives in cheese and other dairy products. The presence of these residues in the human food chain acquires special relevance when considering that triclabendazole sulfoxide (TCBZSO), the first metabolite of TCBZ, presents embryotoxic potential
in vitro in mouse and zebrafish (lowest observed adverse effect concentration for lethality; LOAEC<sub>lethality</sub> = 10 µM; [13]). Therefore, our aim was to evaluate the possible risk for human embryonic development derived from maternal consumption of milk and cheese containing TCBZ derivative residues during gestation.

Figure 1. Life cycle of *Fasciola hepatica* and *Fasciola gigantica* [1].
Immature eggs are discharged in the biliary ducts and in the stool. (1) Eggs become embryonated in water (2), eggs release miracidia (3), which invade a suitable snail intermediate host (4), including the genera *Galba, Fossaria* and *Pseudosuccinea*. In the snail the parasites undergo several developmental stages [sporocysts (4a), rediae (4b), and cercariae (4c)]. The cercariae are released from the snail (5) and encyst as metacercariae on aquatic vegetation or other surfaces. Mammals acquire the infection by eating vegetation containing metacercariae. Humans can become infected by ingesting metacercariae-containing freshwater plants, especially watercress (6). After ingestion, the metacercariae excyst in the duodenum (7) and migrate through the intestinal wall, the peritoneal cavity, and the liver parenchyma into the biliary ducts, where they develop into adults (8). In humans, maturation from metacercariae into adult flukes takes approximately 3 to 4 months. The adult flukes (*F. hepatica*: up to 30 mm by 13 mm; *F. gigantica*: up to 75 mm) reside in the large biliary ducts of the mammalian host. *F. hepatica* parasites can infect various animal species, mostly herbivores (source [1]).
1. Presence of triclabendazole-derivative residues in milk and cheese samples

TCBZ-derivative concentrations in milk and cheese samples were obtained from a study carried out on a dairy farm situated in Cajamarca, Peru [10]. In this study by Imperiale et al., seven female Holstein dairy cows between 490 and 630 kg weight were orally treated with a single dose of 12 mg TCBZ/ kg b.w. (Fasinex®, TCBZ 10%, Novartis). Milk samples were collected prior to treatment and at 6, 12, 24, 36, 48, 60, 72, 84, 96, 120 and 144 h post-treatment. Approximately 50 mL of milk were collected after homogenization of the whole milk yield of each cow. The remaining milk production of all experimental animals collected at 12, 24, 36, 48, 60 and 120 h post-treatment was pooled and processed to produce creamy cheese. The extraction procedures to quantify TCBZ and its metabolites in milk (0.5 mL) and cheese (0.5 g) samples were carried out following modifications of previously described methods [10, 14, 15]. Drug concentrations in experimental samples were determined by HPLC with a UV detector using oxibendazole as internal standard (99.2% purity). Both TCBZSO and triclabendazole sulfone (TCBZSO₂) metabolites but not TCBZ were detected in milk (up to 36 and 144 h post-treatment, respectively). The sum of total residues (TCBZSO and TCBZSO₂) was between 0.6 and 2 µg/mL in pooled milk, and between 1.1 and 20.0 µg/g in cheese.

2. Milk and cheese consumption data

Milk and cheese consumption data concerning the rural population of Cajamarca valley were obtained from two different studies, one from our own group [16] and one from an official Peruvian dietary survey (INEI) [17]. Briefly, in our own study, 36 people from 11 populations located in the rural area of Cajamarca were interviewed for a 24 h dietary recall. To contrast and compare the obtained information, a qualitative food frequency questionnaire was performed by the same interviewer to the same individuals. The official dietary survey of Peru [17] collected information from seven days questionnaires of 1536 households in rural areas and 34698 households in urban areas of Peru. The areas covered in the study included coast, mountains and rainforest.

Milk consumption results reported by these two studies were very different. These differences can be explained by the fact that the INEI
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study covered the whole Peruvian population, while the study from Barenys et al. [16] was performed exclusively in the Cajamarca valley, which is one of the main milk and dairy producer regions in Peru [18]. Besides, the population interviewed, consisted mainly in teenagers (63% of participants were people between 14 and 17 years old), which are described to consume more milk than the adult population [19].

Ideally, the consumption data should be obtained from women at childbearing age, but this consumption values are not available for the whole Peruvian population [17], and the sample size of this group was too small to drive conclusions in the study by Barenys et al. [16]. We have used the values reported in both studies [16, 17] for milk and cheese consumption to further calculate the Estimated Daily Intake (EDI) of TCBZ derivatives.

3. Triclabendazole derivatives estimated daily intake calculation

The results obtained by Imperiale et al. [10] for TCBZSO and TCBZSO$_2$ concentrations in milk and cheese and the contribution of milk and cheese to the diet according to our 24 h dietary recall results, and the INEI survey [17] are detailed in Table 1 and were used to calculate the EDI of TCBZ derivatives per individual, expressed in µg of TCBZ derivatives/kg b.w. per day, as follows:

$$\text{EDI} = \frac{(\text{max milk consumption} \times \text{max concen} \text{r TCBZ derivatives in milk} + \text{max cheese consumption} \times \text{max concen} \text{tr TCBZ derivatives in cheese})}{\text{median woman body weight}}$$

The median weight value for women population was obtained from Ministerio de Salud del Perú [20]. As detailed in Table 1, the EDI of TCBZSO + TCBZSO$_2$ in a worst-case scenario approach was estimated to be 12.0 µg/kg b.w.

$$\text{EDI} = \frac{(180 \text{ mL} \times 2 \text{ µg/mL}) + (12 \text{ g} \times 20 \text{ µg/g})}{50 \text{ kg b.w.}} = 12 \text{ µg TCBZ derivatives/kg b.w.}$$

It is important to remark that there is no study available from the region of Cajamarca indicating which is the real presence of TCBZ derivatives in representative samples in the market and thus, which would be the mean real exposure in the general population. Therefore, the TCBZ derivative values described in Imperiale et al., [10], obtained in only one farm directly after the administration of TCBZ to the animals, were taken to simulate a worst-case scenario and to study its associated risk for human embryonic development.
### Table 1. Triclabendazole derivatives estimated daily intake calculation.

<table>
<thead>
<tr>
<th>Median woman body weight [kg]</th>
<th>Milk consumption [mL]</th>
<th>Cheese consumption [g]</th>
<th>TCBZSO+TCBZSO$_2$ concentration in milk [µg/mL]</th>
<th>TCBZSO+TCBZSO$_2$ concentration in cheese [µg/g]</th>
<th>TCBZSO+TCBZSO$_2$ Estimated Daily Intake [µg/kg b.w.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perú</td>
<td>107$^b$</td>
<td>180.2$^c$</td>
<td>12.0$^b$</td>
<td>8.98$^d$</td>
<td>20$^e$</td>
</tr>
<tr>
<td>Caj</td>
<td>8.0</td>
<td>18.0</td>
<td>2$^e$</td>
<td>20$^e$</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Perú: data from the 5th quintile of the INEI survey [17], milk consumption includes milk and yoghurt; Caj: data from 24-h dietary recall to Cajamarca population [16]; a: [20]; b: [17] (milk consumption corresponding to milk and yoghurt values); c: calculated from g to mL from [16] using milk density=1.03; d: [16]; e: [10].

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### 4. Plasma concentration-ranges after the estimated daily intake vs LOAEC for embryo lethality *in vitro*

In humans, after ingestion of a therapeutic dose of 10 mg/kg b.w. of TCBZ, the obtained maximum plasma concentration ($C_{\text{max}}$) of TCBZSO is 38.6 µM [21]. At present, there is no characterization of TCBZSO $C_{\text{max}}$ reached after direct administration of TCBZSO in humans or in animals, but pharmacokinetic studies with other benznidazoles have compared the reached concentrations of the sulfoxide metabolite after the administration of the parent compound or after the administration of the sulfoxide metabolite itself [22, 23, 24].

For example, albendazole sulfoxide (ABZSO) produced significantly higher ABZSO $C_{\text{max}}$ but not significantly higher area under the curve (AUC) than albendazole (ABZ) in 1-month-old lambs, and significantly higher ABZSO $C_{\text{max}}$ and AUC than ABZ in 8-month-old lambs following administration of 5 mg/kg b.w. of each compound [22]. These differences were comparable to those determined previously in goats [22, 23]. Similarly, the plasma concentration of fenbendazole sulfoxide (FBZSO) was much greater when it was directly administered than when fenbendazole (FBZ) was administered in horses, at 10 mg/kg b.w. each. It is remarkable that FBZSO achieved 25.6 or 35 times greater concentrations (expressed as AUC or $C_{\text{max}}$, respectively) when it was directly administered, than after administration of the parent compound [24]. These studies show that when the sulfoxide metabolite is directly administered, the plasma concentration seems to be similar or higher than when the parent compound is administered at the same doses.

Anyhow, a precise characterization of the $C_{\text{max}}$ of TCBZSO after TCBZSO administration is still missing and would be essential for a correct risk assessment of TCBZSO effects on human development.
Figure 2. Graphical summary comparing TCBZSO results across species and developing time.

Representative pictures of rodent embryos exposed to increasing concentrations of TCBZSO from GD 0 to GD 4 in the preWEC culture (LOAEC_{lethality}= 10 µM) and from GD 9.5 to GD 11.5 in the postWEC culture (LOAEC_{dysmorphogenesis}= 666 µM). Zebrafish embryos were exposed to TCBZSO from 2 hpf to 50 hpf, a developmental period comprising the stages covered by both rodent cultures. No dysmorphogenesis were observed (maximum concentration tested= 50 µM), but TCBZSO was embryo lethal during the first 24 h of culture (LOAEC_{lethality}= 10 µM). Pictures correspond to the developmental time points marked in bold in the x-axis. Pictures of pre WEC embryos are 10 times magnified respect to pictures of post WEC and zebrafish embryos.

Scale bar: 4 mm for post WEC and zebrafish embryos; and 400 µm for pre WEC embryos.

ZFET: zebrafish embryo test (concentrations= 0, 5, 10 and 50 µM); pre WEC: preimplantation whole embryo culture (concentrations= 0, 3, 10, 30 and 100 µM); postWEC: postimplantation whole embryo culture (concentrations= 0, 27, 267 and 666 µM); GD: gestational day; hpf: hours post-fertilization (ref. [13]).
Taking into account this important limitation, after the administration of the TCBZSO EDI of 12.0 µg/kg b.w., the plasma $C_{\text{max}}$ could be calculated following a simplified approach which considers total and instant absorption and no tissue distribution:

$$\text{EDI} = \frac{12.0 \, \mu\text{g TCBZSO}}{\text{kg b.w.}} \times \frac{50 \, \text{kg b.w.}}{2 \, \text{L plasma}} \times \frac{1 \, \mu\text{mol TCBZSO}}{376 \, \mu\text{g TCBZSO}} = 0.80 \, \mu\text{M}$$

Total plasma volume was obtained following the standard weight-based calculation for normal females (0.04L x kg b.w.) [25].

This concentration is still lower than the experimental LOAEC for embryotoxicity observed in in vitro developmental toxicity tests in mouse and zebrafish (LOAEC_{lethality}= 10 µM; [13]; see Fig 2), but the lack of specific pharmacokinetic data after TCBZSO administration and the possible species differences in pharmacokinetics and embryo toxicity sensitivity, pose a serious obstacle to an accurate risk assessment process.

5. Discussion

At present, no MRL has been established for TCBZSO in milk, in cheese or in other dairy products. Likewise, no ADI has been established for TCBZSO. However, there is extensive scientific evidence that flukicide administration to milk producing animals leads to migration of residues to milk and its derived products as cheese, butter and skim milk powder. It is remarkable that pasteurization or heat treatment during spray drying have no impact in reducing these residues [9].

The present study approaches the subject of risk assessment of TCBZ residues in milk and cheese in the diet of a rural population in Cajamarca (Peru). However, several limitations on the current knowledge about exposure characterization, bioavailability of the compound and interspecies differences in sensitivity impede an accurate accomplishment of the risk assessment process.

Limitations on exposure characterization: in section 3 we have calculated the EDI of total TCBZ derivatives considering a worst-case scenario, as the concentrations used for the calculations are from milk and cheese samples obtained from cows after controlled administration of TCBZ [10]. But in a real situation, not all milk and cheese products consumed by the population would contain TCBZ derivative residues.
Currently, there are no studies detailing the prevalence of milk and cheese samples containing TCBZ derivatives in the Cajamarca market. A study comparable to the one performed by [26] where 27.6% of raw milk samples collected from all farms throughout Southern Greece were found to be positive for the investigated benzimidazoles, would be very valuable to better characterize the real exposure scenario.

Besides, in the EDI calculation, the sum of both metabolites (TCBZSO and TCBZSO₂) has been considered, as no concentration values for TCBZSO alone have been detailed. However, the embryotoxic potential of TCBZSO₂ has not been investigated yet and it is a shortcoming of the current approach to consider it as if it would be as potent as TCBZSO and work further on with the EDI value obtained from the sum of both metabolites.

Limitations on bioavailability information: as detailed in section 4, there is no specific pharmacokinetic data about TCBZSO bioavailability after TCBZSO administration in humans, neither in animals. A simplified calculation of $C_{\text{max}}$ has been used for approximation, but real pharmacokinetic data would help to carry a more accurate risk assessment.

Limitations on information about interspecies differences: Embryolethality (LOAEC_{lethality}) from in vitro developmental toxicity tests has been established at 10 µM using the mouse preimplantation embryo culture and the zebrafish embryo models [13]. However, there is growing recognition that to predict human safety, it would be advisable to know if there are species differences in sensitivity towards TCBZSO embryo lethal effects [27], by conducting other studies like parallel human and mouse embryonic stem cell tests.

6. Conclusions

In this study we have calculated the EDI of TCBZ derivatives simulating a worst-case scenario. After a TCBZSO EDI, the expected $C_{\text{max}}$ would be lower than the experimental LOAEC for embryotoxicity observed in in vitro developmental toxicity tests in mouse and in zebrafish. However, our study highlights the current needs of information to carry out an accurate risk assessment for TCBZSO exposure during human development. Specifically, there is a need of:
• A better characterization of TCBZSO presence in commercialized milk and milk-derivatives samples with concrete TCBZSO concentrations.

• A detailed pharmacokinetic profile of TCBZSO concentrations reached after TCBZSO intake.

• More information on human sensitivity towards TCBZSO embryo lethality from human 3D in vitro models.

References


