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IN VIVO BIOCOMPATIBILITY STUDY OF ZIRCONIUM DIOXIDE NANOPARTICLES FOR ITS APPLICATIOIN IN DENTAL TREATMENT

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ABSTRACT

Zirconia is also known as zirconium dioxide (ZrO_2), an inorganic polycrystalline material and is widely used in the field of dental treatment. Due to its mechanical and optical properties, Zirconia has been used in dentistry with a 90% content made up of Zirconia as dental crowns. This Zirconia based ceramics in dental prosthetics leads to cause toxic reactions and delayed allergic responses in oral tissues which may also lead to cause oral cancer. The main aim of our study is to synthesis Zirconia nanoparticles, which has increased surface area and may be less toxic than Zirconia particles in dental treatment. In our present study the synthesis and characterization of Zirconia nanoparticles was performed and the size obtained was 50-70 nm. Biocompatibility study of Zirconia nanoparticles were performed using chick embryo as *In vivo* model system. Different concentrations of Zirconia nanoparticles were tested for the antioxidant enzyme studies in chick embryos. The liver and heart tissues of the embryos were examined for estimation of Glutothione peroxidase, Lipid peroxidase and Superoxide dismutase in the zirconia nanopartilces treated groups (10 PPM, 20 PPM, 40 PPM and 80 PPM). The increased amount of total proteins(35 mg/ml), superoxide dismutase(6.91 Units/mg Protein) and glutathione peroxidase (6.395 Units/mg Protein) and decreased amount of lipid peroxidation (4.99 n mol/mg Protein) was recorded in 80 PPM nanoprticles treated group when compared to that of the control and heart tissue samples of other treatments also.

KEY WORDS: **Dental ceramic materials, Zirconium dioxide (ZrO_2), Toxicity evaluation, Energy dispersive X-Ray spectroscopy (EDX), X-Ray diffractometer (XRD).**

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INTRODUCTION

One of the most difficult areas in dentistry today is the restoration of dental structures with biocompatible materials that are strong enough to withstand the forces of chewing (500- 1000lbs pressure on molar teeth). Recent technology from Germany now offers a material that has overcome most of the pitfalls of present day products. Patients now have a choice of material that is esthetic, strong, pure, biocompatible and capable of being used for single and long span dental bridgework. The material is called Zirconium oxide.[1] Though zirconia has been available for use in restorative dentistry for several years, there has been an increased interest recently in these materials.[2] Zirconia based restorations are quite versatile and can be used for crowns, bridges, and implant abutments in a variety of clinical situations. Although titanium is used in dental implantology, there is a trend to develop new ceramic-based implants as an alternative to monolithic titanium. [3] Due to mechanical and optical properties zirconia has been used in dentistry for the past 10 - 15 years. It is used for fixed restorations as a frame work material and for dental crowns. It is remarkably a robust material able to withstand the wear and tear of everyday use.[4] Zirconia ceramic materials are best able to mimic the appearance of natural teeth. The zirconia systems currently available for use in dentistry include ceramics with a 90% or higher content zirconium dioxide.[5] The type of zirconia used in dentistry is yttria tetragonal zirconia polycrystal (Y-TZP) material, which is a zirconia oxide. Yttria (Y_2O_3) is an oxide of the metallic element yttrium. The yttria is added to the zirconia to stabilize the structure and maintain the material's desirable properties.[2] It is however a little more expensive than the traditional materials. One of the main risks associated with Zirconium based implants is radioactivity. Zirconium at any time can contain a certain number of radioactive isotopes. This can lead to an increased chance of various Oral cancers. Zirconia-based ceramics are being increasingly used in dental prosthetics in substitution of metal cores, which are known to induce local toxic reactions and delayed allergic responses in the oral tissues**.** Patients with artificial teeth are in any case subject to higher dose levels than other members of the population; therefore further efforts should be taken to effectively reduce the content of natural radionuclides in dental ceramic materials.[6] The aim of this study is to identify the effect of Zirconia nanoparticles *In vivo* model to understand the biocompatibility of the material.

MATERIALS AND METHODS

Sample preparation by precipitation method

Zirconia nanoparticles were synthesized by modified precipitation method. [7]

Characterization of zirconia nanoparticle

The synthesized zirconia samples were crushed using mortar & pestle and the samples were given in powdered form for analysis. Structural and Morphological elucidations were checked using XRD and SEM. SEM microstructural analysis was performed using a JEOL JSM-6390 microscope. The powder sample of zirconia was ultrasonically dispersed in ethanol, deposited on a carbon film supported on a copper grid and subjected for SEM analysis.[8] The crystallinity of the nanoparticles was characterized using XRD (Schimadzu, Japan Model XRD 6000) CuKa-radiation with a Bragg–Brentano-arrangement. The sample mounted on Si-single-crystal-wafers and measured under ambient conditions.[9] The quantitative analysis of nanoparticles was carried out by energy-dispersive X-ray spectroscopy (EDAX) (GENESIS 60E EDAX analyser), which identifies the chemical composition. The sample was given in powder form without any coating[10]. The distribution of nanoparticle in solvent was analysed using Particle Size Analyzer (Molvern Zetasize NanoZS90). The zirconia nanoparticle sample was dissolved in 100 ml of 0.9% physiological saline (NaCl) and ultrasonicated for 10 mins at 240 volt. The dispersed zirconia solutions were given for distribution analysis as per. [9]

In vivo studies using chick embryo

Sample collection

Fertilized chicken (BV 380 breed) eggs (n=30) weighing 50-55 gms were obtained from Regal hatchery, Coimbatore, Tamil Nadu, India.

Chick embryo treatment

The eggs were randomly divided into 5 groups (n=6) *viz.* Group I (control), Group II (ZrO_2 nanoparticles 10ppm with 0.9% NaCl), Group III (ZrO_2 nanoparticles 20ppm with 0.9% NaCl), Group IV (ZrO_2 nanoparticles 40ppm with 0.9% NaCl) and Group V (ZrO_2 nanoparticles 80ppm with 0.9% NaCl). Eggs were incubated at standard conditions (temperature 37°C, humidity 60%). Experimental solutions (0.3 ml) were injected to air sack using 1 ml tuberculin syringe in sterile conditions, on 3rd day of incubation. The holes were sealed after injection with wax and incubated at 37°C.[10,11]

Toxicity evaluation

Protein was estimated by the method of Lowry. [12] Lipid peroxidation was assayed by following Brogan method. [13] Superoxide dismutase (SOD) activity was determined by pyrogallol method. [14] Glutathione peroxidase activity was measured by the standard protocol. [15]

Histopathological examination

The cross sectional full thickness liver specimens from each group were collected at the end of the experiment to evaluate for the histopathological analysis. Samples were fixed in 5% buffered formalin, processed and blocked with paraffin and then sectioned into 5 µm sections and stained with hematoxylin and eosin (HE) stains. The tissues were examined by light microscope for epidermal remodeling.

Statistical analysis

Results obtained from three wound models have been expressed as mean ± SD. The data was evaluated by one way ANOVA followed by Student-Newman-Keuls test, $p<0.05$ and $p<0.01$ was considered as significant.

RESULTS

Zirconia Nanoparticle

2.5g of zirconia nanoparticles were obtained by using 6.535g of zirconium oxychloride as precursor.

Characterization of Nanoparticle

Scanning electron microscope (SEM)

SEM analysis was carried over to determine the size of the synthesized Zirconia nanoparticles. The zirconia nanoparticles samples were supported on a copper grid and subjected for SEM analysis at 30000x magnification. The average sizes of the particles were ranged between 50–70 nm. SEM analysis confirms the synthesized Zirconia is nanocrystalline (Fig.1).

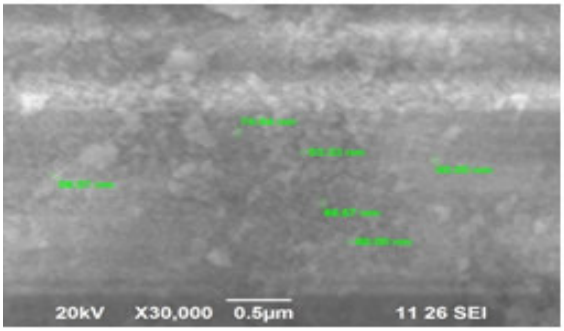

Figure 1
SEM image of zirconia nanoparticles samples

X-Ray diffractometer (XRD)

Structural and Morphological elucidations of Zirconia nanoparticles were analyzed using XRD (Figure 2). The nanoparticles were found to be crystalline in nature with the peak positions at 28.2º, 31.3° and 50.3° corresponding to typical ZrO_2 [111], [200] and [220] planes. The 100% intensity was found at 2Θ value with 28.2º. XRD confirms the presence of monoclinic phase of Zirconia.

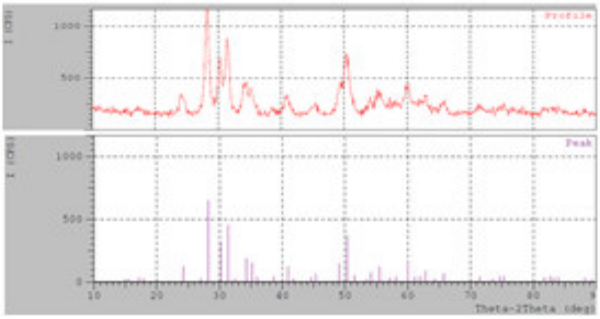

Figure 2
XRD pattern of zirconia nanoparticles

Energy dispersive X-Ray spectroscopy (EDX)

The EDX analysis showed the presence of elemental Zr and O in the sample with a relative proportion corresponding to the ZrO2 particles (Fig 3). EDX Analysis was carried out at 20 kV. The result obtained was 31.17% and 68.73% of Oxide and Zirconium was recorded in the sample

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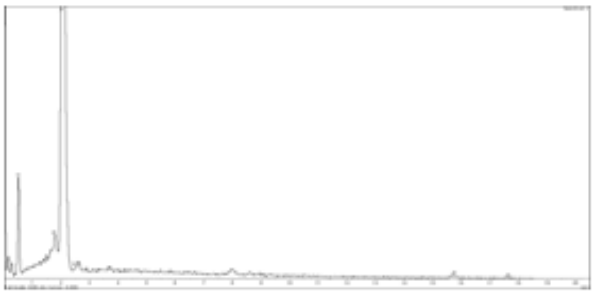


Figure 3
EDAX Profile of zirconia nanoparticles

Particle size distribution

The distribution of nanoparticles was analysized by dispersing zirconia nanopartilces in 0.9% Nacl by ultrasonication. Ultrasonication was carried out for 10 mins at 240 volt. The zirconia solution was distributed based on the size and intensity. 65% intensity was observed for particle size between 10-100nm, 35% intensity was obtained for particle size between above 100nm Distribution percentage was more at particles size less than 100nm(Figure 4).

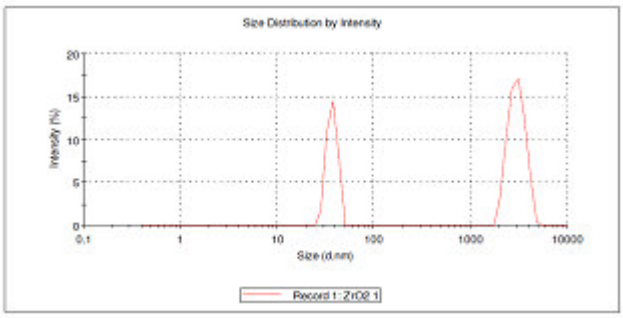


Figure 4
Distribution of Zirconia nanoparticles

Chick embryo dissection
On 19th day of incubation the eggs were opened and embryos were sacrificed by decapitation and liver and heart samples from different groups were collected and stored in PBS.

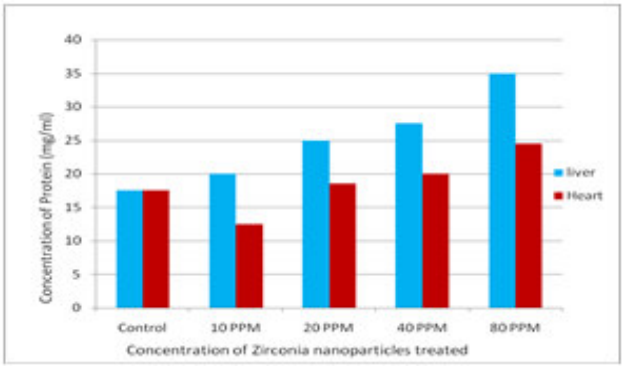
Toxicity evaluation
Oxidative stress is a normal cellular process involved in cellular signaling, though excessive oxidative stress can be harmful. Many studies have shown that exposure to nanoparticles elevates cellular oxidative stress and induce toxicity. In our work, the toxicity of zirconia nanoparticle was analyzed using antioxidant enzymes assay such as Superoxide dismutase and Glutathione peroxidase, non-enzyme assay such as Lipid peroxidation and Histopathological analysis to find out damage in the organ cells.

Estimation of protein
Total protein was estimated in the control and treated groups. Chick embryo Liver and Heart tissue samples were analyzed for total protein. It was observed that the level of protein increased in both the samples, as there was an Increase amount of protein was recorded with the increase in the concentration. The groups treated with 80 PPM of Zirconia nanoparticles showed increased amount of 35±0.03 mg/ml of protein, whereas control group showed 17.5±0.01 mg/ml of protein in the liver sample (Table 1).

Table 1
Estimation of protein in liver and heart

Group	Treatment	Protein Concentration In Liver (mg/ml)	Protein Concentration In Heart (mg/ml)
I	Control	17.5±0.01	17.5±0.023
II	ZrO2 10 ppm	20±0.034	12.5±0.02
III	ZrO2 20 ppm	25±0.02	18.5±0.036
IV	ZrO2 40 ppm	27.5±0.01	20±0.045
V	ZrO2 80 ppm	35±0.03***	24.5±0.05***

The values are expressed means ±SD of 6 replicates. *** indicates significant difference (p<0.001) compared with the control group.



Graph 1
Effect of Zirconia nanoparticles in protein

When considered with the heart sample, groups treated with 10 ppm of Zirconia nanoparticles had 12.5±0.02 mg/ml of protein which is lesser than the protein content of control group sample i.e 17.5±0.01 mg/ml. Groups treated with 20 ppm of Zirconia nanoparticles showed of 18.5±0.036 mg/ml of protein, whereas Groups treated with 80 ppm of Zirconia nanoparticles had the maximum level of protein 24.5±0.05 mg/ml when compared to the control. The amount of protein was increased with the increase in Zirconia nanoparticles concentration (Graph 1).

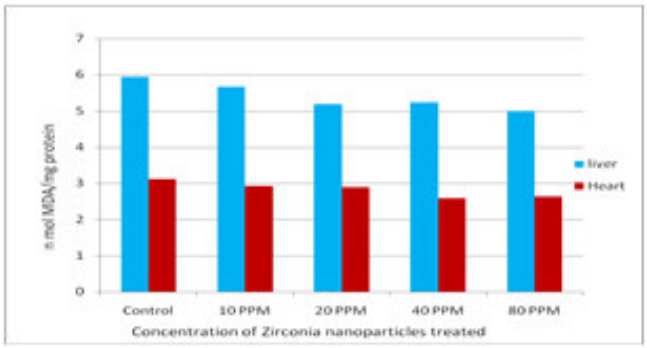
Estimation of lipid peroxidation
Lipid peroxidation level was decreased significantly ($p<0.001$) by administration of ZrO_2 nanoparticles, compared with control in both liver and heart (Table 2).

Table 2
Lipid peroxidation activity in liver and heart tissue of chick embryo

Groups	Treatment	Lipid Peroxidation In Liver (n mol /mg Protein)	Lipid Peroxidation In Heart (n mol/mg Protein)
I	Control	5.942±0.014	3.12±0.019
II	ZrO2 (10 ppm)	5.67±0.02	2.915±0.01
III	ZrO2 (20 ppm)	5.19±0.017	2.89±0.04
IV	ZrO2 (40 ppm)	5.24±0.026	2.58±0.02***
V	ZrO2 (80 ppm)	4.99±0.01***	2.63±0.008

The values are expressed means ±SD of 6 replicates. * indicates significant difference (p<0.001) compared with the control group.**

Lipid peroxidation level was decreased significantly (p<0.001)in the groups treated with ZrO_2 nanoprticles. The liver and heart sample were examined. It was recorded that the concentration of lipid peroxidase was decreased in the treated groups when compared to the control. In liver, maximum level of depletion (4.99±0.01n mol MDA/ g) was recorded in the group treated with 80 ppm, minimum level of depletion (5.67±0.02 n mol MDA/ g) was observed in the group treated with 10 ppm. In heart, the maximum level of depletion (2.58±0.02 n mol MDA/g) was recorded in the group treated with 40 ppm, minimum level of depletion (2.915±0.01 n mol MDA/g) was noted in the group treated with 10 ppm.The administration of ZrO_2 was effective lowers lipid peroxidation (Graph 2)



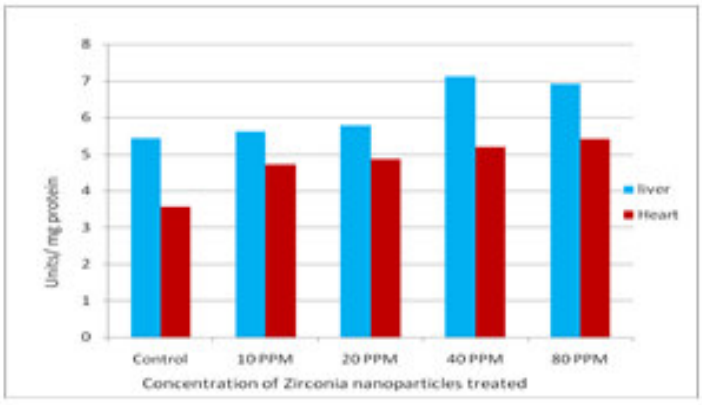
Graph 2
Effect of zirconia nanoparticles on the activity of lipid peroxidation

Estimation of superoxide dismutase (SOD)
Superoxide dismutase (SOD) activity was significantly (p<0.001) increased in the treated groups when compared to that of the control (Table 3).

Table 3
Superoxide dismutase activity in liver and heart tissue of chick embryo

Groups	Treatment	Superoxide Dismutase In Liver (units/ mg Protein)	Superoxide Dismutase In Heart (units/mg Protein)
I	Control	5.44±0.01	3.56±0.003
II	ZrO_2 (10 ppm)	5.618±0.075	4.71±0.004
III	ZrO_2 (20 ppm)	5.774±0.02	4.865±0.009
IV	ZrO_2 (40 ppm)	7.123±0.001***	5.201±0.22
V	ZrO_2 (80 ppm)	6.91±0.002	5.42±0.001***

The values are expressed means± SD of 6 replicates. * indicates significant difference (p<0.001)compared with the control group.**



Graph 3
Effect of zirconia nanoparticles on the activity of Superoxide dismutase

The level of SOD was recorded in the treated groups and in the control. The level of SOD was recorded maximum (7.123±0.001 units/mins mg protein) in the Liver tissue treated with 40 PPM of ZrO_2 nanoparticles. Minimum level of increase (5.618±0.075 units/mins mg protein) was obtained in the group treated with 10 ppm of nanoparticles. In heart tissue, the maximum level of enchancement of SOD (5.42±0.001units/mins mg protein) was recorded in the group treated with 40 ppm, minimum level of enchancement (4.71±0.004 units/mins mg protein) was noted in the group treated with 10 ppm (Graph 3).

Estimation of glutathione peroxidase (GPx)

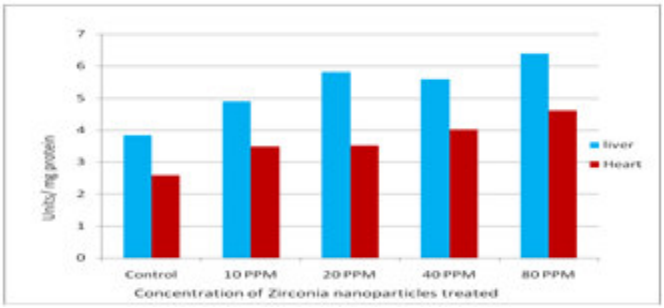
Glutathione peroxidase (GPx) activity was significantly ($p<0.001$) increased by administration of ZrO_2 compared with control in both liver and heart tissue (Figure 9).

Table 4

Glutathione peroxidase activity in liver and heart tissue of chick embryo

Groups	Treatment	Glutathione Peroxidase In Liver(units/mgProtein)	Glutathione Peroxidase In Heart (units/mg Protein)
I	Control	3.84±0.040	2.59±0.01
II	ZrO_2 (10 ppm)	4.89±0.033	3.48±0.022
III	ZrO_2 (20 ppm)	5.81±0.035	3.512±0.023
IV	ZrO_2 (40 ppm)	5.58±0.65	4.019±0.009
V	ZrO_2 (80 ppm)	6.395±0.07***	4.612±0.075***

The values are expressed means± SD of 6 replicates. indicates significant difference ($p<0.001$) compared with the control group.



Graph 4

Effect of zirconia nanoparticles on the activity of Glutathione peroxidase

Glutathione peroxidase (GPx) activity was significantly ($p<0.001$) increased in the treated groups when compared with the control in both liver and heart tissue. The data presented in Table 4 and Fig 9 clearly shows the influence of different concentration of zirconia nanoparticles at 10 ppm, 20 ppm, 40 ppm, 80 ppm in liver and heart tissue. In liver, maximum level of GPx activity (6.395±0.07 µg/mins mg protein) was recorded in the group treated with 80 ppm, minimum level of increase (4.89±0.033 µg/mins mg protein) in activity was observed in the group treated with 10 ppm. In heart tissue, the maximum level of GPx activity (4.612±0.075 µg/mins mg protein) was recorded in the group treated with 80 ppm, minimum level of enchancement (3.48±0.022 µg/mins mg protein) was noted in the group treated with 10 ppm (Graph 4 & Table 4).

Histopathological studies

Histological analysis revealed that in zirconia treated chick embryo there was no inflammation, necrosis, fatty change/ fibrosis noted. This was confirmed by histopathological examinations of liver. Microscopic observations of treated liver showed normal hepatic parenchyma, with well defined hepatocytes cell border (Fig 5(a) & (b)).

A B

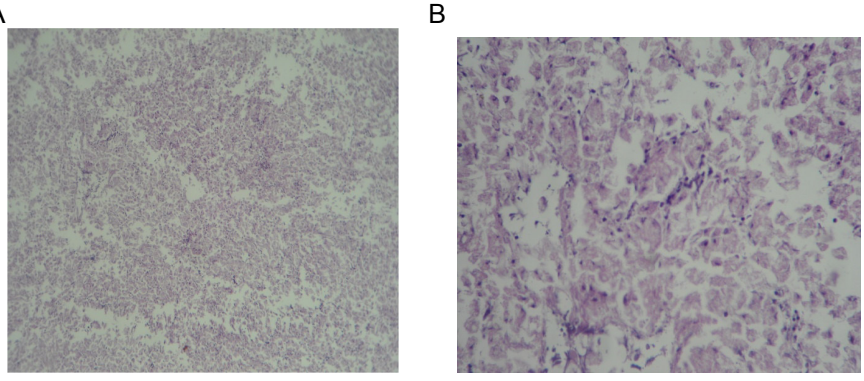


Figure 5

(A) Liver with normal hepatic parenchyma,(B) Liver with normal portal tracts and

(B) hepatocytes with well-defined cell borders

DISCUSSION

From the present study, Zirconia nanoparticles were synthesized. The size ranging from 50-70nm was observed to be less toxic that Zirconia particles used for dental treatment. This may be due to the nanosize and increased surface area. As the size of the particle increases, the level of toxicity increases. The results of our present study are discussed as followsZirconia in dentistry used was synthesized from industrial origin and have contributed to risk assessment. Thus, it is difficult to relate toxicity to specific particle characteristics. Therefore it is desirable to synthesize highly defined and uniform nanoparticles to identify the toxicity of nanoparticles[16]. Zirconia exists in tetragonal, monoclinic and cubic phases. [16] Among all these phases, monoclinic is more stable at ambient temperatures whereas tetragonal and cubic phases are stable at much higher temperatures and observed to be non toxic[7].The average size of the particles synthesized in our present work was ranged from 50-70 nm size and it was proved that the particle synthesized was <100 nm and can be considered as nanoparticles and the structure obtained was crystalline in nature. The reduction in size was mainly due to the change in pH In our present study the pH played an important role for the size reduction. This result was in par with the work done by. [16] Previous researchers reported that the size of the particles may vary due to the effect of pH. [16] The zirconia nanoparticles synthesized in our laboratory ranged between 50 -70 nm size and this was due the pH 9. This confirms that, the pH plays an important role in the size of the particles. The monoclinic phase obtained in this study represents that it is a pure form of zirconia which was obtained at the temperature of 400^{o}C. The XRD results of our study confirms the formation of monoclinic structure of zirconia nanoparticle. Wang et al.[7] reported that the monoclinic phase represents the pure zirconia and change in phase can be achieved by the addition of oxides. It was reported that the temperature plays an important role in the change of the phases with Tetragonal and cubic structure and addition of oxides such as CaO, MgO, Y_2O_3 were used to stabilize the tetragonal and cubic phase of zirconia.[3] EDX analysis of the nanoparticles proved the presence of Zirconium and Oxide in the synthesized particles with very less impurities. The peak obtained through EDX was mainly due to atomic number of the molecules. 31.17% and 68.73% of Oxide and Zirconium are present in the Zirconia nanoparticles. From this result it was confirmed that the synthesized nanaparticles is 100% Zirconium dioxide. Our result was similar to the result of.[10] They also recorded the presence of Oxide and Zirconium.Particle size distribution was done for the Zirconia nanoparticles using Particle size analyzer. 65% intensity was obtained for particle size between 10-100nm, 35% intensity was obtained for particle size between above 100nm. Distribution percentage was more at particles size less than 100nm. The intensity of zirconia nanoparticle dispersed in NaCl is 65% which varies with zirconia nanoparticle dispersed in water is 80%.[9] This particle size distribution size is needed for further toxicological studies for observing the distribution of the particles in animal models. The chicken embryo has been chosen as *In Vivo* model, being independent from external nutrient and water supply, thus comprising a system being directly dependent on *in ovo* provision of experimental compounds. [11] Chicken embryo is a unique biological model because it is independent from mother organism. Moreover, the development of embryos is very fast, intensive and quite well known – described in detail in the standard of Hamburger and Hamilton.[11] Very intensive development of embryos makes them sensitive to even very small amounts of toxic substances. This model is used in medical, toxicological and also nutritional experiment as a primary investigation, often carried out prior to experiments with animals or humans.[19] In our present work the toxicity of zirconia (ZrO_2) nanoparticles was investigated using chick embryo as an *in vivo* model.Oxidative stress is a normal cellular process involved in cellular signaling, though excessive oxidative stress can be harmful. Many studies have shown that exposure to nanoparticles elevates cellular oxidative stress and induce toxicity.[16] In our work, the toxicity of zirconia nanoparticle was analysized using antioxidant enzymes assay such as Superoxide dismutase and Glutathione peroxidase, non-enzyme assay such as Lipid peroxidation and Histopathological analysis to find out damage in the organ cells were performed.In our present study there was a significant reduction in lipid peroxidation in the Zirconia nanoparticles treated groups in both the liver and heart tissues, when compared to the control groups. This result was not in accordance with Chakraborty.[18] They reported that the enhanced lipid peroxidation is associated with the depletion of antioxidants in the liver and heart and which may yield toxic aldehydes that are capable of damaging the membrane proteins. It was interesting to note that the liver and heart samples treated in our study would not have undergone any damage in the membrane even though treated with 80PPM of Zirconia nanoparticles. There was a significant increase in the SOD activity in liver tissue when compared to that of the control and heart tissues. This result varied with the results obtained from Gaurav.[19] They observed the SOD level in Winstar rats treated with Cadmium. There was drastic reduction of SOD level due to the toxic nature of Cadmium. In our study the level increased as the concentration of nanoparticle treatment increases and proved that the ZrO_2 nanoparticle were non toxic and also influences in protecting the tissues from oxidative damage in Chick embryo.[21] Glutathione is a protector against free radicals.[20] The GSH was decreased in liver, spleen and in Mitochondria of the nicotine treated mice. This was due to the toxicity of the nicotine and showed increased level of lipid oxidation products which was associated with the less availability of NADPH required for the Glutathione Reductase activity.[22] It was interesting to note in our present study the GPX activity was significantly increased in the liver than the heart and control group of the chick embryo. This increase in level of Glutathione peroxidase may be due to the conversion of superoxide anion to less dangerous hyperoxide which was further degraded by Glutathione peroxidase to water and oxygen.The above results were in par with the results of Histopathologic examination (Fig 5 a and b). The sectioning of the liver cells in the treatment

group of 80 PPM was found to have normal hepatic parenchyma, normal portaltracts and hepatocytes with well-defined cell borders.

CONCLUSION

From the results observed, the ZrO_2 nanoparticles within the range of 50-70 nm size and the concentration below 80 PPM can be used in Dentistry for dental crowns. The toxicity is mainly involved in the concentration and the size of the particles used. An enhanced activity of SOD, Glutathione peroxidase assays and reduced Lipid peroxidation in chick embryo in treated groups were observed, led to the determination of non toxicity and final confirmation of its use in dental crowns.

CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Piconi C, Maccauro G. Zirconia as a ceramic biomaterial. Biomaterial.1999; 20(1):1-25.
2. Parker RM. Use of Zirconia in Restorative Dentistry. Dentistry Today**.** 2007; 26(3):114-119.
3. Michael H, Sophie V, Philippe V. Zirconia: Established Facts and Perspectives for a Biomaterial in Dental Implantology. J. Biomed.. 2009; 88(2): 519-529.
4. Kreth J, Kim D, Nguyen M, Hsiao G, Mito R., Kang MK, Chugal N, Shi W. The Antimicrobial effect of silver ion impregnation into endodontic sealer against Streptococcus mutants. Open Dent J. 2008; 18-23.
5. Michael H, Sophie V Philippe V. Zirconia: Established Facts and Perspectives for a Biomaterial in Dental Implantology. J Appl Biomater. 2008; 88(2): 519-529.
6. Augusto G, Udo G, Gianpaolo G, Caterina LP, Marie CC, Ivan V. Natural radioactivity in zirconia-based dental ceramics. INIS. 2010**;** 13(2):1-20.
7. Wang JA, Valenzuela MA, Salmones J, Vazquez A, Garcia-ruiz A, Bokhimi X. Comparative study of nanocrystalline zirconia prepared by precipitation and sol-gel methods. Catal Today.2001; 68(1-3): 21-30.
8. Nirmal D, Nalini B, Vijayakumar P. Nanosized high κ dielectric material for finfet. Integr Ferroelectr. 2010; 121(1):31-35.
9. Pabisch S, Feichtenschlager B, Kickelbick G, Peterlik H. Effect of interparticle interactions on size determination of zirconia and silica based systems – A comparison of SAXS, DLS, BET, XRD and TEM. Chem Phys Lett. 2012; 521(1): 91-97.
10. Ruparelia A, Chatterjee C, Siddhartha P, Duttagupta B, Mukherji S. Strain Specificity in Antimicrobial Activity of Silver And Copper Nanoparticles. Acta Biomater. 2008; 4(3):707-716.
11. Zielinska MK, Sawosz E, Chwalibog A, Ostaszewska T, Kamaszewski M, Grodzik M, Skomiał J. Nano-nutrition of chicken embryos-Effect of gold and taurine nanoparticles on muscle development. J. Anim Feed Sci. 2010;19(5): 277-285.
12. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein Measurement With Folin Phenol Reagent. J. Biol. Chem.1951; 193(5): 265-275.
13. Brogan WC, Miles PR, Colby HD. Lipid per oxidation in adrenal and testicular microsomes. Environ Health Persp.1981; 38(4): 105.
14. Nandi A, Chatterjee IB. Assay of superoxide dismutase activity in animal tissues. J Biosci. 1988; 13 (3): 305-315..
15. Flohe L, Gunzler WA. Assays of Glutathione peroxidase. Methods Enzymol.1979;105: 114-121.
16. Huang YW, Wu CH, Aronstam RS, Toxicity of Transition Metal Oxide Nanoparticles. Recent Insights from in vitro Studies. Materials. 2010; 3(10): 4842-4859.
17. Sahu HR, Rao GR. Characterization of combustion synthesized zirconia powder by UV-Vis, IR and other techniques. Bull. Mater Sci. 2000; 23 (5): 349-354.
18. Chakraborty SP, Mahapatra SK, Sahu SK, Pramanik P, Roy S. Antioxidative effect of folate-modified chitosan nanoparticles. Asian Pacific J Trop Biomed. 2011; 1(1):29-38.
19. Gaurav D, Preet S, Dua KK. Protective effect of *Spirulina platensis* on cadmium induced renal toxicity in wistar rats. Arch Appl Sci Res. 2010; 2 (1): 390-397.
20. Yilmaz HR, Turkoz Y, Yuksel E, Orun I. An Investigation of Antioxidant Enzymes Activities in Liver of *Cyprinus carpio* Taken from Different Stations in the Karakaya Dam Lake. J Sci and Tech. 2006; 1 (1): 1-6..
21. Grodzik M, Sawosz E. The influence of silver nanoparticles on chicken embryo development and bursa of Fabricius morphology. J.Anim. Feed Sci. 2006; 15(1): 111–114.
22. Zhou X, Wang Y. Influence of dietary nano elemental selenium on growth performance, tissue selenium distribution, meat quality, and glutathione peroxidase activity in Guangxi Yellow chicken. Poultry Sci. j. 2011; 90: 680-686.