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**Research Article**

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# Evaluation of toxic effect of biogenic silver nanaoparticles on chick embryo

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**ABSTRACT**

*The objective of this study was to examine potential effects of silver nanoparticles on growth and development of chicken embryo. Silver nanoparticles were synthesized from Candida glabrata and the synthesized nanoparticles were characterized. Fertilized eggs were divided into 3 groups and injected inovo; group I (control) – no treated; group II (placebo-1) – physiological saline with 1 injection; group III (Ag) -hydrocolloid of Ag nanoparticles – with 1 injection; group. Nanoparticles of Ag did not influence chicken embryo development. Injection inovo with physiological saline increased activity of asparagine transferase in blood serum and caused hypertrophy of hepatocytes. Injection of nanoparticles Ag showed tendency to restore this negative effect.*

**Key words:** silver nanoparticles, Candida glabrata, chick embryo, toxic effect

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## INTRODUCTION

Nanotechnology bringing the gap between the inanimate and animate nature and aims at understanding the basic principles of biological functional units as well as creating extremely small components at nanoscale in a controlled way with technical materials. Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular strctures.The recent developments in nanotechnology are leading to displays that are cheaper, larger, brighter, and more efficient than current ones. Nanotechnology aims at understanding the basic principles of biological functional units as well as creating extremely small elements at nano-scale in a controlled way with technical materials and interfaces. Nanotechnology has new discipline will probably not only extend our ability to influence the properties of materials in specific ways but also help us to better utilize them, and to integrate nanostructures into complex total systems Noble metals of Nanoparticles like silver shows unique biological properties being potential candidates for a new kind of non-antibiotic stimulators of growth.Nanoparticles of silver and probably palladium show antibacterial properties at the level of about 15ppm [1,2].Moreover, nanoparticles of Ag are documented as anti-inflammatory agents or components ofanti-inflammatory molecules, which do not destroy systemic homeostasis [3,4,5].The unique structure of the crystal lattice of silver allows it tostore atomic oxygen inside the octahedral holes of Ag (0) and probably influences oxygen level in environment [6,7].The antimicrobial and anti-inflammatory properties are definitely enrich cells with oxygen via development of growth can be stimulated of an organism. Furthermore, it is likely that by using“nano-amounts” of these metals, toxicity could be minimized or eliminated and the risk of developing resistant microorganisms can be highly reduced. The objective of this study was to examine potential effects of silver nano particles on growth and development of chicken embryo.

## EXPERIMENTAL SECTION

**Fungal strain**

*Candida glabrata* (MTCC 3986), was obtained from Microbial type culture collection (IMTECH) Chandigarh, India. The strain was maintained on Sabouraud Maltose Yeast Extract Agar (SMYA) slant.

**Synthesis and characterization of silver nanoparticles**

Silver nanoparticles were synthesized from culture filtrate of *Candida glabrata* (MTCC 3986) as described earlier [8].

**Invitro study of Cytotoxicity of Silver nanoparticles on Vero cells and cancer cells showing anticanacer activity.**

**Cytotoxicity assay**

In order to study the antitumor activity of synthesized silver nanoparticles, it is important to determine the cytotoxicity concentration of the synthesized silver nanoparticles. Cytotoxicity test is defined as the upper limit of the extract concentration, which is not toxic to the cell lines. After the addition of the synthesized silver nanoparticles, cell death and cell viability was estimated. The result is confirmed by additional metabolic intervention experiment such as MTT assay.

**Collection of embryonated eggs**

Thirty fertile eggs (50-54 g) from Nandanam poultry station Chennai were collected and incubated at 37.7°C, 60% humidity and automatically turned every hour. Eggs were divided into the two groups (control, nano-Ag) and injected 3 times during incubation on days 5, 11 and 17; the nano-Ag group, with0.2 ml 10 ppm colloidal Silver nanoparticles. The injection holes were sterile sealed and eggs were placed in the incubator at standard conditions. Silver nanoparticles (nano-Ag) used as an antimicrobial agent can stimulate development as well as immunity. This experiment was to evaluate the influence of colloidal Ag nanoparticle on the development status of chicken embryos, particularly on the bursa of Fabricus, Heart, Eye Bursa , Liver (Grodzik and sawoz, 2006). At $20^{th}$ day embryos were taken from shells and immediately sacrificed by decapitation, and embryos and selected organs were weighed and evaluated using Hamburger and Hamilton (1951) –HH standard. Blood was collected from neck vein and after 4 hours centrifugation with 3000 x 15 min, in obtained serum activity of alanine aminotransferase (ALT) and asparagine aminotransferase (ASP) were examined using dry chemistry equipment Vitros DT 60 II, Johnson & Johnson. Liver slices were fixed in 4 % solution of paraformaldechyde and then placed in Phosphate Buffered Saline (PBS). After 24 hours samples were washed in PBS + 0.05% Tween 20 and in methanol from PBT and finally preserved in methanol. Prior to microscopic evaluation the samples were rinsed in the solution of methanol from PBT and PBT and then were freezed and sliced into 9 – 10µm by cryostat and stained with Eosin and Hemotoxylin.The stained cells aer observed in Light microscopes Radicle India Ltd with the magnification of 40X. The data were analysed using mono-factorial analysis of variance ANOVA and the differencesbetween groups were tested by the multiple range Duncan test, using Statgraphics Plus 4.1.Differences with $p < 0.05$ were considered significant.

**Histopathological studies**

The bird was killed and specimens were takenThe specimens included the, liver, heart, Liver, Eye and Bursa. These specimens were sectioned and stained with hemotoxylin and eosin and studied by light microscope.

## RESULTS AND DISCUSSION

Silver nanoparticles were synthesized and characterized previously [9].Synthesized nanoparticles were evaluated against toxic effect Chicken embryo is a biological model independent from external influence, fast developing and easy to maintain. Moreover, it is well known and described in detail in the standard of Hamburger and Hamilton . This model is used in medical, toxicological and alsonutritional experiment as a primary investigation, often carried out prior to experiments with animalsor humans [10,11].In our experiments we demonstrated that hydrosol of Ag given *in ovo* in amount of 200 µl,did not influence mortality, body weight, selected biochemical indices of blood serum of 20 days old embryos (Table 1).The present results also did not show any effects on embryos survival, the embryos from all groups were properly developed, without any abnormalities. Hussein et al.[13] in *in vitro* experiments with rat hepatocytes used nanoparticles of Fe3O4, Al, MoO3 and TiO2 and demonstrated significant detrimental effects at a level of 100 – 250µg/ml. Alanine transaminase (ALT) is an enzyme being present in blood

serum at normal condition, however, an increase of this parameter points on liver (or heart) damage by disintegration of cell membranes. Treatments with hydrosols of Ag/ nanoparticles at levels of 300 µl had no harmless influence on hepatocytes' membrane structure. Asparagine transaminase (ASP) is an enzyme which high activity indicates dysfunction of mitochondrial membrane, especially, of hepatocytes (Table 2).The present results showed that rather the process of injection in general than injection of nanoparticles influenced activity of ASP. We can hypothesize that injection even when was made very carefully, could be stressful for chicken embryo. In the present experiments hepatocyte nuclear profile density was measure as a marker of hypertrophy of the liver cells. Injection of physiological saline, but not nanoparticles, increased hepatocyte nuclear profile density, however, without increasing liver weight

The silver nanoparticles synthesized from *Candida glabrata* was showing the cytotoxicity on the vero cell lines and the Hep2 cell lines at an $Ic_{50}$ value of 1.25 µg and 0.625 µg respectively. Hence, the study says that the silver nanoparticles synthesized from *Candida glabrata* was effective against Hep2 cells at 0.625 µg. at these concentration, they were not lethal to the normal vero cells.Histopathological and ultrastructure analysis is the key method to understand mechanism of human ang animal disease[53-64]Further studies are needed for histopathlogical examination to know about the difference in the apoptopic signs like chromatin condensation, nuclear fragmentation studies.Since we used Eosin and haematoxylin could not predict more histopathological changes in this study, more advanced methods and microscopical observations needed to know the morphology of cell. .Hep2 cell line and vero cell line was purchased from NCCS pune. The cell lines were maintained at $37^0$C at 5% $CO_2$ in $CO_2$ incubator. Cultures were viewed using an inverted microscope to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. Cell monolayer was washed with PBS without $Ca^{2+}/Mg^{2+}$ using a volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1ml per 25 $cm^2$ of surface area. Flask was rotated to cover monolayer with trypsin. Flask was returned to the $CO_2$ incubator and left for 2-10 mins. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. 5 ml of complete media was added and cells were triturated to get the cells in the form of suspension .the cell suspension was centrifuged to get the cell pellet. The cell pellet was diluted and seeded into new flasks. The flasks were incubated in $CO_2$ for 3 day to get monolayer.

Thirty fertile eggs (54-58 g) from Nandanam poultry station, Chennai were collected and incubated at 37.7°C**,** 60% humidity and automatically turned every hour. Eggs were divided into the three groups (control, Placebo,Nano-Ag) and injected 3 times during incubation on days 5, 11 and 17;the nano-Ag group, with 0.2 ml 10 ppm colloidal Ag nanoparticles. On day 20 of incubation the eggs were weighed, opened and the embryos sacrificed by decapitation. The embryo's liver, heart and eyes were weighed and examined.Bursa of Fabricius, heart, liver, eyes were collected and fixed in 4% Formaldehyde in phosphate-buffered saline (PBS; Sigma-Aldrich) overnight at 4°C and in a methanol gradient. The isolated parts were observed by histopathological sections. All the structures and profile of cell nuclei were viewed under light microscopy. On morphologically viewing bursa, liver, heart, eye the administration of nano silver had no effect on Bursa.

**Bursa**

The oval bursa contains canals located between follicles Primary & Secondary canals extend from primary structures. The structure of bursa of fabricius (lymph follicle numbers size,100x) and profile of cell nuclei were viewed by microscopy all were reduced in size but they are not showing much differences when compared to original cells and shape of nuclei not changed in normal. The study BF is the primary site of B lymphocyte immigration and early proliferation. However, the first wave of immigrated blood-borne cells (on day 6.5) are bursal dendritic cells, which produce and secrete "bursa-specific substances"inside the epithelium. These substances contribute to the bursal microenvironment and B-lymphocyte maturation, and play a key role in development of BF. Hypothetically, these ovoinhibitors exhibit a cell signaling activity,probably for B-cell expansion and gene conversion, showing some structural homology to IL-1 Other investigations demonstrated that an anti-bursin monoclonal antibody decreased lymphocyte proliferation, development of follicles and BF weight [9,10,11].Silver nanoparticles(S1) (100 ppm) injected into fertilized eggs on days 5, 11 and 17 of incubation did not influence the development of embryos ,but decreased the number and size of lymph follicles in the bursa of Fabricius.(Figure 3a,b)

**Liver**

No differences was found between the embryos 1-17days postinfection and the controls was observed. No pronounced inflammatory lesion and focal necrosis of the liver were successively observed 24-48 hours and no

granular degeneration as well as no vacuolar degeneration were observed successively. The hepatic cells were not swollen and their shape not changed from polygonal to round. Many vesicles appeared in the hepatic cytoplasm and thus made it appeared loosely foamy. No such Cytoplasm dissolution, ballooning degeneration, together with the marked vacuolation of the hepatocytes were observed in. Normal architecture of monocytes and lymphocytes were observed.No Dehydration, condensation and enhanced cytoplasmic acidophilia occurred in a few hepatocyte cytoplasm, in which round eosinophilic droplets were formed and shared great similarity to that structure observed in the case normal liver (Figure 4a,b)

**Eye**

Histological sectioning and staining showed little difference in nanoparticle treated embryos when compared to control embryos. Slight variations seen in corneal endothelial cell numbers and little or no signs of toxicity on retinal structure, photoreceptor function or aqueous drainage in the eye (Figure 5a,b)

**Heart**

Most of the embryos were found to be normal and no mild hyperemia and hemorrhage seen in their hearts. No granular degeneration of the myocardium, Cross striations of the myocardium; No myocardium underwent karyorrhexis and karyolysis; no small necrotic foci and inflammatory cell infiltration might be observed; Focal pericardit is might developed (Figure 6a,b).

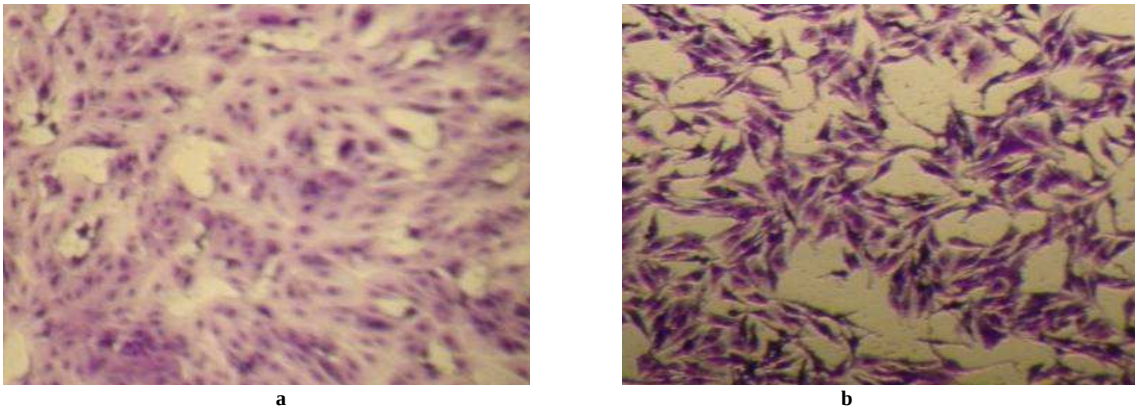
**TABLE 1 Embryos' body weight (g) and weight of selected organs (g/100 g body weight)**

| S.No | Organs | Groups | | |
|---|---|---|---|---|
| | | Control | Placebo | Ag |
| 1 | Egg weight | 53.66 | 55.00 | 54.12 |
| 2. | Embryo | 40.10 | 40.70 | 49.10 |
| 3. | Heart | 0.90 | 0.95 | 0.97 |
| 4. | Liver | 2.00 | 2.18 | 2.20 |
| 5. | Bursa | 1.00 | 0.970 | 1.10 |
| 6. | Eye (Both Eye) | 3.36 | 3.22 | 3.11 |

**TABLE 2. Activities of aminotransferases in embryos' serum at 20 day of incubation**

| S.No | Tests | Groups | | |
|---|---|---|---|---|
| | | Control | Placebo | Ag |
| 1. | Asparagine aminotransferase (U/l) | 87.31a | 188.03b | 170.30b |
| 2. | Alanine aminotransferase (U/l) | 18.10 | 19.25 | 19.65 |

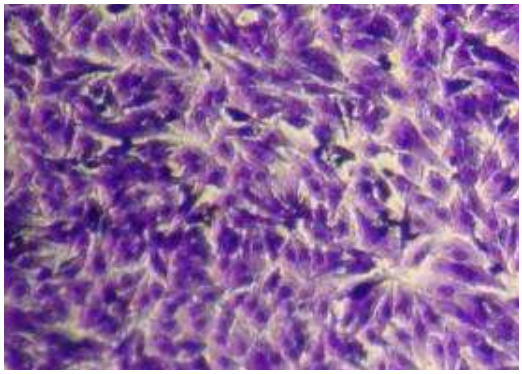
**Figour 1. Untreated Vero cells and treated Vero cells with synthesized silver nanoparticles from fruits of *Candida glabrata* under radical light microscope of 40x magnification**



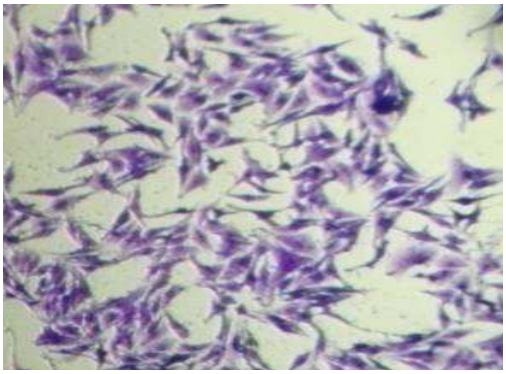
**a** **b**

**Figure 1 a – Untreated Vero cells under light microscope of radical 40x magnification**
**Figure 1b– Action of synthesized silver nanoparticles from *Candida glabrata* Vero cells under light microscope of radical 40x magnification**

**Figure 2 – Untreated Hep 2 cells and treated Hep 2 cells with synthesized silver nanoparticles from *Candida glabrata* under radical light microscope of 40x magnification**

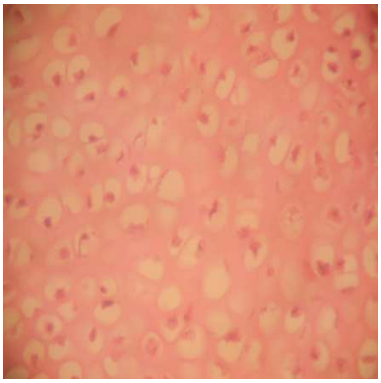


**8 a**

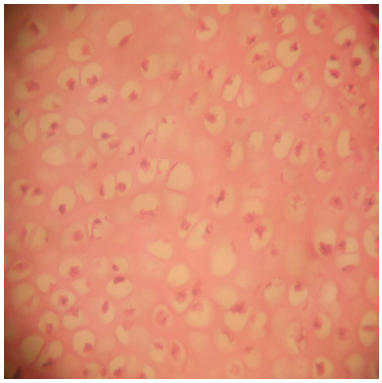


**8 b**

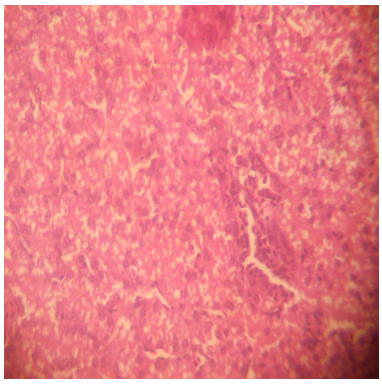
**Plate 2 a – Untreated Hep 2 cells under radical light microscope of 40x magnification**
**Plate 2 b – Action of synthesized silver nanoparticles from *Candida glabrata* on Hep 2 cells radical under light microscope of 40x magnification**



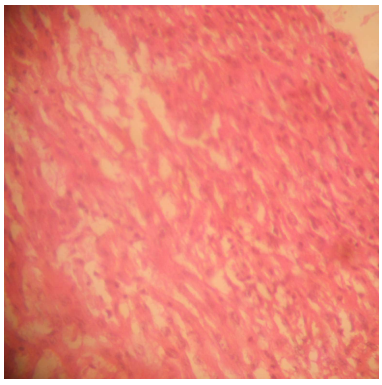
**Control Bursa (40X)**



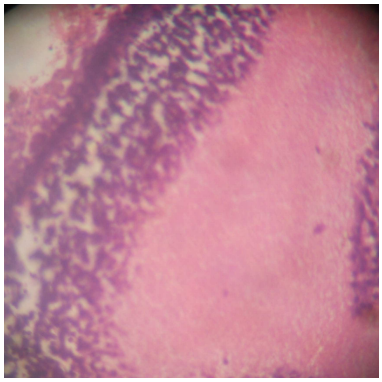
**Bursa (40X) Candida NP treated**



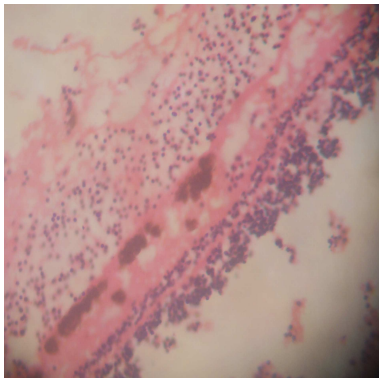
**Control Liver (40X)**



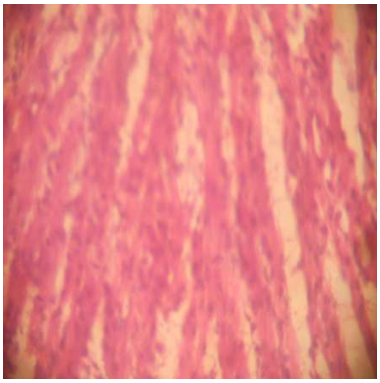
**Liver (40X) Candida NP treated**



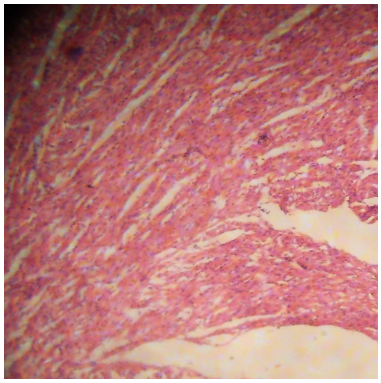
**Control Eye**



**Liver (40X) Candida NP treated**



**Control Heart cells (40X)**



**Heart cells of Candida NP Treated(40X)**

## CONCLUSION

Nanoparticles are being viewed as fundamental building blocks of nanotechnology The application of nanoparticles as delivery vehicles for bactericidal agents represents a new paradigm in the design of antibacterial therapeutics. Silver nanoparticles commonly used for nanomedicine production, are reported to be nontoxic to human but most effective against bacteria, viruses, and other eukaryotic microorganisms at very low concentration. In the prsebt study, toxic effect of silver nanoparticles on chick embryo showed nil toxic effect which would suggests the possible utilization of biogenic silver nanoparticles for the various pharmacological activities.

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