**Introduction**

*Clostridium difficile* is the most common cause of nosocomial infections in the US. The burden of the disease that has significantly increased in terms of incidence, mortality, and recurrence rates. The pathogenic effects of *C. difficile* are mainly secondary to the production of two exotoxins: toxin A (TcdA) and toxin B (TcdB). These toxins monoglucosylate, and thereby inactivate, Rho GTPases of host cells causing several direct and indirect cytopathic effects ultimately leading to colonic death, loss of intestinal barrier function, and neutrophilic colitis. Apart from intestinal effects, toxins contribute to the development of systemic manifestations of CDI. Besides antibiotic consumption, the main risk factors associated with the development of CDI are advanced age, impairment in humoral immunity, renal disease and hypoalbuminemia. An association between low albumin levels and CDI development, disease severity and mortality rates is well known. First hypoalbuminemia was merely considered a consequence of CDI induced protein-losing enteropathy, however subsequent studies demonstrated that hypoalbuminemia is often pre-existing to CDI. The mechanism by which hypoalbuminemia predisposes to the disease is unknown. Preliminary results suggested that HSA is able to reduce the cytotoxic effects of TdBD (Di Bella et al., 2015).

**Aim:** To clarify the molecular mechanism underpinning the protective role of human serum albumin (HSA) towards *C. difficile* toxins, using a combined biochemical and cellular approach.

**Methods:**
- Binding assay: spectrofluorimetry, absorption wavelength 300-400 nm, excitation wavelength 280 nm
- Cell line: human epithelial colorectal adenocarcinoma (CaCo-2) • Citotoxicity tests: MTT test; absorption wavelength 570 nm • Toxins and HSA localization: western blot using anti-*C. difficile* TcdB (ab83066, Abcam, Cambridge, UK) and anti-HSA (H-126, Santa Cruz Biotechnology, Santa Cruz, CA) Ab.

**Results**

1. **HSA binds both TcdA and TcdB.** HSA binds to TcdA and TcdB in a saturating dose-dependent manner. The analysis of data allowed the determination of values of the apparent dissociation equilibrium constant for TcdA and TcdB binding to HSA (i.e., $K = 0.24±0.04 \text{ nM}$ and $K = 0.44±0.12 \text{ nM}$, respectively). Under all the experimental conditions, the Hill coefficient $n$ is 1.0±0.22, as expected for simple systems. The capacity of HSA to bind TcdA and TcdB has been further confirmed by HPLC analyses (data not shown).

2. **HSA exerts a protective role towards *C. difficile* toxins-induced cytotoxicity.** To test the protective effects of HSA towards toxins-induced cytotoxicity, CaCo-2 human epithelial colorectal adenocarcinoma cells were used (extensively used as a model of the intestinal barrier to perform in vitro toxicity tests).

   - Caco-2 cells treatment with 8 μg/ml and 16 μg/ml TcdA or TcdB or with a TcdA-TcdB mixture (each at 8 μg/ml and 16 μg/ml) showed that TcdA did not exert any significant effect on CaCo-2 metabolic activity (data not shown) (Di Bella et al., 2015). In contrast, TcdB caused the typical toxin-induced cytopathic effects, such as shrinking and rounding of cells (panel A), and a reduction of about 60% in cell viability after 24 h exposure (panel B). Similarly, cells treated with both toxins showed an enhanced cytotoxicity compared with cells treated with TcdB only (panels C and D).

To further investigate the protective role of HSA, CaCo-2 cells were treated with both toxins and HSA. As shown in panels B and D, HSA significantly reduced the cytoxin-induced spherical detached shape of CaCo-2.

**Conclusions:** For a long time it has been thought that hypoalbuminemia was merely a consequence of CDI induced protein-losing enteropathy. Subsequently, clinical data collected at the time of admission showed that hypoalbuminemia actually predisposes to CDI. Preliminary data obtained by our group showed that HSA protects enterocytes from *C. difficile* TcdB-induced death. Our results clearly demonstrate that HSA sequester the toxins outside the cell, avoiding their binding to the host cell receptors possibly blocking the cytopathic effects as well as the signaling cascade. Our study contributes to understand the significance of the association between hypoalbuminemia and the development of CDI, its severity and its associated outcomes.

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