



CHROMATIN INTEGRITY IN TESTIS BY MICROSCOPY AND DRUG INHIBITION STUDIES OF RNASE A BY UV VISIBLE SPECTROSCOPY

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Article Received on 10/11/2017

Article Revised on 30/11/2017

Article Accepted on 20/12/2017

ABSTRACT

Chromosomes form the genetic material of germ cells which constitutes the major part of both female and male reproductive system.. Damage to it was caused by the several agents like the drug metosartan which causes severe damage to chromatin leading to male infertility as sperm chromatin is also part of it, so it is necessary to design the methods that prevent the damage immediately. Chromatin imaging by agarose embedding method of DNA localisation from testis formulated in this article through simple fluorescent microscope and imaging techniques Was proved that the drug was toxic to the male reproductive system.

KEYWORDS: chromosomes, metosartan, RNase A, x-ray crystallography, SEM.

INTRODUCTION

Chromosomes forms the genetic material of the cells. There are so many techniques to detect the structure of chromosomes through staining. But the dyes causes damage to the structure of chromosomes. So, using the unstained specimens is more useful than using the stained ones. Chromosomes consist of proteins, DNA and RNA and will organise as nucleosomes. They also organise in to higher order structures such as 30nm fiber and in to solenoid model. Direct exposure of chromosomal material is highly vulnerable to damage compared to the closed hetero chromatin so embedding them in the agarose and visualising them under microscope without any stain proves good design to study the genome integrity in the organs of reproductive systems.

Chromosomes form territories with gene rich regions and also gene poor regions. Some of the chromosomes contain gene rich regions but distributed randomly compared to the gene poor regions. Some of the chromosomes relocate to the periphery and to the interior depending on the response to some drugs which induce DNA damage through non arrangement of DNA in correct position (Bridger 2000;). Where as with drug metosartan micronuclei are formed in the nucleus and whether gama H2AX foci are formed in gene rich chromosomes or not is to be studied.

In cells the protective effects can be observed after drug removal, which is made possible by the finding of distribution of chromosomes in the nucleus at the

original positions and absence of gama H2AX foci in the chromosomes (Bridger 2000). Long stretches of DNA forms isochors which differs in A/T regions especially poor in gene poor regions than the gene rich regions as SINE elements are present in gene rich regions where as LINES are present in gene less regions.

It was found that even the human and porcine chromosomes also show nuclear localisation, where as some cells shows it after exiting from the cell cycle. Some from multicomplexes of chromatin, form the transcriptional factories with some of the enzymes in transcription so inhibition of RNase activity of the enzyme also, have serious impact on the cell genome and even on organs of reproductive system (Bickmore 2013;).

The cell genome is organised as the euchromatin and heterochromatin the former located in the periphery and later in the core of the cell. So, any drug first has the access to euchromatin than heterochromatin also the damage to the euchromatin itself. So, the chromosomal aberrations in germ cells should be considered for each and every drug and also for metosartan. In nucleus chromosomes are distributed uniquely where the other chromosomes is not distributed. Mainly the interphase chromosomes are transferred from the mother to child and the chromosome localisation, how it is maintained is largely unknown (Iyer 2012).

Techniques used to detect unstained chromosomes

Agarose embedding method

The testis was separated from the fat tissue and treated with the drug metosartan, RNase A and minced in lysis medium containing Tris, EDTA and SDS and DNA was isolated from the tissue. Low melting agarose of 1% was mixed with the isolated DNA and spread on a glass slide and allowed to solidify and observed under bright field microscopy.

Chromosomes detection by atomic force microscopy

The protocol was from Tatsuo Ushiki *et al* and the image of AFM; is build up by the interaction between the cantilever tip and the sample and also through forces acting on the sample. Normally the force generated by the cantilever is measured by beam deflection method. A beam is deflected from the rear side of the cantilever and deflection is monitored by the photodetector. Normally dynamic mode was the most preferred mode for imaging of chromosomes in liquid and ambient conditions.

Chromosomes imaging by X-ray diffraction microscope

It is as per the method of Yoshinori Nishino *et al*; Individual chromosomes are purified from HeLa cells and fixed in a compact manner with buffer containing 10mM Hepes- KOH, 5mM MgCl₂ and 0.5% glutaraldehyde for 10min. The fixed cells are spread over on a 100nm thick silicon nitrate membrane by centrifugation in order to adhere the chromosomes to the membrane in acceptable density. The fixed cells are washed in water to remove the salts, air dried and observed under the microscope after drying.

Chromatin imaging by SEM

Testis was isolated and treated with RNase (5mg/ml) 100µl, drug 100µl, both RNase 100µl and drug 100µl minced in water and thin smear is prepared, air dried and fixed in 3% glutaraldehyde solution. It was washed in PBS twice 5 min for each and stained with 5g of aniline blue in 4% glacial acetic acid and dried for 2 months in order to remove water in the sample.

Inhibition studies of Metosartan

RNase A in testis was purified by column chromatography from which 100µl of the sample was taken and incubated with 100µl of RNA for 1-3hrs along with the drug and run on 1% agarose gel with loading buffer and visualised using UV transilluminator.

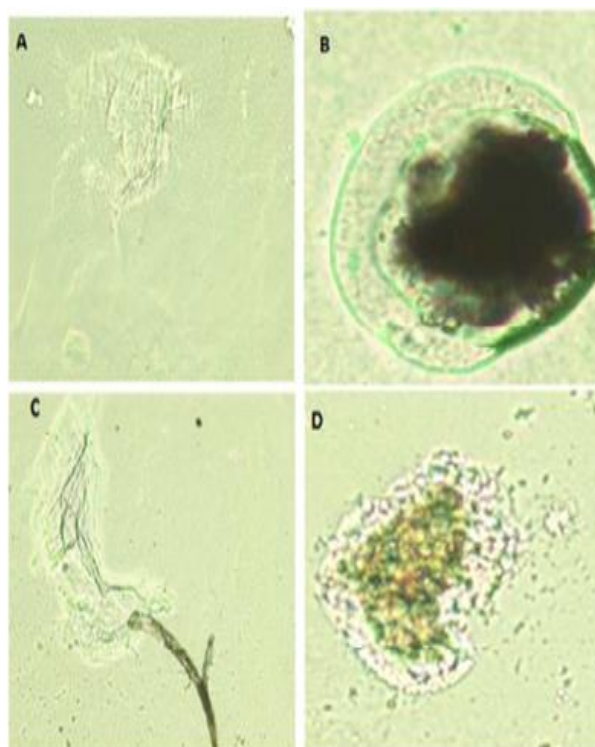
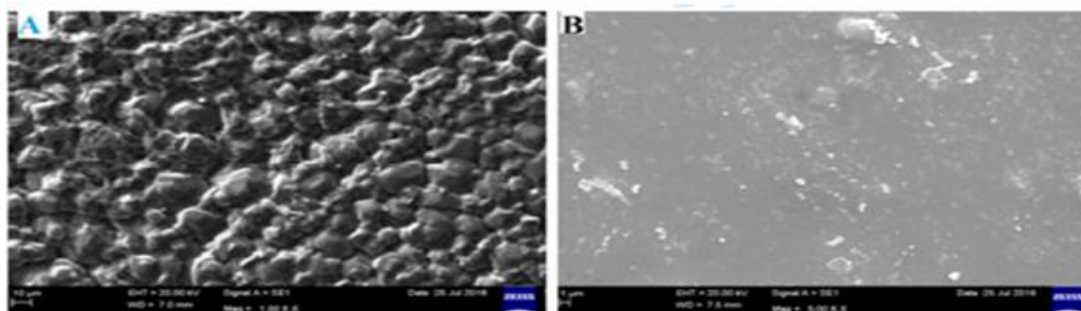


Figure 1: Imaging of unstained chromosomes in testis by agarose embedding method: Testis was collected and treated with RNase A, Drug and RNase A for 30 min, DNA was isolated from it and 100µl of sample was taken and added to 1000 µl of low melting agarose mixed well and slides are prepared thin and after it was examined under fluorescent microscope (A): chromatin of control, (B) chromatin treated with drug metosartan, (C) chromatin of both drug and RNase A treated sample, (D) chromatin of RNase A treated sample.

Figure 1A shows the neat arrangement of chromatin as it is embedded in the agarose has given the three dimensional arrangement and figure 1B shows the green colour which shows most of the chromatin is not damaged but the pink tinch shows the damage in the nucleus and figure 1C shows that the chromatin in sperm head was decondensed and damage to the membrane which leads to male infertility. Figure 2D shows the cleaved chromatin and membrane disintegration of nucleus.



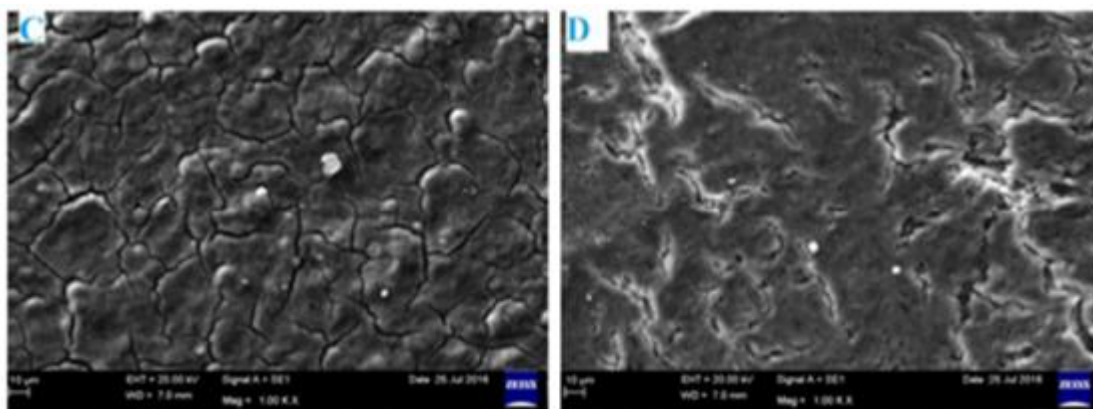


Figure: 2 Imaging of chromatin by SEM. Testis is treated invitro with RNase A, Drug separately and with both drug and RNase A separately. Aniline blue staining was performed and shade dried for 2 months and examined under Scanning electron microscope. (A) chromatin of control. Nucleosomes are clearly organised in a compact manner. (B) chromatin of RNaseA and drug treated sample. Chromatin was completely erased. (C) chromatin of drug metosartan treated cells. Apoptosis was clearly shown with blebbing of the cells. (D) chromatin of RNase A treated sample. Chromatin is cleaved some what as RNA is also one of the part of chromatin.

As RNase A is inhibited by metosartan which causes membrane destabilisation leading to damage to the chromatin of testis. As the chromatin was examined without any stain so the damage due to dyes can be excluded here. Figure 2A shows the clear arrangement of chromatin in to packed nucleosomes. figure 2B there is complete absence of chromatin as testis RNase is not

inhibited by the drug the RNA of chromatin was cleaved completely. Figure 2 C shows membrane blebbing and macrophages presence in the area and figure 2D shows the cleavage of chromatin as RNA is the part of the chromatin. The drug is to be considered for in vivo studies also as invivo is more complex than in vitro.

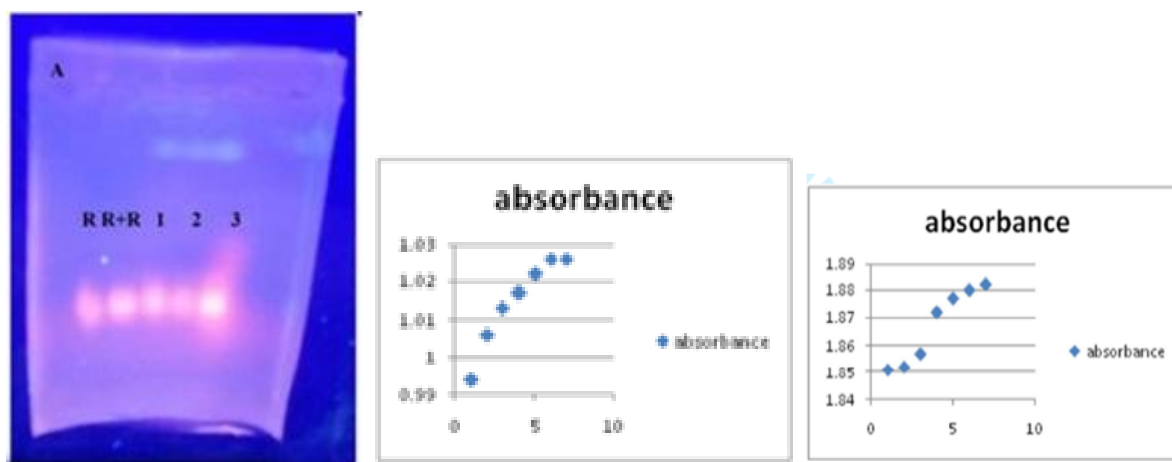


Figure 3A Inhibition studies by agarose gel electrophoresis and uv visible spectroscopy. Agarose gel electrophoresis was performed after incubating RNA as RNaseA+ RNA+ metosartan 100 µl each for 1hr, 2hr and 3hrs and RNaseA+ RNA alone in 2nd lane and RNA alone in first lane was taken. R indicates RNA, R+R indicates RNaseA+ RNA, 1,2and 3 indicates time of incubation in hrs. U.V visible spectrophotometer graph of RNase A along with RNA incubated with and figure 3B with out drug figure 3C for 10 min interval at 260nm.

Figure 3A, 3B, 3C deals with inhibition studies by two different techniques agarose gel electrophoresis and UV visible spectroscopy. In 3B and 3C RNaseA of bovine pancreas which is available commercially is used and in figure A the RNase isolated from testis by column chromatography is used. It is also known that RNase A is the enzyme of pancreas, is inhibited allosterically by the drug as it given the sigmoid curve with Michaels menton graph with UV visible spectroscopy results of time scan of 10 min each for 60 min. As the enzyme was inhibited

allosterically it is important to find out whether through positive or negative cooperativity.

Figure 3A shows that the testis RNase purified by the column chromatography was not inhibited as it is clear from the visible cleaved part of the substrate RNA in the gel in which RNA + RNaseA + metosartan was taken.

CONCLUSION

It was found to be the RNaseA of bovine pancreas was inhibited allosterically where as the RNase of testis was

not inhibited by the drug metosartan and its role in male fertility.

ACKNOWLEDGEMENT

UGC- CSIR is greatly acknowledged for providing JRF.

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