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TITLE. Biosynthesized silver nanoparticles: decoding their mechanism of action in *Staphylococcus aureus* and *Escherichia coli*.

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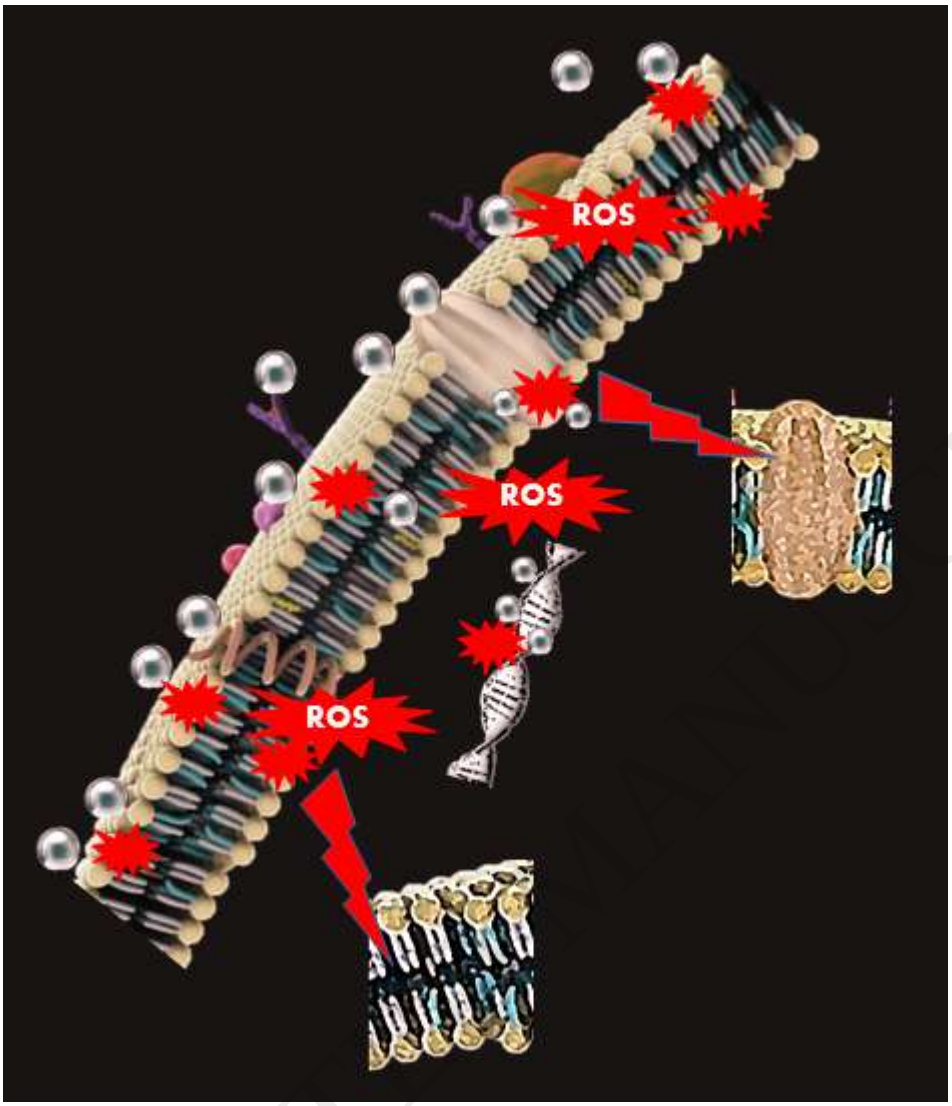
Graphical Abstract



ABSTRACT

The oxidative stress generation in bacteria by the presence of antibiotics (in this case silver nanoparticles (AgNPs)) is already widely known. Previously, we demonstrated that AgNPs generate oxidative stress in *Staphylococcus aureus* and *Escherichia coli* mediated by the increase of reactive oxygen species (ROS). In this work we are demonstrating the consequences of the oxidative stress by the presence of AgNPs; these bacterial strains increased the levels of oxidized proteins and lipids. In addition, it was possible to determine

which reactive oxygen species are mainly responsible for the oxidative damage to macromolecules. Also, we found that the bacterial DNA was fragmented and the membrane potential was modified. This increase in the levels of ROS found in both, *S. aureus* and *E. coli*, was associated with the oxidation of different types of important macromolecules for the normal functioning of cell, so the oxidative stress would be one of the mechanisms by which the AgNPs would exert their toxicity in both strains, one Gram positive and the other Gram negative of great clinical relevance.

[1]**ABBREVIATIONS:**8-OHdG: 8-hydroxy-2′-deoxyguanosine, AgNPs: silver nanoparticles, ANS: 1-anilino-8-naphthalene sulphonate, AOPP: Advanced Oxidation Protein Products, BIP: 2,2'-bipyridyl , CAT: catalase enzyme, dG: deoxyguanosine, GSH: glutathione, MANN: mannitol, MDA: Malondialdehyde, MHB: Mueller Hinton Broth, ROS: reactive oxygen species[1]

Keywords. Oxidative stress, bacteria, silver nanoparticles, macromolecules oxidation.[1]

1. INTRODUCTION

The great progress that has been made in nanotechnology in the last 10 years, has led to a big progress in different fields of study. We can find innumerable studies on the antimicrobial activity of AgNPs in different bacterial genera [(1-4)] in the medicine field but the mechanism by which they exert their action has a long way to investigate. Duran *et al*.[(5)] resume the latest research in the subject, where different mechanisms are proposed, such as the interaction between the plasma membrane and their rupture, interference on the

respiratory chain, free radical generation, oxidation of essential cellular components, among others.

It is already well known that oxidative stress is involved in the toxicity of different antibiotics [6, 7]. It has been seen how different antimicrobials of clinical use produced oxidative stress in different bacterial genera, with the consequent oxidation of essential macromolecules and cell death [1-5, 8]. Determination of the oxidation of macromolecules such as lipids, proteins and DNA is a feasible strategy that allows us to corroborate whether oxidative stress is involved in the antibacterial activity.

It was documented how the membrane potential in *Staphylococcus aureus* was modified in presence of antibiotics that generated oxidative stress, using the probe 1-anilino-8-naphthalene sulphonate (ANS), so it can also be applied as a technique to corroborate the oxidative stress generation[9,10]. Another strategy is the use of ROS scavengers to determine which species is the most involved in the generation or damage caused by ROS. The ROS scavengers can act upon a specific species or on the total of species generated. Tiron is a physical sequestering agent of anion superoxide ($O_2^{\cdot-}$), 2,2'-bipyridyl (BIP) is a chelating agent of Fe^{2+} (in the Fenton reaction), it indirectly inhibits hydroxyl radical (HO•). Mannitol (MANN) is a specific chemical sequestering agent of HO• and the catalase enzyme (CAT) participates in the catalysis of hydrogen peroxide (H_2O_2). Finally, Glutathione (GSH) is a tripeptide that acts as a general redox ROS scavenger [11,12,13].

We observed in previous results that biosynthesized AgNPs that shown antibacterial activity, generated an increase of ROS and reactive nitrogen intermediates (RNI) in *S. aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, being responsible for their toxicity and bacterial death [14]. The aim of this work was analyze the damage of oxidative stress generated

by AgNPs over macromolecules and membrane potential in bacteria, to evaluate AgNPs mechanism of toxicity. According to the above mentioned, we set out to delve into the mechanism of action of AgNPs, by determining markers of oxidative damage, such as proteins, lipids and DNA oxidation and changes in membrane potential, in a Gram positive and Gram negative bacterial gender.

2. MATERIALS AND METHODS

2.1. *Chemicals and reagents*: Luria Bertani broth and the probe ANS from MP Biomedicals (France), silver nitrate ($AgNO_3$), potassium iodide (KI), Chloramine T, Phosphate Buffer Solution (PBS) from Ciccarelli (Argentina), Mueller Hinton broth from Britania (Argentina), Trichloroacetic acid from BioPack (Argentina), thiobarbituric acid, Tiron, GSH, BIP, CAT, MANN, and 8-hydroxy-2′-deoxyguanosine (8-OHdG, ≥ 98%) and deoxyguanosine (dG, ≥ 99%) were all purchased from Sigma-Aldrich (Argentina).

2.2. *Bacterial strains: S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were used for the assays. The strains were grown aerobically in MHB for 24 h at 37°C.

2.3. *Biosynthesis of AgNPs*. The AgNPs were prepared according to the described by Quinteros *et al*. [15]. An overnight culture of *P. aeruginosa* was centrifuged and obtained the supernatant. An $AgNO_3$ solution (10 mM) was added to the supernatant and incubated for 24 h with constant stirring at 37 °C. The AgNPs obtained were characterized by spectrophotometry (on a Shimadzu UV-vis spectrophotometer), transmission electron microscopy (TEM) (JEM-JEOL1120 EXII model microscope operating at 80 kV) and dynamic light scattering (DLS) Delsa Nano C instrument (Beckman Coulter) [16].

2.4. *Advanced Oxidation Protein Products* (AOPP). AOPP levels were determined by spectrophotometric method, which uses the Chloramine T as a witness of the positive control,

which in presence of KI, absorbed at 340 nm. The initial inoculum work was contacted with 0.5 mL of AgNPs (3, 7.5 and 15 pM) or phosphate saline buffer (PBS -pH 7) as control without AgNPs. In a previous study we determined that the maximum ROS generated by AgNPs for *S. aureus* and *E. coli* was 1 and 4 h, respectively [(14)]. From these data, in this case, the samples were taken at different times 0, 1, 4 and 24 h. To each sample 50 µL of KI 1.16 M and 50 µL of acetic acid were added and was measured at 340 nm by spectrophotometric [(17)]. The concentrations of AOPP were expressed as chloramine-Tequivalents per mg of proteins [(18)].

2.5. *Lipid Peroxidation*. Overnight cultures were centrifuged and the pellets were resuspended in PBS buffer (pH 7). 0.5 mL of inoculum was incubated with 0.5 mL of AgNPs (3, 7.5 and 15 pM) or PBS as control. For *S. aureus,* the samples were taken at 0, 1 and 24 h of contact, and 0, 4 and 24 h for *E. coli*. To each sample, 1 mL trichloroacetic acid 35% w/v was added and incubated at room temperature for 20 minutes in darkness. Then, 1 mL of thiobarbituric acid was added and incubated at 80° C for 30 minutes. After cooling in an ice bath, the samples were centrifuged and the absorbance of the supernatant were measured at 535 nm by spectrophotometry. The results were expressed as nM of MDA per mg of protein [(8)].

2.6. *Membrane potential*. Modifications in the membrane potential were determined by measuring the fluorescence of ANS probe [(9)]. The overnight cultures of studied strains were centrifuged and resuspended in the PBS buffer. The inoculum obtained (0.5 mL) was incubated with 0.5 mL of AgNPs (15 pM) or PBS (control sample). According to previous studies, samples were taken at different times for *E. coli* (0, 4 and 24 h) and for *S. aureus* (0, 1 and 24 h) [(11)]. Then, 40 µL of probe (60µM) was added to each sample and incubated for 20 minutes at room temperature. The fluorescence was measured with a spectrofluorometric

(Synergy HT Multi-Mode Microplate Reader - Biotek Instruments, Inc.) with the excitation and emission wavelengths at 360 and 520 nm, respectively. The results were expressed as fluorescence per mg of protein [19].

2.7. *DNA oxidation*. Overnight culture (0.5 mL) were incubated with 0.5 mL AgNPs (15 pM) or PBS buffer as control sample (pH 7) at 37 °C, for 2 h with stirring. DNA extraction was performed using the EasyPure bacterial genomic DNA kit following the manufacturer's instructions [20]. Oxidation of nucleoside dG was determined through the ratio of areas of corresponding peaks attributed to dG or 8-OHdG. Levels of dG and 8-OHdG were quantified by using standards of non-oxidized and oxidized nucleoside, respectively [21].The HPLC system (Jasco chromatograph), consisting of a quaternary pump (PU-2089s Plus), equipped with a Jasco Multiple Wavelenght detector (Jasco UV-2077 Plus) set at 254 nm. Chromatographic separations were performed on a C18 Restek (25 cm x 4.5 mm x 5 µm) column and a guard column was also used, Phenomenex Security Guar Fusion® RP (4 x 30 mm), both thermostatized at 30 ºC. Chromatographic analyses were performed with the mobile phase consisting of citrate buffer in water (pH = 5.1): Methanol HPLC grade (93:7, v/v) mixture at a flow rate of 1 mL/min with 20 µL injection volumes being applied. Retention times were 9.6 min and 13.2 min from dG and 8-OHdG, respectively. The total run time was 15 minutes.

2.8. Interaction nanoparticles-DNA. In order to analyze the interaction between the bacterial DNA and the biosynthesized nanoparticles, variations in the DNA spectrum were observed when the AgNPs were added. The absorbance of DNA was measured between 0.1 and 1 (5 – 50 µg/mL of DNA double strain) in a Shimadzu UV-vis spectrophotometer. First, *E. coli* 25922 DNA was extracted using the EasyPure® bacterial genomic DNA kit following the manufacturer's instructions [20]. To determine the peak corresponding to the *E. coli* DNA,

200 µL of DNA extract was taken (initial sample, 20 µg/mL final concentration) and measured spectrophotometrically, between 200 and 550 nm. Then, 20 additions of 5 µL AgNPs (15 pM final concentration) or PBS buffer (control sample, pH 7) were made consecutively [14]. Each sample was measured spectrophotometrically to observe changes in the peak to bacterial DNA [22, 23].

2.9. *Statistical analysis*. The assays were performed in triplicate. Data were expressed as means ± SD and analyzed by the Student's t-test. **P*<0.5, ***P*<0.05, ****P*<0.005 were used as the levels of statistical significance.

3. RESULTS

3.1. Synthesis and Characterization of AgNPs. The formation of AgNPs was observed by UV-vis spectroscopy. The spectral curve was performed between 200 and 1000 nm. The characteristic peak of AgNPs was obtained at 400 nm (Figure 1A). TEM images show AgNPs with uniform size (approximately 40 nm) and pseudoespheroidal form (Figure 1B). These data were confirmed by means of DLS.

3.2. Advanced Oxidation Protein Products (AOPP). Three concentrations of AgNPs were evaluated at different times and changes in AOPP levels were analyzed. For both strains, AOPP levels were AgNPs concentration dependents (P<0,005) and the maximum AOPP values were observed at the highest concentration of AgNPs evaluated (15 pM) (P<0,005) (Figure 2 A y B). Catalase, GSH and MANN addition produced a reduction in 29%, 50% and 75% in AOPP values for *E. coli* at the time of maximum generation of ROS. For *S. aureus*, it was found that the addition of BIP and CAT reduced the protein oxidation in 35% and 30%, respectively, at the time of maximum generation of ROS (Supplementary data).

3.3. Lipid Peroxidation. Lipid peroxidation was evaluated by determining the concentration of MDA at different concentrations of AgNPs and different times. It was

observed that *E. coli* MDA values were concentration dependent (P<0.005). The maximum value of MDA was found for the highest concentration of AgNPs at 24 h of contact (P<0.005) (Figure 2 C). For *S. aureus,* the concentration of MDA was significantly higher after 24 h of treatment with high concentrations of nanoparticles (15 pM) (P<0.005), whereas no differences were observed between lower concentrations of nanoparticles and the untreated sample (Figure 2 D). The addition of Tiron and GSH, reduced MDA values in *E. coli* in 32% and 47%, in the maximum time of ROS generation (4 h) respect to the control without scavengers. The lipids oxidation was reduced in 46%, 36% and 22% with BIP, CAT and MANN respectively, in *S. aureus*, at the maximum level of ROS (1h) (Supplementary data).

3.4. Membrane potential. In contrast to Gram positive strain, it was found that *E. coli* values of fluorescence increased in comparison with the control without AgNPs. The membrane modifications profiles obtained for both, treated and control samples, were similar. A decrease in the fluorescence levels at 4 h, and an increase at 24 h of contact between AgNPs and *E. coli* cells were observed (Figure 3A). The *S. aureus* strain showed an increase in fluorescence at the maximum generation of ROS. Then, was observed a reduction in the measured fluorescence (Figure 3B).

3.5 DNA oxidation. The quantification of 8-OH-dG inside the cells is the most widely methodology used to evaluate the oxidative damage in the DNA molecule. On the one hand, it has been seen in *E. coli* an increase of 56 % in the level of 8-OH-dG with respect to the control sample. On the other hand, was not observed the peak corresponded to AgNPs, probably because the fragmentation or the destruction of the DNA during assays (Table1).

3.6. *Interaction AgNPs-DNA*. The spectral curve of the DNA between 200 and 800 nm was performed. The ratio between the absorbance measured at 260 and 280 nm,

respectively was 1.8, indicating that DNA extraction was pure, without contaminants like proteins, ARN or others. The first additions of AgNPs (5 µL to 10µL) produced an increase in the absorbance of the DNA (Figure 4A). Then, with the following aggregates (from 15 µL to 130 µL of AgNPs), the spectral curve of DNA changed into another completely different. The peak at 260 nm disappeared. The appearance of two peaks was observed, one small at approximately 250 nm (DNA fragmentation) and another large peak at 290 nm (Figure 4B). Finally, the peak at 250 nm completely disappeared (total DNA destruction) and was observed one peak at wavelengths near 400 nm (Figure 4C).

4. DISCUSSION

The antibacterial activity of AgNPs has been demonstrated in a large number of studies published in recent years [1-4, 24, 25]. However, the exactly mechanism involved in their bacterial toxicity remain unclear. Different ways were proposed, for example, McQuillan *et al*, suggested that the antimicrobial activity of the AgNPs takes place after the dissolution of the cellular membrane in *E. coli* K21,and then the silver ions are responsible for the antibacterial activity[26]. Furthermore, there are a lot of publications that show the formation of ROS as a result of interactions between AgNPs and bacteria [14, 27, 28]. In previous studies, it was found that the AgNPs had antimicrobial activity and generated an increase of ROS in *E. coli*, *S. aureus* and *P. aeruginosa*, leaving to the cells in a state of oxidative stress [15] . In this work, we found that in *E. coli* and *S. aureus* there was oxidative damage in proteins and lipids. Also, the addition of ROS scavengers allowed analyzing which reactive species was responsible for the oxidative damage observed. According to the above, the increase in intracellular ROS levels would be the main responsible for the oxidation of proteins. However, the main difference between both strains was that the increase in protein oxidation

of *E. coli* was gradual during 24 h of contact, with the maximum concentration of AgNPs, and for *S. aureus*, the increase of AOPP was abr1upt during the first hour of contact with AgNPs and after was constant until the end of the assay. At low concentrations of AgNPs, *S. aureus* gradually increases its AOPP levels until getting the maximum at 24 h, while in *E. coli* the values are kept constant over time. In the control sample without NP large amounts of AOPP were quantified, higher than in the treated sample with AgNPs at low concentrations, due to a progressive accumulation in the cells (24 h incubation). An oxidative stimulus (low NP concentrations and long incubation times) produces an incomplete oxidation of the proteins and also an activation of the proteolytic systems of the cells to recover those proteins completely oxidized. However, high concentrations of NPs produce high protein damage by ROS, saturating the proteolytic pathways (samples treated with 7.5 and 15 pM at 0 and 1 h of incubation), so the levels of AOPP are high. Páez et al., demonstrated that the generation of intermediate products of oxidized proteins, such as carbonyls, was related to the generation of AOPP produced by ciprofloxacin (CIP) in *S. aureus*. The AOPP are the products that lead to the regeneration of proteins[17].

As in *S. aureus*, this behavior can be observed in *E. coli* at 4 h of incubation with NPs. The greater stimulus of ROS leads to a greater oxidation of proteins with the consequent degradation by the bacterial proteolysis systems. At 4 hours of contact between *E. coli* and AgNPs, the presence of MANN and CAT reduced the oxidation of the proteins. In *S. aureus*, the addition of BIP and CAT, decrease the AOPP values at the maximum of ROS (1h). These results indicated that probably the HO• was the main species responsible for the oxidative damage observed in both genders.

Respect to lipid peroxidation, we observed that MDA values increase in both strains during 24 h of contact with AgNPs. In *E. coli*, the degree of lipid peroxidation depended on

the incubation time and the concentration of AgNPs, while in *S. aureus,* only observed that the values of MDA increased in the 24 h of contact when the maximum concentration of AgNPs was assayed. This results agrees with Applerot *et al*. [29] where CuO nanoparticles increased MDA values in *E. coli* and *S. aureus*, being more significantly in *E. coli*. The addition of ROS scavengers in *E. coli* produced a decrease in MDA values with GSH and Tiron, in the maximum generation of ROS. In *S. aureus*, the addition of CAT and BIP decreased lipid peroxidation. These results indicated that the main responsible for lipid oxidation were $O_2^{\bullet-}$ and $HO^{\bullet}$ for *E. coli* and *S. aureus*, respectively.

The ANS probe is widely used to observe variations in the potential of the lipid membrane [30].With respect to the effect of AgNPs presence on the membrane potential, in *E. coli* firstly a decrease in the probe fluorescence was observed and then an increase in the fluorescence value at 24 h of incubation with NPs. For *S. aureus,* an inverse fluorescence profile was obtained, there was an increase in the fluorescence in the first hour, and then a decrease in the fluorescence value was observed, until reaching the basal value of the control sample. This observation would indicate that the presence of AgNPs would produce a change in the potential of the membrane. The differential action of AgNPs on both strains may be due to the difference in the membrane composition of Gram positive and Gram negative bacteria. However, there are other parameters to consider, since Páez *et al*.[9] reported that different antibiotics which produced oxidative stress as a mechanism of action, led to a modification in the membrane potential of the sensitive *S. aureus* strain, whereas in the resistant *S. aureus* strain there were not significant changes observed. These results show an integral analysis between ROS generation and oxidative damage in macromolecules. In addition, it was possible to show which is the main species derived from the incomplete reduction of oxygen that produced the damage in lipids and proteins, in both strains.

The DNA damage produced by AgNPs was studied to understand its toxicity in prokaryotic cells [31]. In this work, it was observed oxidation, fragmentation and destruction of DNA bases. It has been described that free radicals can cause oxidative modifications in DNA, including chain breakage and oxidation of its bases[32]. Among oxidized products of DNA damage, 8-OHdG is the most studied because it is easily quantifiable [32].In *E. coli*, it was found that AgNPs produced an increase of 60% in the oxidized base with respect to the control without NPsAg. However, in *S. aureus,* it was not possible to quantify the oxidized and non-oxidized base after incubation with AgNPs. The absence of DNA signal, could indicate the partial or total destruction in *S. aureus* DNA. UV-vis spectroscopy was used to determine DNA fragmentation. It is a simple and useful technique to analyze the interaction of DNA with small molecules [33]. The results obtained in this work are comparable with other studies, where the addition of AgNPs (synthesized from fungi) increased the intensity of DNA peak at 260 nm. This change was produced by the formation of a complex between AgNPs and DNA [34]. Additionally, it is known that the UV-vis spectrum of the guanosine nucleotide can be measured at wavelengths close to 250 nm. So, it is probably that the peak of DNA observed at 250 nm in this work, corresponds to the fragmentation of DNA by the action of AgNPs.

5. CONCLUSION

A biosynthetic product could be obtained with the advantages that this represents, using the culture supernatant of a *P. aeruginosa* reference strain and without the addition of toxic reducing agents. The AgNPs could be characterized by different techniques that confirmed the pseudospheroidal form and uniform distribution. Also, an increase in the levels of oxidized lipids and AOPP was observed in both strains studied. Probably, these changes

produced the modifications in membrane potential of the cells. The damage to the bacterial DNA could be corroborated. It was observed oxidation and fragmentation in the bacterial DNA. Based on these results, cellular damage by the generation of oxidative stress with the consequent oxidation of macromolecules should be considered as the main mechanism of action by which AgNPs exert their antimicrobial activity.

In this way, we make a great contribution with respect to the toxicity produced by AgNPs in bacterial strains of clinical relevance.

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LEGEND TO FIGURES

FIGURE 1. UV-vis spectrum (A) and TEM image (B) of AgNPs biosynthesized.

FIGURE 2. Determination of AOPP and MDA in *S. aureus* ATCC 29213 (A and C) and *E. coli* ATCC 25922 (B and D)incubated with different concentrations of AgNPs or without NPs as the control. **P<0.05, *** P<0.005.

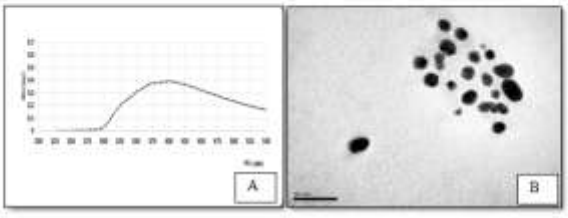
FIGURE 4. Determination of MDA in *S. aureus* ATCC 29213 (A) and *E. coli* ATCC 25922 (B) incubated with different concentrations of AgNPs and without AgNPs as control. **P<0.05, ***P<0.005.

FIGURE 6. Membrane potential determination in *S. aureus* ATCC 29213 (A) and *E. coli* ATCC 25922 (B) incubated with AgNPs and without AgNPs as control.

FIGURE 7.*E. coli* DNA interaction: (A) with AgNPs 5-10 µL, B) with AgNPs 80- 130 µL and C) with AgNPs 150- 210 µL.

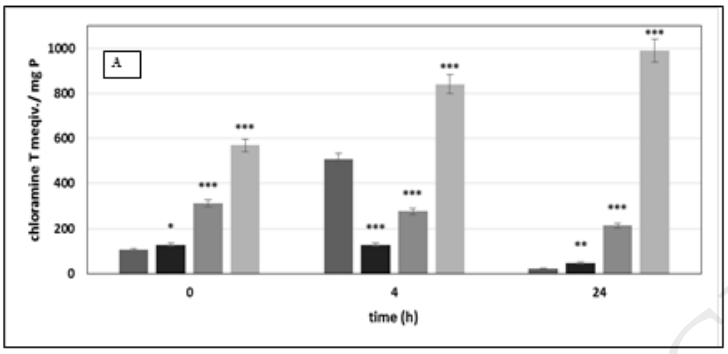
Figr-1

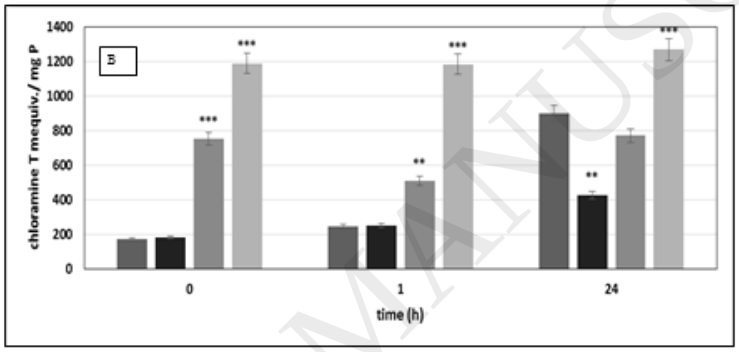
FIGURE 1 Quinteros *et al.*

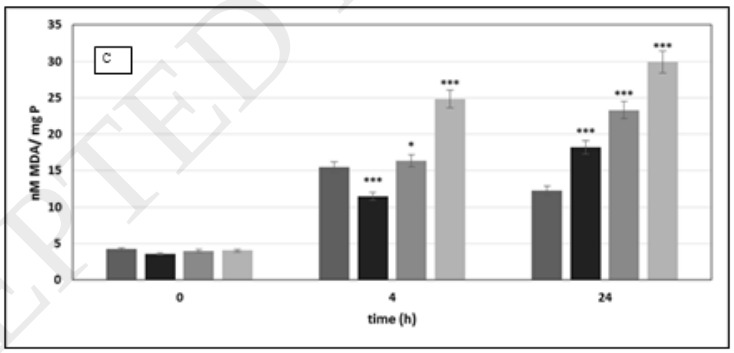


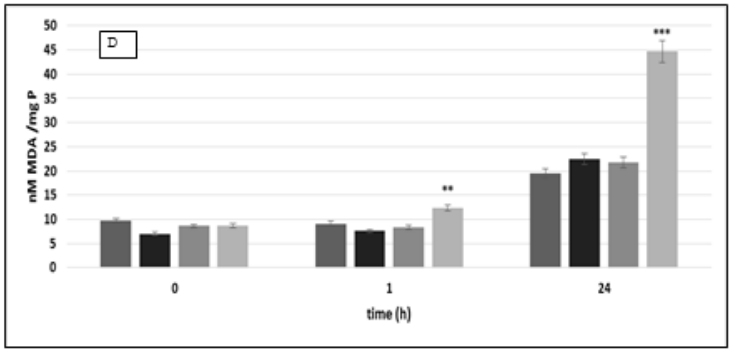
Figr-2

FIGURE 2 Quinteros *et al.*



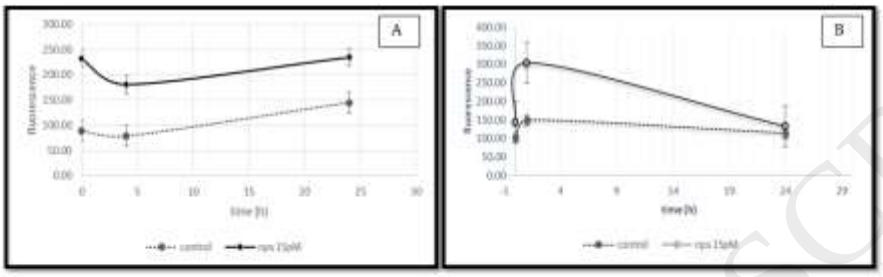






Figr-3

FIGURE 3 Quinteros *et al.*



Figr-4

FIGURE 4 Quinteros *et al.*

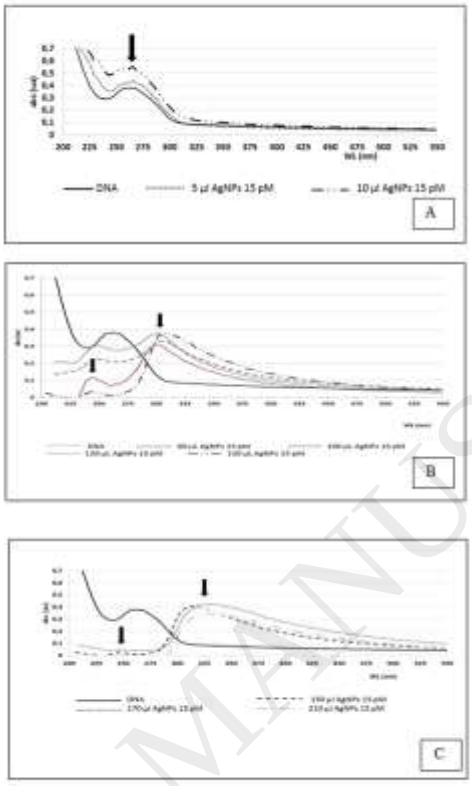


TABLE 1. Relationship between oxidized and non-oxidized nucleoside in *E. coli* and *S. aureus,* with and without AgNPs (15 pM).

	8-OHdG/dG	
	E. coli	*S. aureus*
Control	0,87	0,73
Treated sample	1,39*	-----