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Pilot Study

In-vitro activity of lipoic acid against *Ureaplasma urealyticum* and *Ureaplasma parvum* isolated from women with infections of the urogenital tract. A pilot study

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Several species of *Ureaplasma* bacteria are known to be present in the urogenital tract of humans, in both healthy individuals and symptomatic patients. These pathogens are associated with urogenital tract infections, infertility problems and spontaneous abortion in humans. The present study involved 77 strains of *Ureaplasma* species (*Ureaplasma* spp.), including 21 *Ureaplasma urealyticum* (*U. urealyticum*) strains and 56 *Ureaplasma parvum* (*U. parvum)* strains. Lipoic acid (LA) and its reduced form dihydrolipoic acid (DHLA) are synthesized in all prokaryotic and eukaryotic cells. Research of recent years increasingly points to therapeutic properties of exogenously supplemented LA. In our study, we examined for the first time the effect of LA on the bacteria multiplication and its bactericidal activity against *U. urealyticum* and *U. parvum.* The LA concentrations used were: 1200 µg/ml, 120 µg/ml, and 12 µg/ml. The titer for each strain of *Ureaplasma* spp. was estimated using the color changing units (CCU) assay. For CCU measurements, a series of 10-fold dilutions of each cell culture in 0.9% NaCl (titration) was prepared and 1 CCU/ml was defined as the highest dilution of cells at which color change was detected. The strongest bacteriostatic and bactericidal effect of LA was observed at a concentration of 1200 µg/ml. In contrast, at lower LA concentrations, stimulation of the bacteria multiplication was noted for 14% of the total number of strains tested. Taken together, the current data provide novel findings about potential beneficial antimicrobial effects of LA.

Key words: *Ureaplasma urealyticum*, *Ureaplasma parvum*, lipoic acid

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Abbreviations: CCAL, chitosan-coated liposome containing coenzyme Q10 and lipoic acid; CCU, color changing units; *E. coli, Escherichia coli;* LA, lipoic acid; DHLA, dihydrolipoic acid; ROS, reactive oxygen species; *S. aureus, Staphylococcus aureus*; *Ureaplasma* spp., *Ureaplasma* species; U. *parvum* (Up), U*reaplasma parvum;* U. *urealyticum* (Uu), *Ureaplasma urealyticum*

INTRODUCTION

The *Ureaplasma* genus is the second of two genera of bacteria belonging to the family *Mycoplasmataceae* in the order *Mycoplasmatales*. In 1967, the order *Mycoplasmatales* was incorporated into the class *Mollicutes*. *Ureaplasma* species (*Ureaplasma* spp.) are unique among the *Mollicutes* because they possess a very potent urease which hydrolyzes urea to generate ATP and produces ammonia as a product. The *Ureaplasma* spp. have a genome size ranging from 0.76 to 1.17 million base pairs with a GC content of 27 to 30% and require cholesterol for growth (Razin *et al*., 1998).

Ureaplasma spp. are known to be present in the urogenital tract of males and females, in both healthy individuals and symptomatic patients. Fourteen known *Ureaplasma* serovars have been divided into two species based on phenotypic and genotypic characteristics: *Ureaplasma parvum* (*U. parvum; Up)* and *Ureaplasma urealyticum* (*U. urealyticum; Uu*) (Marovt *et al*., 2015). Uropathogens colonize the urogenital tract mainly via the ascending route. Given the anatomical differences in the structure of the urogenital tract of women and men, it is understandable that *Ureaplasma* spp. are more often identified in women than in men. Epidemiological data indicate that nearly 90% of sexually active healthy women are carriers of *Ureaplasma* spp. (Kim *et al*., 2014). Thus, it is believed that *Ureaplasma* spp. as a component of the microbiota of the human urogenital tract are commensal organisms of low virulence. For that reason, the presence of *U. urealyticum* and *U. parvum* can be considered not only as an infection but also as colonization (McCormack & Rein, 2005).

However, it should be noted that *Ureaplasma* spp. do not cause symptoms if they live in balance with other bacteria. If the population of these microorganisms in the urogenital tract is greater than 10^4 per ml certain health problems may develop and cause symptoms, if left untreated, can lead to serious consequences. There is strong evidence from experimental animal and clinical studies that these pathogens are associated with urogenital tract infections, infertility problems and spontaneous abortion in humans. *Ureapasma* spp. can cause the amniotic sac inflammation resulting in chorioamnionitis, preterm labor and neonatal lung injury. These pathogens appear to also have an etiological role in postpartum infections of mothers and newborns (Kılıç *et. al.* 2004; Liu *et. al.* 2014; Waites *et. al.* 2009; Zhang *et. al*., 2014).

Treatment of *Ureaplasma* infections is exceedingly difficult. The lack of a cell wall confers resistance to all β-lactam and glycopeptide antibiotics. On the other hand, the lack of *de novo* synthesis of folic acid makes the cells resistant to diaminopyrimidines and sulfonamides (Beeton & Spiller, 2016; Tantengco & Yanagihara, 2019). Many authors emphasize that the development of resistance of *Ureaplasma* spp. to tetracycline, fluoroquinolone and macrolides has also been observed (Beeton *et al*., 2009; Biernat-Sudolska *et al*., 2007; Saraçoğlu *et al*., 2018).

Thus, it is reasonable to look for new compounds supporting the therapy of patients with infections of the urogenital tract caused by *Ureplasma* spp. We chose lipoic acid (LA) for our research. LA (1,2-dithiolane-3-pentanoic acid) and its reduced form dihydrolipoic acid (DHLA; 6,8-dimercaptooctanoic) are present in all prokaryotic and eukaryotic cells. In humans and other mammals, LA/DHLA system is essential for oxidation of glucose, other carbohydrates, amino acids and other fuels, and is implicated in the regulation of mitochondrial redox balance. Namely, LA/DHLA system functions as a cofactor essential for five redox reactions: four catalyzed by 2-oxoacid dehydrogenases and the glycine cleavage system. These enzymes include α-ketoglutarate dehydrogenase and pyruvate dehydrogenase from the energy metabolism, branched-chain ketoacid dehydrogenase, 2-oxoadipate dehydrogenase, and glycine cleavage system from the amino acid metabolism. Research of recent years increasingly points to therapeutic properties of exogenously supplemented LA. Currently LA has been shown to be beneficial in type 1 and 2 diabetes by preventing the various pathologies associated with diabetes such as macular degeneration, cataracts, and neuropathy. In many clinical trials LA has been used at a dose between 300–1 800 milligrams per day for diabetes and neuropathy (Dworacka *et al*., 2012; Evans and Goldfine, 2000). Research is also increasingly pointing to LA as an effective drug in the therapy of many diseases other than diabetes and its complications (Bast & Haenen, 2003; Biewenga *et al*., 1997; Bilska and Włodek, 2005; Gorąca *et al*., 2011; Packer *et al*., 1995; Skibska *et al*., 2015).

In our study, we examined for the first time the bacteriostatic (effects on the cell division) and bactericidal activity of LA against *U. urealyticum* and *U. parvum*.

MATERIALS AND METHODS

General. *Ureaplasma* strains isolated in the Chair of Microbiology of the Jagiellonian University Medical College (Kraków, Poland) from women with infections of the urogenital tract were tested in the study. The study involved 77 strains of *Ureaplasma* spp. including 21 *U. urealyticum* strains and 56 *U. parvum* strains.

Detection of *Ureaplasma* spp. The vaginal swabs were collected from each woman and placed in BioMerieux transport media. Next, they were subcultured in liquid and solid PPLO media with 1% urea, prepared in-house and parallelly in Mycoplasma IST 2 kit BioMerieux medium (R2) (BioMerieux, Marcy L'etoile, France). Liquid media and BioMerieux medium test strips were incubated for 72 h at 37°C. Development of a clear red color of samples indicated the growth of *Ureaplasma* strains since hydrolysis of urea with the release of ammonia was signaled by a color change of a pH indicator. Solid media were incubated for 5–7 days at 37°C. In the case of solid media, it was the presence of characteristic brownish colonies that was indicative of *Ureaplasma* spp. (magnification 125×).

Isolation of DNA of *Ureaplasma* spp. After incubation, crude Ureaplasma DNA was isolated from R2 medium by means of centrifugation and denaturation of the sediment in 100 µl of sterile, distilled water at 95°C.

PCR with species-specific primers. The identity of *U. parvum* and *U. urealyticum* was confirmed by amplification of the genome specific regions of these two *Ureaplasma* spp. Primers for *U. parvum* and for *U. urealyticum* were: UPS2c and UPA2c (420 bp), and UUS2c, UUA2c (420 bp), respectively (Kong *et al*., 2000). DNA from reference strains was used as a positive control for identification of *U. urealyticum* and *U. parvum:* serotype IV ATCC 27816 and serotype III ATCC 27815, respectively. Negative control consisted of distilled water. The amplified products were visualized under ultraviolet light after agarose gel electrophoresis containing ethidium bromide (EtBr).

The effect of LA on *Ureaplasma* spp. In the present study, we used the formulation Thiogamma Turbo-Set (Wörwag Pharma, Germany), which contains the racemic LA mixture as a pharmacologically active substance and is used for treatment of diabetes and diabetic neuropathy. The effects of three LA concentrations: 1200 µg/ml, 120 µg/ml and 12 µg/ml on the growth and survival of *Ureaplasma* strains were studied. The highest concentration of 1200 µg/ml used in the studies was also the highest non-toxic concentration of LA for cell culture of the RK13 line, which was previously determined in *in vitro* studies. The titer for each *Ureaplasma* strain was estimated using the color changing units (CCU) assay. For CCU measurements, a series of 10-fold dilutions of each cell culture in 0.9% NaCl (titration) was prepared and 1 CCU/ml was defined as the highest dilution of cells at which color change was detected. The titre was considered to be the highest dilution which produced a color change, when the test result was recorded at a time when color changes were no longer progressive (Poveda & Nicholas, 1998).

In our study, the 18–24-hour bacterial cultures in the growth medium in the absence of LA (control) were titrated by making 10-fold serial dilutions (10^{-1} to 10^{-10}). Titration was carried out in 96-well plates. The titer was read after 24 hours of incubation at 37°C and expressed as CCU/ml.

Test System (two variants)

Bacteriostatic effect of LA (on the cell division). The cultures of each *Ureaplasma* strain were titrated by making 10-fold serial dilutions (10^{-1} to 10^{-10}) in growth medium with the addition of the tested LA concentrations. Titration was carried out in 96-well plates. The titers were read after 24 hours of incubation at 37°C and expressed as CCU/ml. The obtained values were compared with the control titer for a given strain.

Bactericidal activity of LA. The culture of each *Ureaplasma* strain was suspended in medium with the tested concentrations of LA and was incubated for 24 hours at 37°C. After incubation, LA was washed away with physiological saline twice by centrifugation and then titration was performed by making 10-fold serial dilutions (10^{-1} to 10^{-10}) with growth medium without the addition of LA. Titration was carried out in 96 well plates. The titer was read after 24 hours of incubation at 37°C and expressed as CCU/ml. The obtained values were compared with the control titer for a given strain.

Statistical analysis. Statistical calculations were carried out with the STATISTICA 12.0 software (Statsoft Inc., Tulsa, OK, USA). The differences between groups (the comparison of control titers of strains and titers achieved after 24 hours incubation with the test LA concentration) have been analyzed using nonparametric tests: ANOVA rank Kruskal-Wallis test and the chi square test (χ^2). A level of $p \leq 0.001$ was adopted to indicate statistical significance.

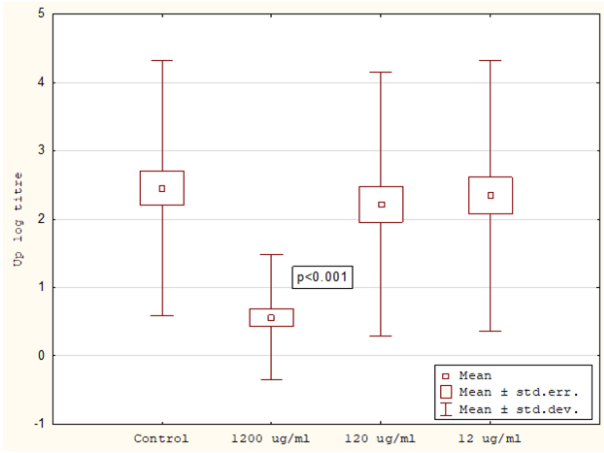

Figure 1. The effect of LA on the cell division of *U. parvum*" (Up).
The graph shows the dependence of log titre Up on LA dose. Data are shown as the mean ±standard deviation. *P*<0.001 *vs.* control sample.

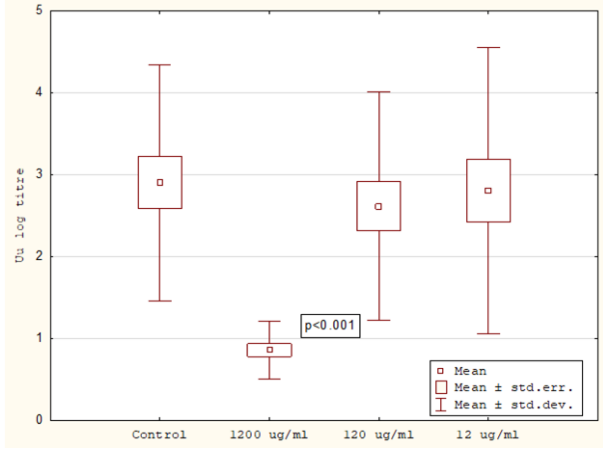

Figure 2. The effect of LA on the cell division of *U. urealyticum* (Uu).
The graph shows the dependence of log titre Uu on LA dose. Data are shown as the mean ±standard deviation. *P*<0.001 *vs.* control sample.

RESULTS

The effect of LA on the cell division of *Ureaplasma* spp.

The obtained results indicate that LA only at the concentration of 1200 μg/ml had a statistically significant (p<0.001) inhibitory effect on cell division of *Ureaplasma* strains compared to the control. Under the influence of LA at a concentration of 1200 μg/ml, titer reduction by over three logarithmic (log) cycles was observed in almost 43% of *U. parvum* strains and in 28% of *U. urealyticum* strains. At lower LA concentrations (120 μg/ml and 12 μg/ml) the titer reduction by three log cycles was observed only in *U. parvum* and these percentages were 2.6% and 1.8%, respectively (Figs 1, 2). Inhibitory effects were observed in 94% of all *U. parvum* and *U. urealyticum* tested strains. At lower LA concentrations, the stimulation of cell division was observed for 10 strains of *U. parvum* and one strain *of U. urealyticum* which represents 14% of the total number of tested strains. Figure 3 shows the percentage of *U. parvum* and *U. urealyticum* strains for which effects of LA on the cell division were demonstrated (Fig. 3).

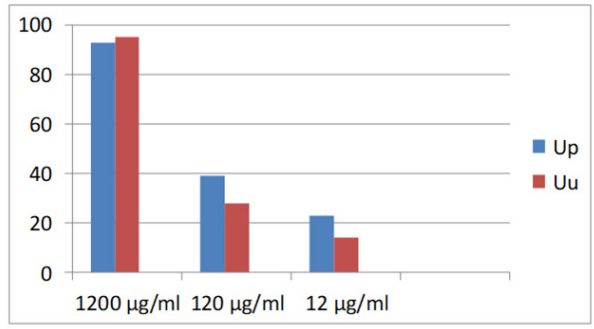

Figure 3. Percentage of *U. parvum* (Up) and *U. urealyticum* (Uu) strains for which inhibition of cell division was found at different LA concentrations.

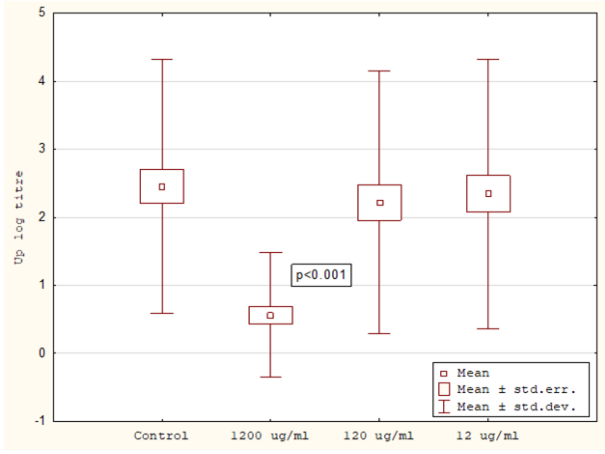

Figure 4. The bactericidal effect of LA on the *U. parvum* (Up) cells.
The graph shows the dependence of log titre Up on LA dose. Data are shown as the mean ±standard deviation. *P*<0.001 *vs.* control sample.

The bactericidal effect of LA on the *Ureaplasma* spp.

The strongest bactericidal effect of LA (altogether on 82% strains of *U. urealyticum* and *U. parvum*) was observed at a concentration of 1200 μg/ml. These results were statistically significant compared to the control (Figs 4, 5). A weaker bactericidal effect of LA was demonstrated for the concentrations of 120 μg/ml and 12 μg/ml, respectively for 39% and 32% of the total number of strains tested. Strains belonging to the species *U. urealyticum* were more sensitive to lower LA concentrations (Figs 4, 5). Figure 6 shows the percentage of *U. parvum* and *U. urealyticum* strains for which bactericidal effects of LA were demonstrated (Fig. 6).

DISCUSSION

In both bacterial and eukaryotic cells, LA is synthesized in a complex multistep process. In humans, defects in the biosynthesis of LA lead to a heterogeneous group of diseases with a wide variety of clinical symptoms. There is no possibility of causal treatment of these diseases, LA supplementation is ineffective (Mayr *et al.*, 2014; Tort *et al.*, 2016; Yi & Maeda, 2005). According

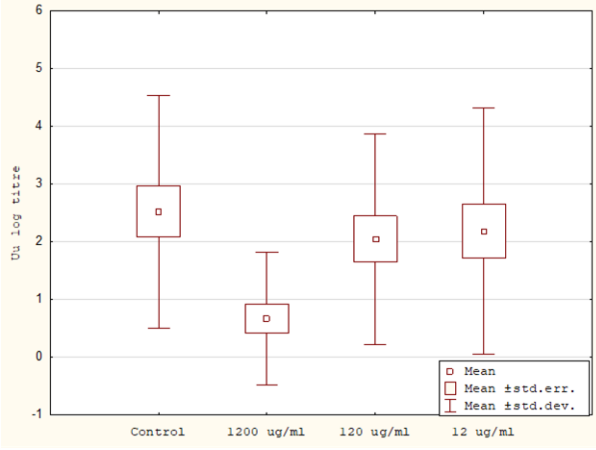

Figure 5. The bactericidal effect of LA on the *U. urealyticum* (Uu) cells.
The graph shows the dependence of log titre Uu on LA dose. Data are shown as the mean ± standard deviation. $P<0.001$ *vs.* control sample.

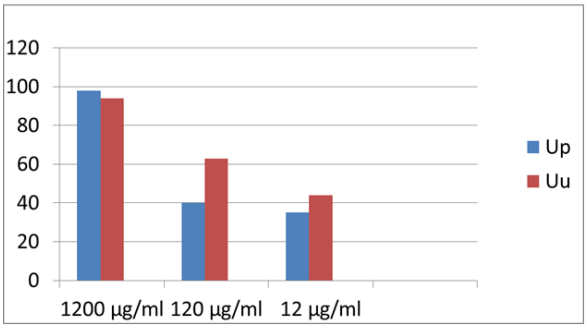

Figure 6. Percentage of *U. parvum* (Up) and *U. urealyticum* (Uu) strains for which bactericidal effects of LA were demonstrated.

to Hiltunen and others (Hiltunen *et al.*, 2009) critical developmental processes require LA to be synthesized *in vivo* in mammalian mitochondria. Thus, in humans and generally in other eukaryotes, LA must be synthesized *de novo*.

It is very different in most bacterial cells which acquire LA through either *de novo* synthesis or scavenging from the environment (salvage), and many pathogens maintain independent LA synthesis and scavenging pathways (Spalding & Prigge, 2010). Thus, the availability of LA in the environment should promote bacterial growth (VanLancker, 2012). Indeed, at lower LA concentrations, we observed a stimulation of proliferation for ten strains of *U. parvum* and one strain of *U. urealyticum,* which represents 14% of the total number of strains tested. However, our research has shown that LA also has antibacterial effect. At a concentration of 1200 µg/ml, it acts as both bacteriostatic and bactericidal agent against most of the tested strains of *U. parvum* and *U. urealyticum*. This type of opposing effect of LA on cells depending on its dose has already been noticed. For example, Dovinova and others (Dovinova *et al.*, 1999) in studies conducted on cultures of L1210 mouse leukemia cells, observed that LA at a concentration of 1 µM increased cell proliferation, i.e., it acted as a growth factor. In contrast, at a concentration of 100 µM, LA acted as an antiproliferative agent. The same authors also showed that the combined single-dose administration of doxorubicin at 5 mg/kg and LA at 16 mg/kg led to a super-additive stimulating effect on survival of leukemic mice (Dovinova *et al.*, 1999). The highest dose of LA used in our own studies on rats was 150 mg/kg divided into three doses per day (Bilska *et al.*, 2007).

The problem of antimicrobial action of LA has rarely been studied. Many more animal studies have demonstrated that LA is highly effective in reducing endotoxin-induced cell damage and suppressing inflammatory response by inhibiting molecular signaling pathways activated by several inflammatory mediators, especially including cytokines and their receptors (Gorąca & Asłanowicz-Antkowiak, 2009; Gorąca *et al.*, 2011; Gorąca *et al.*, 2009; Li *et al.*, 2015).

Although the problem of antimicrobial activity of LA and the mechanism of its antimicrobial action are not well recognized, there are also such data, although scarce, which confirm such LA activity. For example, it was shown that human immunodeficiency virus (HIV) replication was inhibited by LA. The study of Baur et al. indicated that a combined treatment of infected Jurkat cells with azidothymidine, a nucleoside analog inhibiting reverse transcription, and LA resulted in a stronger inhibition of HIV replication than by each drug alone (Baur *et al.*, 1991). It is commonly known that the expression of HIV genes depends, among others, on the activity of the transcription factor NF-κB. It has been shown that LA has a more potent activity in inhibiting NF-κB, and in addition LA completely abolished the initiation of HIV-1 induction by tumor necrosis factor alpha (TNF-alpha) (Merin *et al.*, 1996).

Also noteworthy are the research results showing that LA has a moderate antimicrobial activity against *Cronobacter sakazakii* strains. Those authors demonstrated that LA exerted its inhibitory effect through reduction of intracellular ATP levels in bacterial cells, causing many membrane-disrupting effects and finally increasing permeability of the cell membrane (Shi *et al.*, 2016). In turn, Zao et al. demonstrated that chitosan-coated liposome containing coenzyme Q10 and LA (CCAL) showed a strong antimicrobial effect against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). Those authors also showed that bactericidal effect of CCAL decreased with the decreasing of its concentration, which is also consistent with our observations (Zhao *et al.*, 2018).

On the other hand, in the murine sepsis model caused by *S. aureus,* it was found that upon infection of mice, *de novo* biosynthesis or salvage of LA promoted *S. aureus* survival. However, it was also noted that when both LA biosynthesis and salvage were blocked *S. aureus* was unable to survive (Zorzoli *et al.*, 2016).

Who is right? Many authors increasingly point out that LA is not only involved in intermediate metabolism but has many other major roles in the cell. The LA/DHLA system is characterized by one of the lowest values of the standard biological redox potential (E_0 =–0.29V). Thus, DHLA is able to reduce not only reactive oxygen species (ROS) but also oxidized forms of other antioxidants and therefore is called an antioxidant of antioxidants (Packer *et al.*, 1995). Redox reactions are essential processes of the basic metabolism of all cells. Therefore, the mechanism of pro- and antioxidant imbalance can explain etiopathogenesis and/or development of many apparently distant pathological conditions.

Some authors also highlighted that LA could participate in S-thiolation of proteins according to the following equation:

Protein-$(SH)_2$+LA-(SS)↔Protein-(SH)-S-S-LA-SH↔Protein-(SS)+DHLA-$(SH)_2$

This process is perceived as a molecular "switch" decisive for intracellular redox status of thiols which controls cellular metabolic pathways (Shay *et al*., 2008).

One more concept, so far rarely raised by other authors, should also be mentioned. Namely, in our earlier studies we have shown that LA increases the level of sulfane sulfur in animal tissues and the activity of enzymes involved in its metabolism, although our results also showed that LA itself is a source of sulfane sulfur *in vitro* (Bilska *et al*., 2008; Bilska-Wilkosz *et al*., 2017). Sulfane sulfur is a divalent sulfur atom bonded to another sulfur atom. Sulfane sulfur atom is very reactive and labile. It is interesting because sulfane sulfur can modify cysteine residues in proteins *via* an S-Sulfhydration reaction to produce protein persulfides. This process is seen as one of the ways of covalent modification of proteins that change their activity. Sulfane sulfur is also regarded as a form of hydrogen sulfide (H_2S) storage (Iciek *et al*., 2019). Olson and others (Olson *et al*., 2020) indicated that LA increased both H_2S and polysulfide production in HEK293 cells and that this effect was sustained over several days. Zygmunt and others (Zygmunt *et al*., 2013) demonstrated that anti-inflammatory activity of LA in a mouse model of zymosan-induced peritonitis was associated with an increase in the level of sulfane sulfur in the peritoneal exudates.

Attempts to identify the likely mechanism of antimicrobial activity of lipoic acid against *U. urealyticum* and *U. parvum* led us to formulate the following hypothesis: at low concentrations of LA, the bacterial cell uses it only as a cofactor for metabolically active enzymes and LA acts as a growth factor. At higher concentrations of LA, the cell demand for LA to be used in metabolic processes has already been met and the remaining LA affects cellular proteins through the various mechanisms discussed above, which leads to changes in their activity and, as a consequence, to cell death and/or inhibition of cell proliferation. This type of opposing effect of LA on cells depending on its dose has already been noticed, however, the mechanism of these processes remains unknown. However, this is a pilot study. A detailed understanding of the molecular mechanisms of antimicrobial activity of LA requires further research, including *in vivo* testing.

CONCLUSION

In the present study, for the first time we examined bacteriostatic (effects on the cell division) and bactericidal activity of LA against *U. urealyticum* and *U. parvum*. The strongest bacteriostatic and bactericidal effect of LA was observed at a concentration of 1200 μg/ml. In contrast, at lower LA concentrations stimulation of cell division was observed in 14% of the total number of strains tested.

Thus, the current data provide novel findings about potential beneficial, antimicrobial effects of LA. Obviously, more research is needed, especially *in vivo*. The biggest challenge seems to be solving the problem of the correct dosage of LA. This problem is of course extremely important for any drug, but in this case, there is an additional difficulty due to the type of opposing effect of LA on cells depending on its dose, demonstrated by many authors and by us in this work.

The hypothesis linking the antimicrobial activity of LA with its influence on the transformation of sulfur compounds, as well as its participation in protein S-thiolation processes, perceived as a molecular "switch" decisive for intracellular redox status of thiols which controls cellular metabolic pathways, also requires verification.

So, we have a long way to go. Nevertheless, it can be expected that the results of the future studies will allow for the practical use of LA as a medicine supporting the maintenance of normal microbiota of the human urogenital system.

Conflicts of interest

The authors have declared no conflict of interest.

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