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Paraquat detoxication with multiple emulsions

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ABSTRACT

In this study, we show that detoxifying W/O/W multiple emulsions, prepared with an appropriate extractant/trapping couple, represent a promising technology for quick and safe poisoning treatments, with application to the highly toxic herbicide Paraquat, responsible of poisonings from low-dose exposure leading to several deaths every year. *In vitro* tests led to the choice of an appropriate extractant/trapping couple system with significant detoxication performance. *In vivo* tests showed (i) that rats receiving high doses of Paraquat, then a detoxifying emulsion, presented an increase from 50% to 100% of the MST (median survival time) and (ii) that no mortality was observed during 30 days with rats dosed with emulsions initially loaded with Paraquat at a concentration much higher than the lethal dose, proving the stability and the inocuity of the detoxifying multiple emulsion in the gastrointestinal tract.

1. Introduction

Oral intoxication is a major public health problem. Present day treatments are risky, hard to implement and expensive. The elaboration of new processes allowing quick and safe poisoning treatment is thus a crucial necessity. Many intoxication treatments are only symptomatic and frequently require the administration of specific antidotes. Emptying the stomach with an emetic compound or only a gastric lavage are currently used in standard treatments. Activated charcoal and Fuller's Earth are able to reduce the absorption of toxics when administered within 1 h. Laxatives are also used, but no clinical investigations have shown their efficiency in gastric decontamination (Frochaux et al., 2004). Finally, intestinal irrigation could be applied in some well-defined cases such as the ingestion of drugs with slow or delayed release.

Gastroscopy and gastrectomy are exceptionally used when a gastric aggregate is detected and when all other techniques have proven unsuccessful.

The American Academy of Clinical Toxicology and the European Association of Poison Centers and Clinical Toxicology have published together their recommendations and concluded that unless justified for the patient, the routine utilization of all these treatments is not recommended. This recommendation was reviewed in 2004 and is still being followed (Barceloux et al., 1997; Chyka and Seger, 1997; Krenzelok et al., 1997; Tenenbein, 1997; Vale, 1997).

The elaboration of an efficient treatment enabling the emergency salvation of poisoned patients without side effects is therefore a necessity and represents a real challenge.

Detoxifying W/O/W multiple emulsions (DME) represent a new promising technology for quick and safe poisoning treatment (Grossiord and Stambouli, 2008). The multiple emulsions are vesicular systems where small water droplets (internal aqueous phase, diameter around 1 μm) are entrapped within larger oil droplets (oil phase, diameter around 10 μm), that in turn are dispersed in a continuous water phase (external aqueous phase). Multiple emulsions contain both W/O and O/W emulsions and require at least two emulsifiers to be present in the system, the first (low HLB) to stabilize the primary W/O emulsion and the second (high HLB) for the secondary O/W emulsion.

Mainly used for drug delivery purposes today (Grossiord and Seiller, 1998), multiple emulsions can also be employed for the capture of toxic molecules. The external aqueous phase of the multiple emulsion is in direct contact with the toxic molecule. The extraction process can be enhanced by the addition of an appropriate organic extractant able to form a lipophilic complex with the toxic compound at the external interface (Fig. 1). In this manner, the lipophilic complex diffuses through the oily phase. Upon reaching the internal interface, the complex is broken via a chemical reaction with a hydrophilic trapping agent present in the internal aqueous phase. The toxic is then trapped in the form of an ionic species and the released extractant is again available for a new transport cycle (Trouvé et al., 1982; Durand et al., 1996; Devulapalli and Jones, 1999;

Abbreviations: DME, detoxifying multiple emulsion; WME, control or white multiple emulsion; GF, simulated gastric fluid; PQ, Paraquat.

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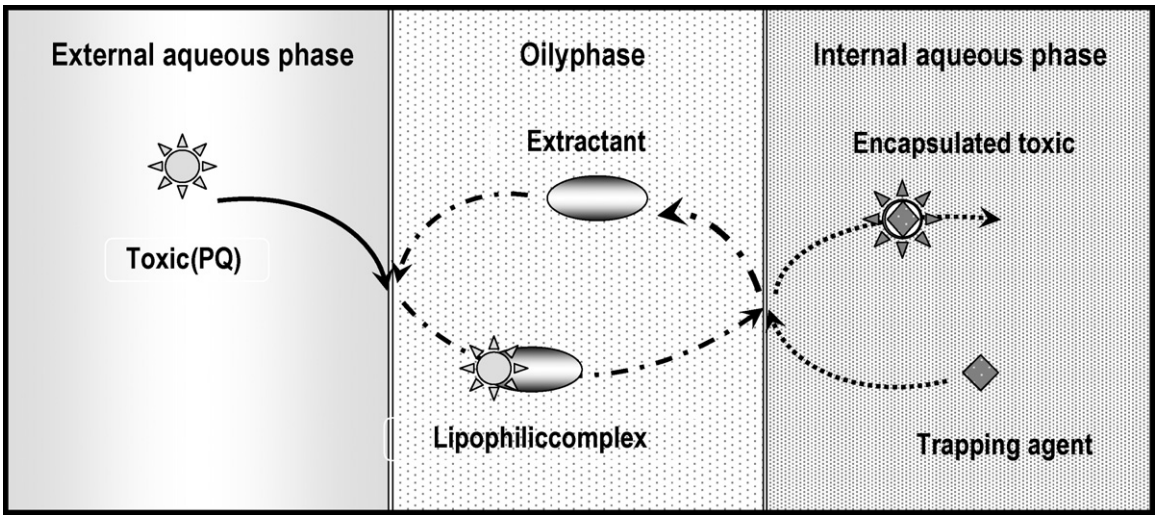

Fig. 1. Transfer of Paraquat in the W/O/W emulsion.

Dobin-Assouly et al., 2002; Hamoudeh et al., 2006; Gamino Arroyo et al., 2008).

When ingested, detoxifying multiple emulsions can therefore capture a toxic from the stomach into their internal aqueous phase. Loaded DME are then naturally removed by excretion, provided their stability is ensured in the gastrointestinal tract.

In the present study, we have designed efficient W/O/W multiple emulsions for *in vitro* and *in vivo* detoxication of Paraquat (trade name of *N*,*N*′-dimethyl-4,4′-bipyridinium dichloride), one of the most widely used herbicides in the world despite of its acute oral toxicity. Fatal Paraquat poisonings from topic or low-dose exposures are still a serious risk (several deaths every year), and yet no antidote is known to date (U.S. National Library, 1995; Wesseling et al., 2001; Sittipunt, 2005).

2. Materials and methods

2.1. Materials

Two different surfactants were used for W/O/W multiple emulsions: a lipophilic one, Abil EM90 (cetyl dimethycone polyol) supplied by Goldschmidt (France) and a hydrophilic one, Lutrol F127 or Polaxamer 407 (ethoxylated propylene oxide copolymer) supplied by BASF (France). Magnesium sulfate ($MgSO_4$, $7H_2O$), sodium chloride, sodium bicarbonate and pepsin were purchased from Sigma–Aldrich (France), hydrochloric acid from Prolabo (France).

The extractant was di-(2-ethylhexyl)dithio phosphoric acid (D2EHDTPA) which was produced, either by acid stripping of the commercial zinc salt ADDITIN RC 3180 (Rhein Chemie), or synthesized by a specific reaction of phosphorus pentasulfide in the appropriate alcohol, according to published procedures (Durand et al., 1996; Dobin-Assouly et al., 2002; Gamino Arroyo et al., 2008). The extractant was diluted in Parleam 4 (hydrogenated polyisobutene), supplied by Rossow and Cie (France). An aqueous solution of Paraquat (1,1′-diméthyl-4,4′-bipyridinium) at a concentration of 362 g/L was supplied by Syngenta (UK). Water was purified through reverse osmosis (MilliQ, Millipore France).

2.2. Animals

All *in vivo* experiments were conducted on male Wistar rats (250–300 g), purchased from Charles River Breeding Laboratories (France). Animals were fed with water and standard food *ad libitum*, for seven days before any experiments took place. Then they fasted for 24 h, with water *ad libitum*. They were housed in metabolism cages to avoid coprophagy and sawdust ingestion. All experiments and procedures were performed following the guidelines of the European Community (86/609/CEE) and the French National Committee (Decree 86/648).

2.3. Preparation of multiple emulsions

The detoxifying multiple emulsions were prepared using a two-step procedure. Firstly, the internal aqueous phase (50 g; NaOH 0.05 M, $MgSO_4$ 1.6%) was mixed with the organic phase (50 g; Parleam 4: 87%; Abil EM 90: 12%; D2EHDTPA: 1%). The mixture was then stirred with a rotor-stator turbine (Ultraturrax) for 3 min at room temperature (13000 rpm), to prepare the W/O primary emulsion.

Secondly, 80 g of the primary emulsion were added to 20 g of the external aqueous phase containing 10% of Lutrol F127 and stirred with a turbine (Rayneri, 500 rpm, 30 °C) for 5–10 min.

Control multiple emulsions (WME) were prepared using the same procedure as DME, but without the extractant (D2EHDTPA) and the trapping agent (NaOH).

In the *in vitro* experiments, both DME and WME were vigorously stirred with different aqueous solutions at controlled pH and Paraquat concentrations.

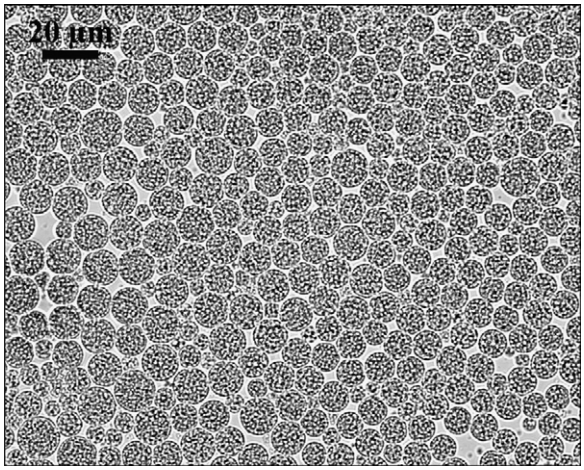

Fig. 2. Multiple emulsion, pH 10; 21 µS/cm.

Table 1
Comparison between white multiple emulsion (WME) and detoxifying multiple emulsion (DME) extraction performances.

Time (min)	% Extraction	
	WME	DME
5	13.2 ± 7.5	46.0 ± 1.8
10	12.2 ± 1.1	46.7 ± 5.9
20	11.0 ± 6.7	42.4 ± 7.2
30	7.4 ± 0.8	45.2 ± 5.4
45	7.4 ± 5.1	44.9 ± 4.2
60	8.2 ± 6.2	38.2 ± 2.3
90	8.9 ± 4.3	39.1 ± 6.1
120	7.5 ± 7.0	38.6 ± 4.0
Average	9.5	42.6
RSD (%)	24.6	8.3

RSD: relative standard deviation.

2.4. Characterization of multiple emulsions

The droplet size, the integrity and the polydispersity of the prepared emulsions were followed by microscopic observation (Leitz Diaplan with CoolSNAP camera, Roper Scientific, France). pH and conductivity of the W/O/W emulsions were determined with a Meterlab PHM 240 (Radiometer, France) and Meterlab CDM 230 (Radiometer, France) respectively.

All these parameters were checked at regular time intervals during 12 months, to verify the long-term stability of the emulsions at 4 °C, 40 °C and at room temperature.

2.5. In vitro Paraquat extraction by DME

The Paraquat solution, supplied by Syngenta, was diluted in either water or simulated gastric fluid (USP 22) to a concentration of 362 mg/L. 20 mL of this solution were mixed with 10 g of emulsion in a beaker at 37 °C under magnetic stirring (450 rpm, Fisher Bioblock 10515). Three samples of 1 mL were recovered at 5, 10, 15, 20, 30, 45 and 60 min and centrifuged for 2 min (550 G, Minispin Eppendorf, Germany). The heavy aqueous phase was removed with a syringe and centrifuged twice again to remove any emulsion trace.

Then 40 μL of this aqueous solution were analyzed after a 1/50 dilution in water or simulated gastric fluid, at 257 nm using a Perkin-Elmer Spectrophotometer Lambda 11.

Each experiment was carried out three times. Concurrently the same experiment was done without Paraquat to measure the absorbance due to the emulsion alone. This value was subtracted from the one obtained with Paraquat.

2.6. In vivo DME efficiency for Paraquat detoxification

Two Paraquat lethal doses LD1 or LD2 (respectively 115 mg/kg and 172 mg/kg) were used, as suggested by literature (U.S. National Library of Medicine, 1995) and confirmed by Syngenta (personal communication).

Animals were first dosed by oral gavage with either LD1 or LD2. After 5 min, the rats were given via oral gavage too, either the detoxifying emulsion DME (12 rats for each lethal dose), or the control emulsion WME (12 rats), or the same volume of physiological serum for the control group (12 rats). We observed the rats' mortality during 30 days; the experiments were repeated six times. Results were measured as the median survival time (MST%) of the animals which was calculated as follows:

$$\mathrm{MST}\,(\%) = \frac{\text{median survival time of the treated rats} \times 100}{\text{median survival time of the non-treated rats}}$$

3. Results and discussion

3.1. Multiple emulsions

The multiple emulsions that were studied were homogenous and stable when stored at room temperature, at 4 °C and 40 °C, for more than 12 months. Both pH (10 ± 0.5) and conductivity (20 ± 2) μS/cm remained constant all the time. The size of the multiple globules was homogenous: 90% of the globule diameters were in the 5–15 μm range (Fig. 2); the internal droplet diameter was about 1 μm.

3.2. In vitro Paraquat extraction and optimization

The efficiency or the extractant/trapping agent system was studied by measuring the extractive performance of DME compared to the control emulsion (WME). Table 1 shows that the Paraquat extraction in the gastric simulated fluid (pH 2) reached 45% with the detoxifying emulsion DME whereas it was only 10% with WME.

This shows the efficiency of the extraction mechanism, which took place rapidly, since the maximum extraction rate was obtained after only 5–10 min.

The influence of several critical formulation parameters was studied *in vitro*, before the *in vivo* tests, i.e. the external pH value, the Emulsion weight/Paraquat solution volume ratio and the Paraquat concentration.

- Influence of the external pH
  The gastric fluid medium was neutralized with sodium bicarbonate for pH 4, 6 and 8, and with sodium hydroxide for pH 9 and 10. As shown in Table 2, Paraquat extraction was improved when increasing the pH. Detoxication was therefore expected to be better in the intestinal medium than in the gastric one.
- Influence of the emulsion/Paraquat (w/v) ratio
  Increasing the emulsion/Paraquat ratio from 1/2 to 6/1 in the simulated gastric fluid increased the extraction rate from 30% to 75% (Table 3). One should note that the rate of increase was decreasing with the emulsion/Paraquat ratio: 1.4 times, 1.9 times and 2 times more when the emulsion/Paraquat ratio (w/v) was 1/2, 2/1 and 4/1 respectively. The extraction rates were similar for emulsion/Paraquat ratio (w/v) of 4 and 6.
- Influence of Paraquat concentration

Table 2
Effect of pH on the extraction performances of DME.

pH	% Extraction vs. time (min)					Average % extraction	RSD (%)
	5	10	20	30	45		
1.2	16.0 ± 0.4	17.5 ± 2.7	19.9 ± 2.9	17.6 ± 1.5	17.0 ± 2.5	17.6	8.1
4	28.2 ± 5.0	30.5 ± 2.6	27.3 ± 6.1	24.7 ± 5.1	25.3 ± 3.2	27.2	8.6
6	32.3 ± 4.7	33.2 ± 3.3	31.4 ± 5.8	29.0 ± 4.8	29.6 ± 3.1	31.1	5.7
8	26.8 ± 1.6	27.5 ± 3.0	29.7 ± 1.4	24.6 ± 6.6	29.4 ± 4.1	27.6	7.4
9	33.0 ± 1.8	36.1 ± 5.9	35.0 ± 7.2	33.2 ± 5.4	34.1 ± 4.2	34.3	3.8
10	33.7 ± 1.3	36.0 ± 3.9	32.2 ± 1.6	30.7 ± 5.2	25.4 ± 3.7	33.3	6.0

RSD: relative standard deviation.

Table 3
Effect of ratio DME/PQ on extraction performances of DME.

Ratio DME/PQ	% Extraction vs. time (min)				Average % extraction	RSD (%)
	10	30	60	100		
1/2	33.4 ± 2.3	36.4 ± 7.9	36.7 ± 6.4	35.0 ± 5.0	35.4	4.3
2/1	47.7 ± 5.0	46.2 ± 7.9	52.2 ± 8.0	49.6 ± 2.0	48.9	5.3
4/1	62.4 ± 4.7	61.6 ± 2.7	70.9 ± 1.0	78.3 ± 6.5	68.3	11.5
6/1	66.4 ± 4.3	65.1 ± 2.2	70.7 ± 1.0	81.3 ± 4.7	70.9	10.4

RSD: relative standard deviation.

Increasing the Paraquat concentration by a factor of 1000 (from 362 mg/L to 362 g/L) in the gastric fluid led to a decrease in the extraction rate (from 60% to 30%) (Fig. 3). This lower rate, obtained for high concentrations of Paraquat, is however significant for man detoxication. The lethal dose for man (50 mg/kg [17]) is indeed reached after the ingestion of only 20 mL of the Paraquat commercial solution (standard concentration of 181 g/L) and a 30% extraction rate is then expected to reduce the concentration under the lethal level.

3.3. Effect of multiple emulsions on Paraquat toxicity in rats

- Multiple emulsions harmlessness and stability

  The harmlessness (inocuity) of detoxifying multiple emulsions was first studied by dosing 12 rats with four successive DME gavages. No toxicity symptoms were observed during 30 days, proving the inocuity of detoxifying multiple emulsions in the gastro-intestinal tract.

  Then DME loaded with high concentrations of Paraquat in their aqueous internal phase (90 g/L) were given to 12 rats which were carefully observed during 30 days. Although the concentration of the entrapped Paraquat was much higher than the lethal concentration, no toxicity symptoms were observed in the rats, demonstrating the high stability of the emulsions in the gastro-intestinal tract. No significant release of Paraquat occurred in the gastro-intestinal tract due to the breakage of the DME multiple globules.

- Detoxifying effect of DME

Several interesting conclusions can be drawn from Figs. 4 and 5:

1) The control group (i.e. rats dosed with Paraquat without any emulsion) showed a 100% mortality rate after 80 h for LD1 (Fig. 5) and after 60 h for LD2 (Fig. 4) One should note that after 40 h, the difference in the mortality rate is significant: 50% for LD1 and 75% for LD2 and is directly related to the Paraquat concentration.

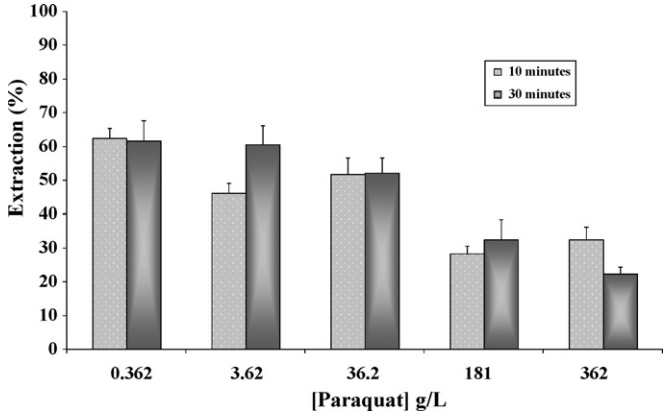

Fig. 3. Paraquat extraction in a simulated gastric fluid: effect of Paraquat concentration, with DME/PQ = 4/1 (w/v). Each point is the mean value for three experiments ($n = 3$).

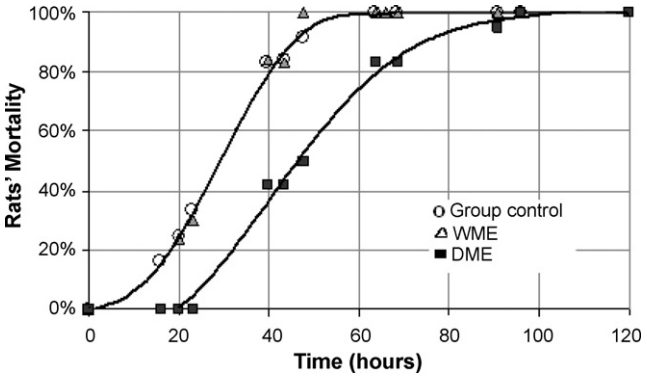

Fig. 4. Mortality rate of the rats after an oral LD2 ingestion of Paraquat without (control) or with ulterior administration of a detoxifying multiple emulsion (DME) or a white emulsion (WME).

2) The results in terms of mortality were the same for the control group and the WME group (Fig. 4). This proves (i) the absence of undesirable effects of WME and (ii) the lack of efficiency when the extractant/trapping agent system was not used. So the mechanism of action which was proved previously (Dobin-Assouly et al., 2002; Hamoudeh et al., 2006) to be an extraction/trapping process, was confirmed by these *in vivo* studies.
3) A significant detoxification effect of DME was observed: a high increase of the median survival time (MST%) was obtained when the rats were treated by DME rather than by WME (increase of 50% for LD1 and 70% for LD2).

The *in vivo* studies in Figs. 4 and 5 were performed with a low emulsion/Paraquat ratio (i.e. 2.5/1 (w/v)). Fig. 6 illustrates the positive detoxication effect when a higher emulsion/Paraquat ratio was used: a 100% increase in MST % was measured at LD2, for a 4/1 (w/v) emulsion/Paraquat ratio. These results were consistent with those obtained in *in vitro* experiments (Table 3). The 4/1 ratio was chosen to respect physiological conditions without

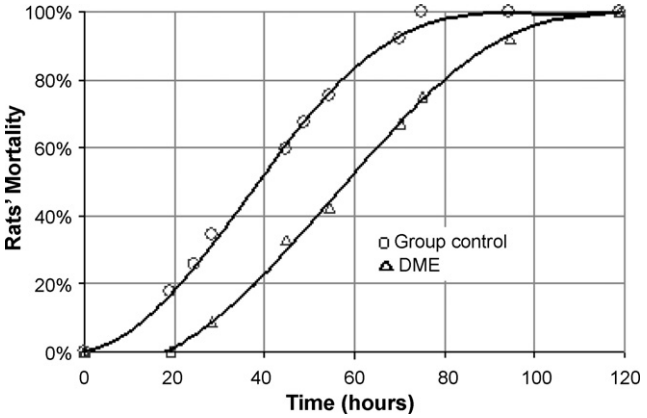

Fig. 5. Mortality rate of the rats after an oral LD1 ingestion of Paraquat, with (DME) and without (control) administration of a detoxifying multiple emulsion.

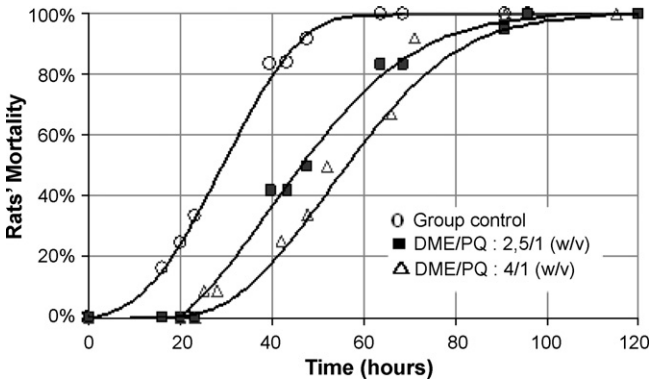

Fig. 6. Mortality rate of the rats after an oral LD2 ingestion of Paraquat and ulterior administration of a detoxifying multiple emulsion (DME): effect of the emulsion/Paraquat ratio (w/v).

inducing stomach distension. On the contrary, the 6/1 ratio was too high, inducing a significant reduction of gastric mobility (data not shown).

4. Conclusion

Both *in vitro* and *in vivo* experiments carried out on Wistar rats demonstrated the detoxifying ability of the DME in Paraquat poisoning. Significant and reproducible results were obtained for two lethal doses, and the following conclusions may be drawn concerning this innovative DME technology: (i) rats receiving high doses of Paraquat via oral gavage (LD1, LD2) and then a Detoxifying Multiple Emulsion presented a significant increase in their survival time, whereas no improvement in survival time was observed with WME and (ii) no mortality was observed during 30 days with rats dosed with DME initially loaded with Paraquat at high concentrations, confirming both the stability and the safety of the DME in the gastro-intestinal tract.

This study demonstrated the powerful extraction/trapping concept involved in DME. Such treatment could increase the survival delay in the case of severe oral intoxications for the installation of emergency care. Complementary studies are in progress for further optimization of DME formulations in terms of osmolarity and pH to still increase their efficacy.

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