EVALUATION IN VITRO DE L'EFFICACITE DU PEROXYDE D'HYDROGENE ET DU SERUM SALE HYPERTONIQUE COMME AGENTS SCOLICIDES

IN VITRO EVALUATION OF THE HYDROGEN PEROXIDE AND THE HYPERTONIC SALINE SOLUTION AS SCOLICIDAL AGENTS

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Résumé

La dissémination per-opératoire des protoscolex représente l'un des principaux problèmes posés au cours de la cure chirurgicale du kyste hydatique. La stérilisation de la cavité hydatique et du champs opératoire par des scolicides reste le moyen le plus utilisé pour prévenir les récidives hydatiques. L'évaluation in vitro de deux agents scolicides les plus utilisés, le peroxyde d'hydrogène (H2O2) et le sérum salé hypertonique (NaCl) a été réalisée avec les protoscolex recueillis à partir de 8 kystes hydatiques pulmonaires humains. Des dilutions de 5% à 50% ont été appliquées à des temps d'incubation allant de 1 à 90 minutes. La solution salée hypertonique s'est révélée peu active. Le peroxyde d'hydrogène a donné de meilleurs résultats ; son efficacité est cependant réduite par la présence du sang dans le liquide hydatique ou au niveau du champ opératoire.

Mots clés : Kyste hydatique, peroxyde d'hydrogène, solution salée hypertonique, protoscolex

Summary

The aim of this study was to determine the *in vitro* effect of two scolicidal agents routinely used in hydatid cyst surgery, hydrogen peroxide (3% H_2O_2) and hypertonic saline (20% NaCl) on protoscoleces obtained from human pulmonary hydatid cysts (n=8).
A suspension of protoscoleces was prepared in its own hydatid fluid and a scolicidal solution was added at 5%, 10%, 20% and 50% dilutions. Viability of protoscoleces was assessed after 1, 2, 5, 10, 15, 20, 30 and 90 minutes exposure. The time at which all protoscoleces were dead was noted.
The use of diluted solutions of 20% NaCl was ineffective after 10 minutes contact. The use of diluted solutions of 3% H_2O_2 was more efficient, being 100% lethal starting from a 0.6% concentration. The efficacy of H_2O_2 depended mainly on hydatid fluid aspects. Previous contact with the blood host during the surgical procedure resulted on a less efficacy.

Key-words : Hydatid cyst, Hydrogen peroxide, hypertonic saline solution, protoscoleces

INTRODUCTION

Cystic echinococcosis is a zoonotic infection caused by the larval form (metacestode) of the tapeworm *Echinococcus granulosus* found in the small intestine of canidae. The eggs of this tapeworm excreted by canidae may infect men causing cystic hydatid disease. This disease is endemic in Tunisia and constitutes a serious public health problem [1]. The liver is infected in about 60% of cases, the lung in about 20%, and remaining organs (kidney, bone, brain, muscles and others) in about 20% [2, 3]. Its treatment remains essentially surgical by the removal of the intact hydatid cyst or the evacuation of the contents followed by cyst excision, partial organ resection or external drainage [4, 5, 6]. To avoid eventual spillage of the cyst content (mainly PS) scolicidal agents are used during the surgical procedure. These scolicides could lower the risk of recurrence by sterilizing the cyst contents before its opening and by protecting the surgical field [5].
Among the various scolicidal agents proposed, H_2O_2 and hypertonic saline solution are the most commonly used [5]. However, their use remains empiric and their action in the operative conditions is rarely estimated. The aim of this study is to determine the scolicidal *in vitro* effect of H_2O_2 in different concentrations and exposure times on PS obtained from different human pulmonary hydatid cysts and second to compare the effects of H_2O_2 to those of hypertonic saline solution.

MATERIALS AND METHODS

Protoscoleces were extracted from eight hydatid cysts of the lung, obtained from patients operated at the department of thoracic and cardiovascular surgery (Abderrahmen Mami Hospital, Ariana, Tunis). The withdrawal of the hydatid fluid and the membrane was practised without using scolicidal agents. The diameter of the hydatid membrane was measured, the volume of the hydatid fluid and the sediment and their aspect were noted (table I). The presence of PS was verified by microscopic observation. Their viability was determined by flame cell activity and vital staining with 0.1% eosin solution.
Two scolicidal agents were tested: commercially available H_2O_2 solution at 10 volumes (3%) and 20% hypertonic saline solution. All cysts were treated by H_2O_2 solution and only 4 were treated by hypertonic saline solution.
In 6 test tubes and for each cyst, a suspension of PS was prepared in each own hydatid fluid and the final concentration reproduced the one found *in vivo* inside the original cyst. The final concentration of hypertonic saline solution in each test tube was 1%, 2%, 3%, 4% and 10% and the final concentration of H2O2 in each test tube was 0.15%, 0.3%, 0.45%, 0.6%, 1.5%. A control tube, where only the hydatid fluid was added to the protoscoleces (PS), was tested in each experience. Viability of PS was assessed after 1 minute, 2 minutes, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes and 90 minutes exposure to the scolicidal agent. At the end of each incubation time, 100 µL PS settled at the bottom of the tube was taken then washed three times in saline solution (0.9% NaCl) and examined for viability. The time at which all the PS were dead was noted.

RESULTS

The use of diluted solutions of 20% hypertonic saline at 5%, 10% and 15% corresponding to a final concentration of 1%, 2% and 3% NaCl respectively was ineffective on PS. At 20% dilution (4% NaCl) only two cysts showed died PS after 10 to 30 minutes contact (table II). The only effective dilution of this scolicide was 50% (10% NaCl) which killed all the PS tested after two minutes contact (table II).
On the other hand, H_2O_2 was more effective at 5%, 10% and 15% dilutions. However the effect is variable according to the cyst. In terms of killing PS, at the lower dilution (5%) corresponding to a final concentration 0.15% H_2O_2, this scolicidal agent was effective after 1 to 5 minutes contact for 4 cysts over 8, 20 minutes contact for one cyst and 90 minutes contact for the other cysts (table III). And when the final dilution of injected scolicide was 10% (concentration of H_2O_2 0.3%), we showed a higher effectiveness after 15 minutes contact except for PS coming from cyst 7 which were still alive after 90 minutes exposure. At a final dilution higher than 15% H_2O_2 (H_2O_2 concentration 0.45%) the scolicidal agent was very effective and all PS were killed after 10 min contact (table III).

Considering 0.15% H_2O_2 effectiveness, we were able to sort the cysts tested in three classes: 4 have very sensitive PS to H_2O_2 action (effectiveness after 1 to 5 minutes contact), one with sensitive PS to H_2O_2 (effectiveness after 20 minutes exposure) and 3 cysts with resistant PS to H_2O_2 action (PS still alive after 90 minutes contact) (table IV). This variability seems to be closely dependant on the presence of blood in the hydatid fluid. In fact the 3 cysts that showed resistant profile had slightly haematic fluid denoting a peroperative contact with the blood host (table 4). No correlation was found between the initial PS viability and the sensitivity to the scolicidal agent (table IV). Indeed, PS from the cyst 7 showing 53% viability were unaffected by H_2O_2 action whereas PS from the cysts 4, 5, 6 and 8 showing viability varying between 90% and 100% were sensitive to the scolicide action (table IV).

If we assume that in operative conditions surgeons routinely inject 20 ml of the scolicidal agent and maintain the contact at least 5 minutes, we could deduce, taking into account the hydatid fluid volume, the scolicide concentration and its expected effectiveness in the cyst according to the experimental results shown in tables 2 and 3. In the shed of these results it appears that in these peroperative conditions, H_2O_2 would sterilize 4 over 8 cysts and the use of hypertonic saline was expected to be ineffective (table V).

Table I : Cyst's description

Cyst Number	Radiological diameter (cm)	Membrane diameter after cyst extraction (cm)	Theoretical volume d3π/6 (ml)*	Volume of hydatid fluid (ml)	Volume of hydatid sand (ml)	Hydatid fluid aspect	PS viability (%)
K1	14	12,5	1022	500	1,5	Slightly haematic	90
K2	6	5	65	50	0,7	clear	35
K3	13,5	8	267	200	1	Slightly haematic	95
K4	12	10	523	300	1,37	clear	90
K5	9	10	523	300	0,95	clear	98
K6	8,5	9	381	120	0,3	clear	100
K7	13	12,5	1022	450	1	Slightly haematic	53
K8	10	11	696	135	0,7	Slightly haematic	100

Table II : Time needed to kill PS by hypertonic saline in various dilutions

Cyst Number	20% hypertonic saline dilution (%) (NaCl final concentration %)				
	5% (1%)	10% (2%)	15% (3%)	20% (4%)	50% (10%)
K2	>90 mn	> 90 mn	> 90mn	30mn	2mn
K3	> 90 mn	> 90mn	> 90mn	> 90mn	2mn
K4	>90 mn	> 90mn	> 90mn	10mn	2mn
K5	> 90 mn	> 90mn	> 90mn	> 90mn	2mn

Table III : Time needed to kill PS by hydrogen peroxide in various dilutions

Cyst number	Hydrogen peroxide dilution (%) ($\%H_2O_2$ final concentration)				
	5% (0.15%)	10% (0.3%)	15% (0.45%)	20% (0.6%)	50% (1.5%)
K1	90 mn	10 mn	-*	5 mn	1 mn
K3	> 90 mn	15 mn	10 mn	1 mn	1 mn
K7	> 90 mn	>1h30	-*	1 mn	1 mn
K4	20 mn	1mn	1mn	1 mn	1 mn
K8	5 mn	1 mn	1 mn	1 mn	1 mn
K2	1 mn	1 mn	1 mn	1 mn	1 mn
K5	2 mn	2 mn	1 mn	1 mn	1 mn
K6	1 mn	1mn	1 mn	1mn	1mn

* : not determined

Table IV : Factors influencing Protocoleces sensitivity to 0.15% H_2O_2

Cyst Number	Time needed to kill the PS	Hydatid fluid aspect	PS viability (%)	Sensitivity to 0.15% H_2O_2
K1	90 mn	Slightly haematic	90	R*
K3	> 90 mn	Slightly haematic	95	R*
K7	> 90 mn	Slightly haematic	53	R*
K8	5 mn	Slightly haematic	100	VS*
K2	1 mn	clear	35	VS*
K4	20 mn	clear	90	S*
K5	2 mn	clear	98	VS*
K6	1 mn	clear	100	VS*

*R : resistant. VS : verv sensitive. S : sensitive

Table V : Estimated PS sensitivity to scolicidal agent in operative conditions*

Cyst number	Hydatid fluid volume (mL)	Deduced scolicide dilution	deduced sensitivity to NaCl after 5 mn contact	Deduced sensitivity to H2O2 after 5 mn contact
K1	500	4%	-**	R
K2	50	40%	R	S
K3	200	10%	R	R
K4	300	6,5%	R	R
K5	300	6,5%	R	S
K6	120	16%	-**	S
K7	450	4%	-**	R
K8	135	13%	-**	S

*results deduced from table 2 and 3, ** : not determined

DISCUSSION

Surgery is the main therapeutic option for hydatid cyst. It must be conservative as far as possible with evacuation of the cyst content and irrigation with a scolicidal agent [8]. Few studies were undertaken to evaluate the *in vitro* effect of H_2O_2 and hypertonic saline as scolicidal agents [9, 10, 11, 12]. In fact, they had usually used ovine hydatid cysts for PS sources [9, 10, 11, 12]. Moreover the PS were used as a suspension in phosphate buffer saline or saline solution (NaCl 0.15 M) or as a crude sediment. In our survey, we tried to reproduce surgical conditions with PS taken from human pulmonary cysts and suspended in their hydatid fluid.

We showed that hypertonic saline solution (20% NaCl) could be effective as a scolicidal agent after 5 minutes contact with PS, unless

its concentration inside the cyst is 10% NaCl which corresponds to a 50% dilution. However, our findings are inconsistent with those of Besim et al., 1998 who found that 10% saline is not effective after 5 minutes or those of Kayaalp et al., 2001 which found 10% saline to be 100% lethal only at the end of 75 minutes [10, 11]. We could explain these differences by the fact that, in our experience PS were handled as if they were in the operative conditions (suspended in the hydatid fluid) unlike Besim et al., or Kayaalp et al., studies who used a crude sediment of PS without any further suspension.

In accordance with Kayaalp et al. results, we also conclude that the scolicidal effect of hypertonic saline depends on the product's concentration rather than the exposure time [11]. So, if surgeons wish sufficient effectiveness from saline, they must increase the concentration. Concentrations less than 10% of saline could be ineffective so 20% NaCl could be used with efficiency if more than the half of the cyst can be emptied and at least with a 5 minutes exposure time. It's however not recommended to use highly concentrated saline solution due to the risks of sclerosing cholangitis in the bile duct (in case of liver cyst) and acute hypernatremia [13, 14, 15].

Discouraged by the dangers and drawbacks of the usual scolicidal agents like formalin or strongly hypertonic saline, many authors have tried and adopted H_2O_2 in surgery of hydatid cysts [9, 14, 16]. The management of the hydatid cysts by 3% H_2O_2 was described by different authors as effective and harmless [9, 17]. Although, Magistrelli et al. had claimed the low efficacy and the complications of H_2O_2 [18]. Our results showed that H_2O_2 efficacy to kill PS after 5 minutes contact was variable depending on its concentration (0.15%, 0.3% or 0.45% H_2O_2). Hydrogen peroxide becomes 100% lethal starting from 0.6% concentration (20% dilution). Moreover, 0.45% H_2O_2 is effective on PS after 10 minutes contact. Our results complement those of Besim et al., who found that 1.5% H_2O_2 was effective after 5 minutes exposure time [10]. They showed that mild H_2O_2 concentration between 0.45% and 0.6% could be effective in terms of killing PS (table 3). The H_2O_2 efficacy mainly depends on the previous contact of the cyst content with blood host, resulting from cyst handling during the surgical procedure. In fact, cysts that had a clear hydatid fluid appeared more sensitive to H_2O_2 than those having a haematic one. Among these lasts, those that showed an abundant moss after addition of H_2O_2 were the most resistant. The inefficiency of the H_2O_2 in presence of blood is not surprising. The presence of metallic ions (iron, cupper, zinc, manganese), organic substances, enzymes of antioxidants systems (superoxide dismutase, catalase, glutathion peroxidase, glutathion reductase and glucose 6 phosphate dehydrogenase) can entail the free radical trapping that induce the activity of H_2O_2. The presence of moss could be in relation with the action of the catalase, present in the blood which consumes H_2O_2. It brings us to insist on the realisation of a rigorous haemostasis of the operative field. Otherwise, surgeons must tempt to avoid bleeding during the intervention. It is indeed often difficult to avoid the bleeding considering the inflammatory reactions induced by the presence of the cyst. It would be better to use hypertonic saline when highly haematic cyst are managed since H_2O_2 could be completely inactivated, NaCl will act by an osmotic mechanism in this case.

Certain variability in sensitivity to H_2O_2 also exists for cysts with clear liquid. This was evident when using 0.15% H_2O_2 (table 4). These differences could be explained by differences in the hydatid fluid composition; this latter may contain plasma components of the host [19] or by variability among PS themselves. This hypothesis can be verified by further experiments like interchanging hydatid fluid.

Lastly, our results showed that scolicide action was closely conditioned by the hydatid cyst volume (table 5). In point of fact, the more the cyst is large, the more the scolicide is diluted and the more this last is ineffective. Hypertonic saline solution is effective from a starting dilution of 50% and H_2O_2 is effective after 5 minutes at 20% dilution or after 10 minutes at 15% dilution. This means that in operative conditions and when H_2O_2 should be used as scolicidal agent, surgeons should estimate the theoretical cyst volume by a $d^3\pi/6$ formula where d is the cyst radiological diameter. Then a fifth of the hydatid cyst content should

be removed, replaced by H_2O_2 and incubated at least for 5 minutes. In order to insure a higher effectiveness the exposure time could be lengthened or several aspiration-injection-re-aspiration cycles could be practiced. We found that practical cyst volumes are 1.5 to 6 times beyond the theoretical ones (table 1), which should give surgeons more secure margins and allow them to use less scolicidal solution.

It is to note that even this study tried to reproduce what happens in surgical condition, *in vitro* survey presents some limits. For example, the assessment of viability of PS, based on morphological tests (flame cells and eosin uptake) can be insufficient. The PS that remained morphologically intact and they have been considered, in our survey, as being still alive after the scolicidal treatment, could have undetectable morphological changes but sufficient enough to prevent their further vesiculisation *in vivo*. In fact, H_2O_2 acts on the macromolecules of the cytoplasmic membrane but also on the DNA, leading mutagenic effects or stopping replications. Further investigations are needed to test viability of PS after *in vitro* scolicidal treatment, by their inoculation to animals in order to confirm their capacity to develop secondary echinococcosis.

Whereas submitted to several interferences, the use of H_2O_2 as scolicide, in different concentrations and incubation time, revealed good efficiency. However prior utilization, it seems necessary to considerate some parameters such as cyst size though deducing the approximate hydatid fluid volume, the scolicide volume and the effective exposure time. During the surgical procedure, bleeding should be avoided as far as possible and the use of hypertonic saline would be an alternative in case of blood loss.

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