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Apoptosis of testis tissue by drug metosartan through activation of intrinsic pathway by the release of Cyt C from mitochondria

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Abstract
Drug metosartan the combination of both metoprolol and telmisartan has proved effective in the treatment of hypertension but the present study deals with the invivo effects of metosartan on testis tissue. It causes release of cytc from mitochondria in both fresh and treated invivo tissue but the marked effect was seen in invivo treated tissue and mitochondrial viability comparision in both drug treated and RNaseA+ drug treated tissue. DNA fragmentation in both RNaseA + drug treated tissue was markedly shown. The combined effects of drug and RNaseA has shown that the apoptosis occurs through extrinsic pathway in testis as DNA is fragmented which is the main cause of apoptosis.

Keywords: Intrinsic pathway, apoptosis, Cyt C, Mitochondria, DNA fragmentation

Introduction
Mitochondria plays a major role in most of the cellular process namely redox maintainance, energy metabolism, ion balance and apoptosis. Apoptosis should be clearly distinguished from the another cellular death process namely necrosis. In case of necrosis the mitochondria lyse where as in case of apoptosis the cells shrink with intact cellular organelles and also mitochondria [1, 2]. Cyt C and AIF (Apoptosis Inducing factor) are the two major proteins release from the mitochondrial membrane but the present study focus on Cyt C.

Cyt C is a 12.3 Kda protein involved in the electron transport between complex III and complexIV in mitochondria. The precursor protein of Cyt C the apocytochrome C was synthesised in cytoplasm on free ribosomes and gets inserted in to mitochondrial membrane through non receptor mediated process.

The mechanism of activation of apoptosis through Cyt C was clearly known now. The apoptosome Apaf known to contain the CARD domain [3] (Caspase Recruitment Domain) at N- terminal which is shared by caspases and ced-4 and WD-40 repeats at C-terminal regions. The process starts by binding of ATP or dATP to the walker boxes present in ced 4 and cyt c binds to WD-40 domains which exposes the CARD region of the Apaf. The Apaf doesn't have the proteolytic activity so the caspase undergoes autocatalysis. Caspase 9 causes cleavage of caspase -3 which causes cleavage of caspase -6, DNA fragmentation factor [4] and poly (ADP-Ribose) polymerase leading to nuclear lamin cleavage and DNA fragmentation leading to the other changes in apoptosis [5].

So, Cyt C release is a irreversible damage to the cell which is mediated in the form of the cell death and present study deals with the effect of drug metosartan through cyt C and RNase A.
Figure: 1

Material and Methods
Experimental design for *in vivo* studies
3 animals each were taken as a group for control, drug treated of 100µl and both RNaseA+ drug of 100 µl treated given through oral gavage for one day and the animals were sacrificed and the following assays were performed.

Agarose gel electrophoresis
1% agarose gel was prepared and the sample was loaded and run on the TAE buffer at 50V for first 1/2hr and at 100V for the rest of the time period. After the run the gel was observed under UV trans illuminator and observed for DNA fragmentation.

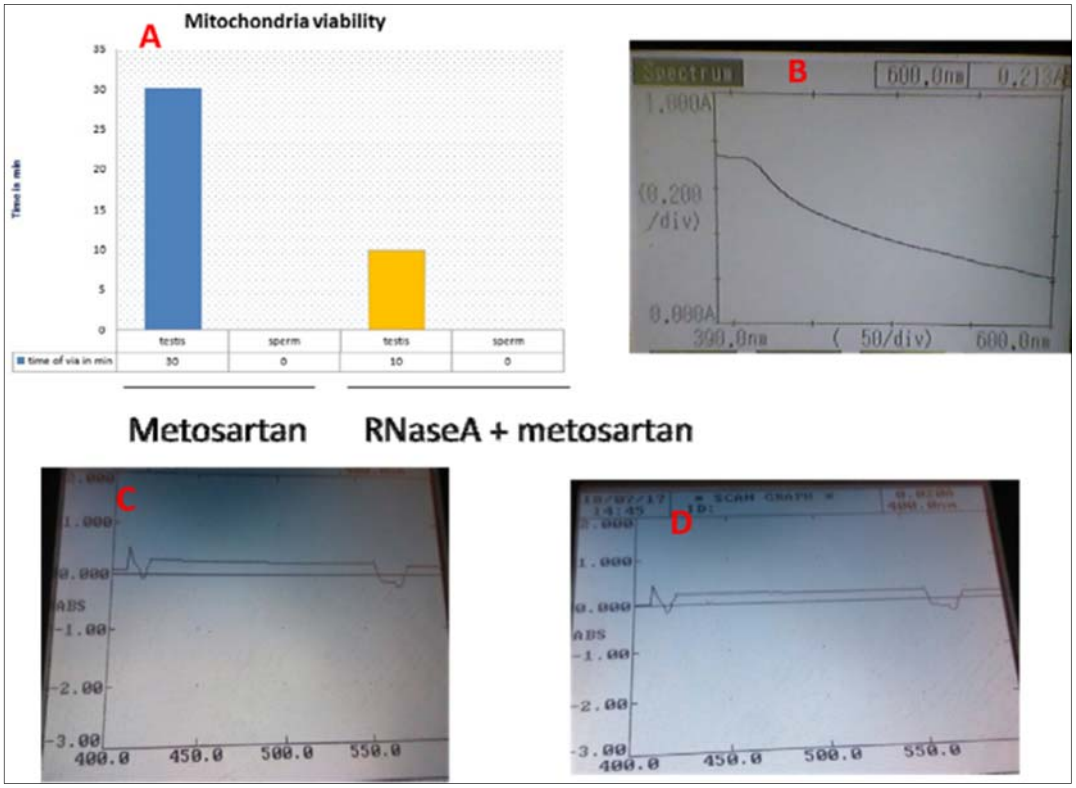

Fig 1: Mitochondria viability and apoptosis in testis and sperm through release of Cyt C. Fig 1A shows that in the drug treated one the mitochondria is viable for up to 30min where a incase of sperm the viability was reduced to 0%. Where as in case of both drug and RNaseA treated one the viability of mitochondria from testis was reduced to 60% and incase of sperm it was as such. Fig 1B is the fresh mitochondria isolated from the testis and tested for release of Cyt C through UV – Visible spectrophotometer. Fig 1C shows the treated testis tissue for release of cyt c and fig 1D shows the release of Cyt C from mitochondria without any treatment.

Figure 2

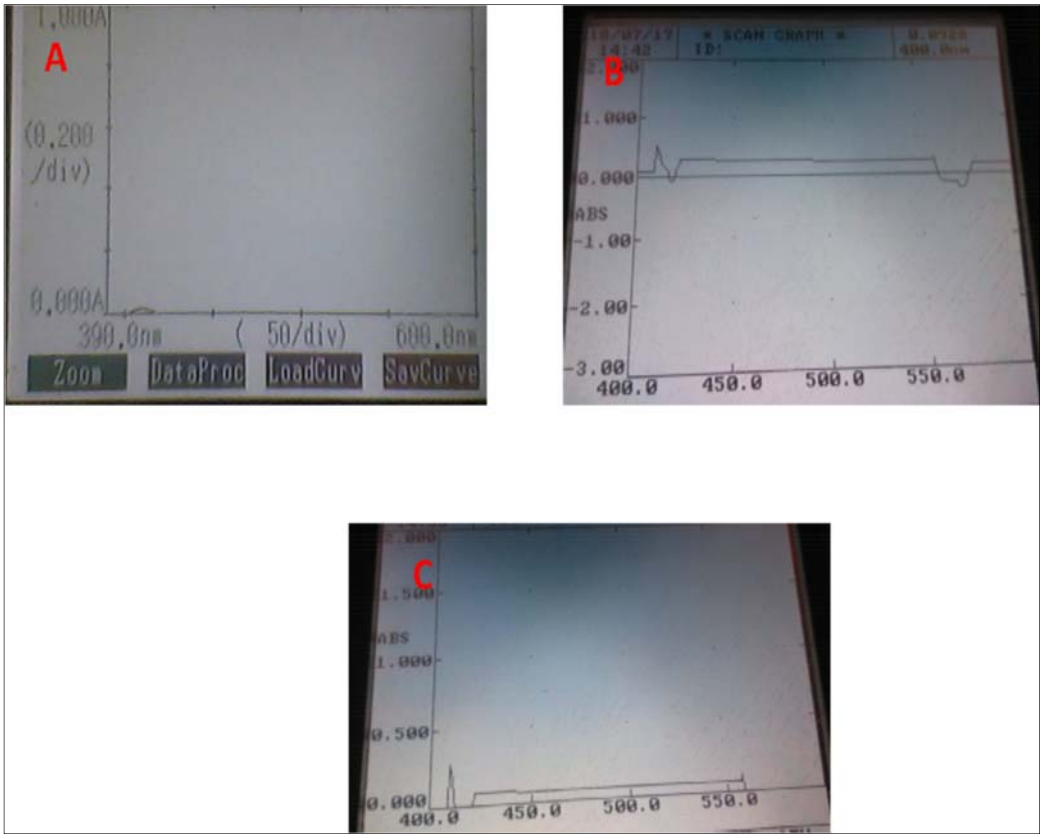

Fig 2: Apoptosis in sperm through release of Cyt C from mitochondria. Fig2A shows the fresh tissue serving as control and the release of Cyt C from the mitochondria is minimal whereas the fig 2B and 2C shows the release of cyt C from the mitochondria of sperm with and without treatment.

Isolation of testicular mitochondria

Testicular mitochondria was isolated by Gazzotti *et al.* (1979) [12]. Following sacrificing the animal testes was collected, decapsulated and minced in the isolated medium containing 250 mM sucrose, 0.2 mM EGTA, 0.1 mM EDTA, 5 mM HEPES KOH (pH 7.4) and 0.1% defatted BSA. The minced tissue was homogenised and centrifuged at 2500rpm for 10min and the supernatant was recentrifuged at 10,000 rpm for 10 min. The pellet obtained was resuspended and recentrifuged for twice at 10,000 rpm omitting the EDTA, EGTA and defatted BSA in the washing medium.

Assessment of mitochondrial viability

The protocol was taken from M. Ghazi-khansari *et al.* (2006) [13]. 1ml of isolated mitochondrial suspension was added to 1ml of janus green of concentration 1PPM and the sample was measured with in 40min at607nm for every 10min via UV- visible spectroscopy and the blank was measured in the same way without any mitochondrial suspension.

Release of cyt c from the mitochondria

The protocol was taken from Robert Eskes *et al.*, (1998) [14]. Mitochondria were incubated for 1 hr in MS buffer (210 mM mannitol, 70mM sucrose, 10 mM Hepes-NaOH, pH 7.4, 0.5 mM EGTA, 5 mM succinate, 5 mM Sodium azide). The reaction mixtures were centrifuged at 10,000 rpmfor 10 min at 40C and the spectrum of supernatant was recorded from 390 to 600nm.

Isolation of DNA

The protocol was from John R. Hofstetter et al; (1997) [15]. Testis was collected after sacrificing the animal and homogenised with the lysis buffer containing 50 mM Tris-Cl, pH 8.0, 100 mM EDTA, 0.125% SDS and 0.8mg/ml of EGTA and 1ml of lysis buffer was added to the homogenate again to increase the yield and incubated over night at 550c and centrifuged at 2000 rpm for 10 min after the addition of phenol: chloroform: isoamyl alcohol (25:24:1) and the aqueous phase was collected and washed with mixture of pci 1ml and chloroform 3ml. Aqueous phase was collected and the DNA was precipitated by chilled ethanol pelleted and dissolved in the water and stored in deep freezer up to reuse.

Results

Figure 1A shows the graph of mitochondrial viability on time scale in both testis and sperm on invivo treatment with drug metosartan and both drug and RNase A. The mitochondria of testis which is drug treated remained viable for up to 30min where as 0% viability in case of sperm after isolation itself. So, it is not clear whether it is due to increased mitochondrial synthesis or due to high activity induction by the drug. Incase of both drug and RNaseA treated animals showed decrease in viability by 60% of mitochondria isolated from testis whereas incase of sperm it remains unchanged.

Figure 1 B shows the release of cyt C from mitochondria in little amount whereas 1C and 1D shows the treated mitochondria to release Cyt C and untreated mitochondria which indicates the apoptosis through release of cyt C from mitochondria by the activation of caspase 9 through Apaf. Oxidised cytC gives absorption peak at 410nm [6] the soret band which is characteristic to heme containing porphyrins whereas reduced cyt C gives peak at 550nm [7]. From the previous studies it was known that cyt C is released in to the cytosol mostly in oxidised form [8, 9] and also the reduced form of cyt C [9] blocks the apoptosis and oxidised form of cytC activates apoptosome whereas the reduced form cannot but replacing the fe of cytC with reduced inactive metals doesn't require the oxidation state to induce apoptosis [9].

From this study we can conclude that the drug metosartan causes release of oxidised cytC from the mitochondria and the reduced cytC has given negative peak which indicates that the cyt C was totally oxidised and triggering apoptosis.

Figure 2 is same as that of fig 1B, 1C and 1D except that instead of testis sperm mitochondria was isolated and fixed.

Figure 3A and 3B shows the arrest of apoptosis by RNase A through preventing the release of Cyt C and also induction of P21 Waf1/cip-1 which is a cyclin dependent kinase inhibitor 1 known to inhibit both P53 dependent and independent apoptosis [10] and known to prevent tumorigenic activity and inhibiting JNK inactivation [11]. but figure 4 shows the DNA fragmentation which indicates apoptosis through extrinsic pathway.

Figure 3

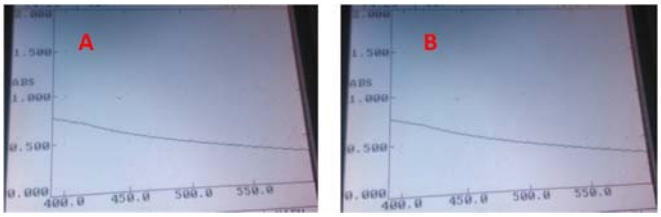

Fig 3: Arrest of release of Cyt C and apoptosis in both drug and RNase A treated invivo. Fig3A and fig3B shows the arrest of cyt C release from mitochondria in testis and sperm. The RNaseA causes the inhibition of apoptosis through p21 waf1/cip1 the cyclin dependent kinase inhibitor activation and inhibition of JNK. Causing the G2 phase cell cycle arrest leading to antitumorigenic effect.

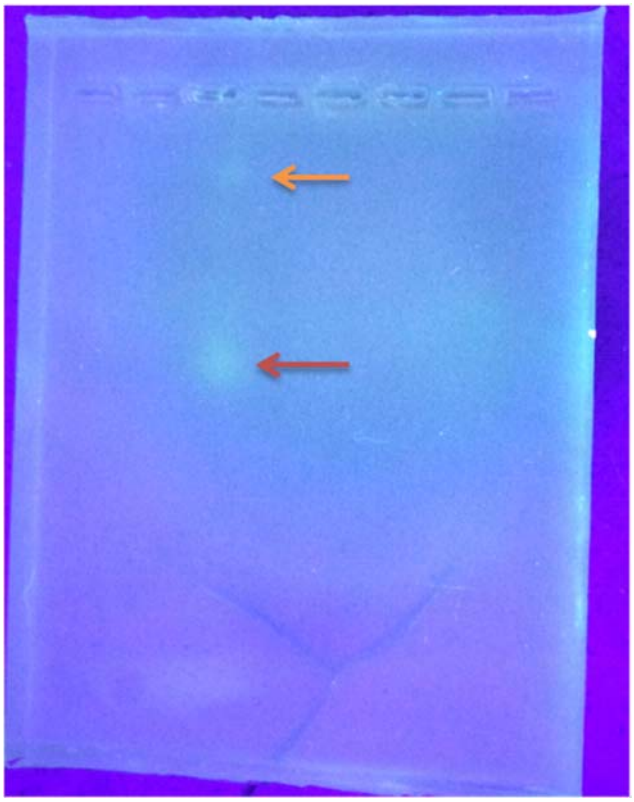

Fig 4: Agarose gel electrophoresis of the sample after DNA isolation from the testis of animal group treated invivo with RNaseA + drug 100mcl each showed DNA fragmentation which is clearly known as that the tissue is undergoing apoptosis which is may be through extrinsic pathway.

Discussion

Apoptosis is one of the major defect seen with the drug metosartan as there is also previous results of telmisartan that induced apoptosis in urinary bladder which has to be rectified. By the results of present study confirmed that the apoptosis of testis tissue is mediated through release of cyt C from mitochondria and treatment of tissue with R Nase A along with drug lead to the arrest of intrinsic pathway but the agarose gel electrophoresis showed the DNA fragmentation which is the indicative of apoptosis lead to conclusion that it activated the extrinsic pathway of apoptosis. As previous studies of eswari beeram *et al.* showed that the drug allosterically inhibits the enzyme so there will be slow release of drug from the enzyme therefore reducing the adverse effects of the drug. Further it was confirmed by the high viability of the mitochondria in drug treated group which indicates its dependence in apoptosis of testis tissue where as in RNase + drug treated group the viability got reduced in testis.

Conclusion

Drug metosartan induces apoptosis in testis tissue through release of cyt C from the mitochondria whereas the RNaseA inhibits the apoptosis through P21 waf1/cip1activation and JNK inactivation.

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Competing financial interest:
The author doesn't have any competing financial interest.

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