A Study of the Effect of Methotrexate and Vitamin A on NOR Expression in Hepatocytes of Male Wistar Rats

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Abstract

The effects of methotrexate (MTX) and vitamin A on NOR expression and histopathology in liver of male Wistar rats were evaluated. Male Wistar rats aged 4 months and maintained in our institution, were used in the present study. Animals were injected intraperitonealy with 3 different doses of methotrexate in alternative days for one week and the animals were sacrificed on the seventh day by using ether. Paraffin sections of the liver were prepared and histopathology as well as AgNOR studies were done. The same procedure was repeated with vitamin A and with both vitamin A and methotrexate. MTX treatment showed disruption of normal hepatic plates, vaculation of the hepatocytes, nuclear variability and enlarged blood vessels. When MTX was given with vitamin A, the latter reduced the damage done by MTX. In AgNOR study, data showed an overall decrease in the total NOR count (numerical) from normal to 12 mg dose MTX. The total count includes small, medium and large sized NORs. A statistically high significant difference was found from normal to 12 mg dose MTX as well as between the doses. The genotoxicity in rats is well documented with high dose of methotrexate (12 mg/kg) compared to low dose (8 mg/kg). Methotrexate treatment reduced the transcription rate and ploidy. Methotrexate reduced the cell proliferation which resulted in the reduction in the NOR count. (**JPBS 2010;Volume 23 No.1:1-7**)

Key Words: *NOR expression, methotrexate, hepatocytes, histopathology*

The nucleolus is the sub-nuclear organelle where ribosomal RNA is synthesized and ribosomes are assembled.[1] Nucleolus contains both chromatin and ribonucleoprotein (RNP) components. The chromatin component contains multiple copies of genes for synthesizing ribosomal RNA and the RNP components consist of pre-ribosomal RNA with a number of ribosomal and non-ribosomal proteins.[2,3]

Nucleolar organizing regions (NORs) are loops of ribosomal DNA present in the nuclei of cells.[4] Transcriptionally active NORs can be selectively stained by a silver colloid technique and visualized as black dots (AgNORs) under an optical microscope.[5,6] Nucleolar organizing regions have strong affinity towards silver and are therefore called AgNORs.[5,7] AgNORs have been proven to be a valuable marker of incipient cell alterations long before these can be detected in routine preparations.[8,9,10]

Silver staining is useful not only for studying the structure of nucleolus but also its variations in the abnormal situations.[11]

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Around 70 NOR associated proteins (NORAPS) have been characterized.[12,13] NORs vary in size and shape according to nucleolar transcription and are closely related to the degree of maturity of the cells [14], the cell cycle and proliferative activity or ploidy in some circumstances.[15,16,17]

The number of silver stained NORs in the chromosome preparation is proportional to the number of acrocentric chromosomes.[18,19] Also, the variations in the intensity of silver staining among chromosomes are mostly due to variations in the number of rDNA gene copies per NOR.[20]

The high incidence of chronic diseases of the liver has aroused strong interest in researching and trying to discover the biomolecular basis. The study of nucleolar organizing regions could be interesting as a prognostic factor for chronic hepatitis and for liver neoplastic disease.[21]

The AgNOR staining method is found to be a useful diagnostic tool to differentiate between normal liver, cirrhosis and hepatocellular carcinoma and also to precisely discriminate between cases of normal liver and Grade I hepatocellular carcinoma.[22]

Methotrexate (MTX) is an antimetabolite, an anteneoplastic agent that inhibits folate metabolism by its effects on dihydrofolate reductase. Since the cytotoxic effect of MTX is not selective for the cancer cells, it also affects the normal tissues that have high rate of proliferation, including the hematopoietic cells of the bone marrow and the actively dividing cells of the genitalia.

Administration of vitamin A (VA) decreases the MTX induced damage to the testicular cells. This protective effect of VA may have clinical applications in cancer chemotherapy.[23] The administration of VA

before high dose MTX may protect against drug induced D-xylose malabsorption in children with cancer.[24] Hence a study was planned to evaluate the effect of agents such as methotrexate and vitamin A on NOR expression of hepatocytes in male Wistar rats. The outcome of the study may be useful in better understanding of the long term effects of MTX and also to overcome or minimize its adverse effects.

Methods

Animals

Male Wistar rats aged 4 months with average weight of 200g were used in the present study. Animals were maintained under controlled conditions of light, temperature and humidity in an air conditioned animal house. The study protocol was approved by the Animal Ethical committee of the Institution.

Table 1. Expression of NORs in vitamin A (VA) and methotrexate (MTX) treated rat's liver. Each value represents mean ± S.D from 6 animals/group. Significant values are represented as*, **, *** ($p < 0.05$, $p < 0.01$, $p < 0.001$) between the control and the drug treated groups**. a, aa ,** a**aa** ($p < 0.05$, $p < 0.01$, $p < 0.001$) between 12 mg MTX vs. VA and VA +12 mg MTX.

Experimental Group	Total	Large	Medium	Small	Regular	Irregular
Control	113.83 ± 2.91	46.66 ± 3.29	27.16 ± 2.03	26 ± 2	54.66 ± 1.97	45.33±1.97
12 mg MTX	60.16± 2.54***	38.66± 2.21 **	31.16± 1.21*	30.16± 1.46*	70.16±1.21***	29.83±1.21***
VA	112.16±4.05^{aaa}	47.16± 3.97^{aa}	27.33± 2.13	25.5± 1.89^{a}	55±2.38^{aaa}	45±2.38^{aaa}
VA + MTX	113.33±3.38^{aaa}	44.5 ± 2.21^{a}	26.83 ± 1.86^{a}	28.66 ± 2.62	55.66 ± 1.88^{aaa}	44.33 ± 1.88^{aaa}

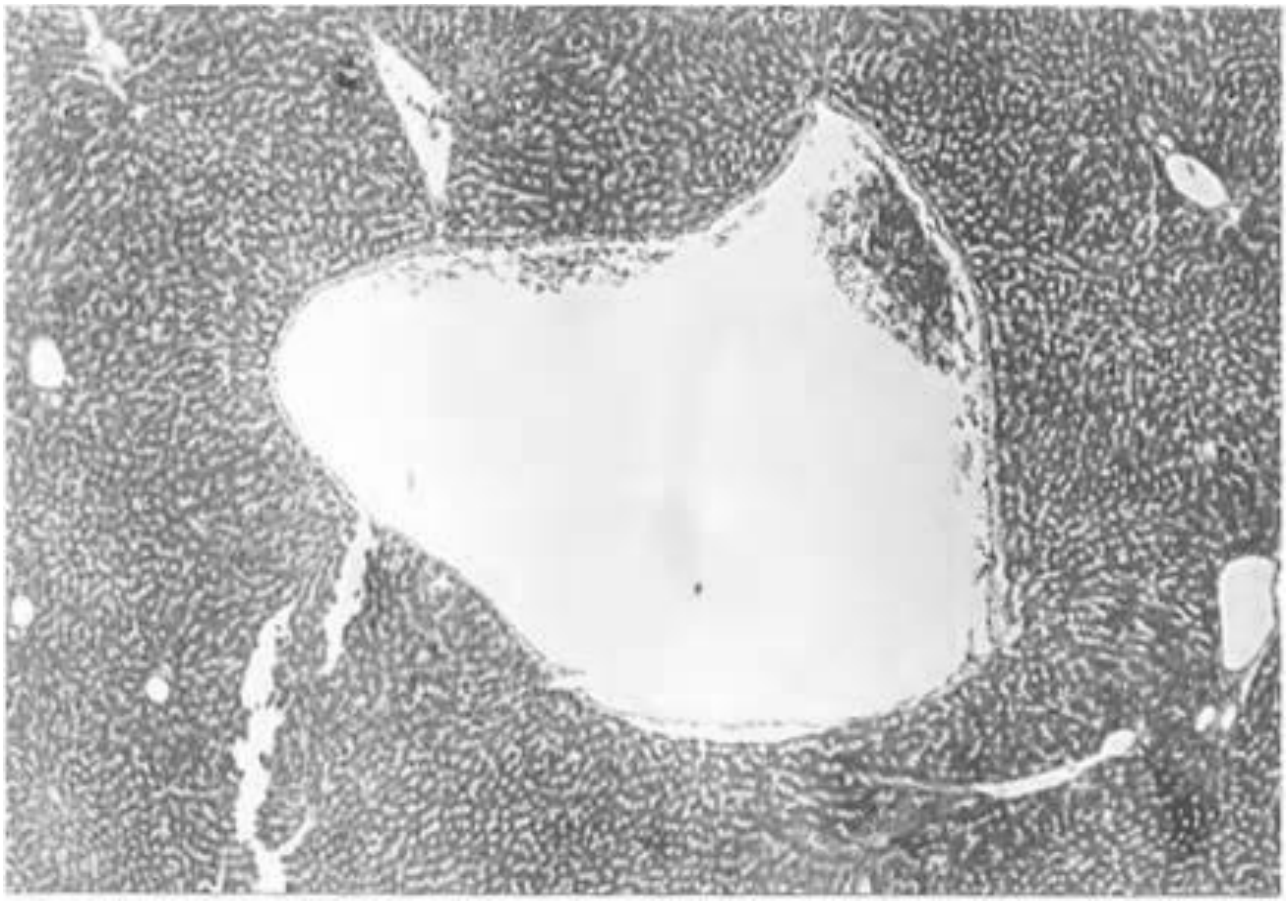

Figure 1. Photograph showing liver of 12 mg MTX treated rat. H & E stain (40 x).

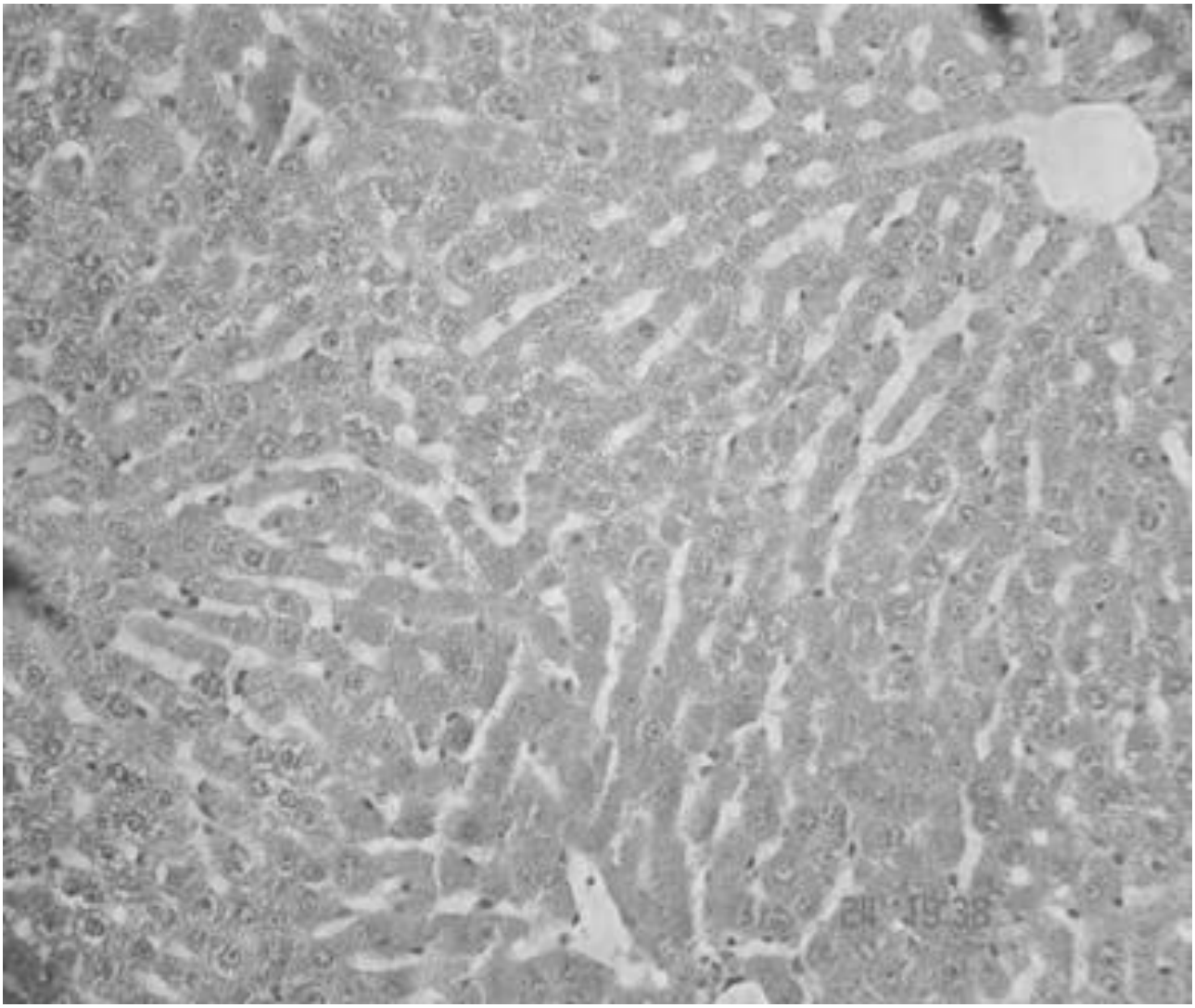

Figure 2. Photograph showing liver of VA + 12 mg MTX treated rat. H & E stain (40 x).

Chemicals

Methotrexate was obtained from NEON antibiotics Pvt Ltd., Thane, India. Vitamin A was obtained from USV Ltd., Mumbai, India. Thiomersal IP 0.01% w/v was used as the preservative. $AgNO_3$ (169.87) was obtained from NICE Chemicals Pvt. Ltd., Cochin. Gelatin was obtained from S. D Fine Chemicals Pvt. Ltd., Mumbai.

Design of Experiment

A total of 36 animals were divided into six groups. Group I served as control and received saline. Group II, III and IV received methotrexate 8, 10 and 12 mg/kg respectively. Group V received vitamin A 5000 IU and group VI received vitamin A 5000 IU and methotrextae 12mg/kg. The same experiment was repeated for NOR study.

Methotrexate administration

Animals were injected intraperitonealy with 3 different doses (8mg, 10mg, 12mg) of methotrexate in alternative days for one week and the animals were sacrificed on the seventh day by using ether.

Vitamin A

Animals were injected with vitamin A (5000 U) intraperitonialy on alternative days for one week and the animals were sacrificed on the seventh day by using ether.

Methotrexate and Vitamin A

Animals were injected with methotrexate and vitamin A (12mg and 5000 IU) intraperitonealy on alternative days for one week and the animals were sacrificed on the seventh day by using ether.

Tissue Processing

After sacrificing the animals, the liver was removed and kept in 10 % formalin for 48 hr (post fixation). Paraffin blocks were made in an embedding bath. Sections of 3-5 microns thickness were cut from the blocks using rotary microtome. The sections were mounted on air dried gelatinized slides and stained with haematoxyline and eosin. Liver histopathology was evaluated with high power(40X).

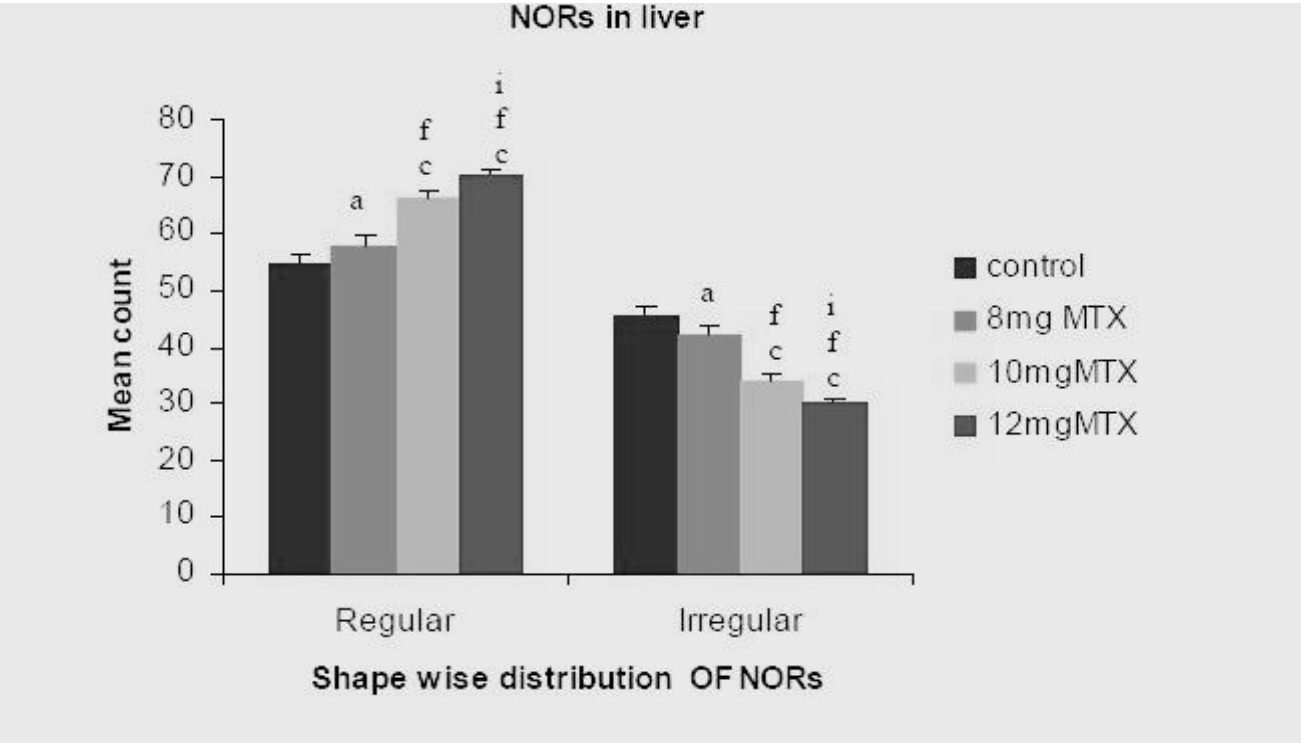

Figure 3. Total count and the size wise distribution of NORs in the control and drug treated groups. Each value represents mean + S.D from 6 animals/group. Significant values are represented as control vs. 8 mg, 10 mg and 12 mg, <0.05= a, < 0.01= b and <0.001=c. Between 8 mg vs. 10 mg and 12 mg - < 0.05= d and < 0.001= f. Between 10 mg and 12 mg, <0.01= h and < 0.001= i (One way ANOVA and Banferroni post test).

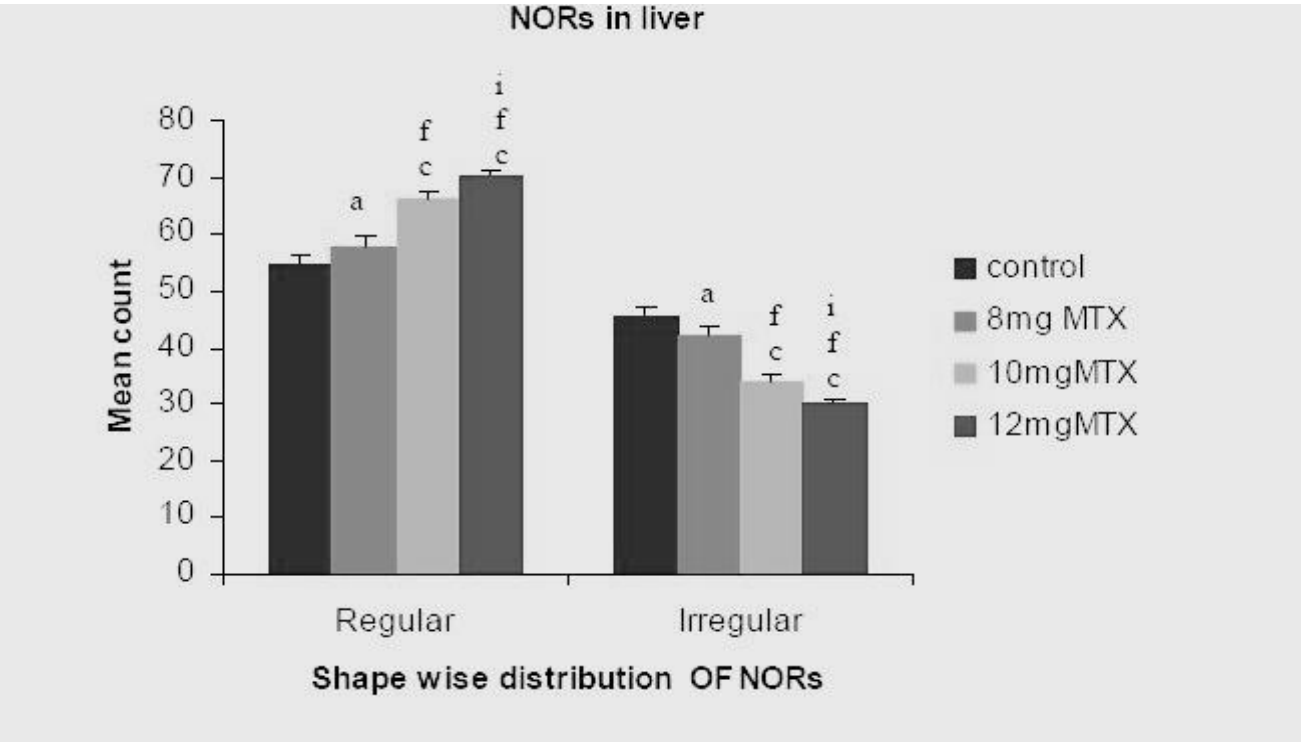

Figure 4. Shape wise distribution of NORs in control and drug treated groups. Each value represents mean + S.D from 6 animals/group. Significant values are represented as control vs. 8 mg, 10 mg and 12 mg - <0.05= a and <0.001= c. Between 8 mg vs. 10 mg and 12 mg - < 0.001= f. Between 10 mg and 12 mg- < 0.001= i.

NOR STUDY

Preparation of $AgNO_3$ solution

To prepare 50% of silver nitrate solution, 5 gm of silver nitrate powder was dissolved in 10 ml of distilled water and filtered through Whatman filter paper 1 in a dark room. The solution was stored in a dark bottle at 4^0 C, and used within one week.

Preparation of Gelatin

One gram of powdered gelatin was dissolved in 49 ml of distilled water and 1 ml of formic acid. To dissolve faster, the mixture was kept in hot water bath at 60^0 C for at least 10 minutes. The solution was stored in a dark bottle at 4^0C and used within one week.

Silver Nitrate staining

A rapid staining and de-staining method developed by Dhar et al. in our lab was used throughout the study.[25]

Microscopic observation

Standard protocols were followed for recording the number and quantitation of the size and shape of AgNOR dots.[26]

NOR count

Areas with minimal cell overlap and no artifact were demarcated for counting. Weak or dark stained slides were not evaluated. The NORs appeared as black dots within the orange colored nuclear background. The dots were defined as discrete homogenous silver precipitates with well defined edges. Overlapped dots with well defined edges were counted as greater than one when these appeared on viewing through different focal planes. Dots lying in a group with indistinguishable boundaries were treated as one dot. Dots outside the nucleolus were not considered. Observations were completed within a week of staining; otherwise the stain will fade on prolonged storage. Counting was done using oil immersion at 400X.

NOR size

The size of the NOR was measured using ocular micrometer (calibrated with stage micrometer). These groups were classified into three groups on the basis of their diameter (small<=1 μm, medium>1 and <=3μm and large > 3 μm).

NOR shape

On the basis of the shape, NORs were classified into regular (with round or oval well-defined margin) and irregular (with irregular serrated margin) dots. In each category, the size of all dots was documented.

Statistical analysis

Data on mean NOR count; size wise distribution of dots was tested using one way ANOVA. Since the test of homogeneity of variance showed high significance between different groups, data were reanalyzed using square root transformation. For each dose and distribution, mean NOR count, standard deviation, f-ratio and p-value were computed.

Results

The histopathological study of methotrexate as well as the dose wise expression of NORs in the liver were considered as the parameters in the present study. Data was collected and analyzed on the basis of the following parameters.

Histopathology

MTX treatment (in all the doses) showed disruption of normal hepatic plates, vaculation of the hepatocytes, nuclear variability and enlarged blood vessels (figure 1). When MTX was given with vitamin A, the changes were reversed (figure 2).

Expression of NORs in the liver

Total count of NORs

Data in figure 3 showed an overall decrease in the total NOR count (numerical) from normal to 12 mg dose MTX. The total count includes small, medium and large sized NORs. A statistically high significant difference was found from normal to 12 mg dose MTX as well as between the doses.

Large sized NORs. > 3μm

Data in figure 3 showed an overall numerical decrease in the large sized NORs from control to 12 mg dose MTX. A statistically significant difference was found between control and the different doses. But there was no statistical difference between 8 mg, 10 mg and 12 mg doses of MTX.

Medium sized NORs >1 μm to 3μm

Medium sized NORs showed a statistically higher in the control than the group treated with 12 mg dose MTX. However, there was a numerical decrease between control and 10 mg dose MTX.

Small sized NORs < 1 μm

Small sized NORs showed a numerical increase from control to 10 mg dose MTX and thereafter a decrease between 10 and 12 mg doses of MTX. The data showed a statistically high significant difference from control to 10 mg dose and between the doses.

Regular shaped NORs

Data in figure 4 showed a numerical increase in the values from control to 12 mg dose MTX. And it showed a statistically high significant difference from control to 12 mg dose as well as between the doses.

Irregular shaped NORs

Data in figure 4 showed a numerical decrease from control to higher doses and a statistically high significant difference from the control to the higher doses as well as between the doses.

When vitamin A was given along with methotrexate, the NOR expression was reversed. The

count was significantly increased in the VA+MTX treated group when compared to 12 mg dose of MTX treated group. The count was almost close to those of the control group (Table 1)**.**

Discussion

Methotrexate (MTX) is an antimetabolite widely used in cancer chemotherapy, which can cause intestinal mucosal injury. The antimitotic effect of MTX is known to give rise to malabsorption syndrome. It inhibits the enzyme dihydrofolate reductase (DHFR) which is required for DNA synthesis and cell division.[27] MTX is a novel xenobiotic inducer of rat liver and intestinal sulfotransferase. MTX therapy is associated with liver damage, both acute (notably after high dose) and more seriously in chronic cases (generally after long term administration).[28,29,30] Hepatic fibrosis and cirrhosis may develop without signs of hepatotoxicity and leads to eventual death. Reversible evaluation of hepatic enzymes may occur in some patients.[31] The administration of MTX increased mortality in patients with primary biliary cirrhosis.[32]

The main objective of the study was to determine whether the AgNOR count increases or decreases with different doses of MTX. Now it is evident that MTX decreased the total count of AgNORs. A decrease in the AgNOR count was due to the inactive proliferation of cells (anti-proliferative activity of MTX). The inactive cell proliferation was because of the blockage in the 'S' phase of the cell cycle by MTX. MTX decreased the cell ploidy, resulting in a real decrease in AgNOR bearing chromosome. Here, MTX reduced the transcriptional activity of the cells. That was another reason for the reduction of AgNOR count.

The irregularity of NORs refers to the aggregation of more than one NORs in a site, thus reflecting greater transcriptional rate. However, MTX reduced the transcriptional activity and hence the decreased AgNOR count in high dose group compared to the control group. Vitamin A reversed the effect of MTX on AgNOR count.

Another objective was to examine the histopathological changes induced by MTX treatment on hepatocytes. We found that MTX treatment showed disruption of normal hepatic plates, vaculation of the hepatocytes, nuclear variability and enlarged blood vessels. Vitamin A protected these damage produced by the MTX administration. Our study confirms that vitamin A minimizes the genotoxic and cytotoxic effect of MTX.

The conversion of MTX to its major extracellular metabolite, the 7-hydoxymethotrexate, takes place in the liver, where it is oxidized by a soluble enzymatic system. Inside the cells, MTX is stored in polyglutamated form.[33] Long-term drug administration can cause the accumulation of MTX polyglutamates and decrease folate levels.[34] The presence of higher levels of polyglutamates causes a longer intracellular presence of the drug, and this has been suggested as a mechanism for MTX hepatotoxicity.[35]

Conclusion

In the present study, NOR data was categorized into large, medium and small dots as well as regular and irregular types of NOR dots. The total NORs, irrespective of their shapes and sizes, followed a progressively decreasing pattern that correlated with the dose of the MTX. All the above findings may be useful in better understanding of the long term effect of MTX and also to overcome or minimize its adverse effects.

Conflict of interest

"None to declare"

References

1. Busch H, Sametana K. Nucleus. In: The Nucleolus. New York Academic press, 1979, vol.8, p.285-317
2. Attardi G**,** Amaldi F. Structure and synthesis of ribosomal RNA. Annu Rev Biochem. 1970; 39:183-226.
3. Olson MO, Thompson BA. Distribution of proteins among chromatin components of nucleoli. Biochemistry. 1983;22(13):3187-3193.
4. Fawcett DW. The cell. In: A textbook of histology. New York: Chapman and Hall, 1994, 12th edn, p. 1-56.
5. 5.Ploton D, Menager M , Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and in the visualization of the argyrophylic proteins of the nucleolar organizer region at the optical level. Histochem J. 1986; 18:5-14.
6. Lindner LE. Improvement in the silver staining technique for nucleolar organizer regions (Ag NOR). J Histochem. 1993; 41; 439-445.
7. Fakan S**,** Hernandez-Verdun D. The nucleolus and the nucleolar organizer regions. Biol Cell. 1986;56(3):189-205.
8. Schwint AE, Gomez E, Itoiz ME, Cabrini RL. Nucleolar organizer regions as markers of incipient cellular alterations in squamous epithelium. J Dent Res. 1993;72:1233-1236.
9. Schwint AE, Savino TM, Lanfranchi HE. Marschoff E, Cabrini RL, Itoiz ME. Nucleolar organizer regions in lining epithelium adjacent to squamous cell carcinoma of human oral mucosa. Cancer. 1994; 73: 2674-9.
10. Schwint AE**,** Folco A, Morales A, Cabrini RL .Itoiz ME. AgNOR mark epithelial foci in malignant transformation in hamster cheek pouch carcinogenesis. J Oral Pathol Med. 1996; 25: 20-24.
11. Howell WM. Selective staining of nucleolar organizer regions (NORs). In: The cell nucleus, Busch H, Rothblum L, eds. New York Academic Press. 1982; XI : 89-142.
12. Hubbell HR, Rothblum LI, Hsu TC. Identification of a silver binding protein associated with the cytological silver staining of actively transcribing

nucleolar regions. Cell Biol Int Rep. 1979;3(7):615-22.
13. Crocker J. Nucleolar organiser regions. Curr Top Pathol. 1990; 82:91-149.
14. Mamaev N, Mamaeva S, Liburkina I, Kozlova T, Medvedeva N, Makarkina G. The activity of nucleolar organizer regions of human bone marrow cells studied with silver staining. I. Chronic myelocytic leukemia. Cancer Genet Cytogenet. 1985;16(4):311-320.
15. Derenzini M, Pession A, Farabegoli F, Trere D, Badiali M, DehanP. Relationship between interphasic nucleolar organizer regions and growth rate in two neuroblastoma cell lines. Am J Pathol. 1989; 134(4):925-932.
16. Leek RA**,** Sarraf C E, Alision MR. The relationship of AgNOR size and number to proliferative status in a range of renewing and neoplastic tissue. J.Path. 1990;161; 342-362.
17. Suresh UR, Chawner L, Buckley CH, Fox H. Do AgNOR counts reflect cellular ploidy or cellular proliferation? A study of trophoblastic tissue. J Pathol. 1990; 160(3):213-215.
18. de Capoa A, Ferraro M, Menendez F, Mostacci C, Pelliccia F, Rocchi A. Ag staining of the nucleolus organizer (NO) and its relationship to satellite association. Hum Genet 1978; 44(1):71-77.
19. Miller DA, Dev VG, Tantravahi R, Croce CM, Miller OJ. Human tumor and rodent-human hybrid cells with an increased number of active human NORs. Cytogenet Cell Genet. 1978; 21:33-41.
20. Warburton D**,** Henderson AS. Sequential silver staining and hybridization in situ on nucleolus organizing regions in human cells. Cytogenet Cell Genet. 1979;24(3):168-175.
21. Bufo P, Losacco T, Latorre V, Parisi G, Santacroce L. Assessment of hepatocyte AgNORs expression in chronic hepatopathy. [Article in Italian] Clin Ter. 2000;151(6):417-419.
22. Siddiqui MS, Soomro IN, Kayani N, Muzaffar S, Hasan SH. Assessment of nucleolar organizer regions (NORs) in proliferative conditions of the liver. Pathol Res Pract. 1999; 195(6):421-426.
23. Yuncu M, Eralp A, Koruk M, Sari I, Bagci C, Inaloz S. Effect of vitamin A against methotrexate-induced damage to the small intestine in rats. Med Princ Pract.2004; 13(6):346-352.
24. Dagdemir A**,** Yildirim H, Aliyazicioglu Y, Kanber Y, Albayrak D, Acar S. Does vitamin A prevent high-dose-methotrexate-induced D-xylose malabsorption in children with cancer? Support Care Cancer. 2004; 12(4):263-267.
25. Dhar PK, Kumar MR, Nayak S et al. A rapid silver staining and destaining technique for the nucleolus organizer region. Biotech Histochem. 1995;70(6):302-303.
26. Crocker J, Boldy DA, Egan MJ. How should we count AgNORS? Proposals for a standardized approach. J Pathol. 1989; 158(3):185-188.
27. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. N Engl J Med. 1983; 309(18):1094-1104.
28. Zachariae H. (1980). Methotrexate induced liver cirrhosis. Br.J.Dermatol. 1980; 102: 407-412.
29. Klaber MR. Prospective study of liver biopsies in patients with psoriasis receiving methotrexate therapy. Br J Dermatol. 1982; 107 (suppl.22); 48.
30. Doroshow JH, Locker GY, Gosterland DE. Occular irritation from high dose of methotrexate therapy pharmacokinetics of drugs in liver fibrosis. 1981;48: 2158-21620.
31. Gong Y, Gluud C. Methotrexate for primary biliary cirrhosis. Cochrane Database Syst Rev. 2005;20 (3):CD004385
32. Chladek J, Martinkova J, Sispera L. An in vitro study on methotrexate hydroxylation in rat and human liver. Physiol. Res. 1997; 46: 371– 379.
33. Galivan J, Nimec Z, Balinska M. Regulation of methotrexate polyglutamate accumulation in vitro: effects of cellular folate content. Biochem. Pharmacol.1983; 32:3344–3347.
34. Kamen BA, Nylen PA, Camitta BM, Bertino JR. Methotrexate accumulation and folate depletion in cell as a possible mechanism of chronic toxicity to the drug. Br. J. Haematol.1981; 49: 355–360.
35. Kremer JM, Galivan J, Streckfuss A, Kamen B. Methotrexate metabolism analysis in blood and liver of rheumatoid arthritis patients. Arthritis. Rheum. 1986;29: 832– 835.