Induction of chromosomal aberrations and associated developmental inhibitions in Drosophila melanogaster by hydroxyurea.

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Abstract Hydroxyurea, also known as hydroxycarbamide, is a medicine that is currently used as an anti-cancer chemotherapeutic agent. The antitumor activity of hydroxyurea is based on its ability to inhibit DNA synthesis. The drug has also proved its efficacy in the treatment of sickle cell disease owing to its ability to increase the level of fetal hemoglobin and a compensatory reduction in sickle hemoglobin that prevents sickling of red blood cells and reduces vaso-occlusion and hemolysis. Hydroxyurea has exhibited teratogenic effects in animals. However, the teratogenic influence of this drug in human is controversial as some studies have shown that the use of this drug during pregnancy yielded no congenital abnormalities in newborns. To resolve this issue it is imperative to see the effects of this drug on development experimentally in an animal model. *Drosophila melanogaster*, a popular model organism has an extensive genetic homology with human. Furthermore, fundamental biological mechanisms and pathways that control development and survival are conserved across the evolution between human and this fly**. Thus in the present study an attempt has been made to assess the effect of hydroxyurea on the development of *Drosophila melanogaster* to appraise the probable outcome of the exposure of this drug during in human development. The drug exhibited a dose dependent negative effect on two important aspects of the development of the fly such as- time needed for metamorphosis and progeny number. Inhibition of development of the fly was accompanied with notable abnormalities in larval polytene chromosomes like-constriction, asynapsis, ectopic pairing etc. We speculate that larval chromosomal abnormalities inhibited the expressions of many genes needed during the course of development. Owing to extensive genetic homology of this fly with human and its extensive use for developmental and toxicological studies, it is possible that the use of this drug during pregnancy may impose analogous negative influences on human development as shown by the flies.**

Key word : Drosophila melanogaster, Hydroxyurea, larva, pupa , chromosomal constriction, ectopic pairing, asynapsis

Introduction

Hydroxyurea (HU) is a myelosuppressive drug developed as an anticancer agent to treat leukemia, melanoma and ovarian cancer. It interferes with the DNA synthesis during the S phase of the cell cycle by interfering the conversion of DNA bases by blocking ribonucleotide reductase, thereby preventing the conversion of ribonucleotide to deoxyribonucleotide[1,2]. HU is an ideal drug for sickle cell anemia also[3]. Sickle cell anemia is a chronic blood disorder in which a defective hemoglobin is produced due to the production of abnormal beta-globin (β- globin) polypeptide resulting from a point mutation of the sixth codon of the β- globin gene on the

short arm of chromosome 11 that substitutes valine for glutamic acid[4,5]. Loss of negatively charged glutamic acid in β- globin polypeptide results in its altered mobility during electrophoresis[6]. The association of this abnormal β-globin with normal alpha-globin (α-globin) chain forms sickle hemoglobin (HbS). It normally carries the oxygen but when oxygen unloaded in the tissues, under low oxygen tension, it is polymerized into long fibres that structurally distort the red blood cells (RBCs) into a sickle shape. The RBCs become less flexible and undergo permanently damaged due to repeated deoxygenation cycles. The sickle RBCs become sticky and adhere to endothelium and clump together plugging micro-vessels[4,5]. As a result the blood circulation is blocked and oxygen can not reach nearby tissues causing severe pain called crises and may cause organ damages[7]. In neoplasia therapeutic efficacy of HU is brought about by its cytotoxic effects that blocks the DNA synthesis leading to cell death[8]. HU increases the synthesis of fetal hemoglobin (HbF) in most patients with sickle cell disease resulting in considerable increase in HbF containing RBCs[9]. Thus this drug inhibits the polymerization of hemoglobin S and improves vaso-occlusive manifestations and hemolysis[10]. The possible mechanism by which HU increases HbF is that the drug can act as a nitric oxide (NO) donor and NO by stimulating the soluble guanylyl cyclase activates gamma-globin (γ-globin) gene expression and subsequent γ-globin chain synthesis necessary for HbF production[11]. Although HU does not offer the cure for this disease but it improves the quality of the lives of the patients by offering benefits like fewer pain crises, reduced need for blood transfusion, and increased life span etc.[12]. In spite of many beneficial effects, HU has been reported to have clastogenic effects, teratogenic and mutagenic effects[13-15.] However, other study have indicated the low mutagenic effects of this drug in vivo[16,17]. Although HU showed teratogenic effects in animals, its teratogenic influences in human is controversial[18-20]. In one study[19], comprising 31 pregnancies with HU exposure that ended in 24 live born infants, exhibited no major malformation. Chromosomal analysis of the newborns was normal in 6 out of 7 studied cases and one case showed inherited inversion of chromosome. In an another study[20], patients exposed to HU therapy for sickle cell disease also delivered live infants with no congenital abnormalities. To resolve this controversy, dose dependent effect of HU on an experimental animal that models human may provide some clues on the possible outcome of exposure of this drug during human pregnancies. Thus in this present study we have made an attempt to evaluate some aspects of the reproductive outcomes of exposures of medically used HU in different stages of development in a dose dependent manner by using a model organism *Drosophila melanogaster* (*D.melanogaster*)[21]. In addition, polytene chromosomes of the third instar larvae of this fly were examined to understand whether developmental abnormality, if any existed, was accompanied with chromosomal abnormalities or not. *D. melanogaster* and mammals, including human, exhibit substantial similarities in DNA and protein sequences. In conserved functional domains, the homology can reach 80 to 90% or higher. In addition, nearly 75% of disease-related genes in humans have functional orthologs in this fly. The degree of conserved biology and physiology between human and *D. melanogaster* makes this fly as an extremely valuable tool in the drug discovery process also[22.] Thus it seems possible that the experimental results obtained from this fly in response to clinically used drugs may indicate analogous outcomes in human development.

Material and methods

Chemicals: Hydroxyurea (Hydroxycarbamide, E.R.Squibb & Sons Ltd,Uxbridge, England), agar, sodium chloride (NaCl, Sisco Research Laboratories, India), potassium chloride (KCl, Sisco Research Laboratories, India), calcium chloride ($CaCl_2$, Sisco Research Laboratories, India), yeast (Kothari fermentation and biochem Ltd, India) glacial acetic acid (CH_3COOH, Sisco Research Laboratories, India) propionic acid (CH_3CH_2COOH, Sisco Research Laboratories, India), methyl paraben (methyl-p-hydroxybenzoate, Loba-Chemie, India), Ethanol (Merck Specialities Private Limited, India), lactic acid ($CH_3CH(OH)CO_2H$, Sisco Research Laboratories, India), orcein (for microscopy, Loba-Chemie, India) were used for this study.

Fly culture

Five adult male and five adult female *D. melanogaster* were kept for mating in each 50 ml glass culture vial with 10 ml standard *Drosophila* culture medium containing agar, maize powder, molasses, yeast, propionic acid and antimicrobial agent methylparaben with varying concentrations of HU (0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml). Control vials also contained five male and five female flies and the medium contained all the above mentioned ingredients but lacked HU. Five repetitions in each concentration of HU and control were used for experimental purpose. Cultures vials were maintained at $25^{O}C$ in a B.O.D. As soon as the culture vials showed the initial appearance of pupa, the breeding male and female flies were removed from the vials.

Preparation of polytene chromosomes of *Drosophila* for microscopic examinations:

Third instar larvae exposed to different concentrations of HU as well as control larvae were used for the isolation of polytene chromosomes. Larvae after being harvested from culture vials were washed thoroughly in

ringer solution and transferred on a grooved slide in the same solution. With the help of a dissecting microscope, a pair of salivary glands was dissected out from each larva. Three pairs of such dissected glands were removed from third instar larvae exposed to each concentration of HU separately on a glass slide. Aceto-alcohol (1:3 ratio of glacial acetic acid and ethanol) was added to them drop wise for two minutes for fixation. After fixation, the glands in slides were stained with aceto-orcein for 15 minutes keeping them covered under a petridish. Following this, stain was carefully absorbed by a filter paper from the slides and glands were washed with a few drops of 50% acetic acid to remove any excess stain. Excess acetic acid was absorbed with a filter paper and 1-2 drops of lacto-orcein was added on the glands on glass slides. A cover glass was mounted over the glands and excess lacto-orcein was absorbed by a filter paper. Uniform and gentle thumb pressure was applied on the cover glass above the glands to squash them for proper spreading of polytene chromosomes.

Data Analyses: Data were analyzed in following three aspects:

i. Assessment of intermediate time (in hour) needed for the initial appearance of a particular stage of development:

It was done by counting the number of hours required for the developing flies to reach a particular stage of development considered under our study i.e. third instar larva, pupa or adult from the time (hour '0') during which parental male and female flies were released into each culture vial for mating.

ii. Assessment of the number of representatives individuals of pupa and adult stages:

It was done by counting the total number of pupae and young adults generated in each culture vial till 100 hours from the initial time of their appearances.

iii. Assessment of chromosomal rearrangements:

It was performed by high resolution microscopic (Olympus Microscope, Japan, Model L-200A) examination of the polytene chromosome preparations in the glass slides obtained from third instar larvae of control and test groups. Photographs of various chromosomal aberrations were taken at X100 magnification by a camera (Nikon, Japan, Model- EH-53).

Statistical Analyses:

Numerical values were expressed as the mean ± standard deviation (SD). 't' test was performed to - i) determine the deviation of the test group, if any, from the controls with respect to number of hours required for initial appearances of third instar larvae, pupae and young adults from hour '0' ii) deviation of the test group, if any, from the controls with respect to total count of representative individuals of developmental stages like pupa and adult up to 100 hours from their initial time of appearances.

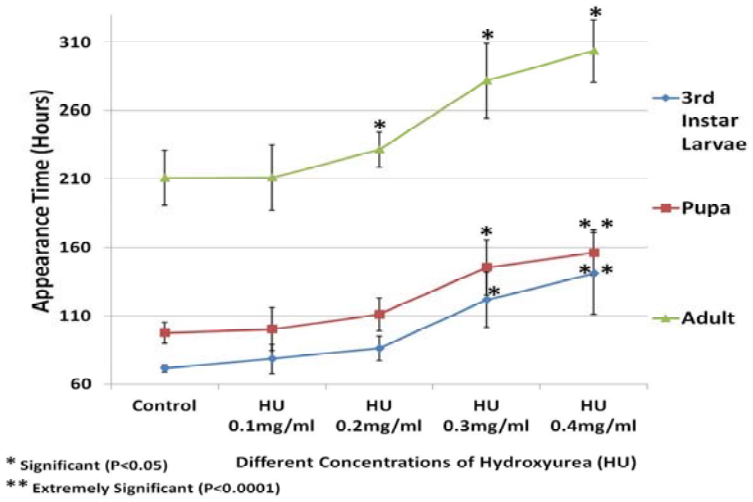
Results

i. Assessment of intermediate time (in hour) needed for the initial appearance of a particular stage of development:

Figure 1 shows that compared to controls, number of hours needed for the initial appearances of pupae and young adults from hour '0' increased in cultures with the increasing concentration of the drug but significant increases were noticed mainly in cultures exposed to HU concentration of 0.3 mg/ml and 0.4 mg/ml only. However, significant delay in the appearance of third instar larvae was initiated in cultures exposed to HU concentration of 0.2 mg/ml. Levels of significance of deviations in developmental time course in response to the exposure of flies to these concentrations of HU gives the p value ≤ 0.05, which is under accepted level of statistical significance at 95% confidence level and 9 degrees of freedom.

Figure 1. Time course of developmental stages of *Drosophila melanogaster* exposed to varying concentrations of HU. Data are represented as the number (Mean +/-SD) of hours required for initial appearances of the particular stages of development of *Drosophila melanogaster* from '0' hour during which adult male and female flies were released into the control and test culture vials for mating.

Figure 1



ii. Assessment of the number of representative individuals of pupal and adult stages:

Number (mean +/- SD) of representative individuals of pupa and adult stages generated up to 100 hours from their initial time of appearances, has been shown in Figure 2. The figure reveals that, compared to controls, significant reduction in number of pupae and emergent adults was intiated in the fly cultures exposed to HU concentration of 0.2 and 0.1 mg/L respectively. However, hallmark of developmental inhibition (P=<0.0001) was observed in cultures exposed to HU concentration of 0.4 mg/L where, compared to control, metamorphosis of developing flies into adult was reduced by approximately77% .

The percent reduction of population of pupae and emergent adults compared to controls in response to exposure to varying concentration of HU has been shown in the **Table 1.**

Figure 2: Population data of *D. melanogaster* exposed to varying concentrations of Hydroxyurea(HU). Data are represented as the number (Mean +/-SD) of pupae and young adults appeared up to 100 hours from their initial time of appearances in control and test cultures.

Figure 2

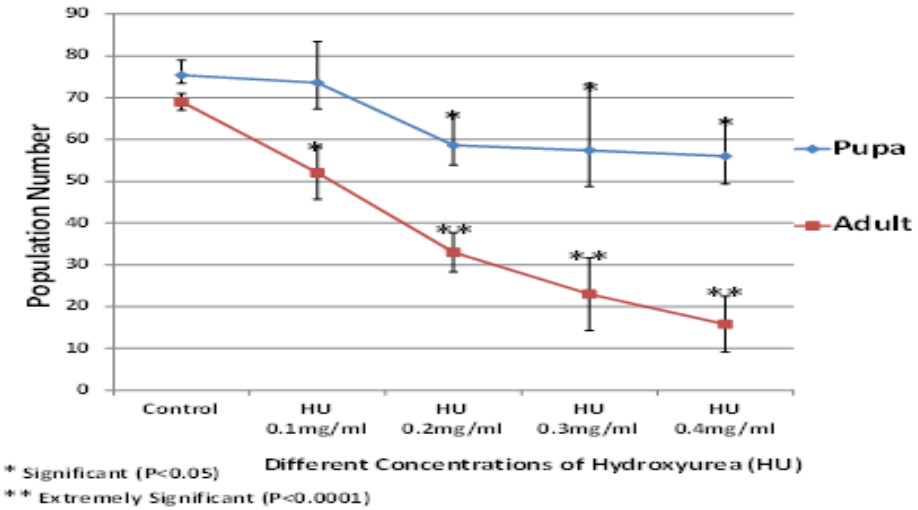


Table1. Percent reduction of population of pupae and emergent adults compared to controls in response to exposure to varying HU concentration.

Concentration of HU	Percent (%) reduction of pupa population	Percent (%) reduction of emergent adult population
0.1mg/ml	2.4	25
0.2 mg/ml	22.3	52
0.3 mg/ml	24	67
0.4 mg/ml	76	77

iii. Assessment of chromosomal abnormalities:

Polytene chromosomes prepared from the third instar larvae of the flies exposed to HU at all concentrations (i.e. 0.1mg/ml, 0.2mg/ml, 0.3mg/ml and 0.4mg/ml) exhibited a number of chromosomal rearrangements. However, occurrence of chromosomal aberrations at HU concentration of 0.1 mg/ml was very low. Chromosomal abnormalities were represented by asynapsis, ectopic pairing, constriction etc (Figures 3,4,5,6 and7). Frequency of chromosomal rearrangements in larval polytene chromosomes was highest at HU concentrations of 0.4mg/ml followed by decreasing HU concentrations.

Figure 3 Ectopic pairing in polytene chromosome in response HU concentration 0.3 mg/ml.

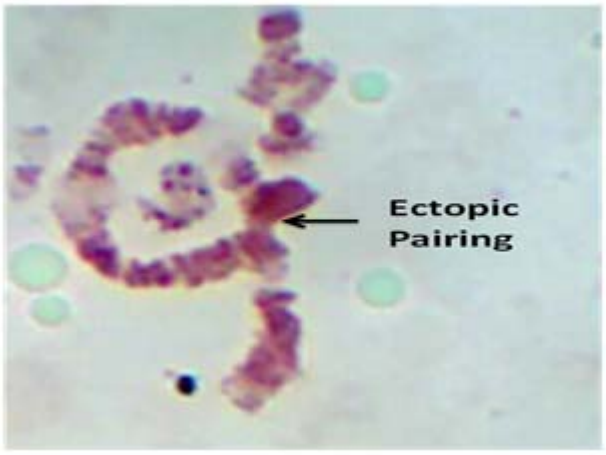


Figure 4 Asynapsis in response HU concentration 0.3 mg/ml.

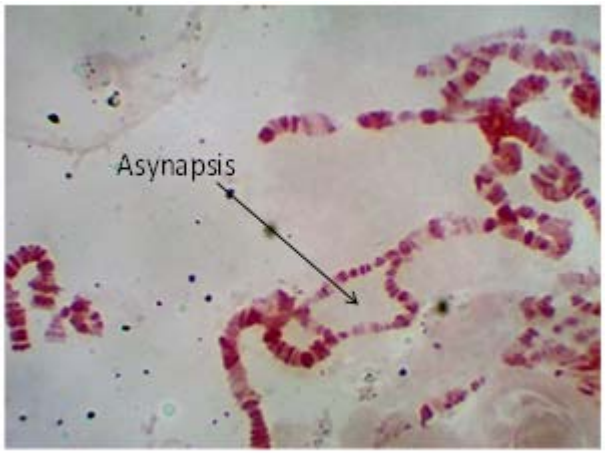


Figure 5 Ectopic pairing, constriction in response HU concentration 0.3 mg/ml.

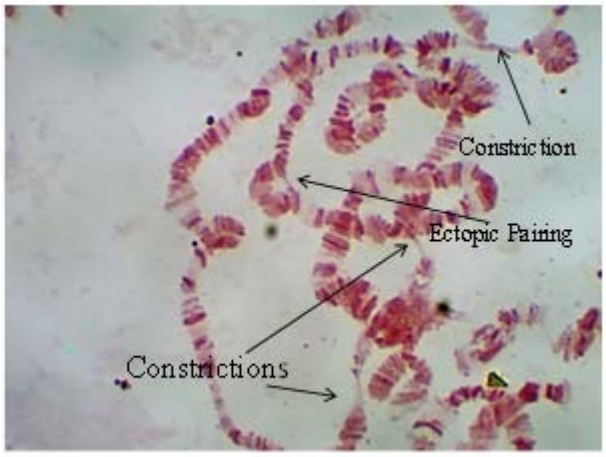


Figure 6 Ectopic pairing in response HU concentration 0.4mg/ml.

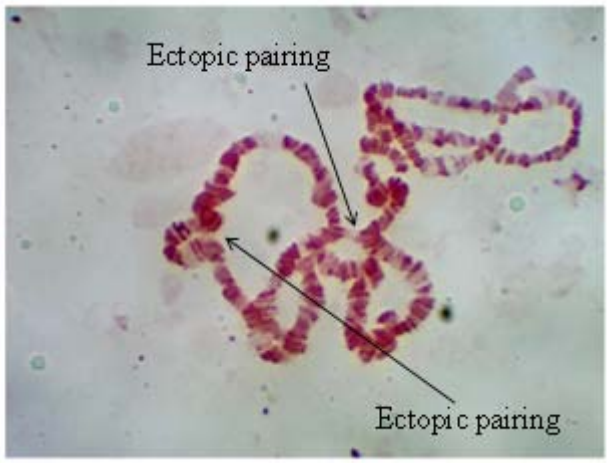
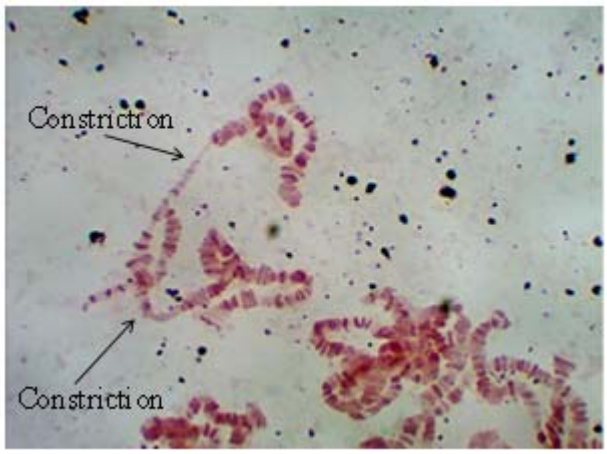


Figure 7 Chromosomal constriction in response HU concentration 0.4 mg/ml.



Discussion: In this study an assessment of dose dependent effects on development of a drug i.e. HU, which is used for treating cancer and inherited blood disorder like sickle cell anemia, was made by using the model organism *D. melanogaster*. It was observed that the drug caused dose dependent delays in metamorphosis as well as reduction in number of emergent adults of this fly. However, significant delay in the initial appearance of pupa and emergent adults was observed in cultures exposed to HU concentration of 0.3 ad 0.4 mg/ml of

which later concentration conferred highest significant impact. Although the significant delay in the initial appearance of the pupa and adult stages commenced at HU concentration of 0.3 mg/ml, significant reduction in pupa and emerging adult number were initiated by further lower HU concentrations of 0.2 and 0.1 mg/ml respectively. This indicates that, compared to controls, most of the developing flies under the exposure of HU could not tolerate even these lower concentrations of HU which caused the paucity of their metamorphosis. In addition, metamorphosis into adult stage was drastically reduced with the increasing doses of HU (Table 1). The developing flies which survived the challenge of these two HU concentration, suffered the significant delays in metamorphosis. In other words, HU exerted a distinct negative impact on the development of this fly. After extensive search we could find of only one report regarding the experimental evaluation of the impact of this drug on the development of *D.melanogaster* [23]. In that study, flies were exposed to only two concentrations of HU viz, 0.1 and 0.25 mg/ml. When we compared our result with the results of the aforesaid study, it was observed that reduction of emergent adult population in our case was many folds higher than the reduction of adult population reported in that study. We speculate that the differences in tolerance of HU by the flies might be due to the strain differences of *D. melanogaster* used by us and the above mentioned group, since it has been reported that strain differences in *Drosophila* make differences in their tolerance for chemicals[24].

Mutagenic potential of chemical and physical agents can be evaluated by the extent of chromosomal lesions induced by them and experimental observation of polytene chromosomes of dipterans especially *Drosophila* provides us an opportunity to see these effects in an magnified way due to the enormous size of this chromosome[25]. In our study, examination of the polytene chromosomes in the third instar larval stage of *D. melanogaster*, exposed to varying concentration of HU exhibited several chromosomal rearrangements in the forms of constriction, ectopic recombination, asynapsis etc. These kinds of chromosomal abnormalities were found to be of very negligible occurrence in case of controls. Although we found a few chromosomal structural abnormalities in larvae exposed to HU at 0.1 mg/ml concentration, significant occurence of these chromosomal aberrations was evident at HU concentrations 0.2 mg/ ml. that was increased further with increasing doses of HU.

One abundant rearrangement that we observed was the constriction of polytene chromosomes. Due to the inhibition of DNA replication, constrictions in chromosomes may appear and the sites become fragile that facilitate their breakages[26,27]. As it has been mentioned earlier that HU interferes with DNA replication, occurrence of such chromosomal constrictions were quite possible in our study that might ultimately repress the developmental gene expressions in *Drosophila* larvae. In asynapsis, homologs of polytene chromosome fail to pair with each other. As homologs remain attached with each other by fibrillar connectors[28] or bundles of microfilaments[29], we speculate that chemical interaction of drugs with these structures may cause their distortion rendering the separation of homologs in polytene chromosomes. This separation of homologs may provide severe impacts in larval gene expressions as it can cause the disruption of trans-interaction (transvection) of enhancer and promoter elements that occurs during their paired state [30]. Ectopic pairing, which was observed in our larval chromosomes, occur through linkages made by heterochromatin threads between regions of sequence homology present either at different regions of the same chromosomal arm or in different arms of a chromosome. If the linkage occurs between adjacent regions of sequence homology, the intermediate portion of the chromosome bulges out to form a toroidal structure. It is possible that due to this linkage, discrete homologous nucleotide sequences in a chromosome might unite to produce a new gene sequence [25, 31]. We speculate that such nascent gene sequence might code for an unusual protein not normally found in *Drosophila* larvae and proved to be detrimental for development of the flies. We speculate that HU hindered the normal expression of genes required for development of the fly by inducing deleterious rearrangement of chromosomes. Asyanapsis possibly caused inhibition of interactions of controlling elements for gene expressions in the larvae. Ectopic pairing might be responsible for creating new genetic sequences [25] the product of which was deleterious for embryonic development. Constrictions of polytene chromosomes in larvae exposed to HU might result into chromosomal breakage as they represent the fragile sites on chromosomes [26]. This might result in disruption of the expression of genes needed for fly development. Thus it seems probable that wide range of larval chromosomal abnormalities those appeared in the polytene chromosomes of *D. melanogaster*, in response to HU exposure, might cause serious inhibition of the expression of the genes needed development of the fly. *D. melanogaster* serves as an important model for developmental biology [32] and toxicological studies [33]. Furthermore, owing to extensive genetic homology between human and *D.*

melanogaster, the experimental results obtained by using this fly raises an alarm regarding the negative impacts of HU exposure during human pregnancies that may bring about chromosomal abnormalities in developing embryo leading to deleterious effect on offsprings. child births.

Conclusion

Our present study revealed that HU exerted negative impact on the development of *D. melanogaster,* a model organism, in a dose dependent manner. Although the drug is very useful in treating cancer and hereditary blood disorders, our study has proved that it exerts genotoxic effects on eukaryotic chromosomes. Chromosomal damages in turn may have deleterious effects on the expressions of genes needed for the normal development. Owing to substantial genetic homology between human and *Drosophila,* results of our study strongly indicates the possibility of obtaining analogous outcome in human due to the exposure of HU during development. Thus the need of exercising cautious use of this drug during human pregnancy is strongly recommended.

Conflict of Interest

The authors declare no conflict of interest.

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