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HORMONES CAN INFLUENCE ANTIBIOTIC SUSCEPTIBILITIES EVEN IN MONO- AND CO-CULTURE CONDITIONS

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Abstract: *Pseudomonas aeruginosa* and *Staphylococcus aureus* are known as important nosocomial infectious agents also their co-infections are commonly seen in some patient groups. It is well known that host factors such as hormones have roles in modulation of growth, pathogenesis and susceptibilities to antimicrobials. In our study, the influences of norepinephrine (NE) and melatonin (MEL) on antibiotic susceptibilities were examined in mono and co-culture conditions. Methicilin resistant *Staphylococcus aureus (*MRSA) ATCC 43300 and *Pseudomonas aeruginosa* ATCC 27853 were investigated to determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of ciprofloxacin and gentamicin in the absence/presence of NE (0.0017 and 0.04µg/mL) and MEL (6 and 60 pg/mL) by microdilution method in mono and co-culture. It was found that hormones decreased (among 2-64 fold) MIC and MBC values of both antibiotics for MRSA. However, it was shown that hormones had no effect on MIC values of both antibiotics for *P. aeruginosa*. MIC and MBC values of both antibiotics for co-culture were found to be reduced compared to monoculture of MRSA; were found to be increased compared to monoculture of *P. aeruginosa*. Whereas, hormones decreased MIC values of both antibiotics in co-culture conditions. Our results suggest that both hormones decreased MIC values and it seems that hormones could influence antibiotic susceptibilities in a strain-dependent manner.

Keywords: *Pseudomonas aeruginosa* and *Staphylococcus aureus* mono and co-culture, norepinephrine, melatonin, MIC, MBC, gentamicin, ciprofloxacin, antibiotic susceptibility

1. Introduction

It is well known that methicilin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* are the most common hospital-acquired infectious agents; and their co-infections are very prevalent and harmful in some clinical outcomes such as cystic fibrosis, chronic wound infections, ulcers, surgical site infections (Dalton et al., 2011; DeLeon et al., 2014; Murray et al., 2014; Nguyen and Oglesby-Sherrouse, 2016; Briaud et al., 2020b). In recent years, there are many studies focusing on alterations of biological properties of *P. aeruginosa* and *S. aureus* when they are co-existed (Yang et al., 2011; Kim et al., 2015; Ping et al., 2017; Alves et al., 2018; Kahl, 2018; Wijesinghe et al., 2019; Briaud et al., 2020b; Yung et al., 2021); also for treatment of these complex infections, development of some alternative treatment

strategies are gained importance (Kahl, 2018; Yung et al., 2021).

On the other hand, many studies have shown the effects of host hormones, as environmental factors in the host body, on bacterial physiology during the infectious processes (Plotkin and Viselli, 2000; Plotkin et al., 2003; Lyte Mark, 2010; Lyte, Mark, Cryan, 2014; Gumus et al., 2017; Gümüş et al., 2019; Gonçalves et al., 2020; Boukerb et al., 2021; Engelsöy et al., 2021). These studies highlighted a new concept which is known as microbial endocrinology, a bi-directional interaction between host and microbe through interkingdom signaling based on neuroendocrine factors of human and sensor molecules of microorganisms (Lyte Mark, 2010; Lyte, Mark, Cryan, 2014). According to previous studies, microorganisms sense and respond to hormones and regulate their self-growth, virulence, antimicrobial susceptibilities (Freestone et al., 2007; Fteita et al., 2014; Sandrini et al., 2014; Yang et al., 2014; Gumus et al., 2017; Gümüş et al., 2019; Truccollo et al., 2020; Engelsöy et al., 2021; Lyte et al., 2021).

From past to present, most of these studies focused on the interactions between norepinephrine and microorganisms (Belay et al., 2003; Bansal et al., 2007; Freestone et al., 2007; Lyte Mark, 2010; Yang et al., 2014; Lyte, Mark, Cryan, 2014; Sandrini et al., 2014; Xu et al., 2015; Bearson, 2016; Boyanova, 2017; Gümüş et al., 2019; Truccollo et al., 2020; Lucca et al., 2020). It has been reported that norepinephrine affects the behaviors of microorganisms via regulating iron accessing and/or triggers of norepinephrine-induced auto-inducers production (Freestone et al., 2007; Li W, Lyte M, Freestone PP, Ajmal A, Colmer-Hamood J, 2009; Lyte Mark, 2010; Lyte, Mark, Cryan, 2014; Sandrini et al., 2014).

Another mammalian hormone melatonin, have a capacity of binding some metal molecules, such as iron, zinc and copper; it was hypothesized that in this way, melatonin influences microorganism' cytoplasmic regulation (Zhou et al., 2016; Liu et al., 2020; He et al., 2021).

In the present study, we aimed to investigate the roles of norepinephrine (NE) and melatonin (MEL) on antimicrobial susceptibilities of methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* in single and co-culture conditions.

2. Materials and Methods

2.1 Bacteria

We investigated the susceptibilities of MRSA ATCC 43300 and *P. aeruginosa* ATCC 27853 to ciprofloxacin (CIP) and gentamicin (GN) in the presence/absence of NE and MEL using the microdilution method.

2.2 Hormones

We examined two concentrations of norepinephrine (**LNE:** 0.0017 and **HNE:** 0.04μg/mL)] and melatonin (**LMEL:** 6 and **HMEL:** 60 pg/mL) according to their physiological blood levels.

2.3 Antibiotics, Determination of Minimum Inhibition Concentrations (MIC)

MRSA and *P. aeruginosa* were grown in Mueller Hinton broth at 37 °C for 24 hours to provide overnight culture. The initial concentrations of bacteria were arranged to approximately 10^7 CFU/mL. Minimum inhibition concentrations were determined by microdilution method. To obtain a co-culture condition, MRSA and *P. aeruginosa* were added into the same well simultaneously.

Two-fold serial concentrations were prepared between 2.5- 1280 μg/mL for ciprofloxacin and gentamicin in 96-well plates. (CLSI, 2021) CIP and GN were selected

because they are broad-spectrum antibiotics and used widely.

Hormones (20 µL) and bacteria (as co-cultured or separately) (20 µL) were added to wells. The plates were incubated at 37 °C for 24 hours to examine whether the presence of hormones could influence the MIC values. MIC values were considered as the lowest concentration of the antimicrobial agent which inhibited the growth of the microorganism. The experiments were repeated three times independently and all conditions were analyzed twice.

2.4 The Determination of Minimum Bactericidal Concentrations (MBC)

After determining MIC values, 10µl of the contents was aspirated from the wells which showed no growth according to MIC results and inoculated on Mueller Hinton Agar. The plates were incubated at 37 °C for 24 hours. The plates were examined for the presence/absence of the growth of the bacteria. The MBC values were determined as the lowest concentration of the antimicrobial agent which inhibits the growth of bacteria.

3. Results

In our study, the effects of hormones on MIC values of ciprofloxacin and gentamicin for MRSA and *P. aeruginosa* were determined separately and together.

MIC and MBC values of ciprofloxacin and gentamicin for MRSA were 320 and 160 µg/ml, respectively without hormones. As shown in **Table 1**, hormones decreased the MIC values of both ciprofloxacin and gentamicin for MRSA strain. The alterations of MIC values were almost 2-64 fold. Although MBC values of gentamicin were found to be reduced in the presence of hormones, ciprofloxacin did not change except for high concentrations of melatonin.

Table 1. MIC and MBC values of ciprofloxacin and gentamicin (µg/ml) for MRSA with and without hormones

Antibiotics	without hormones	HNE	HMEL	LNE	LMEL
		MIC values			
Ciprofloxacin	320	160	80	160	80
Gentamicin	160	≤2.5	≤2.5	≤2.5	≤2.5
		MBC values			
Ciprofloxacin	320	320	160	320	320
Gentamicin	160	≤2.5	20	10	≤2.5

HNE: high norepinephrine, **LNE:** low norepinephrine, **HMEL:** high melatonin, **LMEL:** low melatonin

Table 2. MIC and MBC values of ciprofloxacin and gentamicin (µg/ml) for *P. aeruginosa* with and without hormones

Antibiotics	without hormones	HNE	HMEL	LNE	LMEL
		MIC values			
Ciprofloxacin	2.5	2.5	2.5	2.5	2.5
Gentamicin	2.5	2.5	2.5	2.5	2.5
		MBC values			
Ciprofloxacin	2.5	2.5	2.5	5	2.5
Gentamicin	2.5	2.5	2.5	5	2.5

HNE: high norepinephrine, **LNE:** low norepinephrine, **HMEL:** high melatonin, **LMEL:** low melatonin

Table 3. MIC and MBC values of ciprofloxacin and gentamicin (µg/ml) for MRSA+ *P. aeruginosa* in co-culture conditions with and without hormones

Antibiotics	without hormones	HNE	HMEL	LNE	LMEL
			MIC values		
Ciprofloxacin	20	10	2.5	5	2.5
Gentamicin	40	2.5	2.5	2.5	2.5
			MBC values		
Ciprofloxacin	40	160	2.5	40	40
Gentamicin	40	2.5	2.5	5	5

HNE: high norepinephrine, **LNE:** low norepinephrine, **HMEL:** high melatonin, **LMEL:** low melatonin

MIC and MBC values of ciprofloxacin and gentamicin for *P. aeruginosa* were determined as 2.5 µg/ml without hormones. No alterations were found in MIC and MBC values of ciprofloxacin and gentamicin. On the other hand, the presence of norepinephrine at low concentration was found to be 2-fold increased the MBC values of both antibiotics (**Table 2**).

In our study, we also examined MIC and MBC values of antibiotics in co-culture conditions with and without hormones. In the absence of hormones, MIC values of ciprofloxacin and gentamicin were determined as 20 and 40 µg/ml, respectively; MBC values of both antibiotics were determined as 40 µg/ml. Furthermore, it was found that hormones decreased (2-16 fold) MIC values of both antibiotics and MBC value of gentamicin was found to be reduced (8-16 fold) in the presence of hormones, in co-culture conditions. Whereas high melatonin decreased MIC value of ciprofloxacin, high norepinephrine increased it. There was no alteration of MIC values of ciprofloxacin in the presence of low hormone concentrations (**Table 3**).

As clearly seen in **Table 1** and **Table 2**, MIC and MBC values of antibiotics for monoculture were found to be changed compared to co-culture conditions. MIC values of ciprofloxacin and gentamicin (320 and 160 µg/ml, respectively) for *S. aureus*-monoculture were found to be reduced (ciprofloxacin and gentamicin were 20 and 40 µg/ml) compared to co-culture conditions. Similarly, MBC values of both antibiotics (ciprofloxacin and gentamicin were 320 and 160 µg/ml) decreased for *S. aureus* strain compared to co-cultures' MBC values (ciprofloxacin and gentamicin were 40 µg/ml) (**Table 1**). On the other hand, it was found that MIC and MBC values of ciprofloxacin and gentamicin (2.5 µg/ml) for monocultures of *P. aeruginosa* were found to be increased compared to co-culture conditions (**Table 2**).

4. Discussions

Lyte and Ernst introduced the concept of microbial endocrinology, a bi-directional interaction between host and microbe through interkingdom signaling based on neuroendocrine factors of human and sensor molecules of microorganisms which have coexisted and known each other for millions of years (Lyte M and Ernst S., 1992; Sharaff and Freestone, 2011). Since then, many studies have shown the effects of host hormones, as environmental factors in the host body, on bacterial physiology during the infectious processes (Kornman and Loesche, 1982; Plotkin and Viselli, 2000; Lyte Mark, 2010; Alves et al., 2014; Gumus et al., 2017; Gümüş et al., 2019; Truccollo et al., 2020; Vidaillac et al., 2020; Engelsöy et al., 2021).

Beyond many physiological features of bacteria, antibiotic susceptibility can also be modulated by hormones helping bacteria for

better growth recovery following antibiotic treatment. Freestone et al., 2012 have indicated that *P. aeruginosa* exposed to sub-inhibitory concentrations of tobramycin in serum-SAPI medium still growth when NE was added. This effect has also been observed in *S. epidermidis* (Freestone et al., 2016). Ambrose et al., 2018 concluded that NE in combination with levofloxacin increased the rate of *Escherichia coli* replication provides an opportunity to enhance the bactericidal effect. On the contrary, it was reported that NE markedly decreased antibacterial activity of tigecycline against multidrug-resistant *Acinetobacter baumanii* strain, but had only a slight effect on the activity of colistin (Inaba et al., 2016).

A mammalian hormone, melatonin (MEL) plays roles as antioxidant, anti-inflammatory, and immunomodulatory; there is a limited number of studies about the possible effects of MEL on microbial behaviors (Tekbas et al., 2008; Bishayi et al., 2016; Zhou et al., 2016; Lee et al., 2018, 2020; Chen et al., 2019; Ganganna et al., 2021). Wiid et al., 1999 suggested that for the treatment of tuberculosis, dosing isoniazid simultaneous with MEL provides at least a threefold increase in the efficacy of the drug *in-vitro*. Kilinçel et al., 2019 observed similar results for antifungals against Candida species; they have shown that MEL in combination with antifungals reduces the MIC values of antifungals. Liu et al., 2020 determined that MEL exhibits synergistic activity with colistin against resistant pathogens both *in-vitro* and *in-vivo*.

Consistent with earlier studies as mentioned above, in our study, we found that NE and MEL, each at two different concentrations, decreased MIC values of ciprofloxacin and gentamicin, and decreased MBC value of gentamicin for MRSA strain. However, MBC value of ciprofloxacin was found to be reduced in the presence of only MEL at high concentration.

For *P. aeruginosa,* there wasn't any difference in antibiotic's MICs. For MBC of both antibiotics, only an increasing was found to be detected in the presence of NE at low concentration. Considering these results, indicating possible effects of hormones on antimicrobial susceptibility depend on hormones' concentrations, antibiotics and bacterial species tested.

MRSA and *P. aeruginosa* are commonly identified as causative agents of polymicrobial infections. Antibacterial treatment of polymicrobial infections can be a challenge, thus there are many studies investigating microbial behaviors and interactions during the polymicrobial infectious processes of *S. aureus* and *P. aeruginosa* (Beaudoin et al., 2017; Orazi and O'Toole, 2017; Radlinski et al., 2017; Tognon et al., 2017; Alves et al., 2018; Kahl, 2018; Briaud et al., 2019, 2020a; Orazi G, Jean-Pierre F, 2020; Yung et al., 2021; Camus et al., 2021).

Although the pathogens are studied in pure culture, it is well known that microorganisms within diverse communities are actively responding to each other. These interactions between species can affect pathogenic behaviors such as virulence, biofilm formation and antibiotic tolerance (Korgaonkar et al., 2013; Vega et al., 2013; Briaud et al., 2019, 2020b). Therefore, our study was carried out to assess antibiotic susceptibilities of MRSA and *P. aeruginosa* against ciprofloxacin and gentamicin also in co-culture conditions. Whereas, MIC and MBC values of both antibiotics for co-culture were found to be reduced compared to monoculture of MRSA; were found to be increased compared to monoculture of *P. aeruginosa*.

There are many studies examining possible mechanisms of alterations in antibiotic susceptibilities in microbial co-existence (Michelsen et al., 2014; Beaudoin et al., 2017; Radlinski et al., 2017; Briaud et al., 2019;

Trizna et al., 2020; Dehbashi et al., 2021). In such a study, it was shown that antibiotic resistance increased via composing polymicrobial biofilm (Beaudoin et al., 2017). In another study, it was found that *S. aureus* co-cultured with *P. aeruginosa* in a wound-like medium, had higher tolerance to tetracycline and gentamicin compared to its single species culture but tolerance to ciprofloxacin was reported to be not changed. Besides, the antibiotic tolerance of *P. aeruginosa* was not changed (DeLeon et al., 2014). Although has not been clarified the specific mechanism, some authors suggested that excreted enzymes of *P. aeruginosa* could produce several aminoglycoside-modifying enzymes (Poole, 2005; DeLeon et al., 2014), which could have inactivated the gentamicin in the co-culture which protects both species.

All these kinds of studies suggest that today, to manage antibiotic resistance, a global health concern, inter-kingdom interactions can be used as a target for new antibacterial therapeutics.

It is still unclear whether NE and MEL have some effects on antibiotic susceptibilities of co-cultured microorganisms. To our knowledge, this is the first report aimed to investigate the effects of hormones on microbial behaviors in co-culture conditions. We found that hormones decreased MIC values when bacteria were grown together. While MBC values of both antibiotics were reduced in the presence of MEL at high concentrations, MBC value of ciprofloxacin was increased in the presence of a high level of NE. On the other hand, low levels of hormones did not affect MBC values of ciprofloxacin. All hormones also decreased MBC values of gentamicin.

Conclusions

In conclusion, our study clearly indicated that, NE and MEL affect antibiotic susceptibilities of MRSA and *P. aeruginosa* strains in mono and co-culture. These possible effects of hormones on bacterial susceptibilities are needed to be investigated furtherly, especially which could be useful for developing new approaches for the treatment of infectious diseases.

Conflict of interest

The authors declare that there are no conflicts of interest related to this article.

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