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DUAL ROLE OF RNASE A AND METOSARTAN -EXAMINATION IN INVITRO & INVIVO CONDITION

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Abstract: -

Metosartan consists of two components Metoprolol and telmisartan. Telmisartaninhibits RNase A in rat testes by uncompetitive inhibition whereas Metoprolol forms DNA adducts and reduces sperm count. Metosartan decreases testes viability and sperm count was found to be high in metosartan treated rats compared to Metosartan +RNase A group. RNase A induces Netosis inboth invivo andinvitro conditionin both RNase treated group and as well as RNase A+ Metosartan. Deep cold storageof mitochondria inducesexpression ofcyt P450genein testesmitochondriabut not in sperm.

Keywords: - *Metoprolol, Netosis, Uncompetitive inhibition, DNA adducts, Cyt P450*

INTRODUCTION:

DNA adducts are the structures formed by chemotoxic agents that bind DNA which may be the drugs also. From previous reports DNA adduct inducing drugs are generally used to treat cancer. From previous reports of mine showedmetosartan inhibits RNase A so we proceed to know the individual components of metosartan which inhibits RNase A but surprisingly we came to know that Metoprolol induces DNA adduct formationprovedwhen treated with DNase. Telmisartan is a angiotensin ii type I receptor blocker which hasabsorption maxima at 296nm (1) whereas Metoprolol is a β-blocker and has maximum absorption at 226-228nm (1). There are three types of cell viability loss, namely apoptosis, necrosis and Netosis. Apoptosis is a programmed cell death sometimes followed by necrosis.Netosis is the infiltration of neutrophilsfollowed by NET formation in the tissue. It is also known as autophagy and occurs during inflammation, infection or under condition of nutrient deprivation,especially known as autophagy.Previous reports of mine showed aspermia when treated with metosartan in invivo and teratozoospermia ininvitro and invivo also. Morphological defects are absent with RNase A+ metosartan and also at cellular level to some extent as the inhibition is transient and RNaseA is active up to 2 hrsand again after 3hrs. Metoprolol affects sperm count and telmisartan induces ds break formation in DNA when given as metosartan. Inhibition index of drug was found to be >1 which indicates drug-drug interactionsand also the mol.wtof RNase A in testes of rat was found to be 24kda approximately, where asin case of human it is around 27kda(2)and found to be similaras that of humanpancreatic RNaseproved by Hoechst 33342 taggedwithhuman specific antibodies through technique of immunoflouresence(3).RNase A is a monomer but the enzyme shows cytotoxic activityonly in oligomer state(4). So, the binding of telmisartanto RNase A is with positive cooperivityand it isalsoa negative modulator as it inhibits the drug. CytP450 is expressed in cell organelles mitochondria and endoplasmic reticulum. In present study the deep cold storage induced expressed of Cyt P 450 in mitochondria and may be involved in clearance of oxidative stress.

Results:

RNase A normally induces apoptosis in cancerous cells but in testes tissue the RNase A induces autophagy or Netosis with neutrophil infiltration forming extacellular traps with fragments of chromatin and histones. In autophagy and as well as Netosis the cell membrane is intact(5)anddoesn't stains with viability dyes. Netosis is also seen with RNase +drug treated tissue but it is not up to the mark. In case of both treated tissues Netosis is seen by examination using SEM indicated with arrow marks. In figure C trypan blue staining resulted in film formation stained with blue. So, it is difficult to know whether the cellsare viable or not as metosartanincreases the membrane permeability.

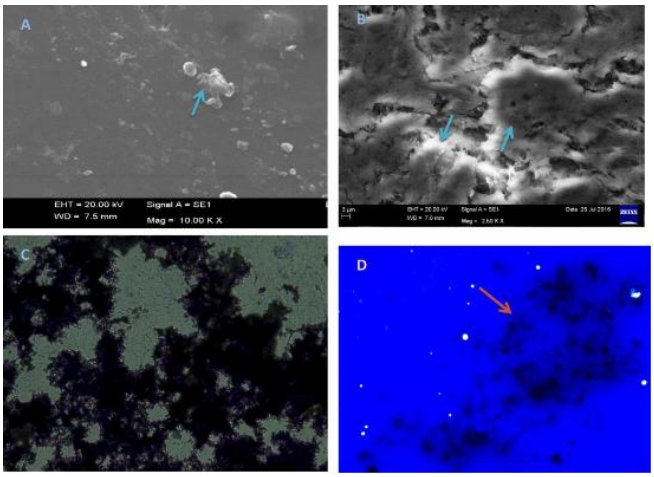

Figure: 1 Tissue examination by SEM and cell viability of tissue treated with metosartan bystaining withtrypan blue. (A) Tissue treated with both metosartan and RNase A invitro. The arrow indicates neutrophil with extracellular traps. (B) Tissue treated with RNase A invitro. The arrowsin the figure indicateNeutrophil extracellular traps with chromatin fragments and proteins like histones. (C) Tissue treated with Metosartan (100µg/100µl) invivo. Most of the tissue is dead due to apoptosis. (D) Rats treated with both Drug+ RNase A invivo. The arrow indicates Netosis.

As metosartan induces aspermia and azoospermia sperm morphology abnormalities were studied usinggeimsa staining. Treatment with metosartan induced morphological defectsof sperms like interlinking, acrosomes reactions and broken middle sperms(fig.2A). In case of RNaseA+ metosartan treated group the sperms are not seen with any morphological defects.But treatment with RNase A+ Metosartan resulted in DNA fragmentation but arrest of apoptotic cell death which clearly indicates Netosis in testes tissue.There are reports on Metoprolol on reduced sperm count with drug interactions. Tel misartan in hibits RNase A by Uncompetitive inhibition (fig. 2B) and shows positive cooperitivity towards RNase A (fig. 2A). Telmisartan duration of action is 24hrs. Whereas Metoprolol duration of action is about 12 hrs. Metoprolol is cleared after the duration of action and the experiments of invivo was performed after 24 hrsand the cells are intact even after 24hrs soMetoprolol is the component responsible for cytotoxicity in testes when given in individual dosage. Telmisartanis the one that changes permeability of membrane in mitochondria as the drug is a reversible inhibitor and inhibits release of cyt C(6)as it inhibits RNase A

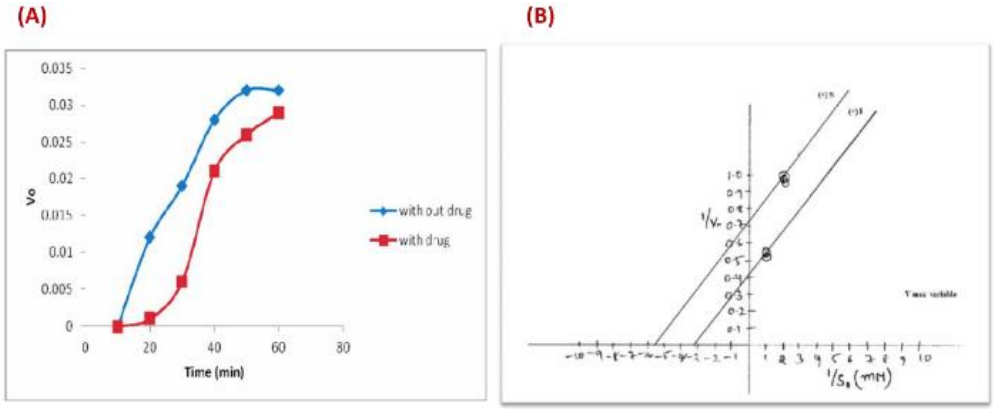

Figure :2 Enzyme Kinetics of pure RNase A in the presence and absence of drug. (A) Michaelis-Menton graph of RNase A. (B) Lineweaver-Burk plotof RNase A.

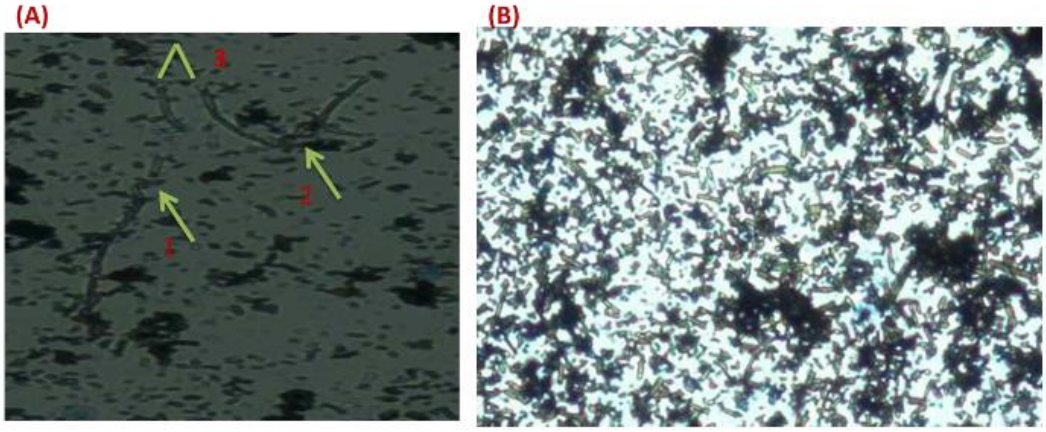

Figure : 3Morphological defects of sperm treated with drug metosartan and Metosartan + RNase A. (A) metosartan treated rats. (B) RNase+ Metosartan treated rats. In figure A the arrow 1 indicates broken sperm, arrow 2 represents interlinked sperms and arrow 3 indicates acrosomes reacted sperms. Where as in figure B there is no morphological defects associated with sperms treated with RNase A+ Metosartan.

Metosartan forms adducts with DNA as Metoprolol (fig. 4)gives maximum absorbance at 226-228nm. The invivo treatment of metosartan +RNaseAresulted in DNA adduct formation with absorption maxima at 265-268nm and treatment with DNase resulted in absorption peak at 226-228nm.

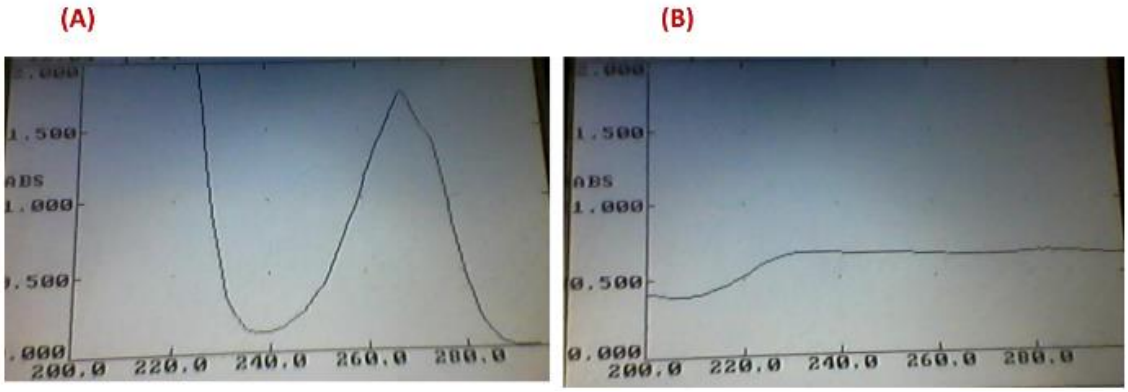

Figure: 4DNA adduct formation with Metoprolol in male rats treated with drug metosartan+ RNase A. (A) DNA absorption spectrum, (B) DNA absorption spectrum treated with DNase.

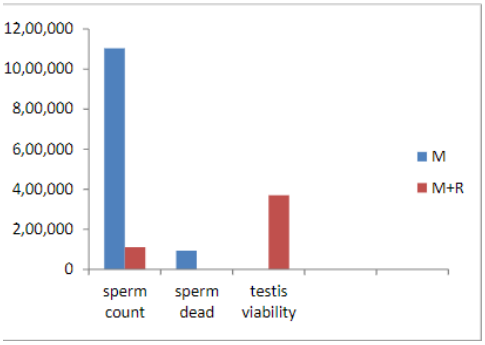

Figure : 5Graph of sperm count and viability of sperm and testes in male rats treated with drug metosartan and RNase A + Metosartan. where M and M+R in the figure indicates Metosartan and Metosartan + RNase A

Sperm count was drastically reduced in M+R group compared metosartan treated group but all the produced sperms are viable in M+R group. In case of testes viability metosartan is extremely toxic to the tissue where as some cells are viable as they excluded the trypan blue but RNase A induces Netosis so that cells may leads to false results with trypan blue (fig; 5) as the membrane is intact (fig.1B).The numberof cells viable in M+R treated group is 3,70,000 where as in metosartan it is taken as almost zero(fig.5).

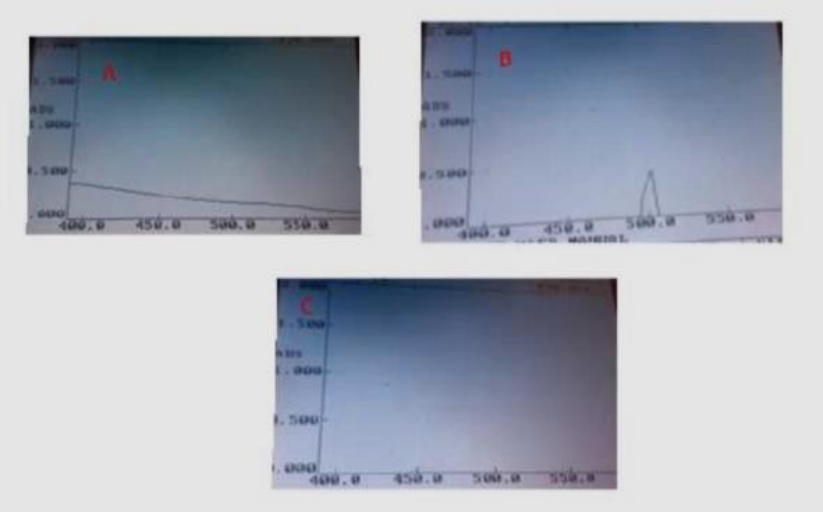

Figure: 6Study of effect of oxidative stress on rat testes mitochondria. (A) control rat testes mitochondria isolated at normal temperature. (B) rat testes mitochondria isolated and subjected to cold storage for about to one week. (C) Rat sperm Mitochondria isolated and subjected to cold storage about to one week. In testes, mitochondria specific expression of protein cyt P450 is seen.

Deep cold storage causes oxidative stress as Electron transport chain is diverted to produce heat instead of production of ATP leading to generation of free radicals.In case of testes mitochondriaspecificexpression of cyt P450 is seen(fig.6B) and its role in the organelle in testes is uncertain. In case of sperms the sperm head consists of antioxidative enzymes so the free radicals are neutralised thereby preventing oxidative stress.

Discussion:

Metosartan as an anti-hypertensive agent is well known but the effect of the drug on the nucleases is to be considered know during medication. However the drug inhibits RNase A by reversible inhibitonwhich begins after 2hrs and ends by completion of 3hrs(7)so from the data available the constituents of metosartan can acts as both H+donor and acceptor so, the drug binds to Angiotensin type I receptor may be with hydrogen bonding. The drug inhibition is transient which can be explained by low amounts of NETs in RNase A + metosartan treated rats.From previous reports of mine metosartan inhibits RNase A by Uncompetitive inhibition because the enzyme binds onlyto ES complex and inhibits the activity of the enzyme proved by agarose gel electrophoresis(8) and RI is also one of the inhibitorto be considered underinvivo conditionsto get a clear data.Metoprolol has the cytotoxic activity in testes which is responsible for the low sperm count(9)and sperm viability. DNA adducts was formed by many drugs especially cisplatin and oxaliplatin(10,11)preventing DNA repair and has negative effect on genome integrity of cancer cells. In present study Metoprolol is able to form DNA adducts which give absorption maxima at 265-270nm.The DNA used is Pure as digestionwith DNase resulted in loss of peak and appearance of absorption maxima at 223-226 nm which is metosartan. Cyt P450 is expressed in all the cells and especially in cell organelles E.R and mitochondria. In E.R the protein is involved in removal of external genotoxic agents where as in mitochondria is involved in removal of internal toxic compounds. In present study deep cold storageof mitochondriainduced expression

of Cyt P450 which is involved in removal of free radicals produced due to oxidative stress. By this I conclude that Metoprolol can be used as a genotoxic agent for cancers like prostate, sperm cancer and testicular carcinomas.

Materials and methods:

Invivo studies:

Both RNase A (1mg/ml) and metosartan (1mg/ml) were given to male wistar rats in both invidividual and combined doses by oral gavage.

Trypan blue staining:

0.2 ml of 0.4% trypan blue in water, 0.2ml of Hanks balanced salt solution and 0.2ml of homogenised tissue solution was mixed and in which 0.1 ml of the mixture was loaded on to neubar chamber and the viable cells are counted. Similarly, 0.4% trypan blue was also used to stain the testes smears on the slide.

Giemsa staining:

Semen was collected from epididymis, smears were prepared, air dried and stained with giemsa for 5min and washed for 2-3 min not exceeding more than 10 minutes and observed under microscope.DNase : 1mg/ml

Enzyme kinetics:

1ml of RNase was mixed with 1ml of RNA and readings wererecorded at 260nm. Similarly thereadings are recorded after addition of 1ml of metosartan and enzyme kinetic plots are plotted with the data.

Isolation of mitochondria from testes:

The Mitochondria were isolated from testes tissue after and before cryopreservation. .The rat testes were homogenated in isolation medium [250mM sucrose, 0.2 mM EGTA, 0.1mM EDTA, 5mM HEPES-KOH (PH 7.4) and 0.1%defatted BSA] using mortar and pestle. The homogenate was centrifuged at 10,000Xg for 10min. The collected supernatant was further centrifuged at 10,000Xg for 10min and the pellet obtained was resuspended and pelleted twice in the isolation medium without EGTA, EDTA, & defatted BSA. It was considered as mitochondrial pellet.(Amaral, et al.,2008)and subjected to cold storage at 40c and spectr was recorded at 400nm -550nm.

Scanning electron Microscope:

Aniline blue stained slides (Hammadeh et al., 1996)were shade dried for 2 months and examined using SEM.

Isolation of DNA:

DNA was isolated from RNase A+ metosartan treated group (Hofstetter et al1997).The rat testes were homogenized in lysis buffer (50 mM Tris-Cl, pH 8.0, 100 mM EDTA, 0.125%

SDS) using mortar and pestle, and further 1ml of the lysis buffer was added to the collected supernatant and incubated for overnight at 550C. To the lysate an ml of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added and centrifuged at 2000rpm for 10min.Aqueous phase was collected and washed once again with 1ml of phenol: chloroform: isoamyl alcohol mixture and 3ml of chloroform. Aqueous phase was separated and DNA was precipitated with cold ethanol. Precipitated DNA was pelleted, collected and stored at -200C. Spectrum of the isolated DNA sample was recorded from 200-300nm and the same sample was performed for spectral analysis after treatment with DNase.

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