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Microscopic Observation of the Effect of ZnO Nanoparticles Synthesized from Different Precursors on Eukaryotic and Prokaryotic Cells

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Abstract: ***In this study, the zinc oxide prepared from three different precursors: zinc sulfate heptahydrate, zinc nitrate hexahydrate and zinc acetate dihydrate, has been tested in vitro on different cell types belonging to eukaryotic and prokaryotic: erythrocytes, leukocytes, Candida albicans and Bacillus BS3 who is thermophilic bacteria, sporulating and multiresistant to antibiotics. The effect of ZnO nanoparticles on the above cells was observed directly via optical microscopy. The results show a separate action of each oxide at the various cells studied. Tests conducted on cells of human blood show that ZnO synthesized from the zinc acetate dihydrate is the most hemolytic oxide. It causes a complete hemolysis of erythrocytes and a total alteration of leukocytes. A total inhibition of the growth of Candida albicans was observed in the case of ZnO synthesized from zinc sulfate heptahydrate. Bacterial cells BS3 even if they are spore forming, are multiresistant to antibiotics and high temperatures. They were eradicated by the three aforementioned synthetic oxides losing their shape, changing their size, their mode of association, Gram stain and ability to reproduce.***

Keywords: Zinc oxides, blood cells, Candida albicans, BS3, microscopy.

1. Introduction

Nanotechnology is now widely considered one of the promising technologies of this century [1-3]. Thanks to special properties due to their small size, the zinc oxide nanoparticles are used in many fields. In the field of health and hygiene as a bactericidal agent [4-8] and antifungal [9,10], in the treatment of wastewater for recycling [11], as an antimitotic agent observed in the rootlets of seeds [12], and in food as a preservative [13]. The objective of this study is to test the effect, directly in vitro, of nanoparticles of metal oxide ZnO synthesized from three precursors [4] on different types of cells:

- The cells of human blood: erythrocytes and leukocytes which have a crucial role in sustaining life and human health; it is a test of tolerance of human cells to these oxides;
- Fungal cells: Candida albicans isolated from a vaginal candidiasis;
- Bacterial cell BS3: Gram-positive bacillus, spore-forming, multiresistant to antibiotics, β-lactamase-producing and thermophilic [14]. The choice of this bacterium based on its exceptional ability to withstand not only the chemical bactericidal agents but also the physical and environmental factors.

2. Materials and Methods

The preparation of zinc oxide nanoparticles was carried out from three different precursors: zinc sulfate heptahydrate, zinc nitrate hexahydrate, and zinc acetate dihydrate according to the methods described by Zegaoui et al. [4].

Table 1: Notation adopted for the oxides prepared from different precursors [4]:

Sample	Notation	Precursor used
	ZnO(S)	ZnSO4, 7H2O
ZnO(X)	ZnO(N)	Zn(NO3)2, 6H2O
	ZnO(A)	Zn(CH3COO)2, 2H2O

1) The effect of ZnO synthesized from three different precursors on the red cell (hemolysis test):

The fresh human blood was collected on a sterile tube containing sodium citrate as anticoagulant in a volume to four volumes of blood. Packedred blood cells are subjected to four washes with sterile physiological saline water [15]. 1mg / ml of each oxide dissolved in sterile physiological saline is mixed with 50µl of packedred blood cells.The control tube is free of oxide. The preparations are then incubated in the dark for 1 hour at room temperature.They are centrifuged at 1500 rotation / min for 3 minutes.The observation of hemolysis is performed directly with the naked eye. Each test is repeated three times.

2) The effect of ZnO synthesized from three different precursors on leukocytes:

In test tubes, 1 ml of fresh human blood, collected in EDTA is mixed with 1 mg of the oxide to be tested. The control tube is free of oxide. The preparations are incubated in the dark for 1 hour at room temperature. Thin smears films were formed, after homogenization of the blood, on clean slides followed by May-Grünwald-Giemsa staining (MGG). An observation is made under an optical microscope at a magnification x 1000 to illustrate the behavior of leukocytes regarding the tested products. Each experiment is repeated three times.

3) The effect of ZnO synthesized from three different precursors on Candida albicans:

A colony of Candida albicans, collected on a solid Sabouraud's medium, is inoculated into a tube containing the same liquid medium, followed by incubation for at 37°C for 24h. 1ml of the sterile Sabouraud's medium liquid is mixed with 1mg oxide to be tested and 50µl removed from the stock culture of Candida albicans. The control tube contains no oxide. The preparations are incubated in the dark at 37 ° C for 24 hours. A drop of suspension is deposited between slide and cover slip. Quantification of the yeast is carried out by observation with an optical microscope at a magnification x 400. Growth score is determined and recorded for each slide as compared to the control. The number of repetitions is three times.

4) The effect of ZnO synthesized from different precursors on the bacterium BS3

BS3 bacterium is a thermophilic bacillus, Gram-positive, at an optimum growth temperature of 50 ° C. This bacterium has been isolated in the city of Meknes in Morocco [14]. It is a bacterium gifted of particular resistance to heat, pH, and salinity. BS3 is sporulating bacterium, multi-resistant to antibiotics and producer of the most thermophilic β-lactamase [14]. This highly resistant bacterium was used in the test of the above zinc nanoparticles to show their antibacterial effectiveness. From a culture of BS3 of 24h on Muller Hinton broth, we collected 1ml of the bacterial suspension in a series of sterile tubes in which we added 1 mg of zinc oxide nanoparticles to be tested.The tube as a control is free of zinc nanoparticles. These preparations were incubated at 50 ° C and in the dark for 3 hours. The number of repetitions is three times. Test tubes are centrifuged and the pellets are spread on slides. After Gram staining, observation is carried out under an optical microscope at x1000 magnification. A bacterial culture on Muller Hinton agar was carried out subsequently for each test taking as inoculum previous old culture 18h, this test confirms the complete degeneration of bacterial cells.

3. Results and Discussion

1) Hemolysis Test

This test is performed to find out the in vitro effect of ZnO, synthesized from various precursors, on human erythrocytes. The results show a hemolytic effect of ZnO(A) following a red blood cell lysis thereby releasing hemoglobin, the tube has a pigmented red supernatant and a white pellet exploded erythrocytes (Photo B Figure 1). ZnO(N) causes a slight hemolysis compared to the control (picture C Figure 1). However, ZnO(S) has no effect on hemolysis of erythrocytes tested. They retain their intracellular hemoglobin content comparable to that of the control (picture A Figure 1).

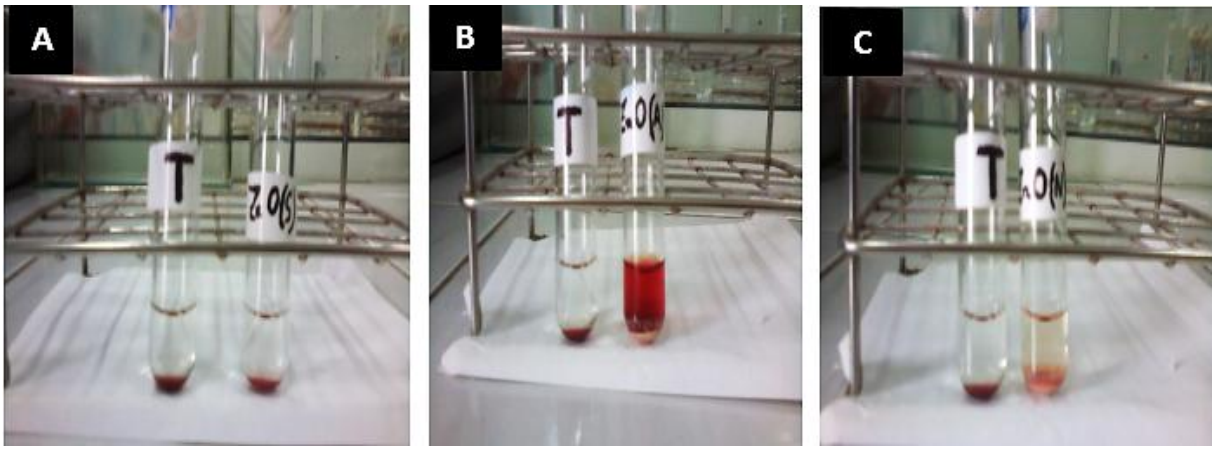

Figure 1: Tests carried out in the presence of hemolysis ZnO(S) (picture A), ZnO(A) (picture B) and ZnO(N) (picture C). The control tube is shown in the photos with the letter T.

These results allow us to infer that the zinc oxide synthesized from zinc acetate is acting adversely on the integrity and permeability of the erythrocyte membrane, most likely by acting on the parameters involved in membrane transport. The erythrocyte has a high hemoglobin content, which is near saturation, giving it a high oncotic pressure. This natural water call to the red blood cell, giving it a shape of a biconcave sphere and may break (hemolysis) in the absence of active pumps Na+ and K+ driving back ceaselessly water [16, 17]. Breakage of these pumps may cause a massive influx of water into the middle intra cytoplasmic and cause a red blood cell lysis. ZnO(S) produces no hemolysis, the float remains clear. However ZnO(N) causes a slight hemolysis compared to the control.

This difference in biological activity of the three oxides was observed on other types of plant prokaryotic cells and eukaryotic [4, 12]. It can be explained by a difference in the shape and size of the synthesized nanoparticles. The observation of zinc oxide by TEM (Transmission Electron Microscopy) shows that ZnO prepared from zinc acetate is composed of grains about 40 nm that are uniform hexagonal shape. The ZnO prepared from zinc nitrate is present in an elongated shape which the length can reach 800 nm and a diameter of approximately 100 nm in average. For ZnO prepared from zinc sulfate, the oxides are highly agglomerated and are in clusters form of large grains and having an average size greater than 100 nm [4].

2) Test on leukocytes

This test is performed in order to realize the in vitro effect of ZnO, synthesized from various precursors, on human leukocytes. Figure 2 shows the optical microscope observation of the blood smear. It confirms the hemolytic

effect of ZnO synthesized from zinc acetate dihydrate; the red blood cells appear pale and drained of hemoglobin and the leukocytes are completely deformed and altered, their nucleus has granular inclusions (picture B Figure 2).

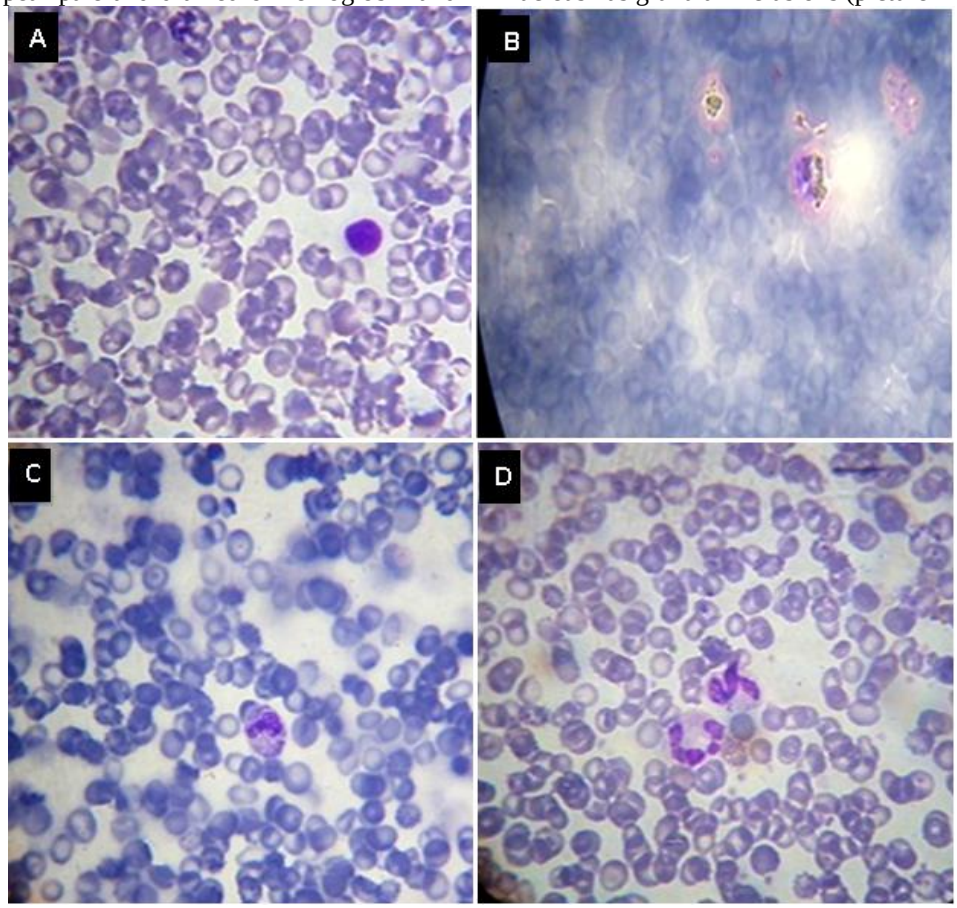

Figure 2: Photos of stained blood smears with MGG. A: test with ZnO (S); B: test with ZnO (A); C: test with ZnO (N); D: control test.(X1000 Magnification).

The control shows a smear with normal red blood cells and two intact polynuclear neutrophils (image D Figure 2). The smear mounted from the blood treated with zinc oxide synthesized from zinc sulphate heptahydrate, shows a normal preparation with the red blood cells and an intact lymphocyte (image A Figure 2). The blood treatment by zinc oxide synthesized from zinc nitrate hexahydrate shows a small attack on the red blood cells whereas the leukocytes keep their normal appearance (picture C Figure 2). This eukaryotic and human cells tolerance test to these three types of zinc oxide, allows us to rank according to their degree of toxicity and increasing their effective efficacy on microbial growth. Either respectively ZnO(S), ZnO(N) and ZnO(A), indicating that ZnO(S) has no toxic effect on blood cells. The selectivity of zinc oxide of the action on the prokaryotic and eukaryotic cells has been already reported by Reddyet et al [18] which showed that human T lymphocytes were more resistant to the toxicity of ZnO compared with Escherichia coli and Staphylococcus aureus.

3) The effect on Candida albicans

Candida albicans cause vulvovaginal candidiasis manifested by clinical signs that are especially vaginal and vulvar itching [19]. They affect 75% of women at least once in their lives (with relapsing forms in 10% of cases) [20]. These fungi are the majority of the severity of nosocomial infections. Epidemiological studies from 1990 show that Candida yeasts ranked fourth in the infectious agents responsible for nosocomial septicemia frequency, and the first for mortality [21].

This test is performed to demonstrate the in vitro effect of ZnO, synthesized from various precursors, on Candida albicans. The results obtained clearly show the total eradication of this pathogenic microscopic fungus in its treatment by ZnO(S) (picture A Figure 3). Whereas at a concentration 1mg/ml of ZnO(N) and of ZnO(A) (picture B and C figure 3), the presence of this fungus persists, but at a lower density and a smaller size compared to control that shows budding yeast associated with pseudomyceliums (image D Figure 3).

These results are consistent with tests on pathogens in which the ZnO(S) is the most effective with a bactericidal activity at very low MIC (Minimum Inhibitory Concentration) [4].

By analyzing these results, it is clear that the inhibitory activity of the growth of Candida albicans by the ZnO(S) is the most important. Tolerance of leukocytes and erythrocytes of this oxide and its remarkable antifungal effect made ZnO(S) a non-toxic molecule to blood cells and having a particular antifungal activity.

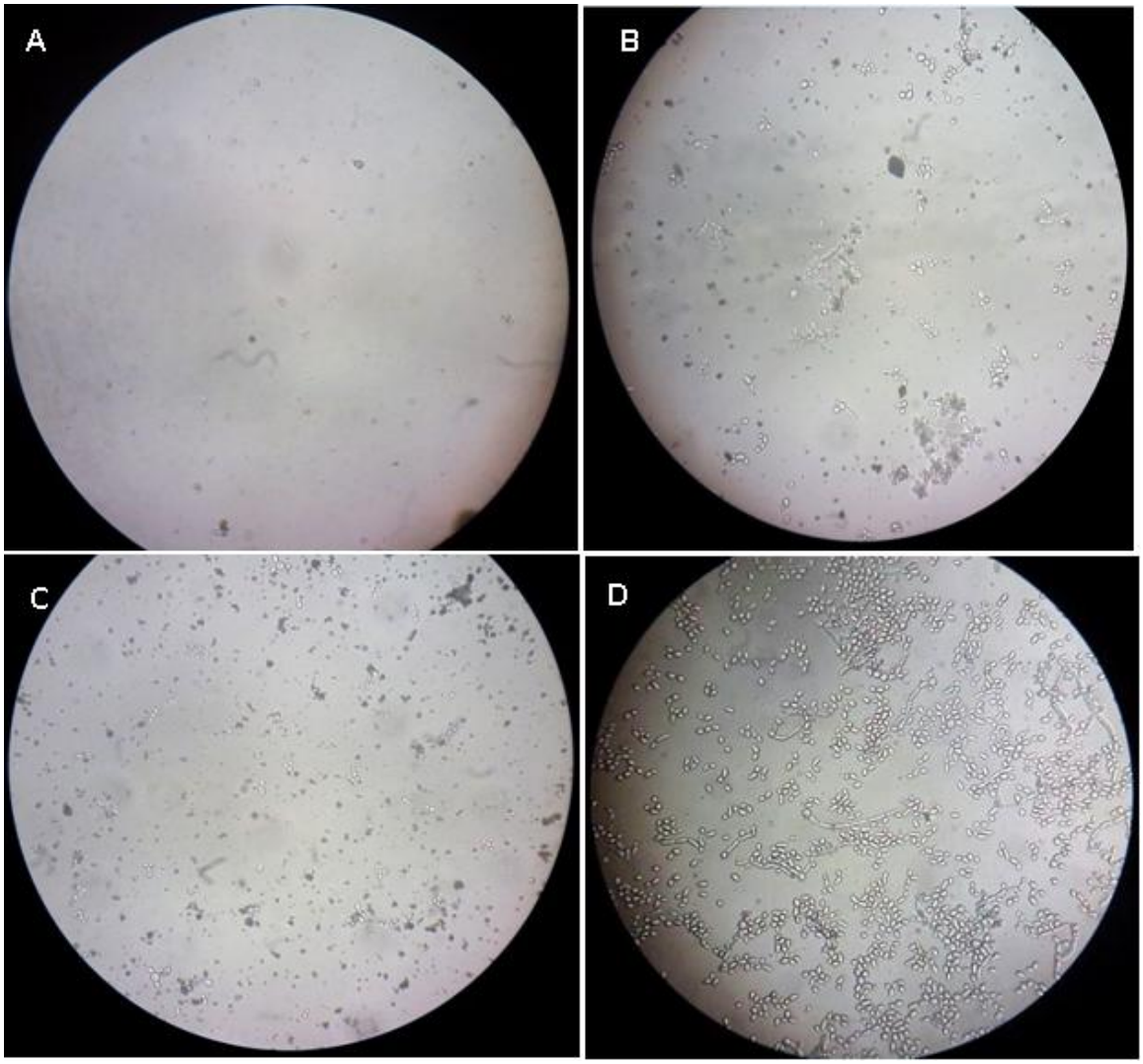

Figure 3: Photos of the direct examination of Candida albicansbetween slide and cover slip. A: test with ZnO (S); B: test with ZnO (A); C: test withZnO (N); D: control test. (X400 Magnification)

4) Sequels on the BS3 bacterium

This test is performed to see the effect in vitro of zinc oxide nanoparticles, synthesized from various precursors, on the morphological behavior of multiresistant and thermophilic bacterial cell BS3. The results obtained show that in the case of the control (picture D Figure 4) the bacterium has a Gram-positive bacillary form. The addition of ZnO(S), ZnO(A) or ZnO(N) in the bacterial culture, shows after 3 hours of incubation changes in the appearance of the bacterial cells. For ZnO(S) there is first a conversion of most of the Gram-positive bacterial cells to Gram-negative, which can be explained by an alteration of the wall leading to the dispersal of the bacterium contained (picture A Figure 4). The cells have a different mode of association of the control, an agglomeration and a tendency towards the formation of clusters of cells tangled and poorly demarcated. Bacterial density decrease indicating an inhibition of growth.

Treatment with ZnO(A) leads to a change in the size of the cells which become smaller, an attack of the bacterial walls displayed by changing the Gram stain and an attack of the bacteria illustrated by empty and pale cells (picture B Figure 4). The test carried out with ZnO(N) shows a change in the shape and size of the bacteria. The cells are inflated and a fluorescent appearance, indicating a change in membrane permeability, intracellular accumulation of zinc oxide and an incoming flow of water. Gram staining is best preserved in this case in most cells, thereby suggesting a different site of action (picture C Figure 4).

The cell viability test confirms a complete eradication of all bacteria; none of them remained alive after 18 hours of incubation following treatment by the various oxides. The bactericidal effect of these synthetic nanoparticles tested in this study on the spore-forming bacterial strain BS3 feature exceptional resistance to antibiotics, the high temperatures and salinity, foreshadows the promising role of these oxides in the clean-up of the environment, food conservation and anti-infective therapy.

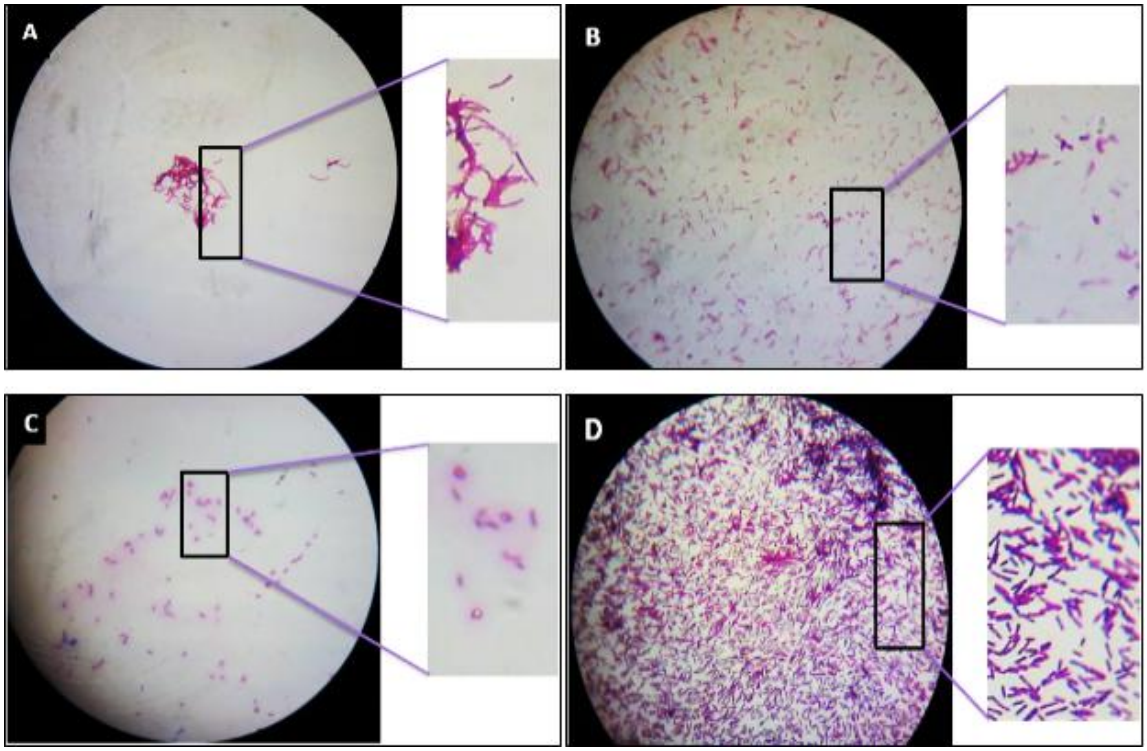

Figure 4: A: Photos of Gram staining of bacteria BS3. A: test with ZnO (S); B: ZnO test with (A); C: test with ZnO (N); D: control test (X 1000 Magnification).

4. Conclusion

The effect of ZnO nanoparticles on eukaryotic and prokaryotic cells tested varies depending on the precursor by which they are synthesized. All the results obtained in this work allowed us to conclude that zinc oxide synthesized by the zinc sulphate heptahydrate has an inhibitory role on the growth of resistant microorganisms and pathogens and shows no toxicity in vitro of the human blood cells, hence its interest in the anti-infective use of food preservation and remediation of the environment.

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