WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Colloidal Silver Exposition increases Heme Oxygenase-1 Activity and Oxidative Stress in Human Lymphocytes

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A B S T R A C T

Colloidal silver is used without restrictions to disinfect fruits, vegetables and water for human consumption. However, also canact as a xenobiotic and antigen stimulating chemical defense, immune and reactive oxygen species, which are involved in chronic degenerative diseases. The heme-oxygenase 1 is a stress-induced isoform enzyme thatattenuatesthe toxic effectsof metals.This study analyzes the effect of colloidal silver on cell proliferation, oxidative stress and heme-oxygenase 1 induction in primary human lymphocytes. The human lymphocytes were exposed to colloidal silver (0.036 µg/mL, 0.36 µg/mL and 3.6 µg/mL) during 0.5, 2 and 24 hours. Cell viability was quantified byMTT andtrypan blue assays. Oxidative stress, heme-oxygenase 1, 8-isoprostane and antioxidant status was measurement by colorimetric methods. Human lymphocytes exposed to colloidal silver inhibitedcellular proliferationand increase oxidative stress dependent on concentration- and time-dependents. The increase of 8-isoprostanes were associated with proliferation inhibition and increases of hydroxides and heme oxygenase-1 at 24 h exposition ($p \leqslant 0.0001$). Fe+ levels were associated withincreases of hydroxides, 8-isoprostanes and HO-1 at 0.5 h of treatment. Colloidal silver induce damage at cellular level, causing proliferation inhibition by oxidative stress and lipid oxidation by the presence of 8-isoprostanes and hydroxides. Oxidative stress caused by colloidal silverinduce the heme oxygenase-1 and catalase activities. Our results indicate that oxidative stress caused by colloidal silverinduce the heme oxygenase-1 and catalase activities, these parameters can be useful tools for studying exposure to xenobiotic such as colloidal silver.

Keywords: Oxidative Stress, Heme Oxygenase-1, Colloidal Silver, 8-Isoprostanes

1. Introduction

Colloidal silver (CS) in commercial presentations is used for fruits, vegetables and water disinfection by population. Little information about the toxic effects ofCS within silver nanoparticles (AgNPs), despite its boom, the precise bioaccumulation place, cellular metabolism and how the body removes them are unknown. In this sense, has been reported that AgNPs can be assimilated via inhalation, dermal or oral, and reach cells and different organs and bio accumulating in them(Foldbjerg et al., 2013; Johnston et al., 2010; Saez-Tenorio et al., 2019; Yang et al., 2010). The organs most affected are the liver, intestinal cells, kidneys, heart and spleen and even the brain, depending on the size concentration and load (Avalos, 2013; Coutiño-Rodriguez, 2015; Coutiño-Rodriguez et al., 2017; Johnston et al., 2010; Rogers et al., 2020). The most commons cellular entry pathways are pinocytosis and passive diffusion, even receptor-mediated endocytosis and can be removed via mucus, sweat, fecal excretion and urine, but mainly via the skin (Yang et al., 2010). The main effect of AgNPs is due to its metal quality, which alters electron transport, as well as the functionality of proteins by strong affinity to functional groups such as sulfhydryl groups or thiols, and attack proteins disulfide bonds, like calcium does, as since 1979 it has proposed (Coutiño-Rodriguez, 1979,

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2012). AgNPs is involved in cell regulation, causing genetic damage indirectly, mainly affecting membrane protein, mitotic spindle, chromosomal and essential in cellular metabolism, producing oxidative stress and free radicals, such induce damage to endomembrane system, cellular organelles: mitochondria and lysosomes, and even the nucleus damaging DNA and RNA nucleic acids (Ahamed et al., 2008; Foldbjerg et al., 2013; Guo et al., 2015; Haase et al., 2012a; Haase et al., 2012b; Shi et al., 2014; Yang et al., 2010), this altering and destabilizing cell homeostasis.

The primary target of AgNPs, in eukaryotic cells are the membranes specially of the mitochondria. In this way, they affect transport interruption of electrons mainly in the mitochondrial respiratory chain, which leads to the interruption of ATP synthesis and, thus, they damage their DNA and nuclear DNA, affecting the chromosomal and mitotic stability and inducing the necrotic or apoptotic cell death, by intrinsic pathway, where caspases 8 and 3 are activated (Ahamed et al., 2008; Asharani et al., 2009a; AshaRani et al., 2009b). In lymphocyte culture colloidal silver induces oxidative stress (Haase et al., 2012a; Haase et al., 2012b). Chronic oxidative stress is related with chronic degenerative diseases and accumulated AgNPs in microglial cells of the brain (phagocytic or macrophages), and their management upon reaching the central nervous system (CNS), where their average biological life is also longer than in other organs. Therefore, prolonged exposures constitute risks consequences and a danger to the important physiological functions of the brain(Yang et al., 2010). In *Drosophila melanogaster* exposed to AgNPs are induced thermal shock protein 70 HSP, catalase and superoxide dismutase enzymes (Ahamed et al., 2008; Foldbjerg et al., 2013; Guo et al., 2015; Haase et al., 2012a; Haase et al., 2012b; Shi et al., 2014; Yang et al., 2010), indicating oxidative stress.

In order to monitoring human exposition to xenobiotic, its fundamental the search of enzymatic markers. Heme-oxygenase 1 (HO-1) is a stress-induced isoform enzyme present in the body, their expression is low and only occurs in the spleen for substrate the heme group. HO-1 is induced by a wide variety of stimuli, including medications, metals, ultraviolet radiation, hypoxia, hyperoxia, ischemia, and H2O2, oxidative stress and glutathione depletion. Therefore, the present study analyzes the effect of CS on proliferation, oxidative stress and HO-1 induction on culture of primary human lymphocytes.

2. Methods

2.1. Human subjects and Cell culture

After informed consent, 10 ml of blood was drawn from healthy volunteers of both sexes in evacuated tubes with heparin (Vacutainer; BD Biosciences). All blood draws were performed between 9:00 and 11:00 a.m. to minimize the effects of circadian variation on end points assayed.Lymphocytes were separated by centrifugation at 1500 rpm for 10 min. The lymphocyte layer and the donor serum were used to make the culture. Cells were cultured in McCoy 5A medium supplemented with 10% FCS (heat inactivated), phytohemaglutinin 2 µg / ml for 40 hours, 100 unit /ml penicillin, 100 µg/ml streptomycin, and nonessential amino acids. All media used for isolation and culture were equilibrated to the target oxygen levels at least 12 h before use.Subsequently, several aliquots were taken for the different treatments exposed to different concentrations of colloidal silver (0.036 µg / ml, 0.36µg / ml and 3.6 µg / ml during 0, 0.5, 2 and 24 hrs.

2.2. Cellular viability and cytotoxic assay

Cells viability was quantified by Trypan Blue assay. After washing twice with PBS, cells were stained with 0.4% trypan blue dye (Trypan Blue, Sigma-Aldrich) for 3 min. Cells were observed and counted in a Neubauer chamber under an optical microscope (Nikon), and the viability was calculated as the percentage ratio of the number of unstained cells relative to the total cells counted. Cytotoxic assay was done with seeded at a density of 1×10^5 cells/well in96-well flat-bottommicroplates (Costar, USA). After treatments, MTT (Sigma-Aldrich, St. Louis, MO, USA)was added to each well in 20 µl (5 mg/ml in PBS) and incubatedat 37 °C for 2 h until purple formazan crystal developed.Subsequently, the MTT-containing medium was removed.One hundred microliters of DMSO were added and incubatedat room temperature for 30 min. The formazan absorbancewas measured at a wavelength of 550 nm on an automaticmicroplate reader (Spectra Max 190 Microplate Reader). The optic density(OD) values of the treated cells were compared with thevalues generated from untreated control cells and reported asthe percentage viability for the control.

2.3. Protein quantification

The cellular lysate was centrifuged at 14000 rpm for 15 min at 4 °C. The Bradford method, standardized in microplates, was used. It was read at an absorbance of 595 nm. in a Spectra Max 190 Microplate Reader, where a standard curve of albumin at a concentration of 8 µg / µl was used. Data are expressed in µg/ml or mg/ml of proteins.

2.4. oxidative stress 8-isoprostane assay

This kit uses a polyclonal antibody to 8-Iso PGF2 to bind competitively to the 8-IsoPGF2 present in the sample by covalent binding. The µM 8-Iso were obtained according to the curve made by following the manufacturer's specifications. The data was corrected per mgof proteins and expressed by µg 8-Iso/mg protein.

2.5. Heme-oxygenase-1 assay

Protein quantification was done with the EIA kit to the manufacture specifications. Briefly, the mouse monoclonal antibody specific for HO-1 is pre-coated in plate wells, where HO-1 is captured by the immobilized antibody and is detected using a polyclonal HO-1 rabbit antibody. Subsequently, the polyclonal antibody is bound by a conjugated horseradish peroxidase secondary anti-rabbit IgG antibody. The assay was carried out using the tetramethylbenzidine substrate, which provides a precise blue color to the amount of HO-1 captured and was expressed in ng of HO-1 / mg of proteins. Activity was carried out byIron Quantification assay using the phenanthroline, which measures free iron in the cell (Fe^{+2}). As a control curve to quantify

iron, iron sulfate 400 µgr / mL was used. Data are expressed in mM of iron/mg of proteins. HO-1 activity was measurement by the indirect bilirubin quantification assay in the supernatant of the samples and bilirubin was detected at 450 nm. Data are expressed in mg of BB / mg of protein.

2.6. Catalase activity assay

Catalase was quantified by the Aebi method (Aebi, 1984), which consists in the quantification of hydroperoxides. 0.01 M potassium phosphate buffer at pH 7.4, potassium dichromate, and Tris were used. 3% hydrogen peroxide was used and from this solution the standard curves of 0.2 M, 5mM and 160mM were established at different concentrations All of this was performed in glass tubes and subsequently was transferred to 96-well plates and read at 570 nm. Values were corrected by protein concentration and the data are expressed in µM of peroxides/mg of protein.

2.7. Statistical analysis

Eight independent experiments were performed. Each experiment was conducted three times and expressed as mean ± standard error. For the significant differences, the unpaired two-tailed t-student statistical test was performed on the raw data before converting them into percentages. Likewise, significant differences are expressed using values of *p≤ 0.1, **p≤ 0.05 and ***p≤ 0.001). GraphPad Prism, version 4.00. and SPSS version 18 were the statistical packages used. Data are expressed in graphs in terms of "mean ± standard error". Correlation tests between the variables by Pearson or Spearman correlations were performed.

3. Results

3.1. Colloidal silver inhibited viability and proliferation on primary human lymphocytes

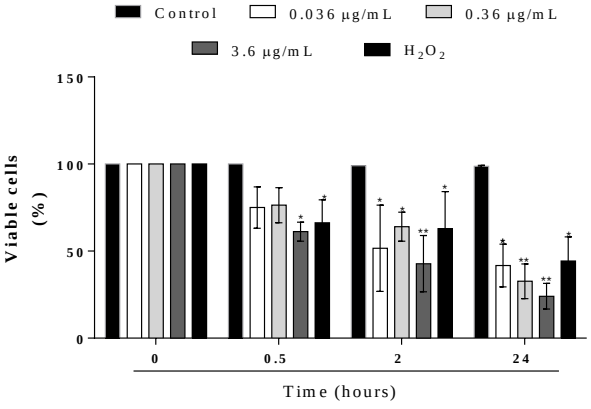

Figure 1A

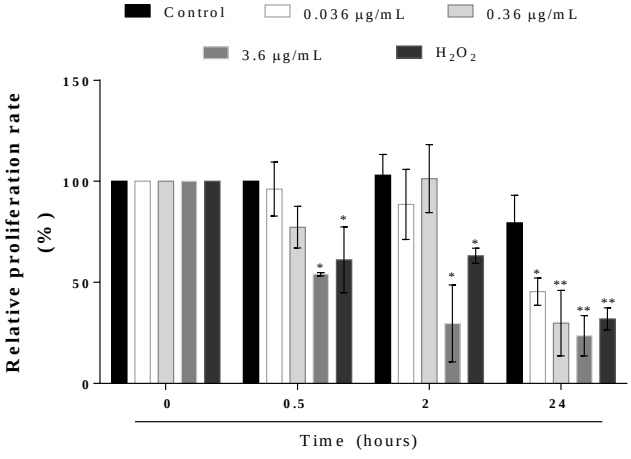

Figure 1 B

Figure 1. Inhibitory effects of colloidal silver on human lymphocytes. A), trypan blue assay; B)MTT proliferation assay. Each assay represents an independent experiment performed in triplicate. Data are presented as the mean ± S.D. n = 6. *P<0.05, **P<0.01 and ***P<0.001 vs control vehicle lymphocytes

In the figure 1A, CS decreases cell viability ($p<0.001$) in all treatment times, with doses of 0.36µg/ml decreased 35% cellular viability ($p<0.002$), and with 3.6µg/ml 65% at 2 h ($p<0.09$). At 24 h of CS exposition, all concentrations significantly decreased upper of 50% of cellular viability ($p<0.0004$). The concentration of 0.36 µg/ml decreased significantly at all times $p<0.05$ vs control cells. In the figure 1B, proliferation reduction was statistically significant at 0.5 h ($p<0.03$), 2 h ($p<0.002$) and 24 h ($p<0.003$) of CS exposition. An increase in mitochondrial activity was observed with 0.036 µg/mL at 0.5 and 2 h of CS exposure. At 24 h of CS exposure proliferation was decreased significantly with 3.6 µg/ ml of CS ($p<0.05$) vs control cells which showed a 20% reduction at 24 h, similar results with trypan blue.

3.2. Colloidal silver effect on oxidative stress and catalase activity on primary human lymphocytes

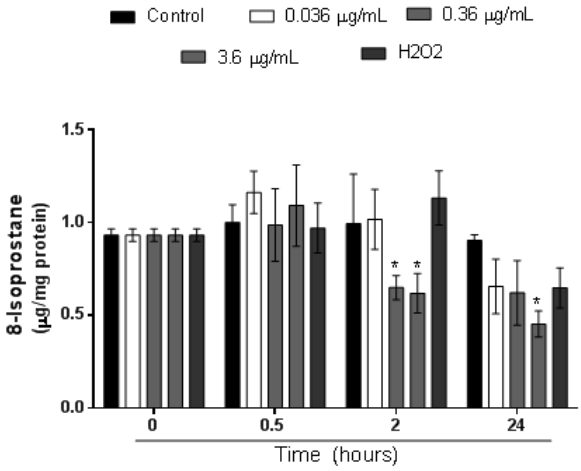

Figure 2A

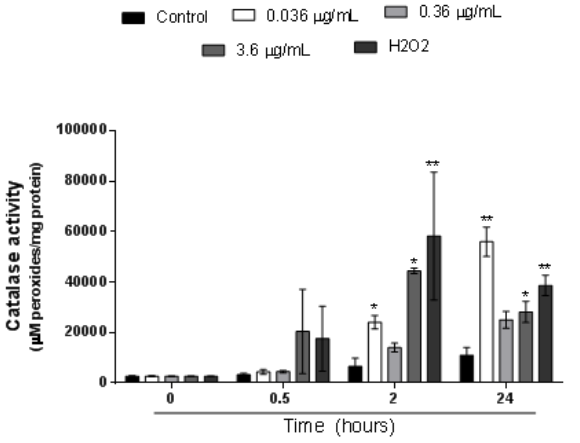

Figure 2B

Figure 2. Effect of colloidal silver in oxidative stress and catalase activity. A), oxidative stress measurement as 8-isoprostane contents; B), Catalase activity. Data are mean ± SD values from five experiments with triplicate determination. *P<0.05, **P<0.01, ***P<0.001 vs control (vehicle) cells.

Oxidative stress was measure by 8-Iso concentration in human lymphocytes exposed to CS, in the figure 2A, showed a no significant slight increase at 0.5 h with all doses of CS. However,with 0.36 and 3.6 µg/mlof CS the 8-Iso concentration decrease after of 2 h treatment ($p<0.01$), until 24 h with 3.6 µg/mL of CS($p <0.004$). The 8-Iso showed a statistical positive association with both MTT and viability assays, (trypan blue R 0.445 $p<0.001$ and MTT R 0.464 $p< 0.001$), respectively. In the figure 2B, catalase activity was induced with3.6 µg/mL doses at 0.5 h, however, at 2 and 24 h a significant considerable increase was detected with all treatments in relation to control cells, been significant with 0.36 and 3.6 µg/ml doses of CS vs control cells ($p<0.001$).

3.3. Effect of colloidal silver on the expression and activity of heme oxygenase-1

In the figure 3A, we show the effect of CS on HO-1 Before and after the different colloidal silver treatments. A similar behavior that of 8-Iso was detected

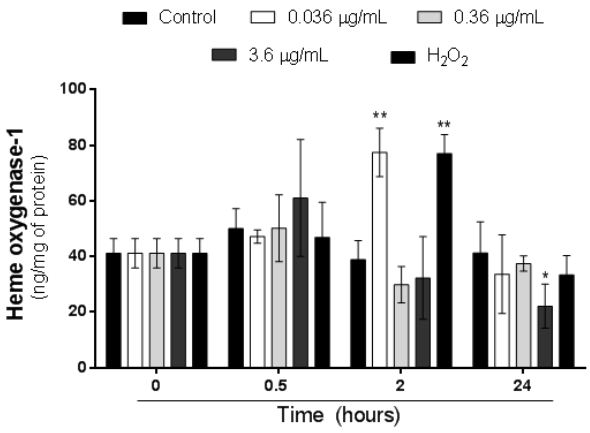

Figure 3A

Figure 3. Effect of colloidal silver in Heme oxygenase-1 activity. Data are mean ± SD values from five experiments with triplicate determination. *P<0.05, **P<0.01, ***P<0.001 vs control (vehicle) cells.

, a mean, at 0.5 h a slight increase was detected (p<0.01 vs control cells), with 0.036 and 3.6 µg /ml doses (p< 0.08). At 2 h exposure, an increase in protein concentration of HO-1 at 0.036 µg / ml (p< 0.02). Although at 3.6 µg/ml of CS decreased HO-1 protein concentration from 2 at 24 h vs controls cells (p< 0.01 at both times).

3.4. Correlation analysis between HO-1 with 8-Iso, iron and catalase activity

Table 1

		HO-1	8-Iso	MTT	Viability	Dose	Time
Catalase activity	Correlation	0.010	-0.238	-0.554**	-0.698**	0.098	0.737**
	p value	0.941	0.067	0.000	0.000	0.456	0.000
HO-1	Correlation		0.878**	0.267*	0.200	-0.210	-0.240
	p value		0.000	0.039	0.125	0.107	0.064
8-Iso	Correlation			0.445**	0.464**	-0.248*	-0.535**
	p value			0.000	0.000	0.056	0.000
MTT (formazan)	Correlation				0.764**	-0.342**	-0.640**
	p value				0.000	0.007	0.000
Cell viability	Correlation					-0.395**	-0.771**
	P value					0.002	0.000

In table 1, we found a strong association between HO-1 with 8-Iso (r = 0.878; p<0.001), p<0.0001) in all used times. In addition, HO-1 was associated with MTT assay(r=0.267; p<0.039). The increase of catalase activity was positively associated with time (r = 0.737; p<0001). On the other hand, catalase activity showed a very high negative association with cell viability (r = -0.698; p<0.001),MTT(r = -0.554; p<0.0001) and marginal with 8-Iso (r = -0. 238; p<0.067). The time show high negative association with MTT (r=-0.640; p<0.001) and viability (-0.771; p<0.001).

4. Discussion

This paper aims to determine the CS damage in cell viability and cellular toxicity on primary human lymphocytes. Our results showed that at different times and concentrations, CS induces oxidative stress and decrease proliferation. It is, therefore, worrisome the undocumented and increased exposure of

the population to derived from silver, including colloidal silver, due to the abuse in human consumption of this compound and its exposure, whose source is unknown(Avalos et al., 2014; Avalos, 2013; Coutiño-Rodriguez, 2015; Coutiño-Rodriguez et al., 2017; Lucerito et al., 2013).

Genotoxic and immune toxic effects of AgNPs associated with oxidative stress have been extensively studied and documented(Ahamed et al., 2008; Asharani et al., 2009a; AshaRani et al., 2009b; Coutiño-Rodriguez, 2015; Coutiño-Rodriguez et al., 2017). However, there is little information about colloidal silver, available in commercial presentations, being used for disinfection of fruits and vegetables and water of human consumption. Our results showed that colloidal silver and AgNPs has similar effects cellular viability and OS induction, mainly at the level of the alteration of plasma membranes, and mitochondria. Thus, an increase and a decrease in mitochondrial activity is observed and similar to that found by other authors in other cellular models with AgNPs(Asharani et al., 2009a; AshaRani et al., 2009b; Sheikpranbabu et al., 2009). The CS effects were time- and concentration- dependent manner, showing a negative association with viability and proliferation, oxidative stress markers as well as with activity of HO-1 and increase of 8-Iso and iron.

Induction of 8-Iso, HO-1 and catalase due to CS effect, only occurred at 0.5 h, with a decrease at 2 and 24 h.. Also, as was expected they show a negative association with viability. The HO-1 activity could regulate negatively the genetic expression of HO-1 and catalase, or increase its degradation and even inhibit its activity. Oxidative stress may destabilize the membranes it appears that iron is the trigger of oxidation damage in the membranes (lipoperoxidation and 8-Iso) CS in lymphocytes increase 8-Iso. Regarding oxidative stress markers, it is the first time thatis found a high statistic correlation between HO-1 induction with the 8-Iso at 0.5h.

These data are very interesting since HO-1 activity was detected in the vast majority or almost all chronic degenerative pathologies (Lucerito et al., 2013) associated with oxidative stress, such as renal failure, and asthma, where an increase in 8-Iso, plasma, saliva and other fluids has been reported. Increased 8-Iso is due to the non-enzymatic and non-specific oxidation of arachidonic, probably by HO-1 activity because of silver. However, the inducible specific oxidation, Cox-2, is probably also induced by the high content of hydroperoxides, and by the decrease in the 8-Iso, because they have a negative correlation, and Cox-2 induction is associated with oxidative stress and inflammatory mediators(Cerella et al., 2010; Sobolewski et al., 2010; Thanan et al., 2012).Perhaps, there is a differential oxidation of arachidonic acid as a function of time and concentration, may be associated with the HO-1 and Iron. First, it may be nonspecific in a short time by producing 8-Iso, related with HO-1, which induce in a long time, the specific enzymatic, by the increase of catalase activity and the decrease of 8-Iso at 2 and 24 h. Cox-2 is also located in the nucleus and associated with the gene expression such as interferon γ, tumor α necrosis factor, interleukin-1, growth factors, etc. mediators related to inflammatory disease, apoptosis and proliferation, although, Cox-2 was not assessed (Cerella et al., 2010; Guo et al., 2015; Thanan et al., 2012).

Correlating analysis with 8-Iso has been reportedpreviously (Lucerito et al., 2013)), in an equal model, regarding the content of TBARS and lipoperoxidation showed a negative association, as well as with catalase activity. This means when TBARS increase and decrease 8-Iso, possibly the initial damage is induction of 8-Iso and HO-1, iron presence, which in turn favors lipoperoxidation. Therefore, an increased production of hydroxides and a decrease of 8-iso that may facilitate an exit of 8-Iso to the medium, or they are involved in Cox-2 induction. Thus, its decrease in the cell is observed. In any case, the initial damage of colloidal silver occurs in lipids of the membrane with production of 8-Iso, HO-1 induction, iron presence and, consequently LPO and hydroxides induction, which agrees with the significant induction at 0.5 h of stress markers (8-Iso, catalase and HO-1), where 8-Iso show particularly a negative correlation as a function of time and concentration, which suggests that at lower concentration, and low time of exposition, 8-Iso increase.

When PC induces an increase inoxidative stress markers, then it represents a serious public health problem, the presence of oxygen reactive species and 8-ISo are the common denominator markers in chronic degenerative non-infectious diseases, such as diabetes, obesity, heart disease, cancer and neurodegenerative diseases, Parkinson's, Alzheimer's, asthma and kidney diseases(Haase et al., 2012b; Shi et al., 2014). We must reflect on the consumption and abuse of colloids and NPs of metals given their interaction and activity with the cells of our organism and provide inform about the reliability of their use, establish control and safety standards in NP derived products, marketed for bactericidal and therapeutic purposes indiscriminately (Avalos et al., 2014; Avalos, 2013; Coutiño-Rodriguez et al., 2017).

5. Conclusion

Oxidative stress caused by colloidal silverinduce the heme oxygenase-1, catalase activities and proliferation inhibition. These parameters can be useful tools for studying exposure to xenobiotic such as colloidal silver.

Acknowledgments

Ávila-Lagunes Lucerito Esmeraldawas supported by graduate of Master of public health from Universidad Veracruzana, fellowshipsfrom CONACyT. This work was submitted in partial fulfillment of the requirements for the Mh.D. degree for Public Health Institute, Universidad Veracruzana.The authors also wish to thank Miguel Laferrele Ortiz for his contribution to this study and proofreading.

Funding

This study had financial support from Public Health Institute POA 2017-19 and CONACyT grant no. CB-2012-01-176513.

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