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Spectrum of bacterial peritonitis in patients maintained on peritoneal dialysis and in vitro assessment of ethylenediaminetetraacetic acid (EDTA) and taurolidine antimicrobial properties

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Abbreviations

3-oxo-C12-HSL, N-(3-oxododecanoyl)-l-homoserine lactone

C4-HSL, N-(butanoyl)-homoserine lactone

AI, autoinducers

AnxV, annexin V

APD, automated peritoneal dialysis

BLI, bioluminescent

CAPD, continuous ambulatory peritoneal dialysis

CIGS, centro interdipartimentale grandi strumenti

CLSI/NCCLS, clinical and laboratory standards institute/national committee for clinical laboratory standard

CQI, continuous quality improvement

CVC, central venous catheter

EDTA, ethylenediaminetetraacetic acid

ESRD, end-stage renal disease

FBS, fetal bovine serum

HD, hemodialysis

HPLC, High-Performance liquid chromatography

ISPD, international society of peritoneal dialysis

IQS, 2-(2-hydroxy-phenyl)-thiazole-4-carbaldehyde

LPS, lipopolysaccharides

MIC, minimal inhibitory concentration

MRSA, methicillin-resistant staphylococcus aureus

OD, optical density

PBS, Phosphate Buffered Saline

PD, peritoneal dialysis

PDC, peritoneal dialysis catheter

PI, Propidio

PMBC, peripheral mononucleate blood cells

PQS, 2-heptyl-3-hydroxy-4-quinolone

RLU, relative luminescence units

QS, quorum sensing

RRT, renal replacement therapy

S., Staphylococcus

SB, saline buffer

SEM, standard error of the mean

SD, standard deviation

RLU, relative luminescence units

TSA, tryptic soy agar

TSB, tryptic soy broth

Abstract

Background: Peritoneal dialysis (PD) is often complicated by bacterial peritonitis, a severe infectious disease associated with high morbidity. Treatment of peritonitis is successful in the majority of cases, but often bacteria producing biofilm are challenging to eradicate because antibiotics are unable to penetrate the biofilm matrix covering the peritoneal catheter. As a result, biofilm-related peritonitis is resolved with catheter removal and switch of the patient from PD to hemodialysis (HD).

Aims: Given the challenges of treating this infection, we developed a research project aimed to broaden the knowledge on the use of lock therapy in a model of biofilm-related peritoneal catheter infection caused by *Pseudomonas aeruginosa*, one of the most virulent pathogens in terms of biofilm formation. This research project aimed to (i) describe the microbiological profile and outcomes of bacterial peritonitis in patients maintained on peritoneal dialysis at the University Hospital of Modena, (ii) assess antimicrobial properties of lock therapy with ethylenediaminetetraacetic acid (EDTA) and taurolidine in an in vitro model of *Pseudomonas aeruginosa* biofilm-related peritoneal catheter infection, and (iii) verify the in vitro safety of EDTA and taurolidine for human peripheral blood mononuclear cells (PBMCs).

Results: Retrospective analysis of 484 patients on PD followed at the University Hospital of Modena from 2000 to 2019 identified 373 episodes of peritonitis occurring in 191 (39.4%) patients. We found an incidence rate of 0.23 episodes/patient/year during these two decades. In line with the literature, the majority of the patients were on continuous ambulatory peritoneal dialysis (CAPD) (61.2%) at the time of the diagnosis of peritonitis. Gram-positive bacteria were the most common (48.1%) microorganisms, although their incidence significantly decreased ($p=0.0017$) over the last 10 years of the study period (2010-2019). *Pseudomonas* species was the etiological microorganism of about one-quarter of the episodes of peritonitis and was the principal cause (21.8%) of catheter removal.

In light of these data, we investigated the effects of two antimicrobial solutions, EDTA and taurolidine, in an in vitro model of a bioluminescent strain of *Pseudomonas aeruginosa* a biofilm-related peritoneal catheter infection. Serial dilutions of taurolidine (0.125%; 0.25%; 0.5%) and/or EDTA (2.5%; 0.75%; 0.25%) were applied (for 24 hours) onto contaminated catheters, first on a 24-hours-old biofilm and then on a previously exposed 72-hours-old biofilm-associated to peritoneal catheter. High-performance liquid

chromatography-mass spectrometry (HPLC-MS) analysis was performed on supernatants from the peritoneal catheter, treated with EDTA and/or taurolidine, to measure the production virulence factors such as autoinducers, pyocyanin and pyoverdines of *Pseudomonas aeruginosa*. The treatment with EDTA and taurolidine did not lead to the eradication of infection yet affected the secretory activity of *Pseudomonas aeruginosa*.
Lastly, in order to translate the use of taurolidine and EDTA in vivo, we evaluated the effect of EDTA and taurolidine on human PMBCs. EDTA (2.5%) and several dilutions of taurolidine (0.125%; 0.25%; 0.5%) were incubated for 24-hours with PBMCs. The cytotoxic essays documented the dose-dependent toxicity of taurolidine on PBMCs, whereas EDTA exerted no cytotoxic effect on these cells.
Conclusions: *Pseudomonas species* peritonitis was the leading cause of peritoneal catheter removal in PD patients followed at the University Hospital of Modena during 2000-2019. In vitro studies evaluated the antimicrobial effect of EDTA and taurolidine, two lock-solutions, on segments of peritoneal catheter contaminated by *Pseudomonas aeruginosa*. Although these solutions showed high antibacterial effects, did not eradicate the biofilm-related infection from the peritoneal catheter. Given that taurolidine cause a dose-dependent cytotoxic effect on human PMBCs cells, further studies are required to better elucidate its mechanism of action.

Riassunto

Background: Il trattamento dialitico peritoneale (DP) è spesso complicato dalla peritonite batterica, un processo infettivo gravato da un'alta morbilità. Alcuni casi di peritonite sono associti ad un'infezione del catetere peritoneale. In questi casi l'infezione è difficile da eradicare perché gli antibiotici non sono in grado di penetrare la matrice del biofilm adeso al catetere peritoneale. Di conseguenza, la peritonite associata al biofilm necessita la rimozione del catetere e il passaggio del paziente dalla PD all' emodialisi (HD).

Scopi dello studio: In considerazione dell'impatto clinico di questo problema, abbiamo condotto un progetto di ricerca volto ad ampliare le conoscenze sull'uso della lock therapy in un modello di infezione del catetere peritoneale correlata al biofilm causata dal batterio *Pseudomonas aeruginosa*, uno dei patogeni più virulenti in termini di formazione del biofilm e produzione di fattori di virulenza. Questo progetto di ricerca ha avuto lo scopo di (i) descrivere il profilo microbiologico e l' outcome della peritonite batterica nei pazienti in DP presso l'Azienda Ospedaliera Universitaria di Modena, (ii) valutare in vitro gli effetti della lock therapy con acido etilendiamminotetraacetico (EDTA) e taurolidina su un modello di infezione del catetere peritoneale da *Pseudomonas aeruginosa* e (iii) verificare la sicurezza in vitro dell' EDTA e della taurolidina su cellule mononucleari di sangue periferico (PBMCs).

Risultati: Lo studio retrospettivo di 484 pazienti con PD seguiti presso l'Azienda Ospedaliera Universitaria di Modena dal 2000 al 2019 ha documentato 373 episodi di peritonite in 191 (39,4%) pazienti. Durante il periodo di osservazione, è stato riscontrato un tasso di incidenza della peritonite pari a 0.23 episodi/paziente/anno. In linea con i dati della letteratura, la maggior parte dei pazienti era in dialisi peritoneale ambulatoriale continua (CAPD) (61,2%) al momento della diagnosi. I batteri Gram-positivi sono stati i principali agenti etiologici della peritonite batterica (48,1%), sebbene la loro incidenza sia significativamente (p=0,0017) diminuita nell' ultimo decennio (2010-2019) del periodo di osservazione. L' infezione da *Pseudomonas* è stato diagnostica nel 23,3% degli episodi di peritonite ed è stata la principale causa (21,8%) della rimozione del catetere nella nostra coorte di pazienti.

Alla luce di questi dati, abbiamo studiato gli effetti di due soluzioni antimicrobiche, EDTA e taurolidina, su un modello in vitro di un'infezione da catetere peritoneale determinata da un ceppo bioluminescente di *Pseudomonas aeruginosa*. Diluizioni seriali di taurolidina (0,5%; 0,25%; 0,125%) e/o EDTA (2,5%;0,75%;0,25%) sono state applicate (per 24 ore)

su cateteri contaminati, prima su un biofilm di 24 ore e poi su un biofilm di 72 ore, precedentemente esposto alle soluzioni antimicrobiche. L' analisi in cromatografia liquida ad alta prestazione-spettrometria di massa, effettuata sul surnatante di cateteri peritoneali contaminati con EDTA e/o taurolidina, ha permesso di misurare la variazione dei fattori di virulenza dello *Pseudomonas aeruginosa*, quando esposto alle sostanze antimicrobiche. I risultati ottenuti da questo studio mostrano che il trattamento con EDTA e taurolidina hanno efficacemente ridotto la carica microbica senza però indurre una completa eradicazione del batterio dal catetere peritoneale. È stato inoltre interessante notare che la pressione esercitata da soluzioni antimicrobiche hanno significativamente influenzato l'attività secretoria del patogeno.

Infine, al fine di traslare l'uso della taurolidina ed EDTA in studi in-vivo, è stato effettuato uno studio di citotossicità con EDTA e taurolidina sui PMBCs umani. EDTA (2,5%) e diverse diluizioni di taurolidina (0,125%;0,25%;0,5%) sono state incubate per 24 ore con PBMCs. I test effettuati ha documentato una tossicità dose-dipendente della taurolidina sui PBMCs, mentre l'EDTA ha mostrato un effetto neutro su queste cellule.

Conclusioni: La peritonite da *Pseudomonas* è stata la principale causa di rimozione del catetere peritoneale nei nostri pazienti seguiti al Policlinico di Modena durante il periodo d' oservazione (2000-2019). Studi in in-vitro hanno valutato l'effetto antimicrobico dell'EDTA e della taurolidina su segmenti di catetere peritoneale con infezione da *Pseudomonas aeruginosa*. Queste soluzioni, sebbene abbiano mostrato un elevato effetto antimicrobico, non hanno eradicato l'infezione da biofilm dal catetere. Dato che la taurolidina (2,5%) ha mostrato un chiaro effetto citotossico dose-dipendente sulle cellule PMBCs umane, ulteriori studi sono necessari per caratterizzare il suo profilo d' azione.

Introduction

End-stage renal disease and dialysis

Chronic kidney disease (CKD) is a chronic and debilitating disorder that affects about 10-15% of the population worldwide[1,2]. CKD is characterized by a progressive evolution toward end-stage disease (ESRD) that coincides with the stage of renal failure[3]. ESRD is a challenging public health problem that is assuming an epidemic diffusion since patients chronically treated with dialysis almost doubled in the last 20 years. A recent epidemiological study has estimated that 3.8 million people are currently receiving dialysis around the world[4] and the prevalence of patients on chronic maintenance dialysis peaks at almost 2000 individuals per million population in the occidental countries[5].

The adequate treatment of ESRD is necessary to prevent death and uremia-associated comorbidities. Care of uremic manifestations due to kidney failure is based on long-term renal replacement therapy (RRT). The modalities of RRT are hemodialysis, peritoneal dialysis, or kidney transplantation. However, it is worth noting that RRT is unable to replace completely normal kidney function. Indeed, the outcome of dialysis therapy in ESRD remains dismal in terms of mortality and morbidity[6]. ESRD patients on maintenance dialysis therapy have a significantly higher mortality rate compared to the healthy population. It has been estimated to be about 20% per year in the United States and 10 - 15% in Europe[7] and is due primarily to the development of cardiovascular disease[8].

Patients' long-term quality of life on dialysis is deeply compromised compared to patients without ESRD. Multiple factors have been identified to worsen physical and psychological wellness[9]. From a practical view, predictors of poor quality of life are categorized into two groups: ESRD- and RRT modality-related factors. About the first group, the quality of life of dialysis patients is greatly impaired than that of the age-matched subjects from the general population, because of the high burden of comorbidity (e.g., diabetes, cardiovascular disease, hypertension) and complications due to ESRD (e.g., diet, depression, fatigue)[10]. The second group of determinants of poor quality of life refers to the intrusiveness of dialysis treatment in daily life[11,12]. Patients undergoing in-center hemodialysis generally receive dialysis treatment two or three times a week for three/four hours per session, a therapeutic program that tends to disrupt their personal and professional lives. Conversely, peritoneal dialysis (PD), the most common home dialysis

treatment option, is characterized by a lower impact on the patient's quality of life.

Peritoneal dialysis

PD is a modality of RRT, which prevalence differs dramatically between regions and countries. The penetrance of this type of dialysis accounts for approximately 11% of patients receiving dialysis[13]. The high cost of disposable material in the low-income countries[14], the high diffusion of public and private HD facilities and the lack of knowledge and experience in managing the care of patients on maintenance PD dialysis are the leading determinants of the low prevalence of PD compared to HD[15,16].

Peritoneal dialysis is performed at home or in any other clean place on its own account or with the help of a caregiver[17]. The procedure of DP consists of instilling fluid, called dialysate, through a peritoneal catheter into the peritoneal cavity. To allow the remotion of fluid and uremic toxins, the dialysate must dwell for a specified period within the abdominal cavity. During the dwell period, solute diffusion and ultrafiltration are the main biological processes leading to the removal of toxins and excess fluid from the body, respectively. The dialysate is then drained and wasted, new fresh dialysate is instilled and the cycle restart afterward.

There are two types of peritoneal dialysis: continuous ambulatory peritoneal dialysis (CAPD) and automated peritoneal dialysis (APD). The modality termed CAPD is performed manually three or four times daily, with the dialysate dwelling in the abdominal for about four-six hours. Alternatively, automated peritoneal dialysis (APD), equipped with a mechanical device, commonly termed "cycler," is used to perform several consecutive exchanges for 8-10 hours, generally during the night[15,18].

At present, the majority of studies indicate that PD and in-center HD have similar survival rates[15,19–21]. However, Lukowsky et al.[22] highlighted a different survival outcome in a study of over 3,718 incident dialysis patients (22,360 HD patients and 1,358 PD patients) who started dialysis between July 2001 and June 2004 at US DaVita dialysis centers. The authors found that PD was associated with 48% lower mortality than HD over the first 2 years of dialysis therapy independently of modality switches or different transplantation rates. Conversely, a recent study showed a survival advantage for HD in ESRD patients with diabetes[23]. Lastly, PD does not appear to favorably affect the outcome of renal

transplantation, although there is a common belief that PD may improve early graft function, by maintaining a valid diuresis and a favorable water balance in PD patients[24].
The survival rate seems also similar between the two modalities of PD: CAPD and APD[25–27]. However, some observational studies reported that APD, exchanging high volumes of dialysate by multiple short overnight depurative exchanges, tends to lower mortality compared to CAPD[28].

Peritoneal catheter

The success of PD relies on a well-functioning peritoneal catheter. The peritoneal catheter is peritoneal access used to dwell the peritoneal cavity with dialysate. It is a single lumen silicone catheter, placed through the anterior abdominal wall. Different types of catheters are available on the market: single or double cuff catheter, a straight or bend inner cuff segment, and a straight or coiled intraperitoneal segment[29]. Although no catheter type is superior to the others, the simple double-cuffed catheter is commonly used across peritoneal dialysis facilities worldwide. This peritoneal catheter is equipped with two Dacron cuffs anchoring the device to the surrounding tissue. Notably, the proximal cuff is anchored subcutaneously close to the exit-site, whereas the distal one is anchored to the *transversalis* fascia of *the rectus abdominis* muscle[30].
To avoid complications such as dialysate leakage, the catheter should be used after two weeks from its placement. This period allows the internal cuff to heal with the *trasversalis* fascia and minimizes the risk of dialysate rich in glucose leaking in the subcutaneous tissue.

Complications of Peritoneal Dialysis.

The complications of PD can be of noninfectious and infectious origin. Noninfectious complications principally include problems related to the catheter, namely catheter malfunction, dislocation and abdominal pain[31]. Infectious complications are a serious problem in PD and included exit-site infection, tunnel infection and peritonitis.
This latter is the most common infectious complication in PD. The international society for peritoneal dialysis (ISPD) guidelines have released the diagnostic criteria of peritonitis in 2016[32]. This complication is present if at least 2 of the following criteria are met:
(1) clinical features consistent with peritonitis, i.e., abdominal pain and/or cloudy dialysis effluent

(2) dialysis effluent white cell count > 100/μL (after a dwell time of at least 2 hours), with > 50% neutrophils
(3) positive dialysis effluent culture

The most common cause of peritonitis is due to contamination of the inner catheter with skin or mucosa bacteria during exchanges or to an exit-site or tunnel infection. Indeed, all exchanges are performed using a sterile procedure[18] to avoid severe bacterial or fungal infections. Further causes of peritonitis are due to colonization of the gastrointestinal tract and hematogenous spread.

Peritonitis is a common and serious infection contributing to the structural and functional changes of the peritoneal membrane, PD technique failure and switch to long-term hemodialysis (about 20 % of cases)[33]. The mortality due to peritonitis is around 5% but it can rise to 10% in some series[34]. The episode of peritonitis negatively impacts mortality up to 120 days after an episode of peritonitis with a greater magnitude during the initial 30 days[35].

As part of a continuous quality improvement program, all PD facilities should monitor the peritonitis rate regularly. Information should also describe the peritonitis rate of specific organisms and drug susceptibilities of the infecting organisms.

At present, there is a substantial variation in the peritonitis rate between countries. Different rates of peritonitis have been found also in the same country as reported in multicenter studies in Australia[36], Scotland[37] and UK[38]. The accepted threshold of the overall peritonitis rate should be no more than 0.5 episodes per year at risk. However, in some outstanding centers, the peritonitis rate is lower and ranges between 0.18 to 0.20 episodes per year[39,40].

Overall, the rate of this complication is decreasing over time. A retrospective study conducted in a single PD center in South Korea reported a significant reduction of peritonitis episodes from 0.57 episodes/patient-year in 1993 to 0.29 episodes/patient-year in 2005[41]. Parallel findings were reported by single-center studies in Brazil[42], Portugal[43] and Taiwan[44]. The epidemiological transition occurred primarily in Gram-positive (Gram +) peritonitis (particularly *Staphylococcus aureus* [*S. aureus*] and coagulase-negative *staphylococcus*), whereas Gram-negative (Gram -) peritonitis rates remained constant. The successful reduction in peritonitis rates was attributed to improvements in PD equipment (introduction of twin-bag connection systems), mupirocin prophylaxis for S. aureus

carriers, fluconazole or nystatin prophylaxis for fungal peritonitis and care of exit-site[45]. Given the important implications of catheter-related infections in PD, further precautions have been introduced in clinical practice. For instance, the peritoneal catheter is oriented inferiorly to prevent the stasis of infected secretion around the exit-site. The distal end of the catheter is attached to an adapter, called a transfer set. The utility of this adapter consists of its replacement, precluding the need for surgical replacement of the entire catheter in case of inadvertent contamination of the distal portion of the catheter system[18].

Biofilm-Associated Peritonitis

Non-complicated peritonitis is generally easily treated with intraabdominal antibiotics for two or three weeks. Severe or relapsing episodes of peritonitis, such as those caused by *Pseudomonas* (*Pseudomonas*) *aeruginosa*, *S. aureus* and Candida are associated with catheter removal and temporary HD[46]. Such infections are the result of bacterial adhesion to biomaterial surfaces and subsequent formation of antibiotic-resistant biofilms that are challenging to treat with intraperitoneal antibiotics. Ex vivo studies on PD catheters removed from patients experiencing peritonitis showed that such catheters were covered by microbial biofilms[47,48] consisting of single-species or mixed-microbial populations[49]. Biofilm is a very effective survival strategy for bacteria to survive in hostile conditions, even under the pressure of antimicrobial substances. Biofilm is a living material composed of matrix embedding sessile and planktonic (free-floating) bacteria that interact with host according to local microenvironmental conditions[50]. The initial event in biofilm formation is the adhesion of microbes to surfaces, such as medical devices made of synthetic material[51–53]. The abundant extracellular matrix embedding these microbial communities hamper adequate penetration into biofilms of intraperitoneal antibiotics resulting in a high rate of relapsing infections which are successfully treated only by catheter removal. Installation of high concentrations of antibiotics into the lumen of the catheter while not in use (lock solution) is a highly promising option for preventing or treating peritoneal catheter-related infections. However, lock solution has rarely been tested in PD patients with peritonitis[54]. According to ISPD guidelines, catheter removal is the only recommended option when intraperitoneal antibiotics fails to eradicate biofilm bacteria[32]. It is worth noting that most of the research studies conducted in the last two decades on catheter-related infections have focused on the use of antibiotic lock therapy for the intravascular catheter. In this setting, antimicrobial lock solution has been proven to be an

effective adjunctive treatment for biofilm-associated central venous catheter-related infections in hemodialysis and oncologic patients[55–58].

Non-antibiotic lock solution

Taurolidine and calcium disodium ethylenediaminetetraacetic acid (EDTA) are two antimicrobial lock solutions. These agents have the advantage to have a broader spectrum of activity and lack of phenomenon of bacterial resistance of the classical antibiotics. Promising anecdotal evidence provides clues about the successful treatment of relapsing peritonitis with taurolidine catheter-locking solution in patients receiving peritoneal dialysis[59–61].

Taurolidine is a synthetic broad-spectrum antimicrobial agent deriving from the amino acid taurine (Fig. 1). It binds lipopolysaccharides (LPS) and neutralizes bacterial exotoxins and endotoxins through methylol groups deriving from taurolidine metabolism[62](Fig. 1). Taurolidine has been used also as an antitumoral agent. The antineoplastic activity of taurolidine has not been fully elucidated, it seems to be nonspecific and may be related to the ability to induce apoptosis and suppress the production of vascular endothelial growth factors[63].

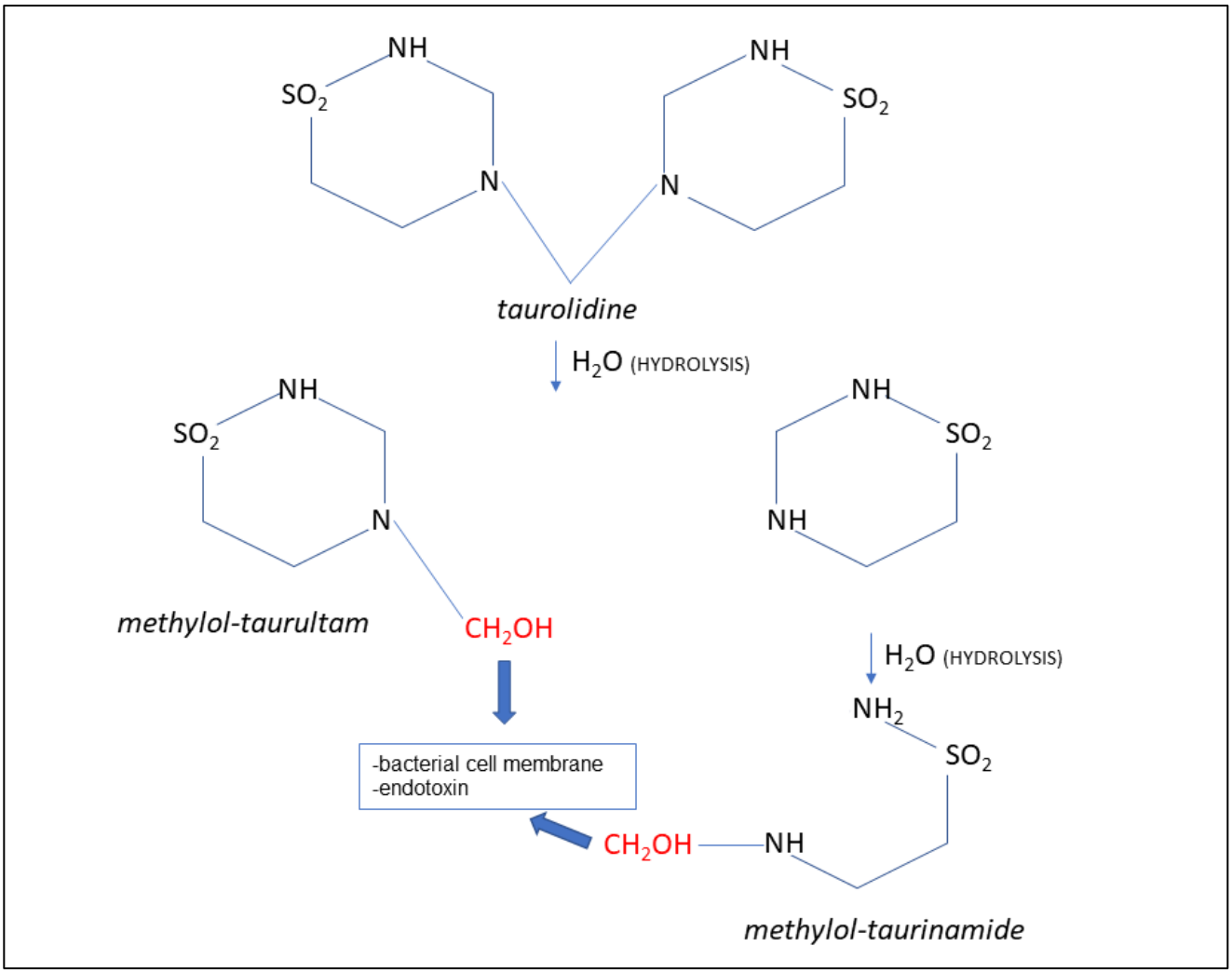

Figure 1. Structure of taurolidine and its major breakdown molecules after hydrolysis. Taurolidine generates methylol-containing fragments (CH_2OH) that have antibiotic and antiendotoxin activities. The amino acid taurine ($C_2H_7NO_3S$) (not shown) is the end-product of the reaction.

Whereas taurolidine has been successful to achieve the eradication of *Pseudomonas aeruginosa* in PD patients with relapsing peritonitis[60,61], there is limited experience on the use of the metal chelator EDTA as a lock solution in PD dialysis. In-vitro studies report an effective antibacterial effect of EDTA when used alone or in combination with classical antibiotics in a model of *Pseudomonas aeruginosa* infection[64–66] and *Pseudomonas aeruginosa* biofilm-related infections[67–69]. EDTA has been extensively used in the hospital setting for the treatment of mercury and lead poisoning. More recently EDTA has been used in veterinary and odontology for treating biofilm-associated infection given its permeating and sensitizing propriety of bacteria cell membrane[70]. The putative mechanism of action of EDTA relies on its chelating capacity of divalent elements[70]. EDTA has been shown to remove $Mg_2^+$and Ca_2^+ from the outer cell wall of bacteria, thereby exposing phospholipids of the inner membrane to the effects of antimicrobials[71]. Hence, EDTA acts principally as a synergistic agent when it is used in conjunction with other antibiotics[70].

Pseudomonas aeruginosa

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic highly virulent pathogen, especially in fragile patients, such as those undergoing peritoneal dialysis[72,73]. Often, *Pseudomonas aeruginosa* is responsible for biofilm-associated infections that require the removal of the medical device to cure the infections. Not only biofilm formation, but a series of other virulence factors such as proteases, toxins, extracellular polysaccharides, yellow-green siderophores (pyoverdine) and phenazines/pyocyanins play a crucial role in *Pseudomonas aeruginosa* infection. Many of these virulent substances are under the control of quorum sensing (QS) a regulatory system that controls gene expression in response to cell density[74]. In Pseudomonas *aeruginosa*, four QS systems have been identified, named LasI/LasR, RhlI/RhlR[75], PqsABCDE/PqsR[76] and AmbBCDE/IqsR[77], that recognize their own signal molecules, named autoinducers (AIs): N-(3-oxododecanoyl)-l-homoserine lactone (3-oxo-C12-HSL), N-(butanoyl)-homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-(2-hydroxy-phenyl)-thiazole-4-carbaldehyde (IQS), respectively. These AIs regulate microbial adhesion, colonization, biofilm formation, microbial dissemination/dispersion, tolerance to immune cells and resistance to drugs and detergents, influencing the course and outcome of the disease[78]. Moreover, AIs affect the release of siderophores, such as pyoverdine, involved in iron removal from the environment, and phenazines, involved in microbial adhesion and

biofilm formation[79,80]. AIs are also involved in oxidative stress, indeed, they interact with molecular oxygen to form reactive oxygen species, like H_2O_2, which modify the redox balance, causing cell injury and death[81].

Note

Part of this chapter has been previously published in the paper "*EDTA and Taurolidine Affect Pseudomonas aeruginosa Virulence In Vitro—Impairment of Secretory Profile and Biofilm Production onto Peritoneal Dialysis Catheters*" (authors: Bruna Colombari, Gaetano Alfano, Christian Gamberini, Gianni Cappelli, Elisabetta Blasi)[82].

Part 1. Microbiological profile and outcomes of bacterial peritonitis in patients on maintenance peritoneal dialysis at the University Hospital of Modena

Aims

A single-center retrospective study has been conducted to describe the epidemiology of the episodes of peritonitis in patients on PD, followed-up at the University Hospital of Modena. This study aimed to characterize the prevalence and the clinical characteristics of peritonitis in a large PD center with a long-term experience of PD. In particular, this epidemiological investigation aimed to define the pattern of infection, causative organisms, and clinical outcome related to *Pseudomonas* species, one of the most redoubtable pathogens in PD patients.

Material and methods

Study design and participants

All information concerning the peritonitis episodes that occurred from January 1st, 2000 to December 31st, 2019 at the University Hospital of Modena, were collected retrospectively from the database software "Peritoenale.mdb". The study included all peritoneal dialysis patients who received care at our center. The observation period ranged from 2000 to 2019 and was subdivided into two decades to evaluate the epidemiological differences over the years. The first decade included all episodes of peritonitis from January 1, 2000 to December 31, 2009 and the second decade from January 1, 2010 to December 31, 2019.

Definition of peritonitis

Peritonitis in PD was defined by two of the following features: (1) clinical features consistent with peritonitis (i.e., abdominal pain or cloudy dialysis effluent); (2) dialysis effluent white cell count >100/μl (after a dwell time of at least 2 hours), with >50% neutrophils; and (3) positive dialysis effluent culture.

A relapse of peritonitis was defined as an infection with either the same organism or culture-negative peritonitis within 4 weeks of completion of antibiotic therapy after a previous episode of peritonitis. Refractory peritonitis was defined as peritonitis that did not respond to antibiotic therapy and resulted in PD catheter removal. Recurrent peritonitis was defined as a separate episode of peritonitis within 4 weeks of completion of antibiotic therapy of a prior episode, but with a different organism. Repeat peritonitis was defined as an episode that occurs more than 4 weeks after completion of antibiotic therapy of a prior episode with the same organism[32].

Diagnosis of peritonitis

White cell count and microbial identification had a key role in the diagnosis of peritonitis in case of clinical suspicion. All microbiology laboratories followed procedures approved by the International Society for Peritoneal Dialysis (ISPD)[32].

To obtain sensitive results from white cell count and culture of dialysate the dialysate should dwell into the peritoneal cavity for two and three hours, respectively. Two methods were used to culture PD effluent in the microbiological laboratory. The first consisted of bedside inoculation of 5 – 10 mL effluent in 2 (aerobic and anaerobic) blood-culture

bottles. It occurred when the microbiology lab of the University Hospital of Modena was closed (during the night shift or holiday). The second consisted of the centrifugation of 50 mL dialysate effluent at 3,000 rpm for 15 minutes, followed by resuspension of the sediment in 3 – 5 mL supernatant and inoculation on solid culture media or standard blood-culture media. Peritonitis rates were calculated as the number of infections by the organism for a time period, divided by dialysis years' time at risk, and expressed as episodes /patient per year[32].

Diagnosis of exit-site infection

According to ISPD 2016 guidelines, exit-site infection was diagnosed by the presence of purulent drainage from the exit-site of the peritoneal catheter, with or without erythema of the skin[32].

Health care delivery

All patients starting PD were trained at the peritoneal dialysis unit of the University Hospital of Modena. The average time is taken to train PD patients ranged between 5-6 days. Additionally, the nurses provided training for patients after hospitalization or peritonitis to document the maintenance of eligibility for PD. Patients with peritonitis were admitted to the hospital only in case sepsis was defined as life-threatening organ dysfunction due to a dysregulated host response to infection[83].

Outcome

Catheter removal, hospitalization and death for peritonitis were evaluated as outcomes.

Data protection

The current study was part of two wider research projects, both approved by the Ethical Committee of Emilia Romagna (839/2020; 507/2021). Informed consent was waived because collected data were aggregated. All data protection policies were followed to maintain the anonymity of the patients, moreover, this study was conducted following Helsinki Declaration guidelines.

Statistical analysis

Continuous data are presented as mean and standard deviation (SD) or median and

interquartile range (IQR) as appropriate whereas categorical data are presented as percentage. Proportions were compared with Chi-square analysis or Fisher's exact test, as appropriate. A p-value lower than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

Participants

From January 2000 to December 2019, 484 ESRD patients on PD were followed at the University Hospital of Modena. The average age of these patients was 64.7±16.4 years and there was a predominance of males (60.7%).

During these two decades, 373 episodes of peritonitis were diagnosed in 191 patients, predominantly male (55.7%) and with an average age of 64.4±16.4 years. The prevalence of peritonitis accounted for 39.4%. The timing of peritonitis diagnosis was heterogeneous, it developed a median follow-up of 2.36 (IQR 1.02-4.43) years from the start of PD. At the time of diagnosis, the majority of them were on CAPD (61.2%). Nine-seven patients (50.7%) experienced multiple episodes of peritonitis. In this group of patients, the number of peritonitis ranged from 2 to 8 episodes (Table 1).

Variables	Patients (n. 191)
Age, yr.	64.4±16.2
Male, n. (%)	107 (55.7)
Modality of PD, n. (%)	
APD	74 (38.7)
CAPD	117 (61.2)
Type of catheter, n. (%)	
One-cuff Vicenza Cath	23 (12.4)
Two-cuff Vicenza Cath	10 (5.2)
Self-locating catheter	13 (6.8)
Swan neck	120 62.8)
Swan neck curled	20 (10.5)
Time elapsed from start of DP to first peritonitis, yr.	2.8±2.2
Patients with more than 2 episodes of peritonitis	97 (50.7)

Table 1. Main characteristics of PD patients with peritonitis

Etiological agent

Data about microbial culture were available only for 345 episodes of peritonitis. The major groups of microorganisms were Gram + (48.1%), Gram – (26.1%), polymicrobial culture (6.1%), mycetes (or fungi) (0.9%) and mycobacterium (0.6%). (Fig. 1). The culture-negative episodes of peritonitis accounted for 19.4%.

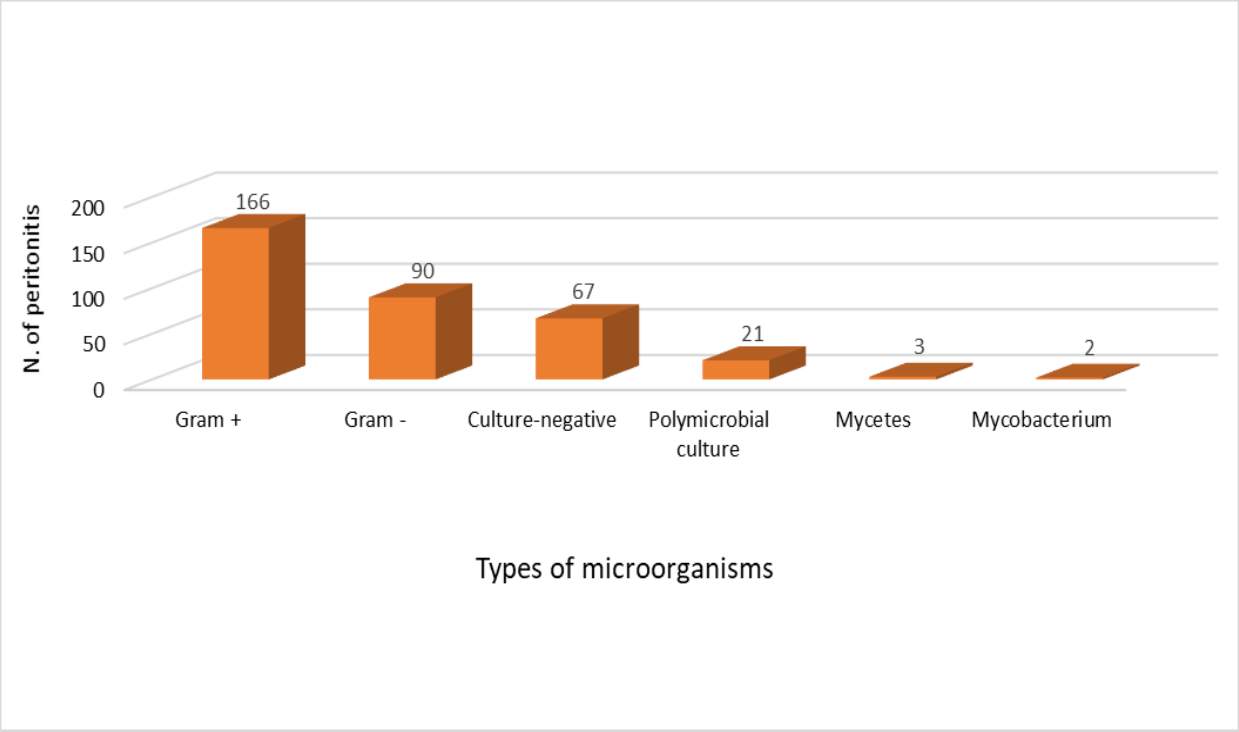

Figure 1. Groups of microorganisms responsible for peritonitis during the observational period

Incidence of infection

Three hundred seventy-three episodes of peritonitis occurred during 1560.4 dialysis-years' at risk. The resulting number of peritonitis was 0.23 episodes/patient-year. To evaluate the different incidence of peritonitis between the first decade (2000-209) and the second decade (2010-2019), the total number of peritonitis was subdivided according to the two periods. The incidence of peritonitis was for 0.29 episodes/patient/year during the first decade and 0.17 episodes/patient/years.

Chi-square analysis was employed to compare the rate of infections between groups of microorganisms. The second decade was characterized by a significant reduction of the rate of Gram+ bacteria causing peritonitis (38.6% vs. 56.5%;p=0.0017). No statistically significant differences were found in the rate of Gram – (p=0.49), culture-negative (p=0.32) and polymicrobial culture (p=0.38) between the first and second decade of the period study. (Fig. 2)

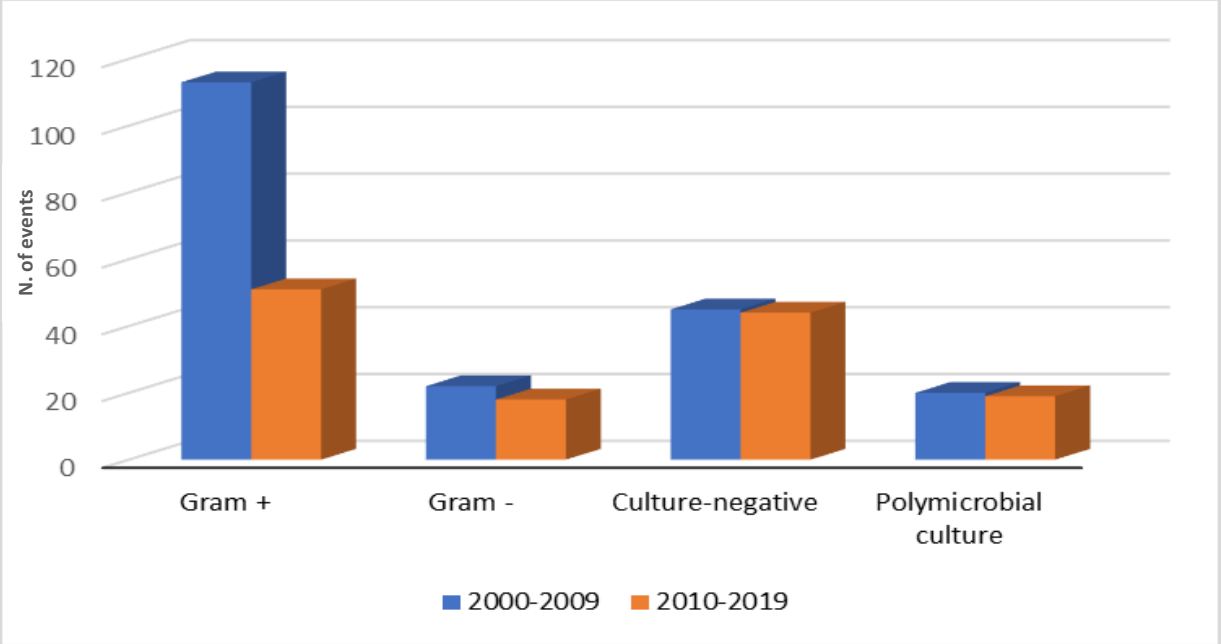

Figure 2. Graphical representation of microorganisms responsible of peritonitis events during the first decade (2000-2009) and second decade (2010-2019)

The most common Gram+ bacteria were *Staphylococcus* (61.4%), *Streptococcus* (15%) and *Enterococcus species* (9.6%). *Staphylococcus* species infections were subdivided in coagulase-negative (58.9%) and coagulase-positive (41.1%) bacteria (principally *S. aureus*). In this latter group, methicillin-resistant *S. aureus* (MRSA) accounted for 29.2% of peritonitis.

Gram- bacteria that caused peritonitis were *Klebsiella species* (25.5%), *Pseudomonas* species (23.3%), E. Coli (22.2%) and *Enterobacter cloacae* (12.2%). (Fig. 3)

Figure 3. The spectrum of bacteria causing 345 episodes of peritonitis

Outcome

The slow resolution of peritonitis led to catheter removal in 32 (8.5%) patients with peritonitis. *Pseudomonas* species (21.8%) infection was the principal cause of catheter removal. This dismal outcome occurred in 30% of all peritonitis caused by *Pseudomonas aeruginosa*. Other microorganisms causing catheter removal were *S. Aureus* (15.6%) and *E. Coli* (12.5%). All the different germs gathered in the group "other" caused a single episode of peritonitis. (Fig. 4)

There were 9 (2.4%) cases of relapse, and 25 (6.7%) episodes of peritonitis were associated with a coexisting exit-site infection.

One hundred twenty (36.5%) episodes of peritonitis led to hospital admission for the severity of the infectious process. Four patients died for the severe consequences of peritonitis, giving a mortality of 2.1% per episode of peritonitis.

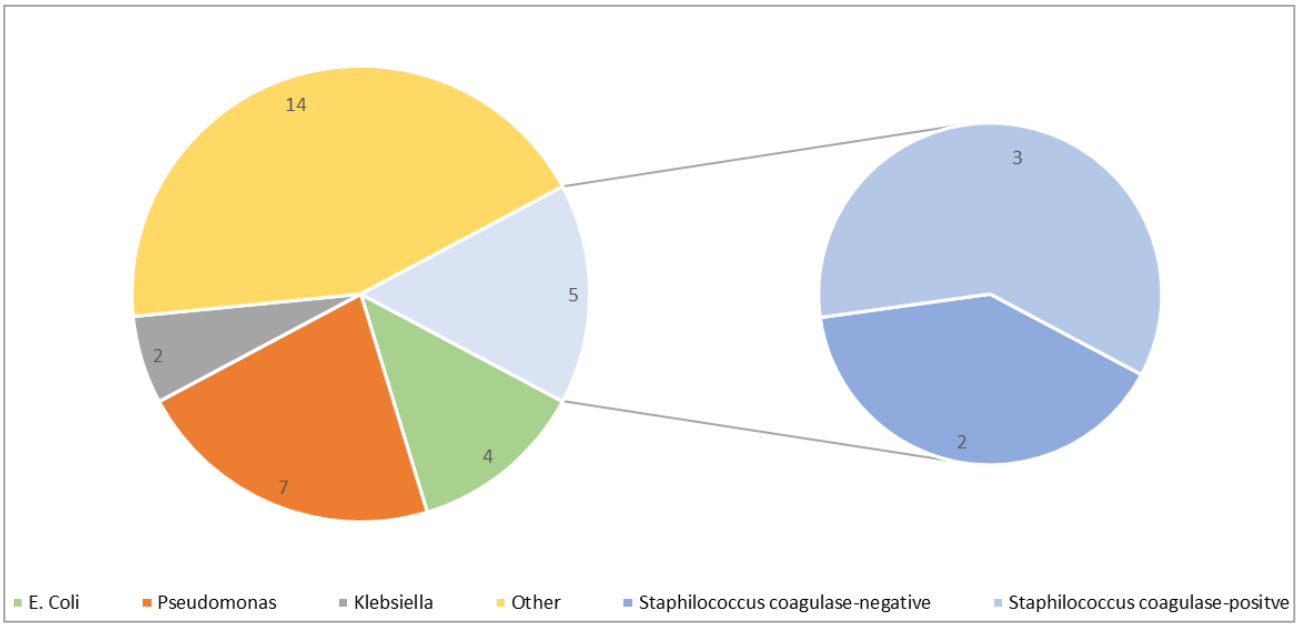

Figure 4. The spectrum of bacteria responsible for catheter removal

Discussion

Infectious risk is high in PD patients, and it is the principal cause of PD failure. The most common infective complication is bacterial peritonitis. This fearsome complication is due to the translocation of bacteria in a favorable milieu. Dialysate is a warmed fluid containing a high concentration of sugar. The entry of bacteria intraabdominal may occur at different levels: during peritoneal exchanges for catheter or bag contamination, hematogenous dissemination[84] and transmigration from intraabdominal organs or the exit-site at the catheter-skin interface[32]. The results of our epidemiological analysis showed that peritonitis was a common complication, occurring in 39.4% of the patients cared for in our center. Nevertheless, the overall peritonitis rates for 2000-2019 accounted for 0.23 episodes/patient/year, lower than the threshold limit of 0.5 episodes per year at risk recommended by the ISPD. More importantly, we noted a decrease in the peritonitis rate during the second decade of the study period (2000-2019). During these years, the resulting incidence rate was 0.17 episodes/patient/year, a finding comparable with the data from patient registries of the most outstanding centers with extensive experience in PD.

Based on our data, we noted that the reduction in the incidence of peritonitis episodes occurred principally by the significant decrease of Gram + infections. The reason behind decreasing the rate of this infection reflected the incomparable progress done in PD (i.e., the introduction of twin-bag connection systems, connectors with a low risk of touch contamination) and the new insight in terms of infection prevention (i.e., mupirocin prophylaxis for *S. aureus* carriers, care of the exit-site, masking and hand washing). This trend, although is not generalizable around the globe, has been reported by different countries[37,42,43,85] and appears a promising step toward reduction of the overall rate of peritonitis.

These results raise the question about which preventive measures in PD have effectively contributed to the decrease of the peritoneal rate over the years. Firstly, we underline that there is a consensus that no single factor can account for the different peritonitis rates between the dialysis units[86]. Based on our data we cannot identify factors affecting this favorable epidemiological trend. Different studies have documented that the use of topical and/or oral antibiotics may reduce exit-site infections and the incidence of peritonitis episodes[86,87]. However, some reports showed that there was no correlation between peritonitis rates and the size of the PD unit[88,89]. Similar results have been reported when has evaluated the number of nurses, time spent by nursing staff retraining and reinforcing

training after an episode of peritonitis[89].

In our study, we found a higher rate of peritonitis in those patients treated by CAPD compared to APD. This result is in line with a randomized control study conducted in the Netherlands showing that PD peritonitis occurred significantly less often in APD versus CAPD patients[90]. However, these data need to be interpreted with caution because recent evidence underlines that the peritonitis risk is not influenced by PD modalities[91,92].

In our center, the spectrum of bacteria that caused peritonitis was similar to previously published series[86,89] that have identified Gram + bacteria (coagulases positive S., *S. aureus* and *Streptococcus* species) as the most prevalent bacteria in PD. About microbial culturing, the average incidence of culture-negative peritonitis was 19.4%, slightly higher than the recommended threshold of 15%[32]. It is worth noting that the studies reporting data about culture-negative peritonitis are of low quality, often limited to small single-center experiences, showing different techniques for sampling and culturing the infected dialysate. A large retrospective series of culture-negative PD-associated peritonitis (435 episodes) conducted in Australia, highlighted that the rate of this event is variable (0%-50%) between dialysis units in the same countries. The interpretation of these data is complex since multiple uncontrolled factors can influence the development of culture-negative peritonitis. In our opinion, the culture-negative peritonitis rate (19.4%) may be considered somewhat in line with the cutoff declared by the international guidelines, given the complexity to obtain standardized microbial culture and the long-time span (20 years) considered in this study.

Our study confirmed that peritonitis is a cause of PD failure. Besides the structural and functional alterations of the peritoneal membrane[93], peritonitis caused catheter removal in 8.5% of our patients. *Pseudomonas* species was the most common cause of catheter removal in our population. *Pseudomonas* species infection is a serious complication of PD and is often associated with a poor response to antibiotics as well as a high rate of catheter removal[94]. The 2016 update of the ISPD guidelines recommend the use of dual antibiotic therapy for 3 weeks[32], rather than a typical course of treatment based on a single antibiotic for two weeks. Catheter removal is generally necessary if concomitant catheter-related peritonitis is suspected because the response to other salvage antibiotics is unsuccessful. *Pseudomonas* species peritonitis is challenging to treat because infectious course frequently evolves in relapsing or refractory peritonitis. Virtually *Pseudomonas*

aeruginosa peritonitis is characterized by biofilm formation that causes biofilm-mediated infections, a form of resistance for bacteria living within biofilms that may survive despite antibiotic treatment. Catheter removal is, therefore, the only therapeutical option to eradicate the source of infection[95–97].

Furthermore, after the diagnosis of peritonitis, one-third of all patients were hospitalized and caused the death of four patients for the development of sepsis shock. Although the mortality rate due to this infective episode appeared quite low (2.1%), frequent episodes of peritonitis can irreparably damage the peritoneum with fearsome consequences such as the early failure of PD as well as the temporary suspension from the waiting list for kidney transplantation.

The main drawback relies principally on the limited amount of information collected. However, we highlight that size of the population and the number of peritonitis events are substantial if we consider the single-center design. According to the aim of the study, we furnish an overview of the epidemiology, microbiology and outcome of peritonitis in one of the largest cohorts of PD patients examined in the literature.

In conclusion, peritonitis is a frequent and challenging event associated with morbidity and, to a lesser extent, mortality. Peritonitis led to a high hospitalization rate and catheter removal with a subsequent switch from PD to HD. Based on our data, the trend of peritonitis events has decreased in the last decade, probably due to the reduction of Gram+ infections. In line with the literature, *Pseudomonas* species is one of the most fearsome bacteria characterized by poor response to antibiotic therapy and the most common species associated with catheter removal.

Acknowledgments

I sincerely acknowledge all the health care workers of the University Hospital of Modena that have contributed to the delivery of care to the patients on peritoneal dialysis from 1995 to today. A special thank is due to Gianni Cappelli, past-Director of Nephrology and Dialysis Unit, and Giuseppe Medici, past-Director of Peritoneal Dialysis Unit at the University Hospital of Modena.

Part 2. Lock therapy with ethylenediaminetetraacetic acid (EDTA) and taurolidine on an in vitro model of Pseudomonas aeruginosa biofilm-related peritoneal catheter infection

Aims

Pseudomonas aeruginosa peritonitis is a challenging infection in clinical practice because frequently causes catheter removal and switch from PD to HD treatment. *Pseudomonas aeruginosa* infection arises from bacterial adhesion to biomaterial surfaces with subsequent formation of antibiotic-refractory biofilms that are challenging to eradicate with intraperitoneal antibiotics. This unmet clinical demand spurred us to investigate the effects of lock therapy on in vitro-model of catheter-related infection caused by *Pseudomonas aeruginosa*. This experimental study aimed to investigate the effect of the lock solutions (EDTA and taurolidine) on the viability of the *Pseudomonas aeruginosa* as well as the secretory profile of this pathogen under the pressure of these two antimicrobial substances.

Part of this study has been previously published in the paper "*EDTA and Taurolidine Affect Pseudomonas aeruginosa Virulence In Vitro—Impairment of Secretory Profile and Biofilm Production onto Peritoneal Dialysis Catheters*" (authors: Bruna Colombari, Gaetano Alfano, Christian Gamberini, Gianni Cappelli, Elisabetta Blasi)[82]

Materials and methods

An in-vitro study was conducted to evaluate the effects of EDTA and taurolidine, two solutions with antimicrobial effects, on a model of peritoneal catheter-associated infection due to *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa

The bioluminescent *Pseudomonas aeruginosa* strain P1242 (BLI-*Pseudomonas*) was used to conduct the current experiment. BLI-Pseudomonas is a bioengineered bacterium expressing the luciferase gene and luciferase substrate under the control of a constitutive P1 integron promoter[98]. The leading characteristic of viable cells of BLI-*Pseudomonas* is the emission of measurable bioluminescence. The bioluminescent signal was measured at certain time points by Fluoroskan Reader (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Relative Luminescence Units (RLU) was a direct measure of the number of viable cells.

Catheter-lock solutions

Sodium calcium edetate (SALF, Pharmacological Laboratory, Bergamo, Italy) is commercialized at the original concentration of 1 gr /10 ml, (EDTA 10% solution). In the current study, it was used at the working concentrations of 2.5%, 0.75% or 0.25%. For the sake of simplicity, sodium calcium edetate is termed EDTA.

Taurosept (Geistlich PharmaTM, Germany) (taurolidine), a catheter-lock solution containing 2% of taurolidine as the antimicrobial component, was used at the working solutions of 0.5 %, 0.25 % and 0.125% in saline buffer (SB). The working dilutions were prepared the day before each experiment.

EDTA and Taurolidine were tested alone or in combination against BLI-*Pseudomonas*

Pseudomonas aeruginosa culture and growth conditions

Bacteria from − 80 °C glycerol stocks were initially seeded onto Tryptic Soy Agar (TSA) plates and incubated overnight at 37 °C; then, a fresh single colony was collected, inoculated into 10 ml of Tryptic Soy Broth plus 2% sucrose (TSB) and cultured overnight at 37 °C. The culture was then washed (centrifuged twice) and inoculated into a fresh medium (TSB). In order to evaluate the bacterial concentration, 100 μl of the microbial suspension was seeded in a 96 well-plate and the optical density was measured at 595 nm (OD_{595}) using the spectrophotometer Tecan

Sunrise™. Through a reference curve the OD values were converted as CFU/mL. For all the experiments, the starting bacterial suspension was adjusted at 10^5 CFU/ml in TSB.

Peritoneal dialysis catheter (PDC) preparation

Twenty-four hours before each experiment, a sterile peritoneal dialysis catheter (PDC) (Argyle™ COVIDIEN™ ManSBield, USA) was cut under sterile conditions, in pieces of 0.5 cm length, as previously described by Kucharikova S. et al.[99], with minor modifications[100]. A maximum of 6 PDC pieces were placed into 1.5 ml microcentrifuge tubes, covered with foetal bovine serum (FBS) and incubated at 37 °C overnight in static conditions, before being used incubated with *Pseudomonas aeruginosa*.

Taurolidine Minimal Inhibitory Concentration (MIC) assay

The MIC assay was performed by the microbroth dilution method according to the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standard (CLSI/NCCLS M7-A6)[101]. According to the experimental protocol, taurolidine was tested at the final dilutions ranging from 0.5% to 0.0075%. In parallel, gentamicin (2 mg/mL) was included as a positive control. A bacterial cell suspension (5 x 10^5 cells/mL in TSB plus 2% sucrose, obtained from overnight cultures, was seeded (100 µL/well) in a 96 U-bottom microtiter-plate and was exposed to saline buffer or treated with scalar doses of taurolidine solution (100 µL/well), afterward. Lastly, bacteria were incubated at 37°C for 24 h with these solutions. The MIC was defined as the lowest concentration that inhibited visible *Pseudomonas* growth.

Protocols for assessing EDTA and taurolidine effects on BLI-*Pseudomonas* growth, adhesion and biofilm formation onto PDC pieces

Different protocols were used to assess EDTA and/or taurolidine effects on several viable BLI-*Pseudomonas*, at various time points, as detailed below.

Protocol for assessing planktonic BLI-*Pseudomonas* cells

BLI-*Pseudomonas* (10^5/ml, 100 µl/well) was seeded in 96 well-plates in SB (control) or in presence of EDTA and/or taurolidine, at concentrations indicated elsewhere. The plate was incubated at 37°C for 1 hour, 6 hours or 24 hours and the bioluminescent signal was measured at each time by Fluoroskan Reader (Thermo Fischer Scientific, Waltham, Massachusetts, USA). RLU values represented the amounts of living cells in treated and control groups.

Protocol for measuring microbial adhesion, growth and biofilm formation

Hereafter, the effects of EDTA and/or taurolidine were investigated on *Pseudomonas aeruginosa* growth in the presence of PDC and on its ability to produce biofilm onto such abiotic surfaces. The biofilm-harbouring PDC pieces were treated once or twice with EDTA and/or taurolidine and kinetic analyses were performed, as summarized in the flow-chart of the study (Fig. 1)

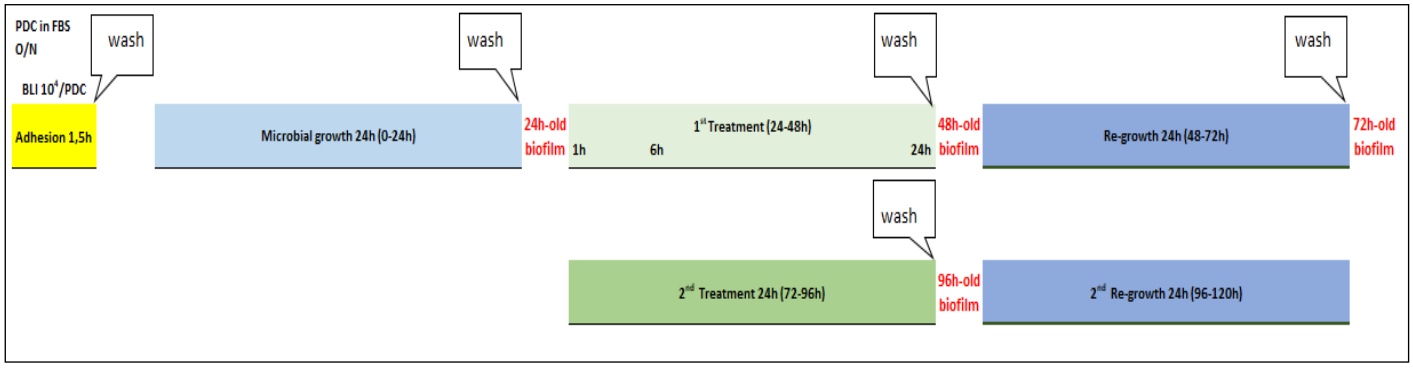

Figure 1. Flow chart of the protocol for assessing taurolidine and/or EDTA effects on PDC-associated biofilm. PDC pieces (0.5 cm; pretreated overnight with FBS) were exposed to BLI-Pseudomonas (105/ml) for 1.5 hours, washed and incubated in fresh medium (0 to 24 hours) to allow biofilm formation. After PDC washing, the 1st treatment with the lock solutions was performed (24 to 48 hours). During that time, microbial growth was kinetically measured, and biofilm was assessed after PDC washing (48 hours). The PDC pieces were further incubated in fresh medium to allow microbial regrowth (48 to 72 hours). Then, the 2nd treatment with the lock solutions was performed (72 to 96 hours). During that time, microbial growth was kinetically measured, and biofilm was assessed after PDC washing (96 hours). Finally, the PDC pieces were further incubated in fresh medium to allow microbial regrowth (96 to 120 hours).

Microbial adhesion (1.5 hours), growth and biofilm formation (24 hours) onto PDC pieces *BLI-Pseudomonas* (10^5/ml in TSB, 180 µl/well) was seeded in 96 well-plates, containing 1 PDC piece/well. The plates were incubated at 37 °C for 90 min (adhesion time). Then, the PDC pieces were washed once with SB at room temperature (RT), transferred into new wells and the RLU was measured to establish the adhesion. Afterward, the PDC pieces (immersed in TSB, 180 µl/well) were incubated at 37°C and the BLI signal was detected to establish microbial growth, hourly up to 24 hours. Later, the PDC pieces were washed with SB at RT, transferred in new wells (containing 80 µl of TSB) and the BLI signal was measured to quantify the 24 hours-old biofilm.

Exposure of the 24-hours-old biofilm to EDTA and/or taurolidine (24-48 hours)

The PDC pieces, harboring a 24-hours-old biofilm, were treated with 100 µl of SB, EDTA and/or taurolidine (at different concentrations) and incubated at 37 ° C for an additional 1hour, 6 hour or 24 hours. Each hour, the RLU was measured and, at the end of the 24 hours treatment, the PDC pieces were washed, transferred in new wells and the BLI signal was assessed again, as a measure of the 48-hours-old biofilm.

Re-growth (48-72 hours) of EDTA and/or Taurolidine-treated biofilm

The PDC pieces, harbouring a 48-hours-old treated and untreated biofilm, were immersed in fresh medium (180 µl/well), incubated for additional 24 hours at 37°C and the BLI signal was again measured at every hour. Lastly, the PDC pieces were washed, transferred to a new well and the bioluminescence signal was measured to evaluate the amounts of viable cells on 72 hours-old biofilm.

Exposure of the 72-hours-old biofilm to a 2^{nd} treatment with EDTA and/or Taurolidine

The PDC pieces, harbouring a 72-hours-old (treated and untreated) biofilm, were exposed to a re-treatment (2^{nd} treatment) with SB, EDTA and/or taurolidine further 24 hours at 37°C. During that time, the microbial growth was kinetically measured up to 96 hours, by BLI assay. Then, the PDC pieces were washed, transferred to new wells and the biofilm (96-hours-old) was measured again. Subsequently, fresh medium (180 µl/well) was added and PDC pieces were incubated for 24 hours at 37°C to kinetically evaluate the microbial re-growth up to 120 hours.

High-Performance Liquid Chromatography-Mass (HPLC) Spectrometric Analysis

High-Performance Liquid Chromatography-Mass (HPLC) Spectrometric Analysis was conducted at the Centro Interdipartimentale Grandi Strumenti (CIGS) of the University of Modena, as previously detailed[102]. HPLC analysis was performed on selected supernatants from samples where *Pseudomonas*-harbouring PDC treated or not with EDTA and taurolidine.

AIs (3-oxo-C_{12}-HSL, C_4-HSL, PQS and IQS), phenazines (1-hydroxidyphenazine, phenazine 1-carboxamide, phenazine-1-carboxylic acid and pyocyanin) pyoverdine (Succ-p-Ser-Y, Succa-P-Ser-Y, PyE and PyD) were assessed in culture supernatants of *Pseudomonas aeruginosa* after the 1st and 2nd lock solution treatment. Before being spectrometrically tested, all supernatants for the analysis were filtered on Amicon Ultra-0.5 10 K centrifugal filter devices and 1:5 diluted with 5% methanol - 0.2% formic acid in MilliQ water. The high-performance liquid chromatography-mass spectrometric (HPLC-MS) instrument used was an UltiMate 3,000 system, consisting of an online degasser, a Binary Pump HPG 3400RS, a Well Plate Autosampler WPS 3000RS, and a Thermostatted Column Compartment TCC 3000RS coupled to a Q-Exactive hybrid quadrupole – orbitrap mass analyzer *via* a HESI-II heated electrospray ion source (Thermo Scientific). Chromatographic separation of a 5 µl sample injection was performed on a Poroshell 120 SB-C18 100 × 2.1 mm ID, 2.7 µm ps column (Agilent) at 30°C and a 0.4 ml/min flow rate. A linear gradient elution scheme was used with mobile phase components being 0.1% formic acid in water (A) and methanol (B). The gradient started at 2% B which was maintained for 0.5 min, then raised up to 30% B in 30 min, and up again to 98% B in 24.5 min. The column was then kept at 98% B for 17.9 min, then starting conditions were restored in 0.1 min and maintained for 19 min pending a successive injection. Electrospray ionization was operated in positive ion mode, using nitrogen as sheath gas (50 arbitrary units), auxiliary gas (290°C, 40 arbitrary units), and sweep gas (3 arbitrary units). The sprayer voltage was kept at 3.8 kV and the tranSBer capillary temperature was set at 320°C. The Q-Exactive was operated in Full MS/dd-MS2 mode. The Full MS scan range was set from *m*/*z* 170 to 1,000 at 70,000 FWHM resolution (*m*/*z* 200). The automatic gain control (AGC) target was set at 1.0×10^6 with a maximum injection time (IT) of 200 ms. Data-dependent MS2 (dd-MS2) acquisitions at 17,500 FWHM resolution (*m*/*z* 200) were triggered for the Top 3 precursor ions following each Full MS scan. The intensity threshold for precursor ion selection was set to 1.0×10^5, then dynamic exclusion was active for 20.0 s. AGC target and maximum IT for the MS2 experiments were set to 2.0 ×

10^5 and 50 ms. Each precursor ion was fragmented using stepped normalized collision energy (NCE) values at 28, 50, and 75 ms.

Compound elution provided peaks appearing in their specific chromatographic traces, showing the abundance of their respective charged ions over the chromatographic run (mass range chromatograms). Using the Thermo Fisher FreeStyle program, the peak areas (adimensional values provided by the software) were used for semiquantitative evaluation, as detailed elsewhere[102].

Statistical analysis

Quantitative variables were tested for normal distribution. Two-way analysis of variance (ANOVA) was used to evaluate the differences between 3 pharmacological treatments (control, EDTA and taurolidine) in 3 different time intervals (1-6-24 hours). Post-hoc Tukey's test was used to test the differences in RLU level of *Pseudomonas aeruginosa* after each treatment compared to control.

One-way ANOVA followed by post hoc Tukey's test was used to compare the mean differences of RLU levels of Pseudomonas aeruginosa on pieces of PCD harboring mature biofilm, after each experiment with EDTA and/taurolidine.

The values in Fig. 1 to 4 and Table 1 are the mean ± standard error of the mean (SEM) of 6 to 8 replicate values obtained from two independent experiments. The data shown in Tables 2,3 and 4 are from a representative experiment, where a pool of triplicates for each condition was processed and analyzed using HPLC-MS.

Funding

This study was not funded by pharmaceutical companies

Results

The antimicrobial activity of taurolidine against BLI-*Pseudomonas* planktonic cells was established according to the standardized CLSI method[103]. As a result, the antimicrobial activity of taurolidine was assessed on 10 different dilutions using the microdilution method. As assessed by visual observation after 24 hours of incubation at 37°C, the MIC was established at 0.0312%.

Subsequently, we employed a previously established BLI-based assay to assess in real-time the effects of the two lock solutions on *Pseudomonas aeruginosa* planktonic cells; serial drug dilutions were prepared to obtain the final concentrations of 2.5%, 0.75%, and 0.25% for EDTA and 0.5%, 0.25%, and 0.125% for taurolidine.

Two-way ANOVA analysis was performed to test the effects of treatments (EDTA and taurolidine) and time of treatment (1,6,24 hours) on the load of planktonic forms of BLI-*Pseudomonas aeruginosa*. Statistical analysis found that the treatments ($p=<0.001$) and time of treatment had a statistically significant effect on the mean level of RLU of *Pseudomonas aeruginosa.* The combined interaction between the type of treatment and time of exposition showed a statistically significant effect on the microbial load of *Pseudomonas* aeruginosa ($p=<0.001$). As detailed in Table 1, after 1 hour of treatment, a non-significant decrease of the RLU levels was observed in treated samples. After 6 hours and 24 hours of treatment (Table 1), the RLU drastically decreased at all conditions tested. Post-hoc Tukey's analysis showed that after 6 and 24 hours of treatment there was a statistically significant reduction of the microbial load of *Pseudomonas aeruginosa* with all tested solutions.

Treatment	RLU at:[b]					
	1 h	*p* value	6 h	*p* value	24 h	*p* value
SB	0.0024 ± 0.0003		0.9310 ± 0.0133		18.8325 ± 0.3815	
EDTA 2.5%	0.0017 ± 0.0001	> 0.99	0.0034 ± 0.0006***	<0.001	0.0001 ± 0.0001***	<0.001
Tauro 0.5%	0.0008 ± 0.0002	> 0.99	0.0004 ± 0.0001***	<0.001	0.0001 ± 0.0002***	<0.001
EDTA 2.5% + Tauro 0.5%	0.0005 ± 0.0001	> 0.99	0.0006 ± 0.00003***	<0.001	0.0007 ± 0.0005***	<0.001
EDTA 0.75%	0.0023 ± 0.0002	> 0.99	0.0070 ± 0.0005***	<0.001	0.0867 ± 0.0805***	<0.001
Tauro 0.25%	0.0005 ± 0.0001	> 0.99	0.0007 ± 0.0001***	<0.001	0.0029 ± 0.0007***	<0.001
EDTA 0.75% + Tauro 0.25%	0.0004 ± 0.0001	> 0.99	0.0013 ± 0.0003***	<0.001	0.0197 ± 0.0018***	<0.001
EDTA 0.25%	0.0022 ± 0.0001	> 0.99	0.1296 ± 0.0079***	<0.001	5.6897 ± 0.3240***	<0.001
Tauro 0.125%	0.0002 ± 0.0001	> 0.99	0.0027 ± 0.0002***	<0.001	0.0357 ± 0.0039***	<0.001
EDTA 0.25% + Tauro 0.125%	0.0005 ± 0.0001	> 0.99	0.0065 ± 0.0003***	<0.001	0.0598 ± 0.0051***	<0.001

Table 1. EDTA and/or taurolidine effects on planktonic BLI-Pseudomonas cells

[a]BLI-Pseudomonas (105/ml; 100 µl/well) was exposed to the indicated doses of EDTA and/or taurolidine or SB (untreated control); after 1 h, 6 h, and 24 h, the BLI signal was measured. The values were expressed as the mean ± SEM of the RLU from six replicates obtained in two independent experiments.

Statistical analysis was performed according to two-way ANOVA followed by Tukey's post hoc analysis.

**** $p < 0.001$*

h, hour (s)

EDTA and taurolidine effects on a 24-h-old biofilm

According to a previously established protocol[100] we initially assessed the ability of *Pseudomonas aeruginosa* to adhere to and produce biofilm on silicone PDC. Briefly, 0.5-cm catheter pieces were contaminated (104 cells/PDC) and incubated at 37°C; then, microbial adhesion (90 minutes), microbial growth (0 to 24 hours), and biofilm formation (24 hours) were measured. As established by RLU determination, BLI-*Pseudomonas* efficiently adhered to the catheter pieces; also, microbial growth and PDC-associated biofilm production significantly occurred, reaching a level of RLU of 2.14 ± 0.01/PDC at time 24 hours, corresponding to 9.8×10^8 CFU/ml, as derived from the reference curve (data not shown). On these bases, the 24-hours-old/PDC-associated biofilm was exposed to EDTA and/or taurolidine and kinetically checked for microbial growth for an additional 24 h. As shown in Fig. 1A, EDTA inhibited microbial growth only after 24 hours and at the highest dose (2.5%); under such conditions, an approximately 1-log decrease in RLU was observed with respect to the untreated control. In contrast, taurolidine allowed a rapid (2 hours) reduction in RLU levels, which persisted throughout the 24-hours observation period. In particular, compared to the control, the BLI signal dropped by about 3 log in taurolidine samples at 0.5% and slightly less (2.5 log) at the other taurolidine concentrations. Similar results were observed using taurolidine alone or in combination with EDTA. When those same samples were tested for the presence of biofilm at time 48 hours, some differences among the groups were observed. As detailed in Fig. 1B, EDTA limited biofilm levels in a dose-dependent manner (2.5% and 0.75% were able to reduce biofilm by 68% and 52%, respectively; only the former produced a significant difference). Instead, taurolidine, either alone or with EDTA, massively reduced biofilm down to barely detectable levels at all the tested concentrations.

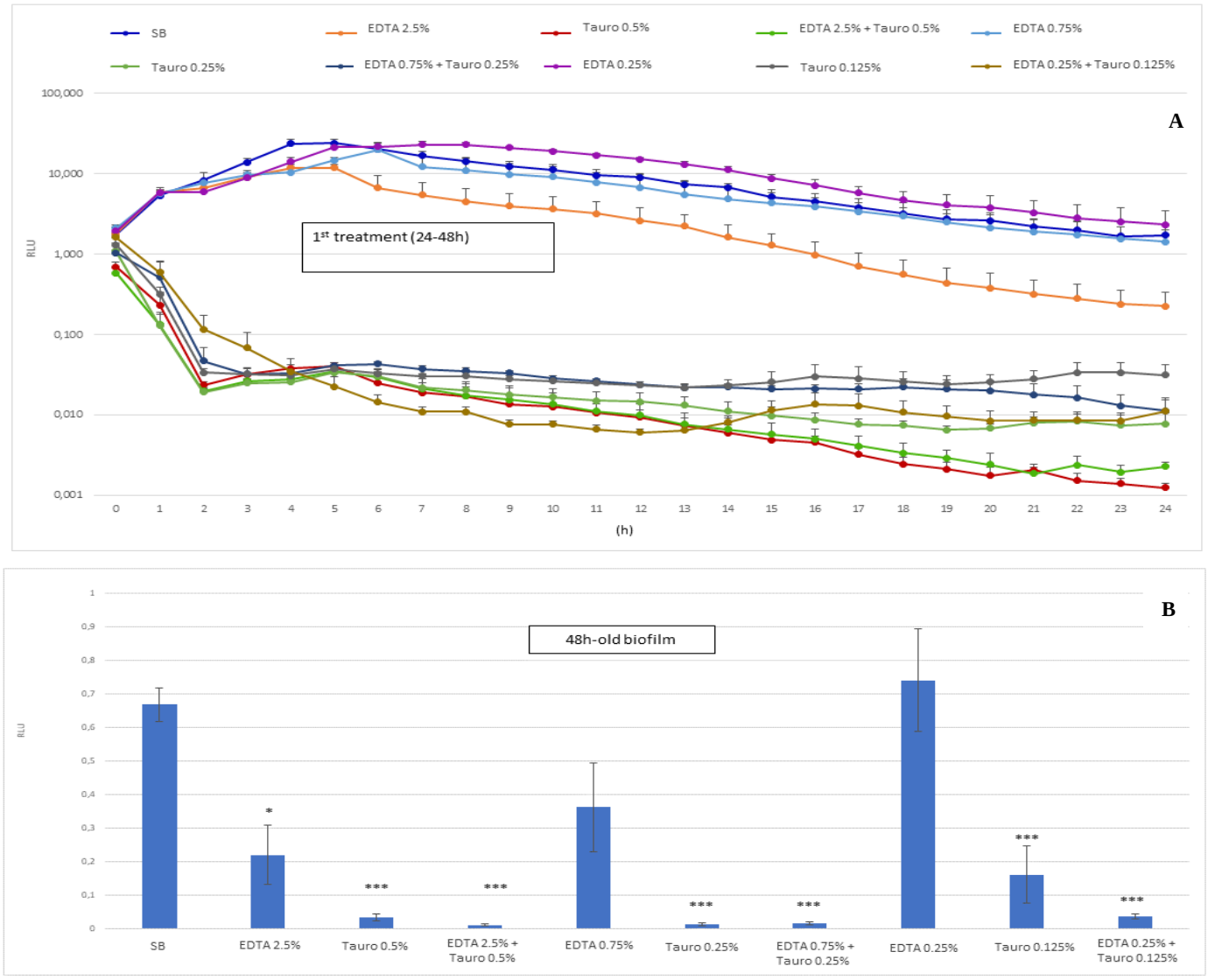

Figure 1. EDTA and/or taurolidine effects on a PDC-associated biofilm: microbial growth during treatment and biofilm production at time 48 hours. PDC pieces (0.5 cm) were contaminated with BLI-Pseudomonas (105/ml) for 24 hours. Then, the PDC-associated biofilm (24 hours-old) was exposed to EDTA and/or taurolidine at the indicated doses from 24 to 48 hours, at 37°C. During such incubation time, the BLI signal was recorded (A) and, at the end of the treatment, the PDC pieces were washed again and the persistent 48-hours-old biofilm was assessed (B). The values were expressed as the mean ± SEM of the RLU of 8 replicates obtained in two independent experiments. Statistical analysis was performed according to one-way ANOVA (p=<0.001) followed by a post hoc Tukey's test.

**, p< 0.05; **, p < 0.005; ***, p < 0.001.*

Effects of EDTA and taurolidine on regrowth (48 to 72 hours) and persistence (72 hours) of PDC-associated biofilm.

Next, we evaluated whether the EDTA and/or taurolidine inhibitory effects on *Pseudomonas aeruginosa* growth and biofilm persisted over time, focusing on the 48- to 72-hours time frame. Thus, at 48 hours, the lock solutions were removed, fresh medium was added to each well containing the PDC pieces and the plate was further incubated at 37°C; microbial growth was kinetically checked (48 to 72 hours) by measuring the RLU. As shown in Fig. 2A, all the groups rapidly regrew consistently, reaching RLU plateau levels comparable to each other in a few hours (in about 6 hours of incubation). Furthermore, at the end of that reading time, each PDS piece was washed and measured for BLI to quantify the residual biofilm (72-hours-old biofilm). As shown in Fig. 2B, we observed non-significant inhibitory effects at the highest doses of either EDTA or taurolidine. At the lowest dose, the combination of EDTA and taurolidine treatment surprisingly enhanced biofilm formation.

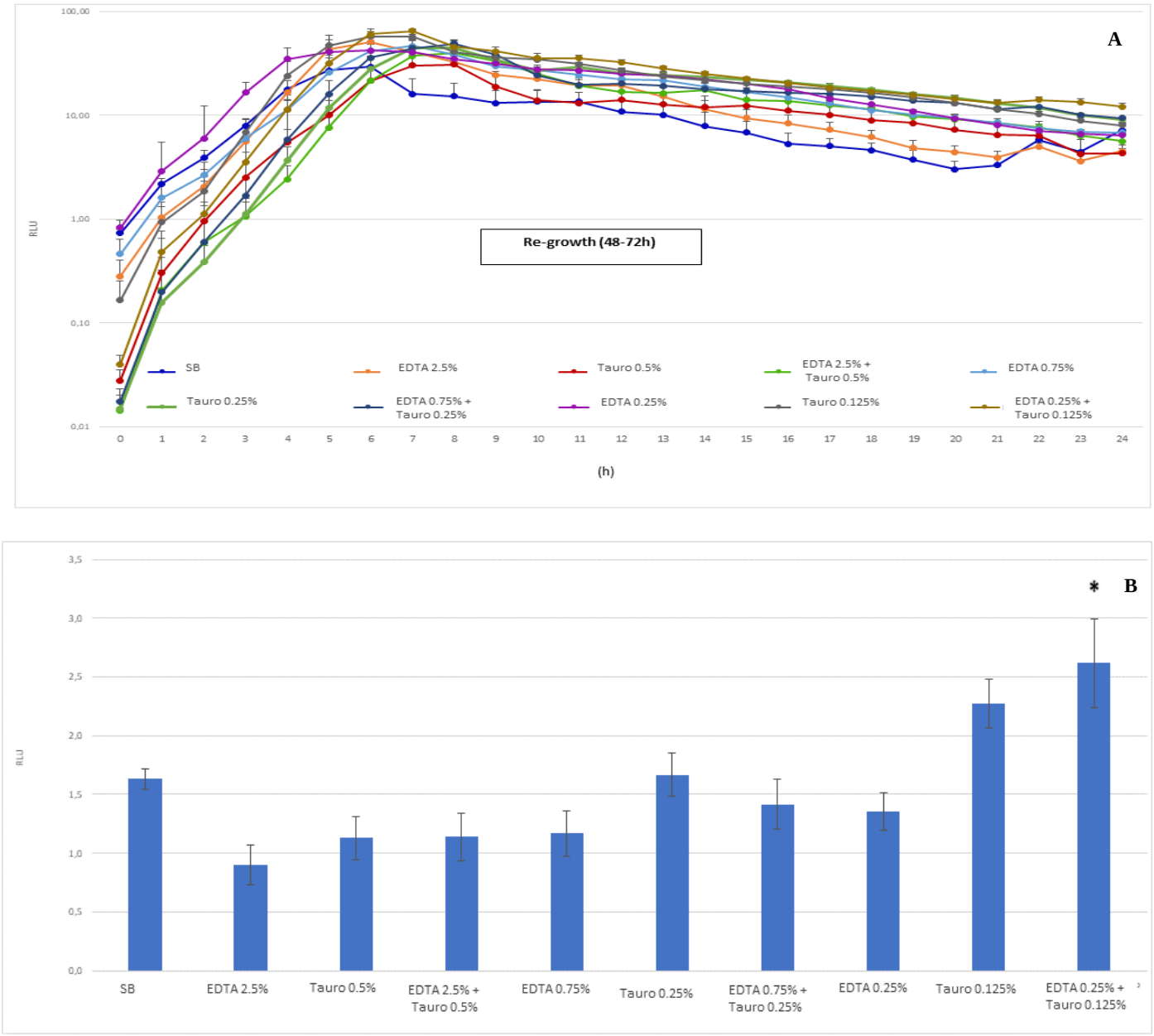

Figure 2. Microbial regrowth after the 1st treatment and biofilm persistence at 72 hours. At time 48 hours, namely, at the end of the 1st treatment, the PDC pieces were suspended in a fresh medium, incubated at 37°C from 48 hours to 72 hours and kinetically checked for microbial regrowth by RLU measurement (A). At time 72 h, the PDC pieces were washed again, and the RLU was measured to quantify the persistent 72-hours-old biofilm (B). The values were expressed as the mean ± SEM of the RLU of 8 replicates obtained in two independent experiments. Statistical analysis was performed according to one-way ANOVA (p=0.001) followed by post hoc Tukey's test.

**, p < 0.05; **, p < 0.005; ***, p<0.001*

Effects of EDTA and taurolidine retreatment (72 to 96 hours) on PDC-associated biofilm

To establish whether a 2nd treatment would strengthen the inhibitory effects of EDTA and/or taurolidine on PDC-associated biofilm, the contaminated catheters were treated twice and further assessed by RLU measurement for an additional 24 hours (operationally from time 72 to 96 hours). The results, shown in Fig. 3A, indicated that EDTA-treated samples returned RLU values similar to those of the control under all the working conditions and at all the time points tested. In contrast, taurolidine caused a drastic and rapid RLU reduction of about 2 log within about 1 h of treatment when used at 0.5% or 0.25% (either alone or with EDTA); such values tended to further decrease, achieving an approximately 3-log difference for the controls (SB), at the latest times.

At time 96 hours, each PDC piece was gently washed, immersed in fresh TSB, and assessed for residual biofilm (Fig. 3B). Also at this time point, the RLU values measured in EDTA-treated samples were similar to those of the control at all the working conditions. Once again, taurolidine treatment alone reduced the RLU in a slightly dose-dependent manner with reductions of 75%, 71%, and 47% when using 0.5%, 0.25%, and 0.125% doses, respectively; the combination of taurolidine plus EDTA showed a similar reduction. Although the treatment with taurolidine alone (especially with taurolidine 0.5% and 0.25%) and in combination with EDTA was successful to disrupt the PCD-associated biofilm of *Pseudomonas aeruginosa,* the reduction of the microbial load of *Pseudomonas aeruginosa was* not statistically significant (Fig. 3B).

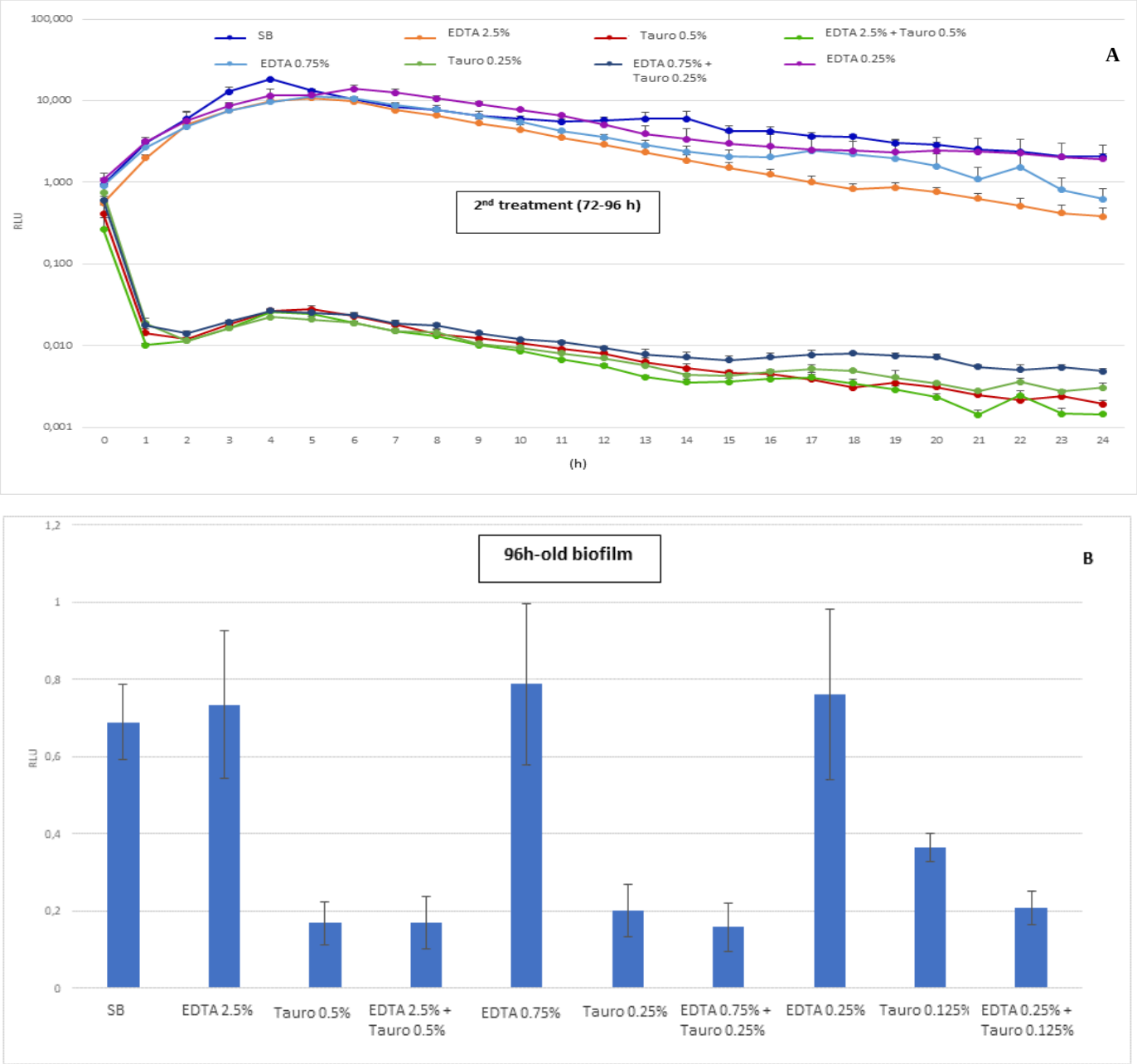

Figure 3. Effects of the 2nd treatment with EDTA and/or taurolidine: microbial growth (72 to 96 hours) in the presence of the lock solutions, biofilm production (96 hours), and regrowth after removal of EDTA and/or taurolidine (96 to 120 hours). At time 72 hours, the PDC pieces were subjected to the 2nd treatment with ETDA and/or taurolidine, at the indicated doses, at 37°C. During such incubation time (72 to 96 hours), the BLI signal was kinetically recorded (A); at time 96 hours, the PDC pieces were washed, and the 96-hours-old biofilm was assessed (B). After that, the PDC suspended in a fresh medium were incubated from 96 to 120 hours to assess the regrowth. The values were expressed as the mean ± SEM of the RLU of 8 replicates obtained in two independent experiments. Statistical analysis was performed according to one-way ANOVA (p=<0.001) followed by post hoc Tukey's test

Lastly, the PDC pieces were incubated again at 37°C and checked for further growth up to 120 hours (Fig. 4). Most of the curves were superimposable to each other, showing an initial increment of the bioluminescent signal during the first 4 hours; the only exception was 0.5% taurolidine with/without EDTA, which allowed a drop of about 2 log; then, the RLU of the two groups increased again, reaching the levels of the other samples, though with slightly different timing.

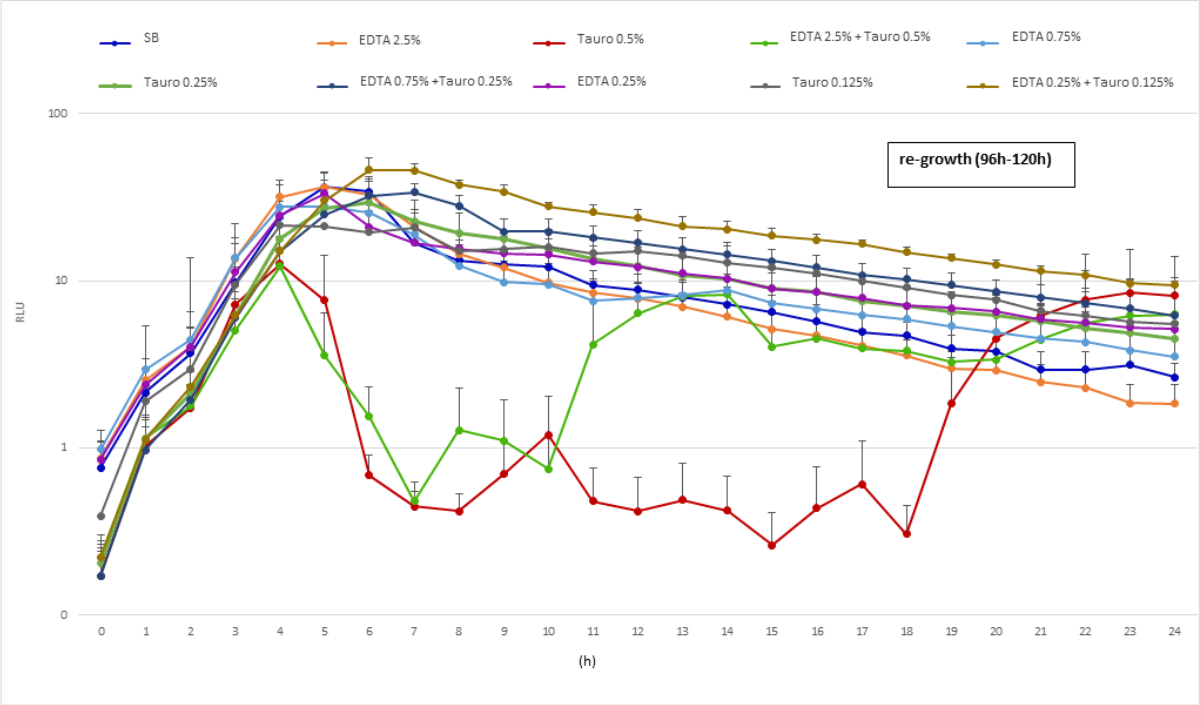

Figure 4 The 96-hours old PDC suspended in the fresh medium were incubated from 96 to 120 hours to assess the regrowth (C). After that, the PDC suspended in fresh medium were incubated from 96 to 120 h to assess the regrowth (C). The values were expressed as the mean ± SEM of the RLU of 8 replicates obtained in two independent experiments.

EDTA and/or taurolidine effects on the secretory activity of PDC-associated *Pseudomonas aeruginosa* biofilm.

Finally, we assessed the effects of EDTA and taurolidine on the ability of PDC-associated *Pseudomonas aeruginosa* biofilm to produce specific secretory products, such as several autoinducers (AIs), phenazines, and pyoverdins, known to be involved in biofilm formation/persistence and virulence[104–106]. Thus, HPLC-MS analysis was performed in cell-free supernatants from PDC-associated biofilm, treated or not with EDTA and/or taurolidine. By chromatogram analysis, the different elution peaks and their areas used for semiquantitative evaluation of the specific products were identified, as previously detailed. Table 2 shows the peak areas and the fold changes in the production of the four AIs, namely, 3-oxo-C_{12}-HSL, C_4-HSL, PQS, and IQS; the analysis was performed after the 1st treatment (upper part of the table), after the 2nd treatment (lower part), and in between (middle part of the table). A consistent presence of all such AI was observed in the supernatants of untreated biofilm (SB). After the 1st and 2nd treatments, the levels of 3-oxo-C_{12}-HSL, C_4-HSL, and PQS were affected, showing a fold change ranging from 0.77 (23% decrease) to 0.001 (99.9% decrease); moreover, the IQS levels dropped to very low or even undetectable levels.

	3-oxo-C12-HSL		C4-HSL		PQS		IQS	
Treatment	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated
24-h-old biofilm								
TSB	1.14E-06		2.73E-07		1.63E-09		7.10E-03	
1st treatment								
SB	1.04E-06	1	2.54E-07	1	1.91E-09	1	2.97E-06	1
EDTA 2.5%	8.02E-05	0.77	5.60E-06	0.22	2.40E-05	0.001	ND	
Tauro 0.5%	3.45E-05	0.33	3.20E-06	0.13	3.67E-07	0.19	ND	
EDTA 2.5% + Tauro 0.5%	ND		2.50E-06	0.10	1.54E-07	0.08	ND	
Regrowth								
SB	2.17E-06	1	2.90E-07	1	4.68E-09	1	5.08E-06	1
EDTA 2.5%	2.43E-06	1.1	2.85E-07	1.00	3.97E-09	0.85	4.11E-06	0.82
Tauro 0.5%	2.75E-06	1.3	2.55E-07	0.89	4.09E-09	0.87	3.49E-06	0.70
EDTA 2.5% + Tauro 0.5%	2.67E-06	1.2	2.34E-07	0.82	3.93E-09	0.84	3.66E-06	0.73
2nd treatment								
SB	1.32E-06	1	2.07E-07	1	8.90E-08	1	4.82E-06	1
EDTA 2.5%	1.04E-06	0.79	2.36E-07	1.1	1.04E-09	1.17	5.22E-06	0.3
Tauro 0.5%	1.27E-06	0.96	1.47E-06	0.1	ND		ND	
EDTA 2.5% + Tauro 0.5%	3.42E-05	0.26	1.25E-06	0.1	4.95E-07	0.06	ND	

Table 2. Secretory profile of BLI-Pseudomonas undergoing two treatments with EDTA/taurolidine (assessment of the four autoinducers [AIs] by HPLC-MS

Supernatants from PDC pieces, contaminated and treated or not treated with EDTA and/or taurolidine, were tested by HPLC-MS. The indicated AIs were investigated. By chromatogram analysis, the different elution peaks were identified, and their areas were used for semiquantitative evaluation of each specific product. The fold changes of the concentration of AIs were calculated with respect to the corresponding SB control. The results shown are from a pool of three replicates of a representative experiment out of two performed.

ND, not detectable

Next, the levels of four phenazines (1-hydroxidyphenazine, phenazine 1-carboxamide, phenazine-1-carboxylic acid, and pyocyanin) were assessed. Table 3 shows that such molecules, abundantly found in the supernatants of the untreated controls (SB), drastically decreased to very low or undetectable levels, whether 1 or 2 treatments had been performed (upper and lower parts of the table, respectively). Furthermore, Table 4 shows the results of pyoverdine release. Also, in this case, the peak areas and the fold change of the four most abundantly detected pyoverdines (Succ-p-Ser-Y, Succa-P-Ser-Y, PyE, and PyD) dropped to undetectable levels in most cases, whether 1 or 2 treatments had been done (upper and lower parts of the table, respectively). Furthermore, as shown in Tables 2, ,3, and ,4, all the above-mentioned secretory products were tested during the regrowth phase, between the 1st and the 2nd treatments (samples incubated in fresh medium from 48 to 72 hours). When assessing the AI and phenazine levels (Table 2 and 3), we observed partial recovery in all the samples; in contrast, the pyoverdines consistently remained at low or undetectable levels (Table 4).

Taken all together, these HPLC-MS data indicated that EDTA and taurolidine treatment greatly impaired most of the *Pseudomonas* secretory potential. Such inhibitory effects were similar (no major differences) whether performing either 1 or 2 treatments; the impairment was transient or persistent, depending upon the secretory product assessed.

Treatment	1-Hydroxidyphenazine		Phenazine 1-carboxamide		Phenazine-1-carboxylic acid		Pyocianin	
	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated
24-h-old biofilm (0–24 h)								
TSB	1.26E-06		1.00E-06		8.58E-08		1.24E-09	
1st treatment (48–72 h)								
SB	1.39E-06	1	2.49E-05	1	9.23E-08	1	1.84E-09	1
EDTA 2.5%	ND		ND		4.13E-07	0.045	8.07E-07	0.044
Tauro 0.5%	ND		ND		2.47E-07	0.027	1.10E-07	0.006
EDTA 2.5% + Tauro 0.5%	ND		ND		2.28E-07	0.025	4.67E-07	0.025
Regrowth (72–96 h)								
SB	2.36E-06	1	2.34E-06	1	8.70E-08	1	2.18E-09	1
EDTA 2.5%	2.36E-06	1	1.95E-06	0,83	9.50E-08	1.1	1.73E-09	0.79
Tauro 0.5%	2.25E-06	0.95	1.67E-06	0,71	8.32E-08	1	1,46E-09	0.67
EDTA 2.5% + Tauro 0.5%	2.28E-06	0.97	ND		9.12E-08	1	1.78E-09	0.82
2nd treatment (96–120 h)								
SB	2.57E-06	1	1.69E-06	1	1.54E-09	1	1.53E-09	1
EDTA 2.5%	1.08E- 06	0.42	1.18E-06	0,7	6.79E-08	0.44	6.57E-08	0.43
Tauro 0.5%	ND		3.22E-05	0,19	2.64E-07	0.02	ND	
EDTA 2.5% + Tauro 0.5%	ND		5.30E-05	0,31	2.85E-07	0.02	ND	

Table 3. Secretory profile of BLI-Pseudomonas following EDTA/taurolidine treatment (assessment of the four phenazines by HPLC-MS)

Supernatants from PDC pieces, contaminated and treated or not treated with EDTA and/or taurolidine as detailed above, were tested by HPLC-MS.

The indicated phenazines were investigated. Using chromatogram analysis, the different elution peaks were identified, and their areas used for semiquantitative evaluation of each specific product. The fold changes of the concentration of phenazines were calculated with respect to the corresponding SB control. The results shown are from a pool of three replicates of a representative experiment out of two performed.

ND, not detectable.

Treatment	Succ-p-Ser-Y		Succa-P-Ser-Y		PyE		PyD	
	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated	Peak area	Fold change treated vs untreated
24-h-old biofilm								
TSB	1.96E- 04		5.20E-04		1.00E-06		2.70E-05	
1st treatment								
SB	1.22E-04	1	5.90E-04	1	1.05E-06	1	5.08E-05	1
EDTA 2.5%	ND		ND		ND		7.40E-04	0.15
Tauro 0.5%	ND		ND		ND		ND	
EDTA 2.5% + Tauro 0.5%	ND		ND		ND		ND	
Regrowth								
SB	2.12E-04	1	7.77E-04	1	3.08E-06	1	1.46E-06	1
EDTA 2.5%	1.97E-04	0.9	ND		ND		7.90E-05	0.54
Tauro 0.5%	ND		ND		6.70E-05	0,22	8.14E-05	0.56
EDTA 2.5% + Tauro 0.5%	ND		ND		ND		6.60E-05	0.45
2nd treatment								
SB	9.30E-03	1	1.07E-04	1	1.61E-05	1	7.16E-05	1
EDTA 2.5%	ND		ND		ND	ND	ND	
Tauro 0.5%	ND		ND		ND	ND	ND	
EDTA 2.5% + Tauro 0.5%	ND		ND		ND	ND		

Table 4. Secretory profile of BLI-Pseudomonas following EDTA/taurolidine treatment (assessment of the four pyoverdines by HPLC-MS)

Supernatants from PDC pieces, contaminated and treated or not treated with EDTA and/or taurolidine as detailed above, were tested by HPLC-MS.

The indicated pyoverdines were investigated. Using the chromatograms, the different elution peaks were identified, and their areas were used for semiquantitative evaluation of each specific product. The fold changes of concentration of pyoverdines were calculated with respect to the corresponding SB control. The results shown are from a pool of three replicates of a representative experiment out of two performed.

ND, not detectable.

Discussion

Peritoneal catheter, as well as medical devices, may be colonized by bacteria which in turn lead to bacterial biofilm formation. This problem poses special challenges in clinical practice because biofilm-related infections are resistant to antibiotic treatment and are often associated with metastatic infections[84]. The use of lock solutions is a useful strategy for catheter-related bloodstream infections[107]. It relies on the use of a high dose of antibiotics or antimicrobial solution within the lumen of the catheter for a certain period whenever the central venous catheter (CVC) is not in use. Clinical experiences on CVC infection treatment and the collection of novel data on real-time monitoring of biofilm growing on medical devices at the University of Modena paved the way for this study aimed to evaluate the anti-*Pseudomonas* efficacy of two lock solutions, EDTA and taurolidine on PD catheters. Both solutions affected the grow of planktonic cells and reduced the microbial load of *Pseudomonas aeruginosa* harbouring mature PDC biofilm. In addition, we documented for the first time the change of the secretory profile of *Pseudomonas aeruginosa* after exposure to EDTA and taurolidine for 24 hours.

Pseudomonas aeruginosa is a common cause of serious peritonitis in patients undergoing peritoneal dialysis[108]. Challenges in treating these cases rely on the difficulty to eradicate infection. The pathogenic potential of this infectious agent is mostly mediated by its strong ability to produce biofilm as well as to release multiple secretory virulence products, in turn tightly controlled by a sophisticated QS network. By an engineered bioluminescent strain, here, we demonstrate that *Pseudomonas aeruginosa* efficiently produces biofilm onto silicone-based PD catheters in vitro; moreover, such biofilm can be affected to a different extent by exposure to two traditional lock solutions, EDTA and taurolidine (alone or in combination), as assessed by real-time monitoring of both microbial growth and biofilm production on such medical devices.

EDTA and taurolidine are two non-antibiotic solutions, already used, in clinical practice, to prevent or treat CVC-related bloodstream infections[40,41]. Percival et al. [69] documented that tetrasodium EDTA is effective in reducing planktonic microbial load and also destroys the established biofilm with the killing of embedded microorganisms. Taurolidine exerts its bactericidal activity by irreversible binding of its methylol groups to the bacterial cell wall and deeply affects microbial fimbriae and flagellae, in turn reducing bacterial adhesion onto epithelial cells and abiotic surfaces[42-45]. These anti-adhesion properties of taurolidine combined with its direct killing activity likely led to impairment of biofilm formation[46,47]. In line with literature data, we documented that taurolidine significantly reduces the viability of *Pseudomonas*

planktonic cells[48,49]. Already after 6 hours of treatment with EDTA and taurolidine, the levels of bioluminescence signal sharply drop with all tested concentrations. Interestingly, a further reduction in microbial load occurs after 24 hours of lock therapy. The combination of the two lock solutions returned data similar to those obtained using taurolidine alone, arguing against additive effects between the two products.

The two lock solutions also affect *Pseudomonas* biofilm, in vitro preformed onto PDC pieces. As kinetically assessed, taurolidine causes a rapid decrease of bacterial load, already evident within the first 2-3 hours of treatment and with all tested doses. Differently, EDTA significantly affects microbial load, but only at the highest concentration (2.5%).

As shown by experiments where the contaminated PDC pieces have been treated, washed and further incubated in a fresh medium, the inhibitory effects of EDTA and/or taurolidine on Pseudomonas were transient. An intense microbial re-growth was observed in the 48-72 hours' time-frame, independently upon the treatment performed. It should be noted that the PDC-associated biofilms initially showed different microbial loads (48 hours), because of the dissimilar antimicrobial activity exerted by the two lock solutions. Then, a similar and time-related bacterial re-growth occurred; indeed, after 5-7 hours, the RLU values reached plateau levels comparable among groups including the control. The highest concentrations of EDTA (0.75% and 2.5%) or taurolidine (0.5%) or the two together showed a trend toward the reduction of 72 hours-old biofilm biomass, without achieving statistical significance. To our opinion, this datum should be further explored because the therapeutic effects of the lock solutions may persist, at least in terms of anti-biofilm activity. To assess the effect of extensive use of lock solutions, contaminated PDC pieces have been exposed to a second treatment (72-96 hours) with EDTA/taurolidine solutions. As detailed above, the inhibitory effects showed a trend similar to the one produced by the first treatment. In particular, microbial growth was deeply affected during treatment and a time-related recovery occurs upon drugs removal; also, the impact on the 96-hours-old biofilm was more clearly evident, closely recalling the one observed after the first treatment. Taken together, these findings provided evidence that EDTA and taurolidine treatment transiently dampened microbial growth without achieving infection eradication.

Increasing data literature underlines the crucial involvement of AIs in *Pseudomona*s species virulence. They affect microbial adhesion and biofilm formation as well as the secretion of many molecules, such as pyoverdine and phenazines. All these molecules have a key role in determining in vivo the course of the *Pseudomonas* infection and the outcome of the disease[31]. To elucidate the mechanism underlying biofilm impairment, HPLC-ESI-MS analysis provided

the first evidence, to our best knowledge, that the secretory profile of PDC-associated *Pseudomonas* biofilm was drastically affected by exposure to the used lock solutions. In particular, when comparing the peak areas of treated and untreated samples, the levels of the well-known Ais (3-oxo-C_{12}-HSL, C_4-HSL, PQS and IQS) were deeply decreased when using EDTA and taurolidine, either alone or in combination. Taurolidine appeared slightly more effective than EDTA in reducing the levels of C_4-HSL and PQS. Upon drugs wash-out, the production of these AIs recovers up to 70-80% in relation to the untreated controls. Thus, in agreement with the microbial load/viability data, also phenazine and pyoverdines production was temporarily impaired by these lock solutions. In agreement with AIs fluctuation, the levels of phenazine and pyoverdin were deeply impaired after the first treatment and returned toward control levels upon drugs removal and microbial re-growth; furthermore, they drop again after the second treatment. To a similar extent, the pyoverdines production drops to undetectable levels upon the first treatment; surprisingly, unlike all the other secretory products, 3 of these pyoverdines (Succ-p-Ser-Y, Succa-P-Ser-Y and PyE) remain below the detection limit during the re-growth period, while PyD levels recover up to about 50% of the control; upon second treatment, all the tested pyoverdines consistently remain at undetectable levels.

Based on these results, we speculate that EDTA and taurolidine solutions transiently affect AI and phenazines production by *Pseudomonas* aeruginosa, while the production of pyoverdines appears irreversibly hampered. Our finding document that EDTA and taurolidine may act disturbing *Pseudomonas* secretory potential, to a wide extent, opens to future in vivo studies on the clinical relevance of these lock solutions by focusing on such novel parameters/targets.

Overall, our data provided in vitro evidence that taurolidine (at all the doses) and EDTA (at 2.5%), or a combination of the two, affected two key virulence traits of *Pseudomonas* species, namely its propensity to produce biofilm onto silicon catheters and its wide spectrum secretory repertoire. In particular, taurolidine and, to a lower extent, EDTA reduce microbial load and biofilm formation onto PDC pieces. Although not achieving eradication, these non-antibiotic solutions may induce a biofilm biomass decrease, likely through destructuration of the matrix. This in vitro notable antimicrobial effect may have a relevant in vivo counterpart; it may result in a reduced risk of PDC-related infections because it may render *Pseudomonas aeruginosa* more susceptible to the antibiotics or the host immune response. Further studies will be needed to verify if such lock solutions could be added to conventional antibiotics to increase the likelihood of clearing a peritoneal infection as well as to verify peritoneal mesothelial cells tolerance to possible contact with lock solutions.

The main limitation of this study is related to the in vitro analysis. We focused only on one type of bacteria, but our data are novel in the setting of peritoneal dialysis, increased our understanding of the efficacy of lock solutions on peritoneal catheter and the pathogenicity of biofilm-related peritoneal catheter infections. To the best of our knowledge, this is the first study that evaluated in-vitro the effects of taurolidine and EDTA on the peritoneal catheter. These findings may stimulate the use of these solutions as a preventive strategy for catheter-related infections.

Conclusion

This study provides new insights into the anti-*Pseudomonas* properties of Taurolidine and EDTA. These solutions impair planktonic cell growth and biofilm production onto peritoneal dialysis catheters. Such inhibitory effects were effective in reducing bacteria biomass and altering the secretory profile of *Pseudomonas aeruginosa*. Mass spectrometry analysis indeed revealed that, upon treatment, also the plethora of virulence factors, commonly released by this pathogen, is profoundly inhibited by taurolidine and EDTA. Further studies are required to document the efficacy of lock solutions in the prevention of catheter-related infections, as well as the potential antimicrobial effect of these substances when combined whit classical antibiotics. A similar effect was seen also on biofilm production/persistence, thus, we suggest that the lock solutions may have a long-term action as

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Part 3. In-vitro cytotoxicity assay of ethylenediaminetetraacetic acid (EDTA) and taurolidine on peripheral blood mononuclear cells (PBMCs)

Aims

In order to elucidate the safety profile of ethylenediaminetetraacetic acid (EDTA) and taurolidine on human cells, in-vitro cytotoxicity assays were conducted using peripheral blood mononuclear cells (PBMC). This research project aimed to verify the in vitro effects of EDTA and taurolidine, two solutions with broad-spectrum antimicrobial activity widely used in clinical practice.

Material and methods

Peripheral blood mononuclear cells isolation

This invitro study was conducted on human peripheral blood mononuclear cells (PBMCs). PBMCs include lymphocytes, monocytes and any other white blood cell with a round nucleus[109]. PBMCs were isolated from a venous blood sample (30ml) collected from a healthy donor into Vacutainer EDTA tubes. PBMC were isolated by Ficoll-Hypaque density gradient according to standard procedures[110]. Briefly, whole blood was diluted with balanced salt solution (PBS) to obtain a 1:2 final dilution and carefully stratified over Ficoll-Hypaque keeping separated the two phases, then it was centrifuged at 1400 rpm for 40 minutes in a swinging bucket rotor at room temperature without break.

We underline that the first part of the experiment was conducted on cryopreserved PBMCs whereas the second part was conducted on fresh PBMCs. The difference in cell preservation may underline the initial different percentage of living PBMCs between cryopreserved and fresh PMBCs.

Expansion of PBMCs

Isolated PBMC were seeded at 1 x 10^6/ml into T25 flasks in a complete cell growth medium. After 48 hours of growth, PBMCs were seeded at 1 x 10^6/ml into 12-well plates and were treated with taurolidine and/or EDTA for 24 hours. Cells cultured in the complete cell growth medium were used as a negative control.

Cytotoxicity assays

PBMCs treated with taurolidine and/or EDTA were stained with Annexin V (AnxV) AF488 conjugate (Thermofisher) in Annexin Binding Buffer for 10 minutes in the dark to detect phosphatidylserine exposure on the cell surface (early apoptotic marker) and with propidium iodide (PI) Ready Flow Reagent (Biorad) to detect late apoptotic/dead cells. PI allows the recognizing of death cells by bindings to the base of DNA when the cellular membrane, disrupted by the apoptotic process, became permeable to this substance.

Samples of PMBCs incubated with taurolidine and/or EDTA were analyzed by flow cytometry (Attune NxT, ThermoFisher): living cells were negative for both AnxV and PI staining, early apoptotic cells were positive for AnxV and negative for PI, whereas late apoptotic/necrotic cells were positive for PI, regardless of staining for AnxV. As a result, gated cells were separated into

four quadrants (Q):

Q1: early apoptotic cells (Annexin positive/PI-negative);

Q2: late apoptotic cells (Annexin positive/PI-positive);

Q3: necrotic cells (Annexin negative/PI-positive);

Q4: viable cells (Annexin negative/PI-negative).

For the sake of simplicity, late apoptotic cells/necrotic cells were termed also "necrotic" or "dead cells" in this study.

Utilized solutions

In the first part of this experiment, cytotoxic tests were conducted using EDTA, taurolidine and a combination of both. The concentration of EDTA and taurolidine used in this first part of the study reflected the concentration utilized in the previous study evaluating the in-vitro effects of these lock solutions against Pseudomonas aeruginosa biofilm-related peritoneal catheter infection.

EDTA, namely sodium calcium edetate, (SALF; Pharmacological Laboratory, Bergamo, Italy), is commercialized at the original concentration of 1 gr /10 ml, (EDTA 10% solution). After 1:4 dilution ratio with "complete cell growth medium", EDTA 2.5% solution was obtained.

Taurosept (Geistlich Pharma, Germany) (taurolidine), a catheter-lock solution containing 2% taurolidine as the antimicrobial component, was used at the working solutions of 0.5%, in "complete cell growth medium".

The original concentration of EDTA and taurolidine are commercialized to treat lead intoxication and prevent catheter-related infection, respectively. EDTA has been also used for treating biofilm-associated conditions in dentistry, medical devices, and veterinary medicine[70].

In the second part of the experiment, different concentrations of taurolidine were tested on PMBCs, in order to evaluate dose-dependent toxicity. The working solution of taurolidine were 0.125%, 0.25 % and 0.5%, in SB. To test the deleterious effect of the solvents, PMBCs were incubated with deionized water 25%, and PBS 25% mixed with a "complete cell growth medium".

As a negative control, PMBCs were incubated in "complete cell growth medium". It consisted of RPMI Medium 1640, supplemented with 10% fetal bovine serum, 1% of Penicillin-Streptomycin, 2mM of Levo Glutamine, 1M HEPES buffer.

All solutions and media were sterile, and all procedures were carried out under aseptic conditions in a sterile environment of laminar flow cabinet.

Statistical analysis

All the processes were conducted in triplicate. The viability of the PBMC was expressed in percentage.

As for the cytotoxic tests, the statistical analysis was performed as follow:

-Grouped percentages of alive, early apoptotic and late apoptotic/necrotic cells in samples incubated with complete cell grow medium, PBS 25%, deionized water 25%, EDTA 2.5%, Taurolidine 0.125%, 0.25%, 0.5% and EDTA 2.5%/taurolidine 0.5% for 24 hours.

The samples of alive, early apoptotic and death cells were compared with controls using Kruskal-Wallis non-parametric test to assess the difference in medians between groups and then with Dunn's test for multiple comparisons. A box plot was used to comparing distributions of the data. It draws amount of cells from the first quartile (lower line) to the third quartile (upper line). A horizontal line goes through the box at the median.

The non-parametric Mann-Whitney test was used to compare the viability of cells after incubation with only two solutions (Taurolidine 0.5% and EDTA 2.5%/taurolidine 0.5%).

P values of 0.05 or lower were considered significant. Statistical analysis was performed using Graph Pad Prism® and Microsoft Office® software.

Funding

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Results

First part of the experiment.

Cytotoxicity assay evaluated the viability of human PBMCs following incubation with taurolidine and/or EDTA. The experiment was conducted on PBMCs incubated with complete cell growth medium (control negative), EDTA 2.5%, taurolidine 0.5%/EDTA 2.5%/ taurolidine 0.5%. (Figure1).

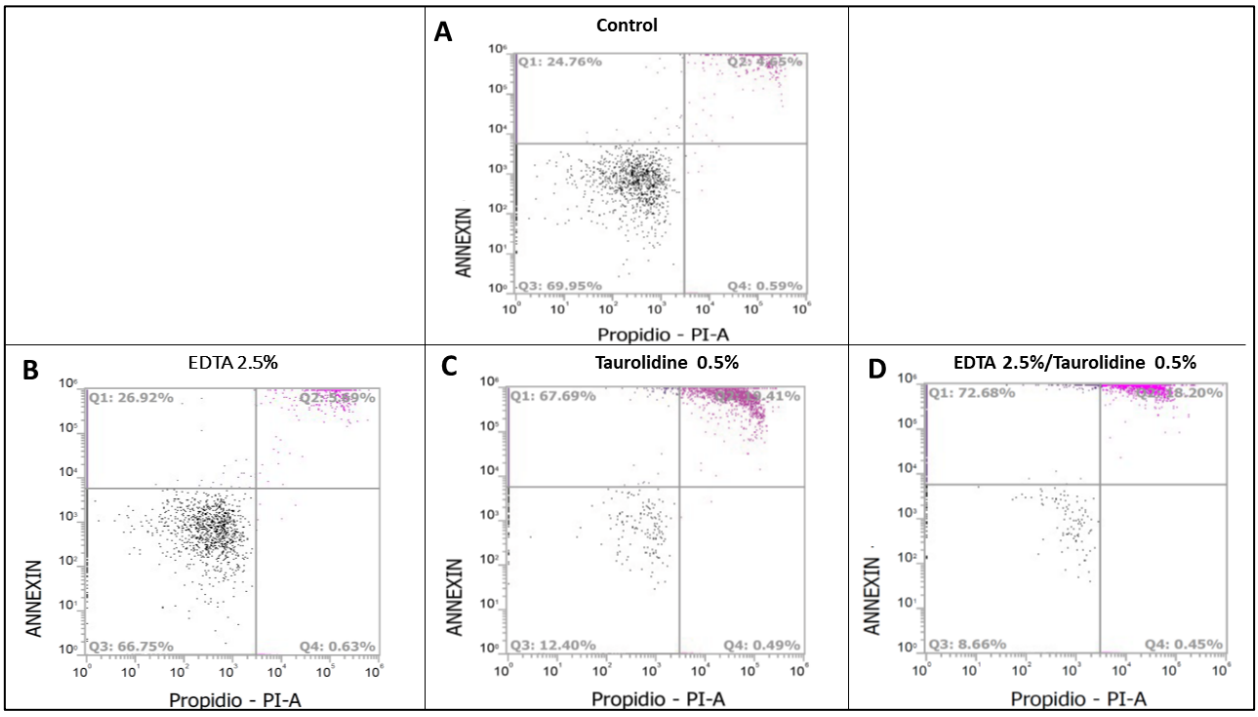

Figure 1. Evaluation of cell viability in PMBC treated with EDTA, taurolidine and EDTA/taurolidine. Cytofluorometry analysis sorted cells into 4 quadrantes: early apoptotic cells (Annexin positive/Propidio [PI] negative), late apoptotic (Annexin positive/PI positive), necrotic cells (Annexin negative/PI positive) and viable cells (Annexin negative/PI negative). A, control; B, EDTA 2.5%; C taurolidine 0.5%; EDTA 2.5%/taurolidine 0.5%. Note that on both coordinates the scale is logarithmic.

As detailed in Fig. 2, the percentage of viable, apoptotic and necrotic PMBCs varied according to the solution utilized during incubation.

Treatment with EDTA 2.5% determined an average percentage of viable, apoptotic and necrotic cells of 67.6%, 25.6% and 6.02%, respectively.

Treatment with taurolidine 0.5% determined an average percentage of living, apoptotic and necrotic cells of 14.5%, 73.3 and 11.6%, respectively.

Treatment with EDTA 2.5%/taurolidine 0.5% determined an average percentage of death, apoptotic and necrotic of 19.6%, 71.2 and 8.7%, respectively.

The negative control showed an average percentage of living, apoptotic and necrotic of 71.5%, 23.9 and 4.6%, respectively.

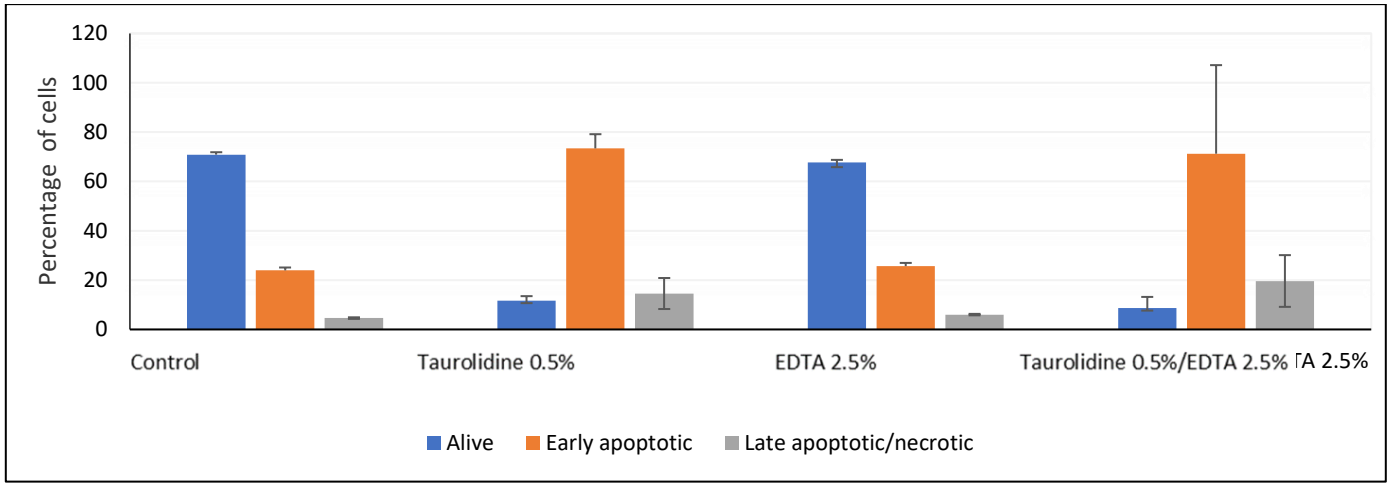

Figure 2. Graphical representation of the amount of living (blue bars), early apoptotic (orange bars) and late apoptotic/necrotic (gray bars) PMBCs incubated with different solutions

Taurolidine 0.5% strongly affected PMBCs viability at 24 hors (14.5% vs 67.6% of control). This effect was seen also when PBMCs cells were incubated with taurolidine 0.5% and EDTA 2.5% (19.6% vs 67.6% of control).

Statistical analysis was performed comparing percentages of living cells incubated with EDTA 2.5%, taurolidine 0.5%, EDTA 2.5%/taurolidine 0.5% and controls after incubation of 24 hours as described above. Kruskal-Wallis test yielded a $p<0.001$ for samples of living cells between groups; Dunn's test for multiple comparisons was statistically significant ($p=0.019$) when comparing controls with PMBCs treated with EDTA 2.5%/taurolidine 0.5%. Nevertheless, a tendency towards a lower percentage of living cells treated with taurolidine 0.5% was detected without reaching statistical significance ($p=0.18$). Results of this statistical analysis are reported in Fig. 3.

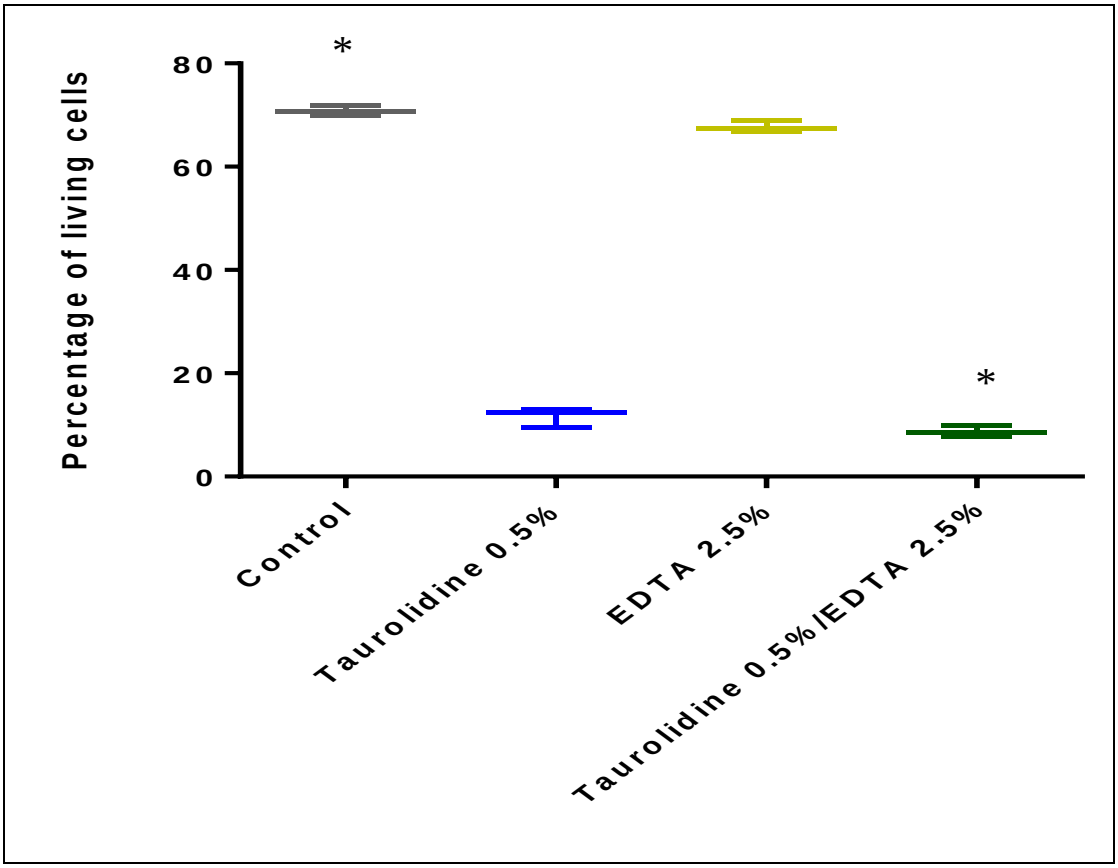

Figure 3. Comparison of percentages of living PBMC cells incubated with different solutions. Kruskal-Wallis test yielded a p<0.001 for samples; Dunn's test for multiple comparisons was significant between PMCB treated with EDTA 0.5%/taurolidine 2.5% and control.

(*) p=0.019

Kruskal-Wallis test yielded a p=0.006 for samples for early apoptotic cells between groups; Dunn's test for multiple comparisons was significant (p=0.038) when comparing controls with PMBC treated with taurolidine 0.5%. Nevertheless, a tendency towards a lower percentage of early apoptotic cells treated with taurolidine 0.5%/EDTA2.5% was detected without reaching statistical significance (p=0.09). Results of statistical analysis are reported in Fig. 4.

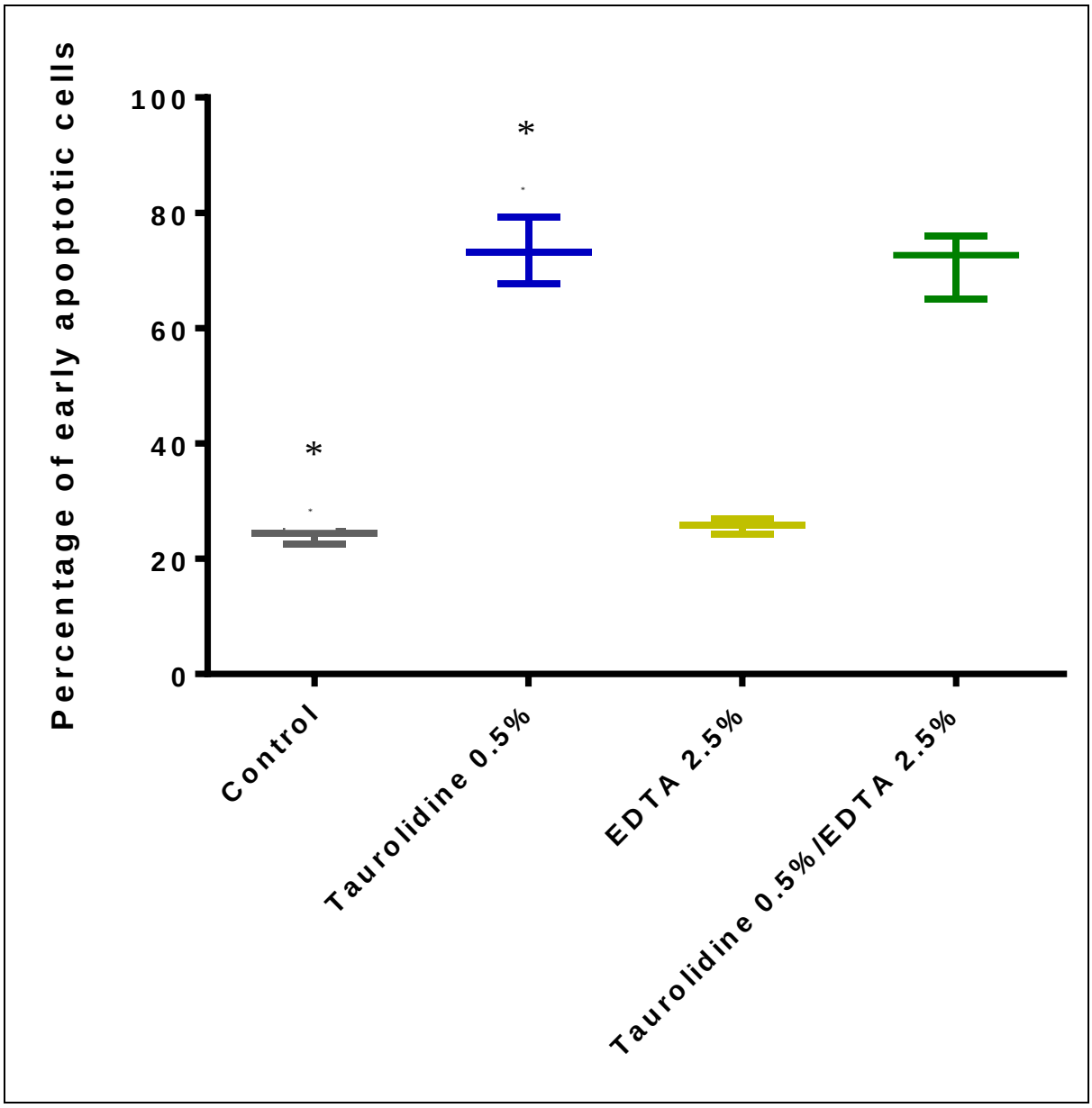

Figure 4. Comparison of percentages of early apoptotic PBMC cells incubated with different solutions. Kruskal-Wallis test yielded a p=0.006 for samples; Dunn's test for multiple comparisons was significant between PMCB treated with taurolidine 2.5% and control

(*) p=0.038

Kruskal-Wallis test yielded a p=0.001 for samples of necrotic cells between groups; Dunn's test for multiple comparisons was significant (p=0.039) when comparing controls with PMBC treated with EDTA 2.5%/taurolidine 0.5%. A tendency towards a lower percentage of necrotic cells treated with taurolidine 0.5% was detected without reaching statistical significance (p=0.18). Results of statistical analysis are reported in Fig. 5.

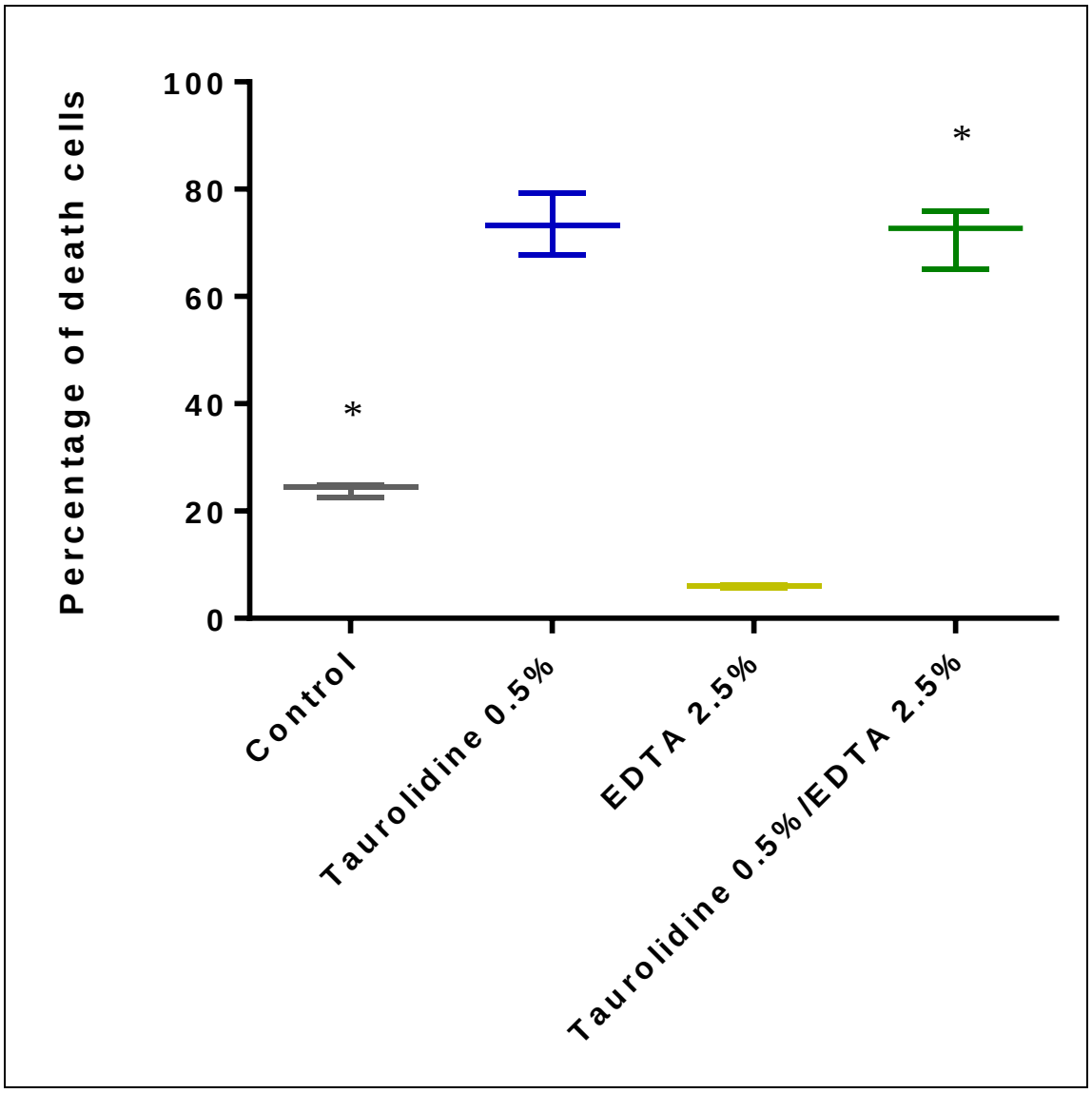

Figure 5. Comparison of percentages of necrotic PBMC cells incubated with different solutions. Kruskal-Wallis test yielded a p<0.001 for samples; Dunn's test for multiple comparisons was significant between PMCB treated with EDTA 0.5%/taurolidine 2.5% and control

(*) p=0.039

Comparison between taurolidine and EDTA/taurolidine

Giving the similar rate of living cells and early apoptotic cells between taurolidine 0.5% and EDTA 2.5%/taurolidine 0.5% groups, Mann-Whitney test was used to evaluate a potential synergic effect of EDTA against PMBCs. Statistical analysis reported no statistically significant differences between the two solutions regarding the rate of living cells (p=0.2) and early apoptotic cells (p=0.7)

Second part of the experiment

Cytotoxicity assay was conducted to assess the viability of fresh human PBMCs following incubation with taurolidine at incremental concentrations. Cells were treated with complete cell growth medium (control), deionized water 25%, saline buffer (or PBS) 25% and taurolidine 0.125%, 0.25%, 0.5%. (Fig. 6).

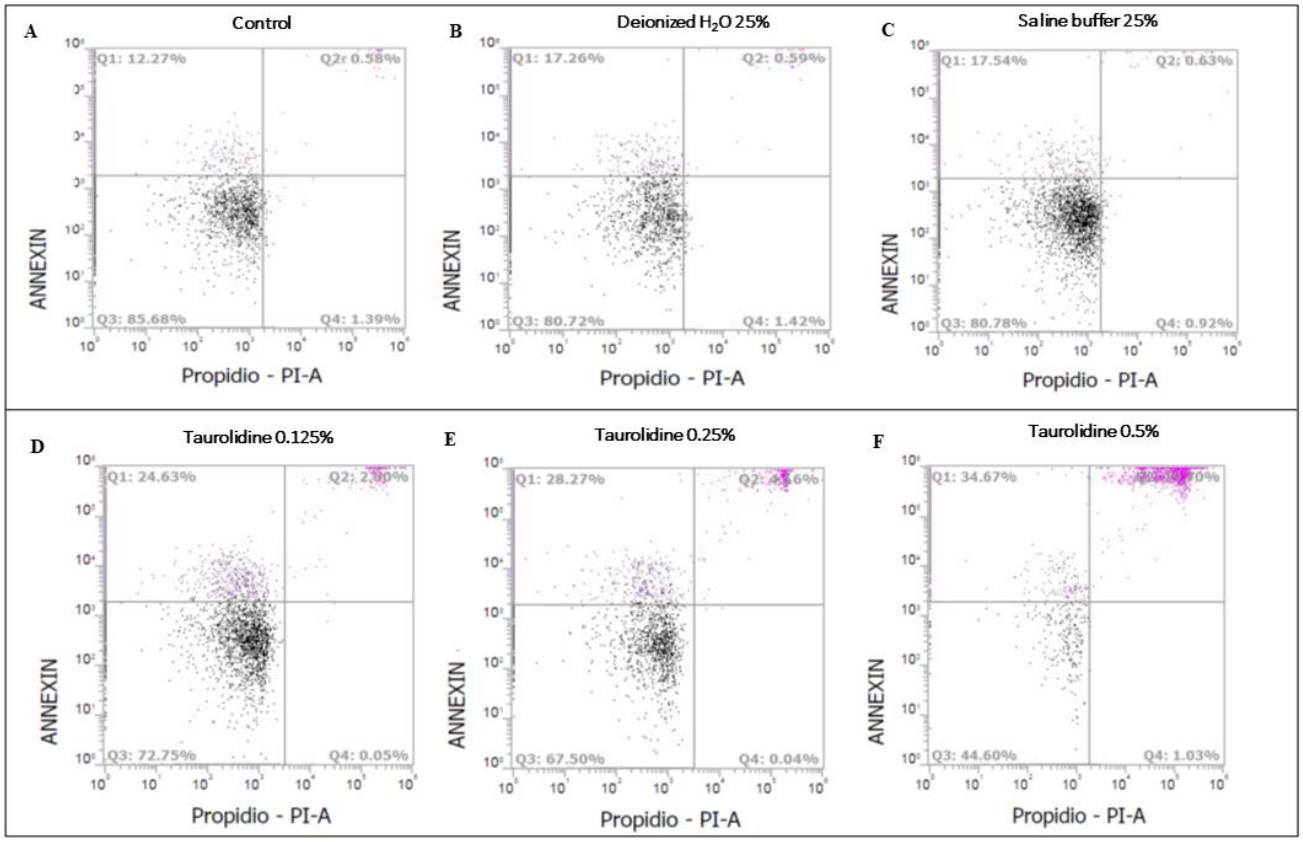

Figure 6. Evaluation of cell viability in PMBC treated with taurolidine. Cytofluorometry analysis sorted cells into 4 quadrantes: 4 quadrantes: early apoptotic cells (Annexin positive/Propidio [PI] negative), late apoptotic (Annexin positive/PI positive), necrotic cells (Annexin negative/PI positive) and viable cells (Annexin negative/PI negative). A, control; B, deionized water 25%; C, saline buffer 25%; D, taurolidine 0.125%; E, taurolidine 0.25%; F, Taurolidine 0.5%. Note that on both coordinates the scale is logarithmic.

As detailed in Fig. 7 cytofluorometry analysis provided a different percentage of viable, apoptotic and necrotic cells in relation to the following incubation solutions:

-taurolidine 0.125% showed an average percentage of living, apoptotic and necrotic cells of 73.2%, 23.7% and 2.4%, respectively

-taurolidine 0.25% showed an average percentage of living, apoptotic and necrotic cells of 66%, 30.1 and 3.8%, respectively

- taurolidine 0.5% showed an average percentage of living, apoptotic and necrotic of 44.9%, 35.4 and 18.7%, respectively

The negative control showed an average percentage of living, apoptotic and necrotic of 86.5%, 11.2 and 0.7%, respectively.

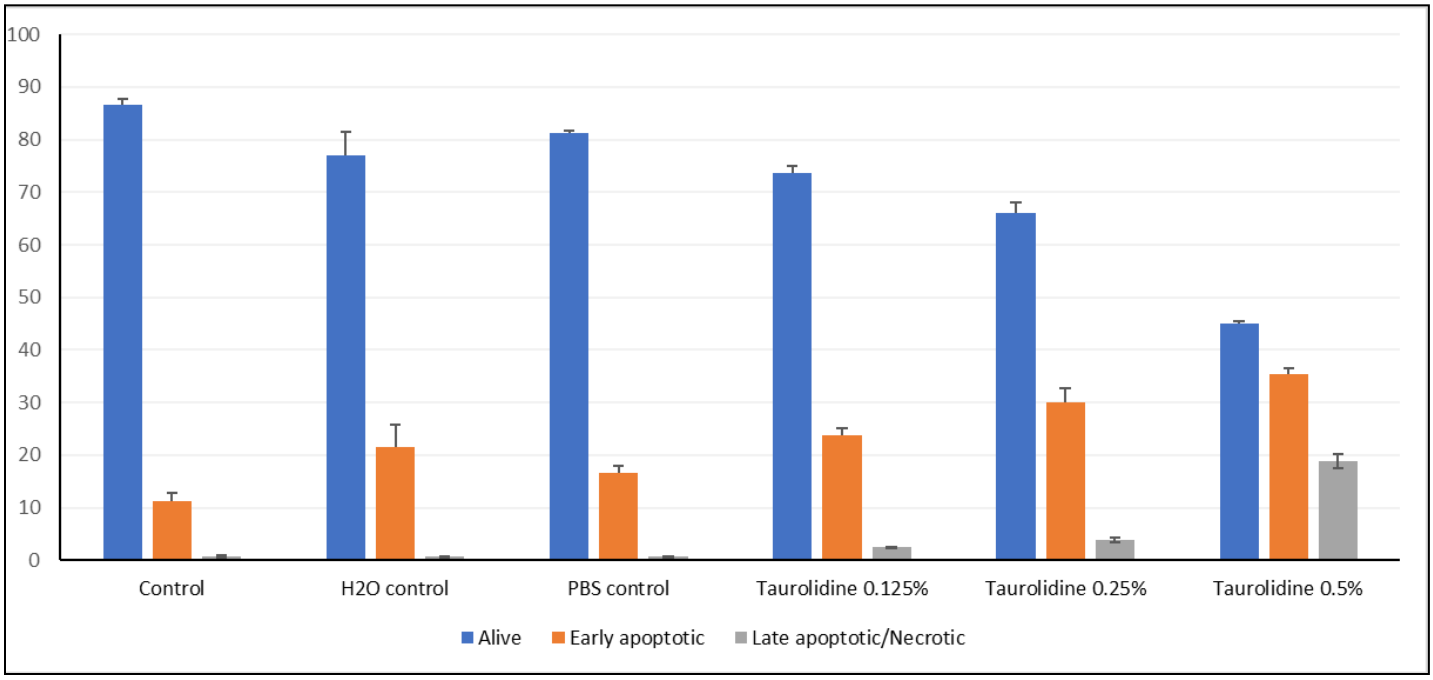

Figure 7. Graphical representation of the amount of living (blue bars), early apoptotic (orange bars) and late apoptotic/necrotic (gray bars) PMBCs incubated with different solutions.

Statistical analysis was performed comparing percentages of living cells incubated with a crescent concentration of taurolidine (0.125%; 0.25% and 0.5%). Kruskal-Wallis test yielded a $p<0.001$ for samples of living cells between groups; Dunn's test for multiple comparisons was significant ($p=0.027$) when comparing controls with PMBC treated with taurolidine 0.5%, the highest concentration of taurolidine tested in this experiment (Fig. 8)

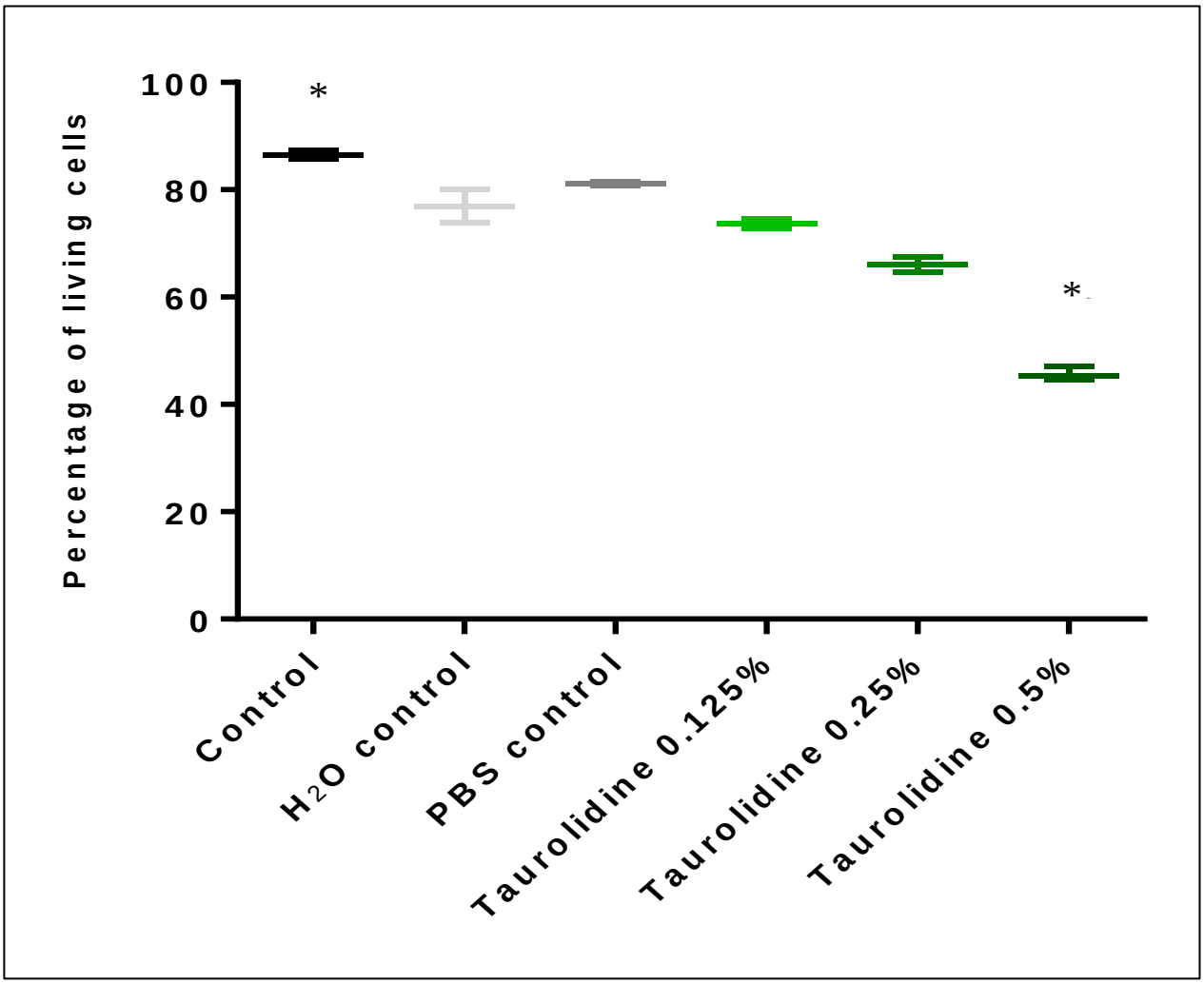

Figure 8. Comparison of percentages of living PBMC cells incubated with different solutions. Kruskal-Wallis test yielded a $p<0.05$ for samples; Dunn's test for multiple comparisons was significant between PMCB treated with taurolidine 0.5% and control.

(*) p=0.027

The rate of early apoptotic cells was statistically significantly different ($p<0.001$). Dunn's test for multiple comparisons was significant ($P=0.02$) when comparing controls with PMBC treated with taurolidine 0.5% (Fig. 9).

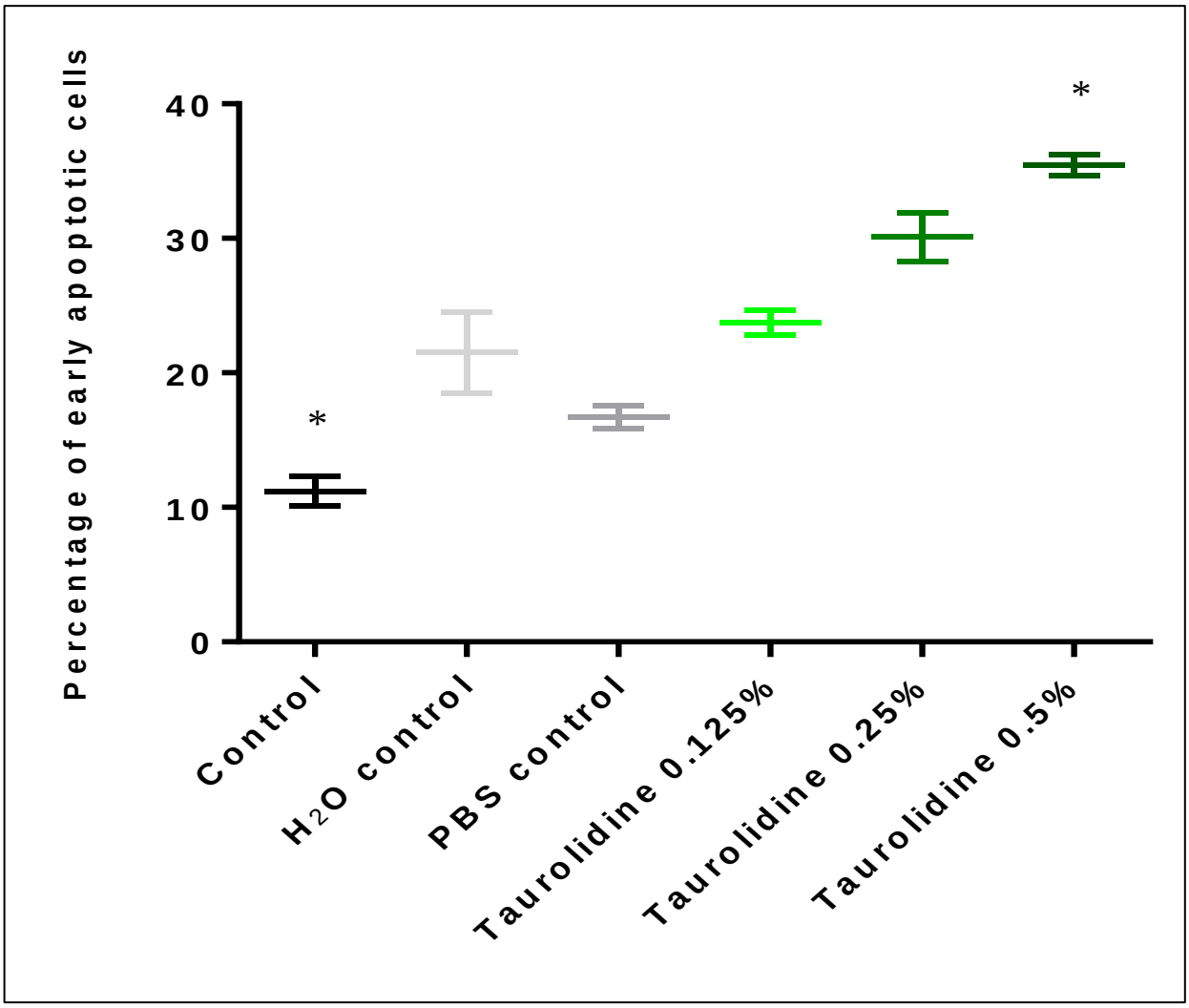

Figure 9. Comparison of percentages of early apoptotic PBMC cells incubated with different solutions. Kruskal-Wallis test yielded a $p<0.05$ for samples; Dunn's test for multiple comparisons was significant between PMCB treated with taurolidine 0.5% and control

(*) p=0.02

Lastly, also the rate of death cells was significantly different after incubation with a crescent concentration of taurolidine (p=0.009). As expected, after taurolidine 0.5% incubation the rate of death cells was significantly greater than control (p=0.043) (Fig. 10).

Lastly, no differences were noted between control and cell treated with deionized water and saline buffer.

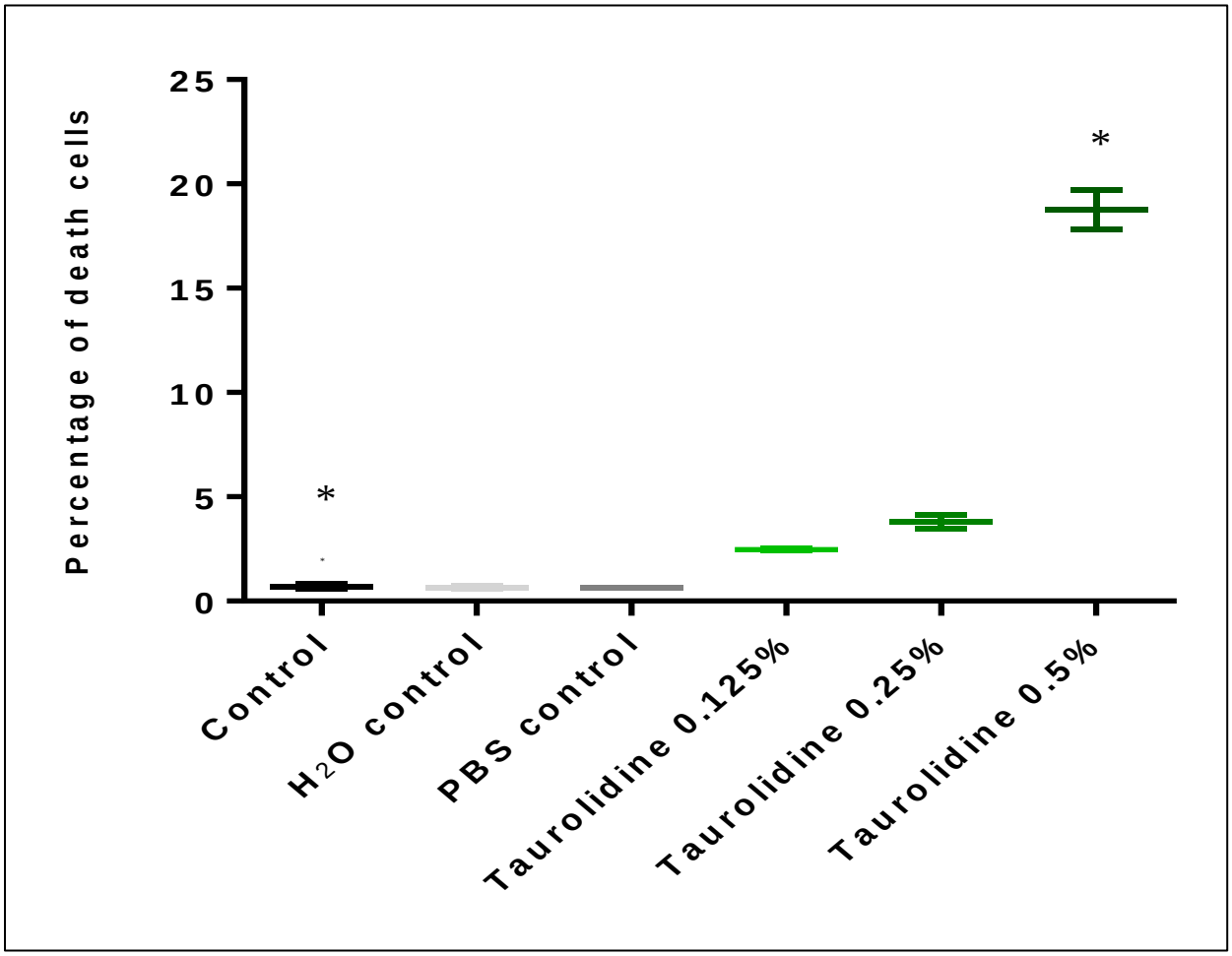

Figure 10. Comparison of percentages of necrotic PBMC cells incubated with different solutions. Kruskal-Wallis test yielded a p<0.05 for samples; Dunn's test for multiple comparisons was significant between PMCB treated whit taurolidine 0.5% and control

(*) p=0.043

Discussion

The findings of this study report novel data on the effects of taurolidine and/or EDTA on human PMBCs. In the first part of the study, we found that taurolidine 0.5% alone or in combination with EDTA2.5% affected the viability of human PMBCs after 24 hours of incubation with these antimicrobial agents. The findings of this cytotoxic assay showed that taurolidine 0.5%, and in a similar way taurolidine 0.5%/EDTA 2.5%, caused cellular damage and cell death compared to control. Notably, taurolidine 0.5% significantly increased the number of early apoptotic PMBC cells compared to the control. A similar effect was recognized when taurolidine 0.5% was combined with EDTA 2.5%, although the effects of this combined solution did not reach statistical significance.

In all the cytotoxic assays, we noted that the administration of EDTA was unrelated to cell injury. A similar rate of cell viability after incubation with EDTA/taurolidine and taurolidine excluded the synergic cytotoxic effect of the chelation agent with taurolidine.

In the second part of the study, we confirmed the cytotoxicity of taurolidine on fresh PMBCs. The trend of the cytotoxic effect was dose-dependent, although cell viability was significantly affected only at the highest concentration of taurolidine. Indeed, a higher rate of apoptotic and death was observed taurolidine 0.5%, the highest concentration of taurolidine used in this experiment. Taurolidine *per se* was the major contributor to cell damage since we excluded the toxic effect of solvents used in inducing cell injury.

Few studies have examined the cytotoxic side-effects of EDTA. In all the experiments conducted until now, EDTA was found to be cytotoxic in a dose-dependent manner but in all cases, the concentration utilized was higher than that commercialized (EDTA 10%) and, therefore, higher than the concentration (EDTA 2.5%) used in the current study[111–114].

Although these results require confirmation in vivo studies, the cytotoxicity of taurolidine cannot remain unnoticed, especially when the cytotoxic effect of this solution occurred with a lower taurolidine concentration (1:4 dilution ratio) than the commercialized vials.

On this issue, the available literature reports conflicting results on the safety of taurolidine in clinical practice. Taurolidine is commonly used as an antimicrobial solution to prevent catheter-related bloodstream infections due to bacteria able to form biofilm on the inner surface of the catheter (biofilm-related infection). For this reason, taurolidine is generally instilled in the dialysis and non-dialysis catheters to prevent catheter-related infections. In support of this

practice, a vast amount of literature is available. In 2016 the Italian group for venous access devices reported that taurolidine may be the most promising lock solution for the prevention of CVC-related bloodstream infections, given the well-known antimicrobial, anti-biofilm properties and the lack of antimicrobial resistance[115]. Liu et al. [116], observed a possible beneficial effect of taurolidine for the prevention of CVC-related bloodstream infections in a meta-analysis including three randomized controlled trials. A recent meta-analysis, including 14 randomized controlled trials, confirmed the previous results and highlighted the significant role of taurolidine for the prevention of CVC-related bloodstream infections[117].

On the other hand, the mechanism by which taurolidine produces a toxic effect on human cells remains in part elusive. Taurolidine act as an antimicrobial agent by causing a chemical reaction with the bacterial cell wall. In addition, taurolidine exhibits the ability to neutralize bacterial endotoxins. Notably, the antimicrobial property of the taurolidine is due to the release of active methylol (hydroxymethyl) groups soon after its metabolization to methylol taurinamide and taurine. These short-acting N-methylol derivatives react with the bacterial cell wall inducing the bacterial cell surface damage. Furthermore, methylol derivates interact with the lipopolysaccharide-protein complex resulting in the neutralization of the bacterial endotoxins.

Over the recent years, taurolidine has been used across different diseases. It has been used for the treatment of peritonitis, either as monotherapy or in combination with systemic antibiotics, leading to significant improvement in patients' outcome[118,119] and as an antineoplastic agent. This latter pharmacologic property of tauroldine is related to its capacity to inhibit the adhesion and growth of tumor cells[120]. Taurolidine has been shown to block interleukin 1 and tumor necrosis factor in PBMCs[121] and promote apoptosis by inducing various apoptotic factors and inhibiting the production of vascular endothelial growth factor[63].

In this setting, taurolidine has been not associated with acute or chronic toxic effects on hematological and biochemical parameters[62]. Ribizzi et al[122], reported taurolidine eliminated viable cancer cells while minimally reducing viable bone marrow cells in an in-vitro model of murine and human tumor cell lines. In light of their results, they suggested a promising role of taurolidine in bone marrow purging.

However, one recent publication reported that long-term high-dose treatment with taurolidine caused severe hepatic injury in a murine osteosarcoma model[123]. Therefore, the authors suggested caution with the use of this drug because data on long-term toxicity were lacking[124].

Concerning the systematic administration of taurolidine, there is only one study that evaluated the side effects associated with the administration of taurolidine (5 gr) intravenously. The subjects noted discomfort at the infusion site, though there were no serious adverse events, probably because of quick degradation of taurolidine products[125].
In 2018, Wouters et al. [126] documented with an "abstract" that taurolidine decreased cell viability of PBMCs and granulocytes in a dose- and time-dependent manner. No data were reported in terms of the mechanism of injury[126].

In daily practice, the use of taurolidine is not associated with any side effects. Generally, an adequate volume of taurolidine is instilled within the lumen of the catheter. Before utilizing the line for dialysis or administration of medication, the volume of taurolidine added to each lumen should be aspirated. If in the event of line occlusion, slow intravascular flushing (not more than 1 mL per 3 seconds) is allowed[127]. Taurolidine manufacturer informs that taurolidine degrades to taurine (amino acid), carbon dioxide and water, once enters the body via the vascular access device. Hence, intravenous administration of a small amount of taurolidine does not seem to raise any specific concerns in humans.
The limited amount of information about the etiological mechanism underlying the cytotoxicity of EDTA and taurolidine is the main limitation of the study. Although these results are partials, they provide new insight regarding its safety. To the best of our knowledge, this is the first study that confirmed the cytotoxic effect of taurolidine reported until now only in the abstract form[126].

In conclusion, taurolidine showed dose-dependent cytotoxicity on human PBMCs. In-vitro toxicity is a concerning issue and should be further explored because the toxic dose used in this in-vitro experiment had a lower concentration compared to the commercialized vials. Since concerning adverse reactions have been reported with intravenous administration of taurolidine in humans, new studies focusing on the causative mechanisms of cell death are warmly welcomed for better understanding the safety profile of this drug.

Acknowledgments

I sincerely acknowledge all the people that have allowed the realization of this research project: Silvia Giovanella, Giulia Ligabue and Professor Gianni Cappelli of the University of Modena and Reggio Emilia, Italy.

General discussion

PD is currently the most common home dialysis treatment option for patients with ESRD. Compared to HD, PD is characterized by less impact on hemodynamic, better preservation of residual renal function as well as a higher quality of life. After adequate training with specialized nurses, DP patients can perform their dialysis treatment at home without attending hospital or dialysis facilities. The Achille's hill of PD is the infectious risk related to the inoculum of bacteria directly into the abdomen cavity with peritoneal exchanges during dialysis. Bacterial peritonitis is therefore a frequent complication associated with mobility and, to a less extent, mortality in this group of patients. The severity of bacterial peritonitis is variable and ranges from mild symptomatic abdominal pain to septic shock. Generally, the aggressivity of the pathogens influences the outcome of the peritonitis. As a result, highly pathogenic germs such as *Pseudomonas aeruginosa* are associated with refractory peritonitis that is frequently treated with catheter removal[32]. Unfortunately, this strategy is associated with multiple implications for the patients, including hospitalization, surgery and switch to HD.

A practical solution could be the instillation of an antimicrobial solution within the lumen of the peritoneal catheter as a lock therapy. Compared to CVC catheter, lock therapy is not regularly practiced in patients on PD because the use of lock therapy precludes the use of the peritoneal catheter. However, anecdotal experiences reported that lock therapy can be an effective therapeutic strategy for the treatment of refractory peritonitis[54,128]. Given the serious consequences of bacterial peritonitis in patients on PD, we developed a research project aimed to (i) investigate the epidemiology and outcome of bacterial peritonitis in our Center at the University Hospital of Modena, (ii) evaluate the effects of lock therapy with EDTA and taurolidine in an in vitro model of Pseudomonas aeruginosa biofilm-related peritoneal catheter infection, and (iii) verify the in vitro safety of EDTA and taurolidine for human peripheral blood mononuclear cells (PBMCs).

The results of our first study showed that 484 ESRD patients on PD were followed at the University Hospital of Modena from January 2000 to December 2019. During this period, 373 episodes of peritonitis were diagnosed in 191 patients. Peritonitis was a common complication in DP, occurring in 39.4% of the patients followed at our center. Notably, about half of them (50.7%) experienced multiple episodes of peritonitis. The overall peritonitis rates for 2000-2019 accounted for 0.23 episodes/patient/year, lower than the threshold limit of 0.5 episodes per year at risk recommended by the ISPD. Based on our data, we noted that Gram + infection was the

principal threat for patients on PD, even though the trend of Gram + infection decreased over the last 10 years of the study period. Although the mortality rate due to this infective episode appeared quite low (2.1%), one-third of all patients were hospitalized for the severity of the infection. Our study confirmed that peritonitis was a cause of PD failure, indeed this infectious episode lead to catheter removal in 8.5% of our patients. Among the bacterial pathogens, *Pseudomonas* species was the most common cause of catheter removal in our population.

In the second study, we evaluated the effects of two solutions with antimicrobial effects (EDTA and taurolidine) on an in vitro model of peritoneal catheter-associated infection due to *Pseudomonas aeruginosa*. A bioengineered *Pseudomonas aeruginosa* strain (BLI-*Pseudomonas*), emitting measurable bioluminescence, was used to experiment. BLI-*Pseudomonas* was seeded on PDC pieces for 24 hours to allow microbial adhesion, growth and biofilm formation. The pieces of PDC, harboring a 24- and 72-hours-old biofilm were treated and incubated with EDTA (0.25%; 0.75%; 2.5%), taurolidine (0.125%; 0.25%; 0.5%) and a combination of two solutions for 24 hours. The results of this study showed that these two lock solutions affected *Pseudomonas* biofilm that developed on PDC pieces. Taurolidine caused a rapid decrease of bacterial load, already evident within the first 2-3 hours of treatment and with all tested doses. Differently, EDTA affected microbial load only at the highest concentration (2.5%) used in this experiment. Unfortunately, the inhibitory effect of EDTA and/or taurolidine on *Pseudomonas* biofilm was transient. An intense microbial re-growth was observed after 24 hours from the exposition to the antimicrobial substances, independently upon the treatment performed. To assess the effect of extensive use of lock solutions, contaminated PDC pieces were exposed to a second treatment with EDTA/taurolidine solutions. Also during this experiment, microbial growth was deeply affected during treatment, but a time-related recovery occurs upon drugs removal.

Then we evaluated the secretory profile of *Pseudomonas* biofilm after exposure to EDTA and taurolidine. To elucidate the mechanism underlying biofilm impairment, HPLC-ESI-MS analysis provided for the first time evidence that the levels AIs, phenazines and pyoverdins deeply decreased following the treatment with EDTA and taurolidine, either alone or in combination. In agreement with the microbial load/viability data, the antimicobial effect of AIs, and phenazines was transient. The levels of AIs and phenazines returned toward control levels upon drugs removal and microbial re-growth. Surprisingly three pyoverdines (Succ-p-Ser-Y, Succa-P-Ser-Y and PyE) remained below the detection limit during the re-growth period. These data document that EDTA and taurolidine may irreversibly change affect the production of pyoverdines secreted

by this *Pseudomonas aeruginosa* and therefore increase its vulnerability to the host and antibiotics.

The third study evaluated the safety profile of EDTA and taurolidine on human cells. Hence, a series of in vitro cytotoxicity assays were conducted on peripheral blood mononuclear cells (PBMCs) exposed to EDTA and/or taurolidine. We found that taurolidine alone or in combination with EDTA significantly affected the viability of cryopreserved human PMBCs after 24 hours of incubation. Notably, taurolidine 0.5% significantly increased the number of early apoptotic PMBCs compared to the control. A similar effect was recognized when taurolidine 0.5% was combined with EDTA 2.5%, although the effects of this combination did not reach statistical significance compared to control. A repeated study on fresh PMBCs documented that the trend of the cytotoxic effect was dose-dependent, indeed, a higher rate of apoptotic and necrotic cells was observed at the highest concentration of taurolidine (taurolidine 0.5%) used in the study. We also documented that taurolidine *per se* was the major contributor to cell damage since we excluded the toxic effect of solvents commonly used for cell sorting (PBS) or as drug additives (deionized water). Furthermore, in all the experiments performed, EDTA did not cause cell toxicity on human PMBCs.

Based on our results, in-vitro toxicity of taurolidine is a concerning issue and should be further explored because the in vitro toxic effects of this substance occurred at a lower concentration (0.5%) than that commercialized (2%). Since adverse reactions have been reported with intravenous administration of taurolidine in humans, new studies focusing on the causative mechanisms of cell death are required to better understand the safety profile of this antimicrobial agent.

In conclusion, bacterial peritonitis is a serious complication of PD treatment for ESRD. *Pseudomonas* species is the most common cause of refractory peritonitis in our cohort of patients. The therapeutic strategies are limited because the formation of biofilm on the PD catheter is a common form of resistance of the *Pseudomonas* species. The use of EDTA and/or taurolidine as a lock therapy might be a promising strategy if associated with anti-*Pseudomonas* antibiotics to eradicate the biofilm-related infection from the peritoneal catheter. However, further cytotoxic studies are required to better evaluate the mechanism of action of taurolidine, since it revealed dose-dependent toxicity on human PMBCs.

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