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Evaluation of the Genotoxic Effect of Hydroxyurea Using Cytogenetics Endpointsin White Mice

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Abstract

The genotoxic and cytotoxiceffects of Hydroxyurea (HU) in bone marrow of male Swiss white mice *Mus musculus* were estimated using the Micronucleus test (mn) in PCEs (Polychromatic Erythrocytes) and DNA damage using Comet assay. Male Swiss white mice were dosed orally in single doses of 250, 500 and 1000 mg. Kg^{-1} b. wt, the first negative control group was given D.W and the control group carrying DMSO. The results showed significant differences in the mean values of immature erythrocytes containing micronuclei in bone marrow, in the groups treated with doses of 250, 500, and 1000 mg. Kg^{-1} b. wt, reached its highest value with the dose (500) mg. Kg^{-1}51.20 ±**4.15,while the mean difference with (250 ,1000)16.0 ±*4.15 and 48.80 ± **4.15,respectively, compared with the negative control 0.81 ± 2.40, and the results showed significant differences in the number of Micronuclei (mni), with the dose (500) 52.40±**4.88 compared with the control group 0.81± 2.40, mean difference micronuclei in with the doses (250, 1000) 16.0 ± 4.88 and 50.40± **4.88 , respectively, compared to the negative control.Results of comet assay showed that the treatment with hydroxyurea caused damage to the genetic material, as the mean difference cell with damaged DNA in bone marrow increased with the dose of 1000 mg. Kg^{-1}, reaching11.40± **1.13,compared with negative control group, which amounted to 8.20 ± 0.80, while the average number of cells with damaged DNA withdosage 250 mg. Kg^{-1} 4.40± **1.13 and with 500 mg. Kg^{-1}7.60± **1.13. The results of the present study indicate that hydroxyurea has both genotoxic and cytotoxic effects in the somatic cells of male Swiss white mice.

Key words: micronuclei, halo estimation, Hydroxyurea, genotoxicity, cytotoxicity.

Introduction

Hydroxyurea was first made in 1869 in Germany by researchers Drestr and Stein [1]. HU is also approved for the treatment of skin cancer and ovarian cancer, as well as treatment of renal cells, urinary bladder, prostate, cervical cancer and other diseases [2]. It is an anti-tumor drug, which has wide uses in the treatment of malignant and non-malignant diseases such as anemia. Sickle-cell anaemia (SCA) and HIV infections, act on the ribonucleotide reductase enzyme to prevent the conversion of ribonucleotides into deoxyribonucleotides [3]. The most common side effects of HU in human patients are leukopenia, anemia and thrombocytopenia [4, 5, 6]. In 1928 an animal study was conducted and it was concluded that exposure to HU leads to leukopenia, anemia, macrocytosis, and high mortality [7]. When the drug HU is taken orally, it is absorbed well within minutes of administration. And it turns into free radicals called nitroxide in the body of the organism. It is then transmitted by spreading to cells where it binds to the tyrosyl

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compound in the active site of the M2 protein unit of the ribonucleotide enzyme that enters the mechanisms of synthesizing nucleic acids, which leads To inactivate the enzyme, and it results in the reduction of the ribonucleotide, and the synthesis of DNA is inhibited selectively, which results in a special effect on the S phase and cuts the cell cycle in the G2 and S stages. The drug also works to prevent the repair of damaged DNA due to chemicals or radiation, thus It provides a potential link between hydroxyurea and the chemicals that lead to DNA damage [8,9].

HU acts as a strong inhibitor of ribonucleotide reductase (RNR), which is a ubiquitous intracellular enzyme that converts ribonucleotides into deoxyribonucleotides, which are necessary for the synthesis and repair of DNA [10]. HU is partially eliminated by the liver as it turns into urea, and is done the bulk is excreted through the kidneys, and it may be excreted through urine without change [11]. Studies have shown that HU causes rapid cell death by initiating an uncontrolled free radical chain reaction. These reactions are capable of rapidly destroying cellular metabolism by activating enzymes and cross-linked DNA and altering membrane function through self-oxidation of lipids. Although they have been used frequently in the treatment of many malignant and non-malignant diseases, prolonged use of HU has been reported as being it causes mutations and influencing effects in many laboratory systems, as well as cytogenetic damage in exposed mice. HU has demonstrated a variety of toxic effects, such as chromosomal abnormalities and cytotoxicity, as well as establishing an effective genotoxicity in cell culture in rodent models [12]. Among the most common side effects of HU are anemia and thrombocytopenia, as the JAK ruxolitinib inhibitor has been associated with decreased growth. Leukocytes and an increased incidence of viral infection, in addition to an increased risk of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [13].

This study aims to assess the genotoxicity and cytotoxicity of Hydroxyurea by using the Micronucleus test and Comet assay technique in male laboratory egg mice.

Materials and methods of work

Laboratory animals used:

In the current study, 60 Swiss egg mice were used, Mus musculus, which were obtained from the Animal House laboratory at the National Research Center, Giza, Arab Republic of Egypt. The age is ranging between (8-6) weeks and weighing from 20-30 gm. The mice were in good health. They were maintained according to a standard diet of casein and clean water daily. The animals were placed under optimal conditions of temperature and humidity.

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-The chemical - the drug Hydroxyurea (HU) used in the experiment:

The drug used was obtained from the local market of Baghdad governorate and it is a treatment imported from the United States (Canada). It consists of boxes containing 100 capsules each box, which is an oral treatment of 500 mg, as this drug was dissolved with DMSO prepared by Sigma Fresh.

Experimental design:

-Transactions

White mice were used in groups consisting of 5 mice for each group as follows:

1- Control (-ve) negative control group (distilled water).
2- Vehicle group (DMSO).
3- Positive control group (Benzo [a] pyrene).
4- Total groups treated with hydroxyurea (250, 500, 1000 mg. Kg^{-1}).

Methods of Work

A test to quantify the micronuclei in immature erythrocytes (PCEs) in the bone marrow of male Swiss white mice:

Animals were sacrificed by separation of the cervical vertebrae after 18 hours from the time of dosing for the negative and positive groups and treated with hydroxyurea and the test was performed according to the method [14]

***Staining of slides,**Slides were stained with May-Gruenwald stain for 7 minutes and then by Giemsa stain for 3 minutes.

***Testing and Calculating Cells**

The prepared slides were tested using a standard light microscope and under (40X) power. 1000 blood cells were counted per animal. The presence of PCEs was investigated, and MNPCEs and micronuclei were counted.

Estimation of DNA damage in bone marrow cells of male Swiss white mice using comet assay:

Animals were sacrificed by separating the cervical vertebrae after 24 hours of dosing time, as this test was performed according to the mediated protocol. [15]

Statistical Analysis:

The Statistical Package of Social Science [SPSS] was used in analyzing the results statistically and by applying an analysis of variance to find the mean values of the differences and conducting a Tuky test to identify HSD the highest significant difference between groups.

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Results and discussion:

Micronucleus test:

The results of the present study showed a significant difference in the mean differences in the number of immature red blood cells containing micronuclei in the bone marrow of white mice (Table 1) treated with HU at doses of 250, 500, 1000 mg. Kg-1 compared to negative control 2.40 ± 0.81, where

If the average value of the differences with the dose is 500 mg. Kg^{-1} and was ** 4.15 ± 51.20 while with the dose being 1000 mg. Kg^{-1} ** 4.15 ± 48.80 and with a dose of 250 mg. Kg^{-1} * 4.15 ± 16.0, and significant differences appeared in the mean differences in the number of minute nuclei (MNi), reaching ** 4.88 ± 52.40, ** 4.88 ± 50.40 and 4.88 ± 16.0 with doses (250, 1000, 500) mg. Kg^{-1} compared to the negative control group which was 2.40 ± 0.81. The results above show the hypothesis of a genotoxic effect and / or HU drug.

Table (1): Micronuclei in the bone marrow cells of male Swiss white mice after treatment with different doses of hydroxyurea negative and positive control groups

Transaction / dose mg.kg- 1 .b.wt	MNiPCEs S.D ± M	MNi S.D ± M	MNiPCEs S.E ± MD	MNi S.E ± MD
CONTROI (-ve)	2.40 ± 0.81	2.40 ± 0.81		
DMSO	7.60 ± 1.12	7.60 ± 1.12		
Benzo(a)pyrene(+)			** 4.15±35.20	**4.88 ± 36.80
HU (250mg/kg)			*4.15 ±16.0	4.88 ± 16.0
HU (500mg/kg)			**4.15 ± 51.20	**4.88 ± 52.40
HU (1000mg/kg)			**4.15 ± 48.80	**4.88 ±50.40

*Significant value at $P \leq 0.05$
*Significant at the level of probability (t-test) $P \leq 0.01$
* (Tukey HSD test) MNiPCEs immature red blood cells containing micronuclei, MNi micronucleus, HU hydroxyurea, DMSO, drug solvent, Benzo (a) pyrene (+) positive control group.

Micronuclei are chromosomal fragments or fragments missing a centromere surrounded by a nuclear envelope, or a whole chromosome surrounded by a nuclear envelope in which the spindle is damaged during the division process. Caused by factors that produce breaks or changes in the mitotic spindle, this elevation reveals a mutagenic effect that can be recognized as a result of genetic instability in non-cancer cells, such as lymphocytes in peripheral blood, and chromosomal damage is used as a marker for the genetic toxic effects arising from drug exposure [16]. The results of a study [17] also showed that using HU stimulates DNA damage and chromosome damage, which induces genetic instability and

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culminates in mutations and carcinogenicity.

The results of a study [18] proved that HU induces the breaking of the single strand of DNA and that the genotoxicity of HU may be due to its effective mechanism of action in the process of cell division, and that these breakdowns in the DNA are the result of inhibition of the enzyme ribonucleotide reduction by HU. Its increase is not dose related, which means that the drug has the ability to induce the formation of PCEs.

The results of [8,19] also showed significant differences in the mean of micronuclei in mice treated with HU if it was attributed to the presence of genetic toxicity resulting from chromosomal fractions through the formation of nitric oxide, which is metabolized from HU, and HU prevents the incorporation of thymidine into the DNA. It directly leads to DNA damage, which leads to a decrease in marrow diffusion. For the treated groups and HU, it caused genotoxicity in the bone marrow of the rats, and the damage was severe at high doses compared to the lower dose [20]. This is what our current study showed in groups of HU-treated mice.

Figure (1) showed a normal immature red blood cell PCE from the bone marrow of a mouse from the negative control group

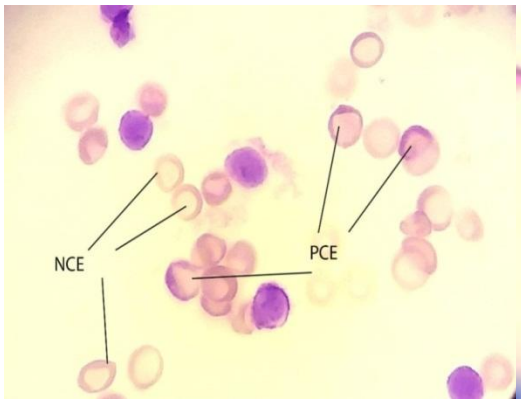

Figure (3) an immature red blood cell (PCE) containing two micronuclei (black arrow) from the bone marrow of a mouse from the group treated with HU at the dose of 1000 mg. Kg^{-1} May-grunwald Gemsa.100X

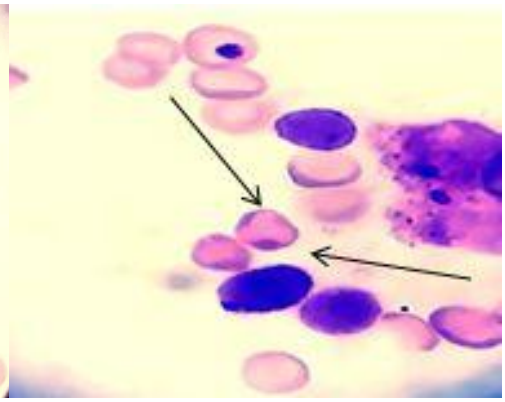

Figure (2) A normal immature red blood cell PCE (black arrow) from marrow bone marrow from the may-grunwald negative control group Gemsa.100X

Table (2) shows the mean values of the differences for cells with damaged DNA and total damage in the DNA of bone marrow cells of white mice in the HU drug treatment and control negative groups. So Comet assay, which is called Gel Electrophoresis Single Cell, is an important technique concerned with studying the biological monitoring of living organisms in detecting single chain fractions of DNA and damage marked in the bases of DNA and repair sites by shearing. Also, the technique of estimating the aura is multi-use, sensitive and fast to determine the damage of DNA and its ability DNA repair at the single cell level. [21]

The results of Aura estimation technique showed that the treatment with HU caused induce the damage of the genetic material, as significant differences appeared in the mean values of the differences between bone marrow cells with damaged DNA related to the dose when compared with the negative control group 8.20 ± 0.80, as it reached **1.13± 4.40 **1.13± 7.60 , **1.13

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± 11.40 in the dose-treated groups (250, 500, 1000 mg). 1 kg of hydroxyurea respectively, while the mean value of the differences for the bone marrow cells with total damage was significant when compared to the negative control group, which amounted to 9.40 ± 1.20, as it reached ** 2.77 ± 11.60, **2.77 ± 23.40, **2.77 ± 36.80 In the treatment totals for the above mentioned doses.

Table (2) the mean values of the differences for the affected cells and the total damage in the bone marrow cells of male white mice in the HU treatment groups and the negative and positive control groups.

Average whole total damage S.E ±MD	Average cells with damaged DNA S.E±MD	Average whole total damage S.D ± M	Average of cells with damaged DNA S.D ± M	transaction dose mg.kg- 1 .b.wt
		9.40 ± 1.20	8.20 ±0.80	**CONTROI (-ve)**
		11.60 ± 1.60	9.20 ± 0.86	**DMSO**
**2.77 ± 48.40	**1.13± 16.60			**Benzo(a)pyrene(+)**
**2.77 ±11.60	**1.13± 4.40			**HU (250mg/kg)**
**2.77 ± 23.40	**1.13± 7.60			**HU (500mg/kg)**
**2.77 ± 36.80	**1.13 ±11.40			**HU (1000mg/kg)**

*Significant value at $P \leq 0.05$
*Significant at the level of probability $P \leq 0.01$(t-test)
* (Tukey HSD test) MNiPCEs immature red blood cells containing micronuclei, MNi micronucleus, HU hydroxyurea, DMSO, drug solvent, Benzo (a) pyrene (+) positive control group. M mean, S.D. standard deviation, MD mean of differences, S.E. standard error

Moreover, the presence of the halo means that there is damage in the DNA, which indicates genetic toxicity. A study [17] also confirmed that the use of HU enhances DNA damage and the occurrence of damage to the chromosomes, leading to genetic instability and culminating in the occurrence of mutations and cancers. Of sudden cardiac arrest, and treatment with HU is responsible for strengthening the chromosomal damage in these patients. In previous studies of patients with SCA using HU, they had a statistically higher damage index in the DNA than a control group, and they were in good health. However, the damage index was not evaluated in patients with SCA without using HU [22,23].

The increase in DNA damage in SCA patients untreated with HU compared to the control group, who are in good health, can be attributed to the chronic inflammation present in the disease with the production of reactive oxygen species, which can cause DNA damage [24]. Also, [25] in the study of the genotoxicity of HU on children with sickle cell anemia showed that there is greater damage to DNA compared to uninfected children. HU compared with the control group, and that the DNA damage on the image of a tail halo depends on the dose used as shown by the results of our current study in groups treated with different doses of HU for

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white mice.It also showed [23] that using HU leads to DNA damage.

The hydroxyurea disrupts the binding of nitrogenous base aggregates by ribonucleotide reduction via the formation of free radical nitroxide that binds the free tyrosyl radical to the active site of the enzyme. Hydroxyurea can also cause site-specific DNA damage by forming free radicals (radicals) such as hydrogen peroxide and nitric oxide [27]. Due to its subtle properties, halo estimation technique is used to assess the ability of any type of eukaryotic cell to resist DNA damage. The halo estimation technique is also used to study the ability of several drugs to cause DNA damage [28].

Exposure of living cells to DNA synthesis inhibitors such as hydroxyurea (HU) or aphidicoline prevents the cell's ability to repair damage to the genetic material and leads to the accumulation of DNA damage and fractures. This helps to discover the harmful effects of hydroxyurea treatment or the automatic or basic damage to DNA [29,30,31].

Hydroxyurea inhibits DNA repair by inhibiting DNA synthesis and affecting the transcription checkpoint on a tape of genetic material [32], thus enhancing the occurrence of cytotoxicity to cells.

Figures (3,4,5) also showed different levels of bone marrow cells for male white mice, class 0 normal cells that do not contain damaged DNA from the negative control group, while class 1, class 2, class 3 cells have damaged DNA from the dose-treated groups. 250, 500, 1000 (mg). 1 kg of HU in varying degrees (mild, moderate, severe) (white arrow) Ethidium bromide, X100

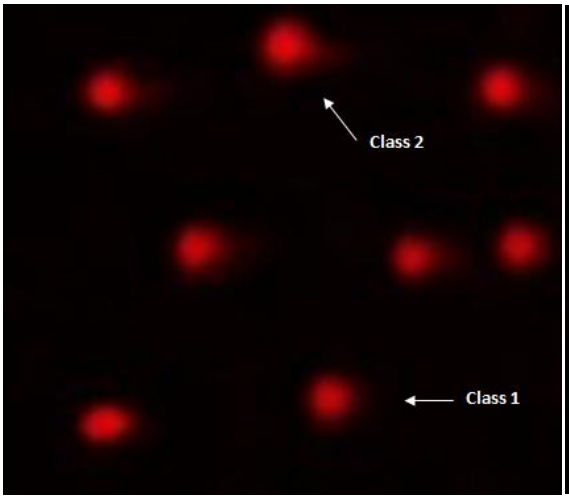

Figure (4) Different levels of damage in the bone marrow cells of male white mice: cells with damaged DNA (class 1 & 2) from treatment with doses of 250 mg and 500 mg.

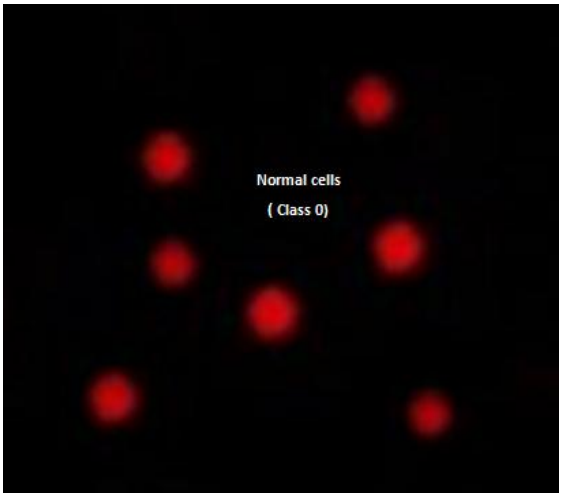

Figure 3 Normal cells without impaired DNA (class 0) from the negative control group

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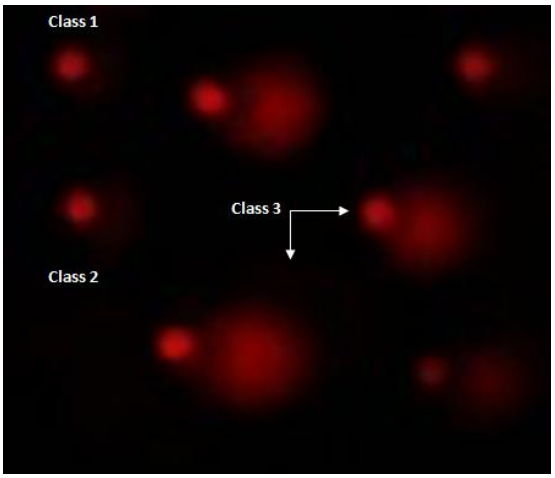

Figure (5) Different levels of damage in the bone marrow cells of male white mice: cells with damaged DNA (class 2 & 3) from treatment with doses of 500 and 1000 mg.

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