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Sodium Tripolyphosphate: An excipient with intrinsic *in vitro* anti-*Candida* activity

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ABSTRACT

Sodium Tripolyphosphate (STPP) is a food additive that is being used in the development of micro and nanoparticles as it induces ionic interactions with chitosan molecules. Although the ability of STPP to inhibit the growth of several food contaminants has been reported, studies on its activity against clinical isolates are scarce. *Candida* spp. are common causative agents of mucocutaneous infections including the vulvovaginal tegument and new therapeutic approaches are needed in order to treat resistant and recurrent cases. The aim of this study was to evaluate *in vitro* both antifungal (anti-*Candida* spp.) activity, and cytotoxicity, on human dermal fibroblasts, of STPP solutions. STPP showed an inhibitory species-dependent activity against several *Candida* spp. strains being particularly active on *C. glabrata*, followed by *C. guilliermondii*. *In vitro*, STPP showed a concentration dependent cytotoxicity. Therefore STPP use, in low concentrations, seems to be interesting in the development of drug delivery systems for the treatment of vulvovaginal candidosis.

1. Introduction

Sodium Tripolyphosphate (STPP), also known as Pentasodium Triphosphate or Pentasodium Tripolyphosphate ($Na_5P_3O_{10}$), is a straight chain derivative from phosphoric acid. It is classified by the Food and Drug Administration as being a Generally Recognized as Safe Substance (GRAS) (FDA, 2006) and is approved to be used as a food additive, along with other polyphosphates, either in USA and in Europe, in a wide variety of foods including fruits, beverages, meat and seafood sole or in combination with other phosphates (EC, 1995). STPP is identified in labels by the food additive code 451 (E451, in Europe) and is able to act as a buffer, emulsifier, texturizer and sequestrant (Zaika and Kim, 1992).

In pharmaceutical technology, STPP has gathered particular interest as an ingredient of chitosan nano and microparticle systems. In 1989, Bodmeier et al. (Bodmeier et al., 1989) first reported drug encapsulation by ionotropic gelation due to the formation of inter and intramolecular crosslinks of the positively charged chitosan mediated by the polyanionic tripolyphosphate. This method has the advantage of being simple, non-toxic, developed at room temperature while avoiding the use of organic solvents. The functionality role of STPP in these systems supports its classification as a pharmaceutical excipient having the advantage of being considered safe since it is used in food industry (Pifferi and Restani, 2003).

Several researchers have applied chitosan–STPP ionotropic interactions, by different techniques, to achieve encapsulation of several drugs like vitamins (Desai and Park, 2005), antioxidants (Hu et al., 2008; Luo et al., 2010), antimicrobials (Anal et al., 2006; Hasanovic et al., 2009), proteins (Ghanem and Skonberg, 2001; Pan et al., 2002) and nucleic acids (Csaba et al., 2009), among many others, and to develop new controlled release drug delivery systems (Buranachai et al., 2010; Ko et al., 2002; Shu and Zhu, 2000; Srinatha et al., 2008).

Studies of the antimicrobial activity of STPP and other polyphosphates, on food contaminants have been conducted suggesting their possible use as preservatives (Firstenberg-Eden et al., 1981; Foster and Mead, 1976; Jen and Shelef, 1986; Lee et al., 1994; Molins et al., 1984; Post et al., 1968; Vareltzis et al., 1997). However, *in vitro* studies of STPPs antimicrobial activity over clinical isolates are very scarce. Interestingly, while there are several publications on antimicrobial chitosan–STPP micro and nanoparticles (Anitha et al., 2009; Du et al., 2009; Qi et al., 2004; Shi et al., 2006)

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the contribution of STPP itself to the overall *in vitro* activity has not been investigated.

STPP, in aqueous solution, was shown to have an inhibitory effect over bacteria in poultry meat (Elliott et al., 1964; Vareltzis et al., 1997), and to retard fungal growth on fresh cherries (Post et al., 1968). It was also shown to exhibit *in vitro* antimicrobial activity upon spore suspensions of *Penicillium expansum*, *Rhizopus nigricans*, and *Botrytis* sp. (Post et al., 1968) and upon pure cultures of bacteria such as *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Jen and Shelef, 1986; Molins et al., 1984), *Listeria monocytogenes* (Zaika and Kim, 1992) and *Moraxella-Acinetobacter* species (Firstenberg-Eden et al., 1981). The inhibitory effect of STPP is stronger against gram-positive than on gram-negative bacteria (Molins et al., 1984). When comparing the inhibitory effect of different polyphosphates on *L. monocytogenes* growth, Zaika and Kim (1992) showed that the longer chain molecules exhibit more inhibitory effect than the short chain molecules such as STPP (Zaika and Kim, 1992), a difference that was also observed in molds (Suarez et al., 2005). Orthophosphates did not show any activity when compared with polyphosphates (where STPP is included) as growth inhibitors of *S. aureus* in liquid media (Jen and Shelef, 1986). In fact, STPP activity following heat sterilization, when hydrolysis of STPP to sodium acid pyrophosphate and tetrasodium pyrophosphate is expected to occur, is diminished, as documented in several articles (Firstenberg-Eden et al., 1981; Jen and Shelef, 1986; Molins et al., 1984) sustaining the importance of phosphates chain size for antimicrobial activity.

Because of their highly charged anionic nature, polyphosphates have the capacity to chelate cations. It has been suggested that the ability to form stable complexes with cations may turn them unavailable for metabolic functions, either by sequestering the cations from the media (Elliott et al., 1964) or by removing essential metals from cation-binding sites within the cell walls of microorganisms (Knabel et al., 1999; Suarez et al., 2005). In fact, several published papers point to a reversal of polyphosphate inhibitory effect after addition of metals considered essential for cellular function, particularly Mg^{2+} and Ca^{2+}, to the growth media of inhibited bacteria such as pseudomonads (Elliott et al., 1964), *S. aureus* (Jen and Shelef, 1986; Lee et al., 1994), *L. monocytogenes* (Zaika and Kim, 1992) and *Bacillus cereus* (Maier et al., 1999).

Vulvovaginal candidosis is one of the most common clinical manifestations of *Candida* spp. infections, affecting 70–75% of women at least once in their lifetime and has an high rate of recurrence (Mardh et al., 2002). *C. albicans*, followed by *C. glabrata*, *C. tropicalis* and *C. krusei* are the main species responsible for this infection (Mardh et al., 2002; Sobel, 2007). Difficulties in the clinical management of this infection, particularly in recurrent forms (usually associated with *C. glabrata*) (Sobel, 2007), are impelling scientists to search for new therapeutic approaches.

We have reported on the *in vitro* effect of a chitosan hydrogel against several *Candida* strains pointing out its possible interest as an active vehicle with antifungal activity for vaginal drug delivery systems (Palmeira-de-Oliveira et al., 2010).

The aim of the present work was to evaluate the activity of STPP by its own against several collection type and clinical *Candida* strains resistant to conventional drugs. STPP cytotoxicity on normal human dermal fibroblasts was also investigated.

To our knowledge this is the first report on STPP *in vitro* activity against fungal clinical strains.

2. Materials and methods

2.1. Chemicals

Sodium Tripolyphosphate (85%), HEPES, L-glutamine, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin G, amphotericin B, streptomycin, RPMI 1640, trypsin and $MgCl_2{\cdot}6H_2O$ were purchased from Sigma-Aldrich (Sintra, Portugal); Fetal bovine serum was purchased from Biochrom AG (Berlin, Germany). Culture media Brain–Heart Broth (BHI) and Sabouraud agar (SDA) were purchased from Difco Laboratories (Detroit, MI, USA).

2.2. Yeasts strains

Twenty-six clinical isolates of *Candida* spp. strains were used in this study corresponding to *C. albicans* ($n = 6$); *C. parapsilosis* ($n = 7$); *C. glabrata* ($n = 5$); *C. tropicalis* ($n = 6$); and *C. guilliermondii* ($n = 2$). Additionally one type strain *C. albicans* 10231 from the American Type Culture Collection (ATCC) was also used. Clinical isolates were obtained from mucocutaneous infections; from patients with severe clinical conditions, including cases of recurrent disease and showing variable degree of resistance to fluconazol, as previously confirmed *in vitro*. These isolates had been characterized to species level using API 32C (BioMérieux, Vercieux, France). The strains were kept frozen in BHI with 5% glycerol at −70 °C until testing. For each experiment, the yeasts were subcultured twice on SDA to assess the viability of the culture.

2.3. Sodium Tripolyphosphate antifungal activity assay

Sodium Tripolyphosphate was dissolved in ultrapure water (Milli-Q; Millipore) and sterilized by filtration through a 0.2 μm-pore-size cellulose membrane filter. Serial dilutions were then performed with RPMI. pH determinations for each dilution were performed using a pH meter (Metrohm 620, Swiss).

The minimal inhibitory concentration of STPP upon *Candida* spp. was determined according to the CLSI reference M27-A3 micromethod, after 48 h of incubation at 37 °C (CLSI, 2008). Yeast growth was visually compared for each concentration with the control sample. Only the 100% MIC value was taken as a result. Minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) were determined after 48 h of incubation at 37 °C. MLC were determined based on the modified protocol proposed by Canton et al. (Canton et al., 2003). Yeast growth was visually compared for each concentration with the control sample. All determinations were performed in duplicate for each assay and three independent experiments were run with concordant results.

2.4. Influence of Mg^{2+} in STPP anti-Candida activity

To determine the influence of Mg^{2+} on STPPs antifungal activity different *Candida* strains were selected and plated in a 96-well plate (cellular density 0.5 McFarland) in contact with the correspondent MIC STPP solution (final concentration) and 1 mM, 10 mM, 100 mM Mg^{2+} solution (final concentration). In a parallel study Mg^{2+} was added 1 h after STPP. Yeast growth was visually compared with the control sample after 24 h and 48 h. Additional controls were performed using (1) the tested Mg^{2+} final concentrations in RPMI without addition of STPP and (2) STPP (MIC) without addition of Mg^{2+}.

2.5. Fibroblast source and growth

To study cytotoxicity we chose normal human dermal fibroblasts (NHDF) (acquired to ATCC – American Type Culture Collection). Cells were routinely maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), HEPES (0.01 M), L-glutamine (0.02 M) and sodium pyruvate (0.001 M) and 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 μg/mL amphotericin B). Experiments were

Table 1
Susceptibility of *Candida* spp. to STPP (MIC: minimal inhibitory concentration; MLC: minimal lethal concentration).

	Strain	MIC (mg/mL)	MLC (mg/mL)
C. parapsilosis	*C. parapsilosis* MC405	12.50	50.00
	C. parapsilosis MC409	12.50	50.00
	C. parapsilosis MC428	12.50	50.00
	C. parapsilosis MC429	12.50	50.00
	C. parapsilosis ART64	12.50	50.00
	C. parapsilosis 011	12.50	50.00
	C. parapsilosis 030	12.50	50.00
C. tropicalis	*C. tropicalis* MC374	12.50	25.00
	C. tropicalis MC375	12.50	25.00
	C. tropicalis MC407	12.50	25.00
	C. tropicalis MC410	12.50	25.00
	C. tropicalis ART41	12.50	25.00
	C. tropicalis ART35	12.50	25.00
C. albicans	*C. albicans* ATCC 10231	6.25	12.50–25.00
	C. albicans MC 440	6.25	12.50–25.00
	C. albicans MC 416	6.25	12.50–25.00
	C. albicans MC 439	6.25	12.50–25.00
	C. albicans MC 437	6.25	12.50–25.00
	C. albicans 28	6.25	12.50–25.00
	C. albicans 30	6.25	12.50–25.00
C. guilliermondii	*C. guilliermondii* ART32	1.56	12.50
	C. guilliermondii ART33	1.56	12.50
C. glabrata	*C. glabrata* MC 369	0.39	25.00
	C. glabrata MC 370	0.39	25.00
	C. glabrata MC 425	0.39	25.00
	C. glabrata MC 426	0.39	25.00
	C. glabrata H30	0.39	25.00

performed in 12-well tissue culture plates with 1×10^4 cells/well. Cells were used on the third passage.

2.6. Analysis of cell viability (cytotoxicity assay – MTT test)

Cell viability was studied by quantifying the extent of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction according to the procedure described by Freshney (Freshney, 2005). Briefly, cells were seeded in 12-well plates (1×10^4 cells/well) in culture medium containing FBS and after 48 h adherence they were treated with five concentrations of STPP (0.39 mg/mL, 0.52 mg/mL, 0.78 mg/mL, 1.56 mg/mL, 3.12 mg/mL) for 48 h, incubated at 37 °C, in a 5% CO_2 atmosphere. Untreated cells were used as control. After the incubation period the media in wells were removed and replaced with fresh media and MTT solution and incubated for 4 h, in the same conditions. Thereafter, media containing MTT were removed, formazan crystals were dissolved and absorbance was measured at 570 nm using a Biorad 550 microplate reader (Benchmark). The extent of cell viability was expressed as the percentage of viable treated cells in comparison with control cells.

In order to evaluate the ability of fibroblasts to recover after contact with STPP we performed another assay where removal of STPP-containing media after the incubation period was done, replacing it with fresh medium, for 48 h. After this period the MTT test was performed.

2.7. Statistics

For cytotoxicity assays graphical results were expressed as the mean ± SEM (standard error of mean) of triplicate of three independent experiments. Comparison of groups was analysed using Student's *t*-test. Differences between groups were considered statistically significant at $p < 0.05$.

3. Results and discussion

STPP showed to be active upon all tested strains in a species-dependent effect. It was particularly active against *C. glabrata* (MIC = 0.39 mg/mL) followed by *C. guilliermondii* (MIC = 1.56 mg/mL) (Table 1). For *C. albicans*, *C. parapsilosis* and *C. tropicalis* MIC values ranged from 6.25 mg/mL to 12.5 mg/mL. MLC values differed from MIC for all tested strains suggesting a concentration-dependent fungicidal effect.

Considering the proposed mechanisms of action for STPP (Knabel et al., 1999; Suarez et al., 2005) and the reversal effect of ions media addition (Elliott et al., 1964; Jen and Shelef, 1986; Lee et al., 1994; Zaika and Kim, 1992) we studied the effect of Mg^{2+}

Table 2
Influence of Mg^{2+} on STPP inhibitory effect (48 h of contact).

Yeast	STPP (mg/mL)	Addition of Mg and STPP at the same time			Addition of Mg 1 h after STPP		
		Mg (1 mM)	Mg (10 mM)	Mg (100 mM)	Mg (1 mM)	Mg (10 mM)	Mg (100 mM)
C. glabrata H30	0.39	+	+	+	+	+	+
C. guilliermondii ART 33	1.56	–	+	+	–	+	+
C. albicans ATCC 10231	6.25	–	+	PP[a]	–	+	+
C. albicans MC 416	6.25	–	+	PP[a]	–	+	+
C. parapsilosis MC405	12.50	–	–	PP[a]	–	–	PP[a]
C. tropicalis ART 41	12.50	–	+	PP[a]	–	–	PP[a]

PP[a] indicates that a white precipitate was formed and covered the well.

Table 3
pH values of STPP solutions after dilution in RPMI medium used for anti-*Candida* studies.

STPP in RPMI concentration (mg/mL)	pH
0	7.0
0.10	7.0
0.39	7.0
1.56	7.1
3.12	7.2
6.25	7.2
12.50	7.4

supplementation on the yeast growth inhibition by STPP solutions, knowing that magnesium seems to be essential for the metabolism of *Candida* spp. including enzymatic activity (Veser, 1987) (Table 2).

When high Mg^{2+} concentrations (100 mM) were obtained simultaneously with STPP concentrations ≥6.25 mg/mL a white insoluble precipitate (probably a Mg-TPP precipitate) was observed. In these cases visual evaluation of yeast growth was hindered. This observation is comparable with other investigators that reported an insoluble calcium polyphosphate precipitate when high concentrations (>3 mM) of calcium ion were added to a 0.1% long-chain sodium polyphosphate glassy solution (Maier et al., 1999).

We observed that the amount of Mg^{2+} required to reverse this inhibitory effect correlates positively with the amount of STPP initially added to the medium (MIC) (Table 2) probably related with differences in Mg^{2+} requirements between species. We have also observed that STPP mechanism of action is time-dependent since MLC showed no fungicidal effect at 12 and 24 h of treatment (data not shown). These results confirm that STPP activity is related with an ion depletion effect that may inhibit yeast growth but only leads to death when ions are not available for metabolism.

To avoid the immediate reaction between STPP and Mg^{2+} and to contribute to the clarification of the possible STPP interaction with the yeast cell wall, a latter (1 h after STPP) addition of Mg^{2+} was tested. Slightly differences were observed. For *C. albicans*, formation of the white precipitate was also observed with the highest Mg^{2+} concentration but it was less extensive and did not completely cover the well allowing for growth identification. This observation suggests that either some molecules of STPP were already chelating other media ions or they were interacting with the cell wall and were not removed by the added Mg^{2+} ions. In this case, however, Mg^{2+} supply to yeasts was not compromised. On the other hand for *C. tropicalis* the latter addition of Mg^{2+} (10 mM) did avoid cellular growth (Table 2) suggesting that Mg^{2+} entrance in the cell is reduced in this situation. Since yeast growth was observed when STPP and Mg^{2+} were added simultaneously, the inhibitory effect of STPP when Mg^{2+} was added latter on may be hypothetically explained by STPP prior interaction with the yeasts wall avoiding recently added Mg^{2+} entrance. Further studies on cell wall composition and on STPP cellular interactions are needed to clarify the differences in behavior of these strains.

Polyphosphates solutions pH has been shown to be an interfering factor on antibacterial and antifungal activity probably due to superior ionization and consequent chelation ability at elevated pH where they revealed better activity (Jen and Shelef, 1986; Suarez et al., 2005). The pH of our STPP solutions after dilution in RPMI was measured. Only a 0.4 variation of pH was observed from culture media to the highest MIC determined (Table 3). Therefore natural pH of STPP diluted solutions are not expected to have interfered with our results.

Although STPP is approved as a food additive the maximum allowed concentrations are an important issue. Our *in vitro* cytotoxicity results show that STPP has a concentration dependent negative effect on the viability of fibroblastic cells. When these

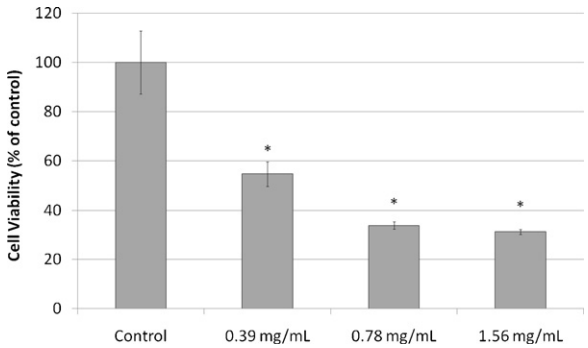

Fig. 1. Cytotoxicity of STPP solutions on fibroblastic human cells after 48 h of contact. * indicates significant difference between control and samples ($p < 0.05$, Student's *t*-test, $n = 9$). Bars represent the mean and the lines represent SEM.

cells were maintained in contact for 48 h with STPP concentrations ≥3.12 mg/mL cell death occurred as verified by microscopic visualization of cell cultured plates. For 1.56 mg/mL, 0.78 mg/mL and 0.39 mg/mL concentrations MTT assay was performed for cell viability assessment and compared with cells in STPP-free culture media (Fig. 1). For 0.78 mg/mL and 1.56 mg/mL STPP concentrations only 34% and 31%, respectively, cellular viability was measured when compared with controls. For the lowest tested concentration (0.39 mg/mL) 55% cellular viability indicates a direct toxicity profile in these conditions. In fact, considering that this *in vitro* study involves a limited culture medium volume supplying cells with nutrients during the assay we hypothesized that this effect could be reversed by replacing the culture medium after 48 h contact with fresh STPP-free culture medium. We concluded that, for the lower STPP concentration, cells could recover from STPP effect (73% viability when compared with cells that didn't contact with STPP solutions) (Fig. 2). However, for 0.78 mg/mL and 1.56 mg/mL STPP concentrations previous contact, cells couldn't recover and significant differences were observed when comparing these viability results with those obtained with cells that had been in contact with STPP lowest concentration. Comparison with control cells show that STPP didn't stimulate cell growth (Fig. 2). Since STPP MIC for *Candida* tested strains ranged from 0.39 to 12.50 mg/mL most of these concentrations are within those we showed to have an *in vitro* cytotoxic effect to normal human fibroblasts, under the tested conditions.

However, since STPP can be used in food individually or in combination with other phosphates up to 5 g/kg (expressed as P_2O_5) (EC, 1995) with no described security issues we believe that the cytotoxicity results we showed are intimately linked with test conditions and related to nutritional deficiencies of tested cells during

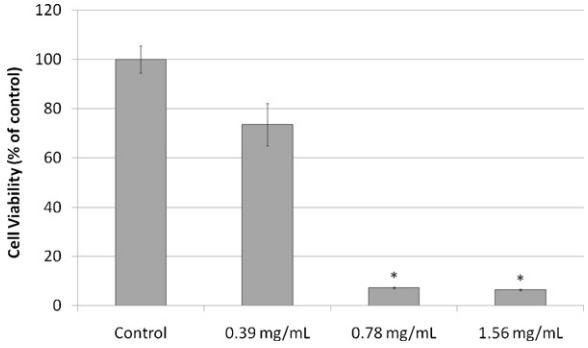

Fig. 2. Recovering capacity of fibroblast human cells after contact with STPP solutions.* indicates significant difference between 0.39 mg/mL and 0.78 mg/mL or 1.56 mg/mL concentrations ($p < 0.05$, Student's *t*-test, $n = 9$). Bars represent the mean and the lines represent SEM.

the first 48 h. Different results would probably be obtained *in vivo* where the tissue organization with nutrient supplies by blood flow is expected to protect these cells from the sequestering effect of STPP. On the contrary, *Candida* yeasts infecting the vaginal tissue depend on medium ions and nutrients for growth and may be affected by STPP. Therefore a possible selective toxicity may be revealed *in vivo*.

4. Conclusion

Our results show that STPP demonstrates antifungal activity against several *Candida* spp. isolated from mucocutaneous infections. However in the tested conditions it was also demonstrated a concentration dependent cytotoxicity on normal human dermal fibroblasts. The use of STPP as an excipient in the development of new drug delivery systems for vulvovaginal candidosis may be interesting if used in low final concentrations as those commonly required for chitosan particles formation and after extensive *in vitro* and *in vivo* testing for antimicrobial activity and particularly for cytotoxicity.

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