Kafrelsheikh University
Faculty of Veterinary Medicine
Department of Animal Medicine

PREPARATION, CHARACTERIZATION AND EXAMINATION OF SOME NANOMATERIALS AS FREE RADICALS SCAVENGERS IN SOME ANIMALS DISEASES

A Thesis Submitted

By

Samr Hamdy Kassem
(B.V.Sc.,Kafrelsheikh University,2007)
(M.V.Sc.,Kafrelsheikh University,2011)

For
The degree of doctor of philosophy in veterinary medical science
(Internal Medicine)

Under supervision of

Prof. Dr. Midhat N. Mohamed
Prof. of Veterinary Internal Medicine
Head of Animal Medicine Dept.
Faculty of Veterinary Medicine
Kafrelsheikh University

Prof.Dr.D.Sc. Mohamed Ali Ahmed
Late Professor of Experimental Physics
Materials Science Lab.(1)
Physics Department
Faculty of Science
Cairo University

Dr. Hossam E.M. Sayour
Senior Researcher of analytical and bioanalytical chemistry
Biomedical Chemistry unit, Chemistry and Nutritional Deficiency Disorders Department
Animal Health Research Institute

2016

List of contents

List of Tables

List of Figures

SUMMARY AND CONCLUSION

Synthesis of nanoparticles with unique characters able to overcome the disadvantages of traditional antioxidants as well as being safe on cells when applied *in vitro* and *vivo* is the main aim of this study. Novel ultrafine spherical yttrium oxide nanoparticles with particle size of 7.78 nm and average particle size 149.5 nm with surface charge of +29.4 were successfully synthesized by a low temperature coprecipitation method. Synthesized NPs characters like thermal profile, particle size, crystalline structure, morphological characters and surface charge were revealed by variable characterization tools as TGA, XRD, DLS, ZP and TEM. Also, the catalytic activity of yttria NPs was examined towards oxidation reaction of hydroquinone to benzoquinone in the presence of the hydrogen peroxide solution which revealed powerful highly specific catalytic activity extend to 48hrs. Synthesized NPs showed degree of agglomeration which will only effect on its antioxidant efficacy but also increase toxicity, so some of attempts performed to solve the problem of agglomeration and increase hydrophilicity of particles.

Grafting polymeric shell on surface of NPs by AA and EGMP polymers considered the most successful trials to produce hydrophilic well dispersed yttria NPs stable for one week and valid for wide range of biomedical applications, N-fluorescein Acrylamide added to shell in polymerization mixture as fluorescent material help in tracking the NPS *in vitro* and *vivo* as novel approach in diagnosis of NPs in cell culture and animal tissues.

The cytopathic effect and survival rate of polymerized NPs *in vitro* on 96-well plate of mouse normal hepatocyte cell line

with four replicate for each concentration of two polymerized NPS with AA and EGMP at wide range of serial dilution for each, NPS capped by AA (1.25-1000 μg/ml PBS) and NPS capped by EGMP (10-1800 μg/ml PBS), in this assay, the particles capped by AA showed cytotoxicity at low concentration of 10μg/ml at which loss its normal spindle shape of control cells being to sloughed and detached over time of examination. On the other hand, the particles capped by EGMP didn't show toxicity even at highest concentration 1800μg/ml that cells keep the same morphological characters compared with control ones.

24hrs post incubation, cell line plate which incubated with two polymerized NPS with AA and EGMP observed with fluorescence microscope detecting green internalized particles representing the penetration of cell membrane and well distribution of particles intracellular. The survival rate of incubated cell culture with two polymerized NPs with AA and EGMP 24hrs post incubation calculated by MTT assay, survival rate of cell line incubated with AA ranged from 99.9 to 100% at concentration ranged from 1.25 to 8μg/ml and ranged from 1.67 to 0.59% at concentration ranged from 10 to 1000μg/ml but survival rate of cell line incubated with EGMP ranged from 99.5 to 99.8% at concentration ranged from 10 to 1800μg/ml.

Depending on *in vitro* results which help in trendy determining of toxic and safe doses, two experiments applied *in vivo,* one to detect toxicity of NPs capped by AA and EGMP at high concentration and another to detect the biocompatibility and bio distribution of EGMP capped NPs. At the first trial, high toxic doses (10, 30 and 50mg EGMP

capped NPs group 1-3) and (2 and 1mg AA capped NPs group 4 and 5) administered intravenous via tail vein for five groups of Sprague-Dawley rats weighed 150g Bwt each consisted of 7animals and one control group, tissue specimens were collected from all experimental NPs groups after scarifying the animals from targeted organs like liver, spleen, kidney and brain collected at time interval 12, 24, 72hrs and 10d after injection of NPs for histopathological examination as well as fluorescence imaging of NPs in tissues.

The liver is known to be a major immunological organ affecting systemic responses in animals in response to xenobiotics, Adverse changes were detected in liver ranged from mild changes as congestion, sinusoidal cell activation and hydropic degeneration in group 1, 2 and 4 to moderate degree in group 3 ended with severe changes in group 5, 12hrs post injection and portal fibroblasts and oval cells hyperplasia was observed in a mild degree in group 1, 4, 5 72hrs post inoculation, the morphological changes begin 12hrs after injection extending 24 and 72hrs declined 10days to be mild changes in most of experimental groups. The differences in hepatic response between EGMP and AA capped NPs not only dose dependent but also the solubility and hydrophilicity effect on the toxicity and bio distribution of NPs, our study noticed decline on severity of lesions even at high toxic doses which referred to surface modification of NPs by core shell polymerization as key factor assess in reducing extension of adverse effects overtime of *in vivo* experimentations of NPs.

Our study able to record adverse changes in brain architecture after high toxic doses injected, adverse changes were detected in brain ranged from very mild neuronal

degeneration and necrosis was observed in group 1 to mild degree in group 2 and group 3 and in severe degree in group 4 with focal neuronophagia and one hemorrhagic area were observed in group 3. Also, mild degree of neurophagia and gliosis in group 1 and 2 and in severe degree in group 3, 4 and 5 with severe sub meningeal hemorrhage and congestion was observed in group 4 and mild acute meningitis was observed in group 5 extended 72hrs and 10days post injection, the severity of response between EGMP and AA capped NPs is clear that severe sub meningeal hemorrhage was observed in group 4 matched with the effect of solubility and hydrophilicity on the toxicity of NPs. The spleen was normal in all experimental groups 12hrs post injection and showed lymphocytic cells depletion varied from mild to moderate degree in all experimental groups 24hrs and 72hrs post inoculation respectively to be mild lymphoid hyperplasia 10days post inoculation in all experimental groups as well as no pathological changes observed in kidney.

Intracellular localization and distribution of fluorescence labeled NPs in tissues was detected by fluorescence microscope 12, 24, 72hrs and 10days after NPs injection. NPs group1 (dose 10mg) were identified in liver specimens 12hrs after inoculation absent after that while in group 2 (dose 30mg) present 24 and 72hrs after injection absent before and after these times respectively, group 3 (dose 50mg) and 4 (dose 2mg) detected 12 and 24hrs post inoculation absent after that while in group 5 (dose 1mg) detected only 12hrs post inoculation not present after that , The NPs was detected in spleen tissues in all groups 12, 24 and 72hrs after inoculation absent after that. Our results revealed brain localization of injected NPs in group 1 was identified in brain tissues 12, 24

and 72hrs after inoculation absent after that while in group 2 not able to pass BBB before 72hrs and 10d , group 4 and 5, NPs detected 12 and 24hrs post inoculation absent after that. Those results showed that EGMP NPs group1 at lowest dose 10mg enter the brain earlier than group 2 at dose 30mg revealing dose differences affecting cellular uptake of NPs as well as solubility of NPs as in group 4 and 5 which not able to persist more than 24hrs post injection differing from EGMP capped NPs, fluorescence microscope investigation of the polymerized YNPs were completely absent in kidney tissues in all groups all over the time.

The second *in vivo* trial performed to determine the biocompatibility and biodistribution of polymeric nanomaterials based on EGMP only in low doses injected intravenous via tail vein for five groups of Sprague-Dawley rats weighed 150g Bwt each consisted of 7 animals, groups 6-10 at doses 0.02, 0.066, 1.0, 0.02 and 0.1mg respectively. The regular findings of liver immune response against any foreign materials were observed by light microscope mainly mononuclear cell infiltrations as macrophage ranged from mild degree in NPs group 6, 9, 7 and 10, and in a moderate degree in group 8 1hrs after injection extend in all experimental groups 24hrs post injection without significant variation in the degrees of severity between groups and finally decline to mild and very mild changes 72hrs and 10 days post injection as those results referred to high solubility and hydrophilicity of NPs as well as the biodistribution and biocompatibility depends mainly on dose of administration and surface characters of NPs.

Histopathological studies were conducted on brain, spleen and kidney sections harvested 1hr, 24,72hrs and 10 days post injection with EGMP capped NPs, all groups revealed no remarkable changes that it is the first study recorded no pathological effects at dose up to 1mg. Intracellular localization and distribution of fluorescence labeled EGMP was detected by fluorescence microscope 1hr, 24, 72hrs and 10days after NPs injection, NPs were absent in liver tissues in group 6, 7, 9 and 10 at all time intervals while present in group 8 at 1hr after inoculation but mildly present 24,72hrs after inoculation. The NPs was detected in spleen tissues in group 7 at low level 1hr after inoculation absent after that, group 8 NPs present mildly 24, 72hrs after inoculation absent before and after that while group 9 NPs mildly present 1hr after injection and 1hr, 24hrs in group 10 and completely absent in group 6, those records referred to greater extension of polymeric coating on surface of NPs resulted in avoiding ready recognition and lateness of uptake of circulating particles in blood by reticuloendothelial organs.

Our study success to record NPs localization in brain at all times intervals at all doses 1hr, 24, 72hrs persist till 10days post injection but the lowest dose (0.02mg) of group 1 detected 24hrs extend to 10days post injection, the lateness in reticuloendothelial uptake of injected NPs due to proper highly extended surface coating give high chance to NPs to remain longer time circulating in blood subsequently ability to pass to brain tissues. The polymerized YNPs with EGMP were completely absent in kidney tissues in all groups all over the time as the same results recorded in trial of toxicity detection of NPs.

The third *in vivo* trial performed to determine the antioxidant efficacy of EGMP capped YNPs, heat stressed model performed on total of 21 rats (heat stressed group) and exposed to 48°C and relative humidity 50±15% for 15 min two times at 5 min interval and assuring stress by measuring internal body temperature (39±1.5 ˚C) and then differentiated to two subgroups 1(heat stressed with NPs injection) and 2 (heat stressed without NPs injection), first one injected with 0.2mg of EGMP capped YNPs and another group not injected with NPs post heat stress exposure as well as another group consisted of 7 rats injected with 0.2mg of NPs pre heat stress exposure (prophylactic group) with the same conditions of heat stressed group. Different samples of blood, serum and tissue homogenate samples were collected at time intervals along the period of experiment to measure the oxidative biomarkers like enzymatic antioxidants (SODs, GPX, GST, GR and TAC) and oxidative byproducts (MDA, PC and 8-OHdG) and liver specimens histopathologically examined 2hrs post NPs injection, prophylactic group and heat stressed ones.

In heat stressed group, significant increase was recorded ($P>0.05$) in SODs activity in erythrocyte lysate and TAC concentration in serum as well as PC and MDA concentrations in tissue homogenate of liver and spleen and 8-OHdG in serum compared with control group however other enzymatic antioxidants as GST, GPX and GR activities erythrocyte lysates revealed significant decrease ($P<0.05$). In prophylactic group, significant decrease ($P<0.05$) in SODs activity and TAC revealed as well as PC and MDA in tissue homogenate of liver and spleen and 8-OHdG concentrations in

serum however other enzymatic antioxidants as GST, GPX and GR activities revealed significant increase (P>0.05).

Significant increase (P>0.05) in TAC concentration, GST, GPX and GR activities as well as significant decrease (P<0.05) in SODs activity, PC, MDA and 8-OHdG concentrations was recorded in heat stressed group with NPs injection 2, 24 and 48hrs post injection compared with heat stressed group without NPs injection at the same time intervals.

Histopathological findings revealed improvement of morphological characters of prophylactic group and heat stressed ones 2hrs post NPs injection compared with heat stressed ones.

The present study was concluded to

- Nanotechnology based theranostics for oxidative stress is successful alternative to traditional antioxidants, our study success in synthesis of unique yttria NPs with particle size of 7.78 nm and average particle size 149.5 nm with surface charge of +29.4 with high catalytic activity as those novel characters give the chance for wide range of biomedical applications.
- Core shell polymerization with EGMP polymer is successful novel approach for the first time to modify NPs characters, increase hydrophilicity, dispersity and control agglomeration over time, surface modification of synthesized NPs is the key factor in maximizing the beneficial effects rather than toxic ones especially *in vitro* and *in vivo* applications. Also, labeling of NPs with fluorescein is successful way in tracking of NPs in

tissues and considered cheap and safe alternative method of diagnosis.

- The cytopathic and survival rate of core shell AA and EGMP based YNPs were detected *in vitro* to be concluded that the difference in response between two NPs depending on the hydrophilicity and surface charge of NPs mainly.

- The morphological characters of NPs as surface characters, size, shape, surface charge as well as dose and route of administration are collectively factors not only effect on toxicity of NPs *in vivo* but also on biodistribution and compatibility of NPs as this study first recorded the ability of EGMP capped NPs to pass BBB as real challenge at all low doses up to 1mg with persistence up to 10days without pathological changes. Also, depending on those novel characters, lateness in reticuloendothelial uptake of circulating NPs in blood give the chance to enter other organs is achieved.
- The synthesized NPs success to perform double purposes, one prophylactic and another therapeutic antioxidant one on heat stressed lab animal model to overcome the wide limitations of traditional antioxidants and able to face the cascade reaction of free radicals generation and rescue cells from harmful effect.