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Molecular Study of Acute Toxicity of Biosynthesized Copper Oxide Nanoparticles in Adult Zebra Fishes

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Abstract: ***Copper oxide nanoparticles are widely used in biomedical, electronic and catalytic fields. The evaluation of potential hazards and toxicity is necessary to ensure aquatic and human ecosystem do not get affected. Zebrafish (Danio rerio), are used numerous applications in research and preclinical studies. Zebra fish have the advantage of similarity in genetic homology with humans (70%). Due to this, it is widely used in toxicological studies. This study describes the expression of β-actin, Tumour Necrosis Factor-α (TNF-α) and induced Nitric Oxide Synthetase (iNOS), inflammatory genes due to the effect of biosynthesized copper oxide nanoparticles from Coleus amboinicus in adult zebra fishes. Further the study focuses on the acute toxicity which is induced by copper oxide nanoparticles in adult zebra fishes. Isolated genes were characterised by using UV transilluminator. However copper oxide nanoparticles have antimicrobial property, they cause oxidative stress in human genes. This study concludes that inflammatory response was triggered by biosynthesized CuO nanoparticles in adult zebra fishes. In this study, the expression analysis helps in limiting the dosage of copper oxide nanoparticles in drug development.***
Keywords: ***Copper oxide nanoparticles, Danio rerio, β-actin, Tumour Necrosis Factor-α, induced Nitric Oxide Synthetase.***

I. INTRODUCTION

Nanoparticles play a vital role in industrial development due to their ever expanding range of applications due to their versatile modifications in sizes, chemical properties and shapes [21]. In recent years, copper nanoparticles are being used in various fields that include drug delivery, organic synthesis and agriculture. In copper based nanoparticles, copper may be present in oxidation states, such as solid metal state (Cu0), Cuprous ion (CuI), Cupric ion (CuII). Cu0 is a highly conductive elemental copper which is used in synthesizing Cu_2O nanoparticles. $Cu(OH)_2$ nanoparticles are used as antimicrobial agents [33]. In recent years, CuO nanoparticles are used extensively in commercial products which include gas sensors, antimicrobial agents, electric conductors, etc [15].In drug delivery, the nanoparticles surface is modulated based on its applications. Nanotoxicity also addresses a regulatory aspect of growing explosion [21]. To design or to develop a medicine for humans, preclinical screening with animal model is essential for the study of biotoxicity of nanoparticles and biodistribution [1]. At present, a short-term test is being performed to find the acute toxicity of chemicals in fish, especially on juvenile (or) adult zebra fish. Over the last few years, animals used in acute toxicity are reduced due to implementation of three Rs (Reduction, Refinement, Replacement) [16]. Compared to mouse and chick models, zebra fish has many advantages due to genetic homology with humans [1]. The innate system of zebra fish has neutrophils and macrophages as hematopoietic leukocytes which form the first line of defence against mortalities, diseases and pathogens. The zebra fish hematopoietic system has a strong negative effect on copper [17]. Today, in various researches the zebra fish is used as a genetic model which includes chemical genetic screening, proteomes, toxicology, cancer research and infectious diseases. The study of zebra fish in high-throughput screens of small molecules is useful for studying gene expression for toxicogenic studies. Thus, using biotechnology tools on zebra fish will contribute to novel knowledge in new drug discovery [30]. The genotoxic risk in zebra fish is increased due to high intake of CuO nanoparticles. The exposure of CuO nanoparticles in aquatic organism causes teratogenicity [15]. Syed and Coombs studied the similarities of copper metabolism among fish and mammals [34]. In zebra fish, the effect of CuO nanoparticles leads to oxidative stress, cell death, DNA damage in animals and it is a key toxic nanoparticle to humans [15]. TNF-α and iNOS plays a major role in self propagation in neuroinflammation. Although a few studies show that increased protein expression of TNF-α and iNOS at carrageenan induced inflamed paws in mice. TNF-α and iNOS carry many cellular processes which include differentiation, inflammation and apoptosis during activation of TNF receptor 1and 2. TNF-α controls gene expression through transaction pathways [18]. PCR is one of the techniques of microarray data to monitor gene expression variation in all genes. This method has an advantage of knowing quantitation of changes at mRNA levels (10^7folds) and one can obtain wide ranging of data in a single experiment [22]. A 50% lethal concentration (LC_{50}) is the statistically calculated concentration of any material which leads to death of half of the members of target species which receive it [7].In this study, expression of iNOS and TNF-α genes has been studied in adult zebra fish using CuO nanoparticles which is synthesized from *Coleus amboinicus.*

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II. MATERIALS AND METHODS

A. Zebra Fishes and Maintenance

Adult zebra fishes that are healthy and devoid of any malformations or infections were chosen for the study. Prior to initialization of the experiment, fishes were acclimatized in laboratory conditions according to Organisation for Economic Co- operation and Development guidelines (OECD, 203). These Fishes were grouped and separated in various tanks for the treatment with Copper oxide nanoparticles biologically synthesized using *Coleus amboinicius* leaf extract already available in Virtis Biolabs Pvt Ltd, Salem, Tamilnadu.

B. LC_{50} Determination of the Given Sample

Zebra fishes of interest were weighed and separated in various tanks for the study. The test concentrations of biosynthesized copper oxide nanoparticles varied from 0.5 mg / L, 1.0 mg / L, 1.5 mg / L, 2 mg / L and 2.5 mg / L of the CuO nanoparticles. Finally, the dosages were calculated for 2 L to analyse acute effects and mortality. Also fishes used as control, maintained in separate tanks all the fishes were under treatment for 7 days.

C. Dissection Of Fishes For Muscle Tissues

Each fish was slowly anesthetized by adding ice chips into the water until the temperature reaches 12 ° C. When the fish remains still to any external stimuli, then those were taken for dissection. The muscle tissues were removed carefully and stored in Phosphate-buffered Saline for DNA isolation.

D. Isolation of Genomic DNA Procedure

Muscle tissue weighing 100 mg was taken in a pestle and motor, and was homogenized in a well by freezing using liquid nitrogen. The ground tissues were dissolved in 1.2 ml digestion buffer and incubated overnight (12-18hrs) in a water bath maintained at 55 ° C. Equal volume of Phenol-Chloroform-Isoamyl alcohol mixed in the ratio 25:24:1 was added and mixed gently and centrifuged at 3000 rpm for 5 min at room temperature. The aqueous phase was carefully removed without disturbing the middle or bottom layer and transferred to a new tube and ½ the volume of 7.5 M ammonium acetate and twice the volume of absolute ethanol was added, mixed well and centrifuged at 3000 rpm for 5 min at room temperature. The pellet was rinsed using 70% ethanol and the ethanol was discarded. The pellet was kept for air drying. Once dried, the pellet was dissolved in 30 µl nuclease free water for further processes.

E. Agarose gel Electrophoresis: (Mohamedet Al., 2011)

1) *Preparation of the Gel:* An appropriate quantity of agarose was put into an Erlenmeyer flask. Agarose gels were prepared using a w/v percentage solution. Most gels ranged between 0.5% - 2%. The volume of the buffer should not be greater than 1/3 of the capacity of the flask. Running buffer was added to the agarose-containing flask and mixed. The most common gel running buffers is TAE. The agarose/buffer mixture was melted. At 30s intervals, the gel was mixed and this was repeated until the agarose has completely dissolved. Ethidium Bromide (EtBr) was added to a concentration of 0.5µg/ml. The agarose gel was allowed to cool. The gel tray was placed into the casting apparatus and the melted agarose was poured and then the comb is placed into the gel mold to create the wells. The agarose was allowed to set at room temperature. The comb was removed and placed the gel in the buffer system.
2) *Setting Up of Gel Apparatus and Separation of DNAFragments:* A loading dye was added to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration. The power supply was programmed to desired voltage (1-5 V/cm between electrodes). Enough running buffer was added to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel. The leads of the gel box were connected to the power supply. The lid was removed. The DNA sample(s) were loaded into the gel. An appropriate DNA size marker should always be loaded along with experimental samples. The lid was replaced to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). When electrophoresis was over, the power supply was turned off and the lid was removed from the tank. The gel was removed and any excess buffer from the well was drained and the gel was placed in a paper to absorb any excess buffer. The gel was exposed to UV light under UV illuminator. DNA bands showed up as orange fluorescent bands. A picture of DNA bands was taken.

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F. Inflammatory Marker Expression Through PCR Amplification

1) Primers: For the current study iNOS, TNF α and β-Actin primers were used. Inflammatory markers such as TNF-α and iNOS (induced Nitric Oxide Synthase) were designed with National Center for Biotechnology Information Primer BLAST tool

2) PCR Amplification: For PCR amplification TAKARA©'s EMERALD PCR master mix (India) was utilized. Amplification was carried out for 30 cycles and the resulting PCR product was resolved in 1% agarose gel, and the results were recorded.

III. RESULTS AND DISCUSSION

A. LC_{50} Survival Rate

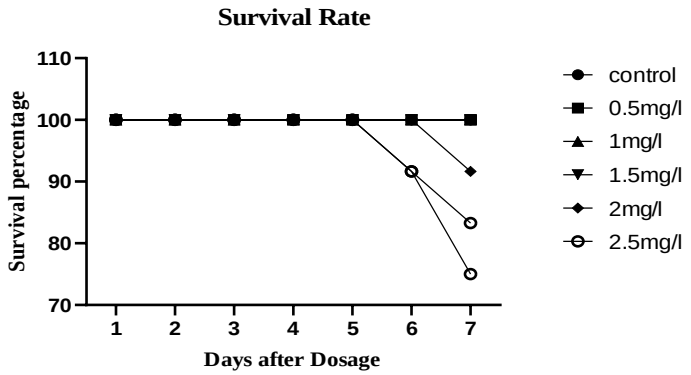

Fig : 1 Survival Rate of Adult Zebra Fishes After Exposure of Green Synthesized CuO Nanoparticles

To study the acute toxicity of green synthesized CuO nanoparticles adult zebra fishes were exposed to various concentrations of CuO nanoparticles for 7 days. The survival rate of adult zebra fishes were studied by treating with 0 mg / ml, 1 mg / ml, 1.5 mg / l, 2 mg / l and 2.5 mg / l of green synthesized CuO nanoparticles. In the treatment, dosages of 2 mg / l and 2.5 mg / l of green synthesized CuO nanoparticles has resulted in death. Till 5thday, the survival rate is not reduced. On the 6th day, the survival rate has been reduced to 91.6% in the sample which is treated with 2.5 mg / l of green synthesized CuO nanoparticles. On the 7th day, the survival rate reduced to 91.6% in the sample which is treated with 2 mg / l of green synthesized CuO nanoparticles and on the same day the survival rate reduced to 83.3% and 75% in the sample which is treated with 2.5 mg / l of green synthesized CuO nanoparticles.

B. Isolated DNA – Quantitative Analysis

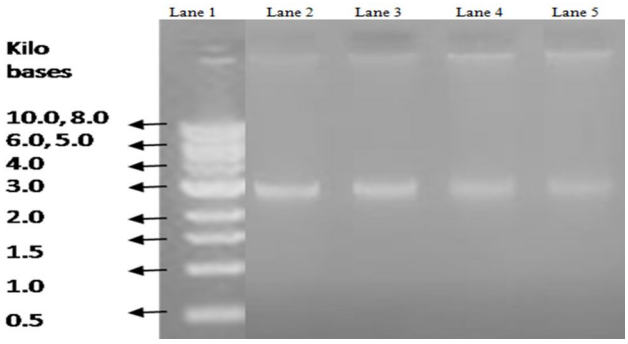

Fig : 2, Qualitative Analysis of DNA of Treated Zebra Fish Model

Lane 1 – 10 Kb DNA Ladder; Lane 2 – Control; Lane 3 – Sample (0.5 mg/L); Lane 4 – Sample (1.5 mg/L); Lane 5 – Sample (2.5 mg/L)

Isolated DNA samples run in 1% agarose and all the DNA bands in the lanes were noticeable and observed. (Fig. 2) shows that there is no degradation in the isolated DNA samples.

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C. Gene Expression Studies of Copper Oxide Nanoparticles in Adult Zebra Fish Model

1) β-actin

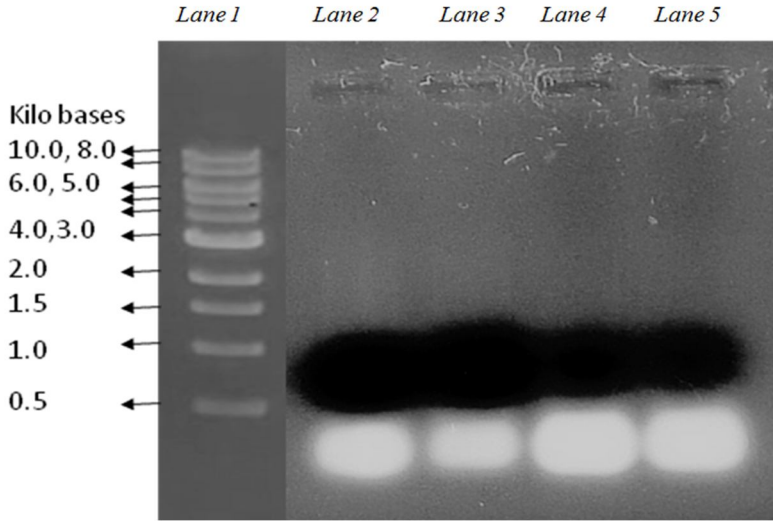

Fig : 3. β-actin Gene Expression of Treated Zebra Fish Model
Lane 1 – 10 Kb DNA Ladder; Lane 2 – Control; Lane 3–Sample (0.5 mg/L); Lane 4 – Sample (1.5 mg/L); Lane 5–Sample (2.5 mg/L)

A perfect housekeeping gene should be stable under disease state or experimental studies, the gene expressed in the cells and tissues of interest do not show any change. In gene expression experiments the most widely used reference gene is β actin, which encodes a structural protein of cytoskeleton [26]. The PCR product has been run in 3% agarose (Fig. 3) indicates that the house keeping gene (β actin) between various treated concentrations and as control. Treatment bands exhibit that β actin was stable upto 2.5mg / L with reference to control.

2) TNF-α

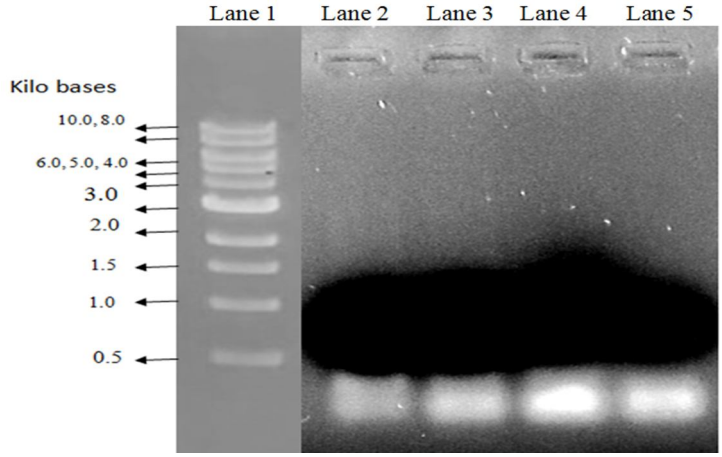

Fig. 4 TNF-α Gene Expression of Treated Zebra Fish Model
Lane 1 – 10 Kb DNA Ladder; Lane 2 – Control Lane 3–Sample (0.5mg/L); Lane 4 – Sample (1.5mg/L) Lane 5–Sample (2.5 mg/L)

For pro inflammatory cytokine production, TNF-α is considered as “master regulator” a powerful pro inflammatory agent and is suggested as a central player in inflammatory cell activation. TNF-α is produced by activation macrophages which play a vital role in chronic inflammatory disease development [24]. TNF-α signaling pathway activates apoptosis as cellular responses are triggered by it [14]. (Fig. 4) indicates the effect of biosynthesized CuO nanoparticles on inflammatory marker (TNF-α) expression between various treated concentrations and control samples. From this gene expression studies, it indicates that biosynthesized CuO nanoparticles induce oxidative stress in adult zebra fish upto 2.5 mg / L.

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3) iNOS

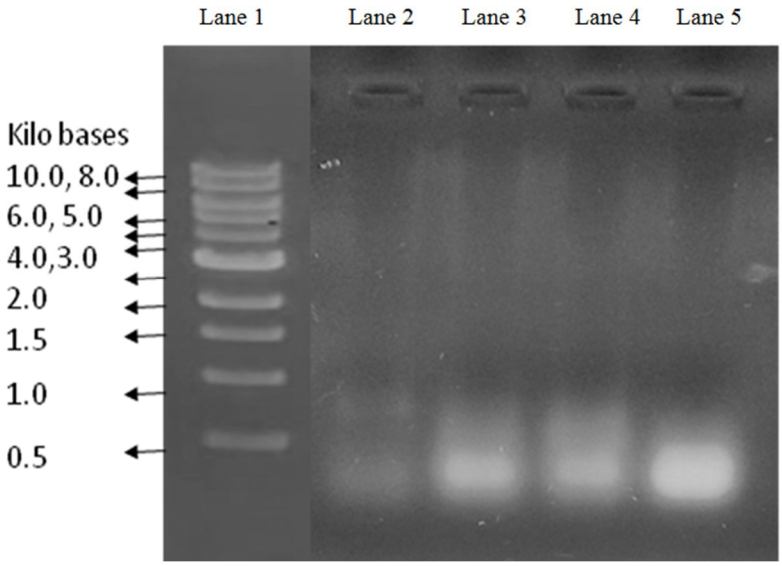

Fig: 5 iNOS Gene Expression of Treated Zebra Fish Model

Lane 1 – 10 Kb DNA Ladder; Lane 2 – Control Lane 3–Sample (0.5mg/L); Lane 4 – Sample (1.5mg/L) Lane5–Sample (2.5 mg/L)

As a defence mechanism iNOS produce large amounts of nitric oxide which is toxic and proinflammatory. The effect of nitric oxide in immune regulation include interaction with cell signaling pathways like JAK/STAT or MAPK, cAMP, cGMP, G-protein dependent signal transduction pathways which leads to programmed cell death [14]. (Fig. 5) indicates the effect of green synthesized CuO nanoparticles on inflammatory marker (iNOS) expression between various treated concentrations and control. From the gene expression studies, it indicates that CuO nanoparticles trigger oxidative stress in adult zebra fish upto 2.5 mg / L.

IV. CONCLUSION

As nanoparticles are used in drug delivery systems, toxicological studies are necessary to be done. Among metal oxide nanoparticles copper oxide (CuO) has been given special attention due to its antimicrobial activity. CuO nanoparticles are synthesized from the leaf extracts of *Coleus amboinicus.* After the exposure of green synthesized CuO nanoparticles on adult zebra fishes, LC_{50} survival rate of sample was estimated as reduction from 83.3% to 75% on 7^{th} day. DNA was isolated from the sample and amplified with iNOS, TNF α and β-Actin primers using PCR. The expression of iNOS and TNF α genes were found in biosynthesized CuO nanoparticles treated adult zebra fishes by qualitative analysis. It can be concluded that the inflammatory response was triggered by biosynthesized CuO nanoparticles in adult zebra fishes. This will be helpful in altering the dosage of CuO nanoparticles in drug development.

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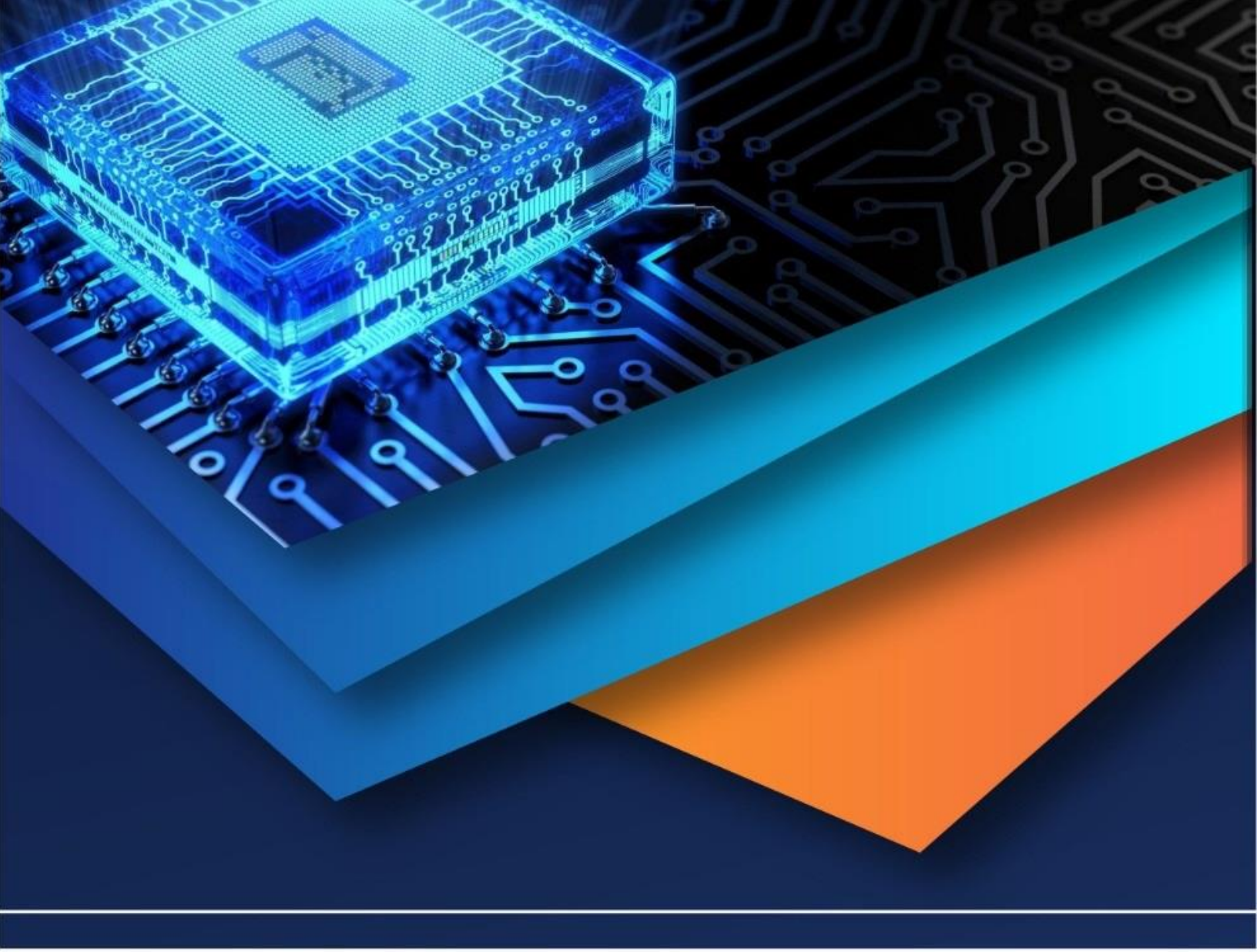

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