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EFFECT OF CAFFEINE ON RING CHROMOSOME X LOSS AND NON-DISJUNCTION IN UNDERNOURISHED *DROSOPHILA MELANOGASTER* ADULTS

Rodolfo Félix,* María Esther de la Rosa, Judith Guzmán, and Robert Fernand Mouton**

Departamento de Radiobiología y Genética, Instituto Nacional de Energía Nuclear, México.

ABSTRACT

The mutagenic effect of caffeine has been demonstrated in several animal and plant species, including human cells in tissue culture. For such studies, whose aim is to find gene-controlled repair mechanisms, *Drosophila melanogaster* with its well-known genetics is a suitable test system. The induction of recessive lethals in *Drosophila* by feeding the larval with special food, has been reported by several investigators and refuted by others.

An improved method was used for detecting X or Y chromosome loss and non-disjunction in both sexes of *Drosophila melanogaster* by identifying the exceptional progeny recovered. Females from the stock y^2w^a/sc^8Y; e/e were collected as virgins and stored in a complete food medium or in an incomplet food medium i.e., without yeast, which is the main nutritive component of the *Drosophila* food. The stock which provided males had an sc^8Y chromosome and a closed ring X^{c2} chromosome with the markers yellow and Bar.

Continuously well-fed flies showed no increase in X or Y chromosome loss or in non-disjunction whereas those adults fed with normal nutrient with caffeine added, showed significantly higher frequencies of X or Y paternal chromosome loss. No change was found in maternal X chromosome loss or in the non-disjunction of sex chromosomes in both parents. On the other hand, undernourished adults gave paternal X or Y chomosome loss rates which were significantly higher than those obtained in the well nourished groups.

The joining of the broken ends of sperm chromosomes is normally delayed until fertilization is over, hence, undernourishment seems to affect the repair mechanism contained in the egg, due to modifications in the physiological environment of the oocytes, as discussed below in this paper.

The undernourishment of females fed on nutrient with caffeine added and mated to normally fed males produced a decrease in the rate of paternal sex chromosome loss, as compared to undernourished females without caffeine.

The data obtained can be interpreted according to the hypothesis that mutational enhancement by undernourishment, as well as the contradictory effects of adding

* Present Address: Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México.

** Former expert of the International Atomic Energy Agency commissioned to the Departamento de Radiobiología y Genética del Instituto Nacional de Energía Nuclear, México.

caffeine to complete and incomplete media are associated with some restriction in the fertilized egg on the joining of the paternal chromosome ends produced previously by breakage in the sperm.

RESUMEN

Se ha demostrado el efecto mutagénico de la cafeína en multitud de especies animales y vegetales, incluyendo a las células humanas cultivadas en medios artificiales. Para tales estudios, cuyo propósito es la identificación de mecanismos de reparación genéticamente controlados, *Drosophila melanogaster*, con su genética tan ampliamente conocida, constituye un sistema muy apropiado. Algunos autores han inducido mutaciones letales recesivas mediante la alimentación especial de las larvas de *Drosophila*, mientras que otros no lo han logrado.

Se aplicó un método mejorado para la identificación de los individuos provenientes de cigotos en los que faltan el cromosoma X o el Y, así como de los individuos provenientes de la no disyunción cromosómica en ambos sexos de *Drosophila melanogaster*, mediante el reconocimiento de los descendientes que difieren en su fenotipo, del característico de las líneas empleadas. Se aislaron hembras vírgenes de la línea y^2w^a/sc^sY; e/e, que se pasaron a medio alimenticio completo o incompleto. En el medio incompleto se excluyó la levadura, que es el componente nutritivo principal para *Drosophila*. La línea de la que se aislaron los machos es de la composición genética sc^sY, con su cromosoma X en forma de anillo cerrado: X^{e2}, dicho cromosoma es además portador de los marcadores *yellow* y *Bar*.

Entre los descendientes de las moscas que se alimentaron normalmente, no se observaron pérdidas mayores de cromosomas o incremento de la frecuencia de la no disyunción, mientras que el alimento completo al que se agregó cafeína a concentraciones mayores que la de 0.08%, produjo una pérdida significativamente mayor del cromosoma X paterno. No se registró incremento alguno de la frecuencia espontánea con que se pierde el cromosoma X materno, o en la no disyunción cromosómica en ambos sexos. Por otra parte, los adultos desnutridos dieron descendencia que presentó una frecuencia significativamente mayor en la pérdida de los cromosomas sexuales paternos que la producida en los grupos bien nutridos. Considerando que la unión de los extremos rotos de los cromosomas espermáticos es normalmente retardada hasta concluida la fecundación, la desnutrición parece afectar los mecanismos de reparación del óvulo.

La desnutrición de las hembras alimentadas con medio con cafeína y cruzadas con machos alimentados normalmente, produjo un descenso de la frecuencia de la pérdida de los cromosomas sexuales de origen paterno, en comparación con la que tiene lugar en las hembras mantenidas en el medio nutritivo incompleto sin cafeína.

Los datos obtenidos pueden interpretarse según la hipótesis de que el incremento en la frecuencia de la pérdida de cromosomas producida por la desnutrición, así como los resultados contradictorios obtenidos al aplicar la cafeína en el medio completo y en el medio incompleto, están asociados con algún efecto sobre la restitución en el óvulo fertilizado, de los extremos rotos de los cromosomas paternos, originados en las rupturas previas que tienen lugar durante la espermatogénesis.

INTRODUCTION

Caffeine has long been known to be radiomimetic to plant chromosomes (Kihlman *et al.*, 1971), to bacteria (Demerec *et al.*, 1950, 1951), to ascomycetes (Fries, 1950) and to *Ophiostoma* (Zetterberg, 1959). This purine (1, 3, 7 - trimethylxantine) is also effective in breaking chromosomes of human cells in tissue culture (Ostertag *et al.*, 1965). Attempts to demonstrate the mutagenicity of caffeine in the mouse (Demerec *et al.*, 1951; Lamy, 1947) have not led to conclusive results. Induction of

recessive lethal mutations by larval feeding treatment in *Drosophila* has been reported by some investigators (Andrew, 1959; Ostertag and Haake, 1966) and refuted by others (Yanders and Seaton, 1962; Alderson and Khan, 1967; Clark and Clark, 1968).

Andrew (1959) reported that caffeine behaved as a weak mutagen when included in the food medium at a concentration of 0.25%. A weak mutagenic effect was also observed after injection of a caffeine solution into adult males. A 24-h brood interval was used but there was no clear indication of the existence of a particularly sensitive period during spermatogenesis. Although Andrew carried out only two experiments, they were both on a substantial scale and a conclusion of weak mutagenicity appeared justified, at least for the Canton-S strain used in her experiments.

Yanders and Seaton (1962) were unable to confirm the mutagenicity of caffeine when included in *Drosophila* food medium. They suggested that the lack of agreement with Andrew's (1959) data might indicate strain differences in susceptibility, or perhaps the confounding of Andrew's results as a consequence of the recovery of clusters of lethals. Andrew makes no reference in her paper to the occurrence of lethal clusters in her experiments. Her culturing schedule was such that clusters could have been detected, had they occurred.

Alderson and Khan (1967) raise the question of whether RNA and ribonucleotides present in the usual *Drosophila* culture medium might exert an antimutagenic effect and thus mask the mutagenicity of caffeine. Differences in the composition of culture media used in different laboratories might thereby account for discrepant reports. They tested the effects of 0.05% and 0.075% caffeine on larvae in axenic culture kept from 48 to 240 h. on a chemically defined medium. Purine and pyrimidine sources were omitted from the medium, save for the added cafeine. Different concentration levels of added RNA were tried in various experiments. However, no evidence could be obtained to suggest that caffeine is able to induce recessive sex-linked lethals in male larvae. The results therefore agree with those of Yanders and Seaton (1962).

After the appearance of the work of Yanders and Seaton, it was decided to repeat Andrew's work on a more extensive scale. Part of this work was carried out in the Zoology Department of the University of Tasmania from 1962-1963, but as it did no prove possible to obtain clear evidence that caffeine can induce sex-linked lethals in *Drosophila*, the data have not previously been published. However, in view of the reports by Ostertag and Haake (1966) and Mittler *et al.* (1967), which suggested that caffeine may be mutagenic after all in *Drosophila*, we have decided to publish this short report of our experiments made in order to show the effect of caffeine on the frequencies of non-disjunction and chromosome loss in both males and females, testing a standard medium from which yeast was omitted, considering that the high concentration of RNA and nucleotides in the yeast contained in the normal *Drosophila* food medium might mask the effect of caffeine.

The rationale of the experiments described in this paper is as follows. The absence of dose fractionation effects in X-rayed mature sperm indicates that processes leading to chromosomal rearrangements are active only after the sperm has inseminated an egg (Muller, 1940). Such processes might depend, at least in part, upon factors present in the egg, suggesting

that the repair machinery in the oocytes plays an important role in the formation of chromosome aberrations in postmeiotic germ cells.

Further evidence substantiating the above thesis came from exposure-fractionation studies which showed interaction of breaks produced in spermatids (in males) with those produce in mature spermatozoa (in inseminated females) (Sobels, 1972).

All these findings strongly suggest that the repair machinery in the oocytes plays a vital role in the formation of induced chromosomal aberrations in post-meiotic male germ cells. It should therefore be possible, by suitably modifying the genotype of the females and/or the physiological environment of the oocytes, to influence the magnitude of the genetic damage induced in the male germ cells. Some work along these lines has been carried out by Kaufmann (1946) who exposed eggs to near-infrared radiation at the time of their fertilization by x-irradiated spermatozoa; he found that such a combination of treatments led to an increase in the frequency of chromosomal rearrangements as detected by cytological analysis.

MATERIALS AND METHODS

The following experiments were designed to test the effects of caffeine in *Drosophila* adults under different nutritional conditions. The recorded genetic events were the frequency of X or Y chromosome loss in males, chromosome non-disjunction also in males, and the chromosome X loss and non-disjunction in females. For this purpose an improved method for detecting non-disjunction and X or Y chromosome loss was applied, that gives particularly reliable information on the origin of the exceptional progeny recovered.

Virgin females were taken from one stock with an sc^8Y chromosome which includes the wild type allele at the y locus, to avoid the existence of any secondary exceptions from XXY mothers (y^2w^a/sc^8Y; e/e). The tester ring stock which provides males also had the sc^8Y chromosome and the closed ring X, X^{c2}, with mutants yellow and Bar ($X^{c2}yB/sc^8Y$). The expected offspring from this type of mating are yellow-Bar females and white-apricot males. The appearance of exceptional y^2w^a males indicates either loss of the ring X chromosome from the paternal stock, as well as non-disjunction in males. Bar females and white apricot females result from the presence of two X chromosomes and a Y chromosome by non-disjunction in males and females, respectively. Yellow-Bar males are XO males which indicate the loss of the maternal X chromosome. The mating system for the experiment is as follows:

P.	Treated adults:	♀ y^2w^a / y^2w^a	x ♂ yB / sc^8Y
F 1.	Normal progeny:	y^2w^a / yB	(♀ $y^2B/+$)
		y^2w^a / sc^8Y	(♂ w^a)
	Non-disjunction in ♀ :	y^2w^a / y^2w^a / sc^8Y	(♀ w^a)
	X loss in ♀ :	yB	(♂ yB)

Non-disjunction in ♂ : y^2w^a / yB / sc^8Y, (♀ B/+)

Non-disjunction or X loss in ♂ : y^2w^a (♂ y^2w^a)

RESULTS

Experiment I. Effect of caffeine added to the normal nutrient of adults. Newly emerged males and virgin females were aged for six days in separate cultures containing normal nutrient with caffeine added at several concentrations. After this treatment they were mated and transferred to mass culture bottles with standard culture medium that included sugar, agar, cornmeal and brewer's yeast. Transfers to fresh food bottles were made every two or three days, so that separate broods would be obtained covering a 14 day post-treatment period.

The results obtained are summarized in Table I where it can be seen that there is an increase in paternal X or Y chromosome loss in the groups treated with caffeine. The X^2 distribution table gives significant P values for the caffeine concentrations of 0.08, 0.10, and 0.20%.

There was not any noticeable difference between the control and the caffeine treated groups with regard to the non-disjunction of sex chromosomes in both sexes and to chromosome loss in females. In all the groups such events were of remarkably low occurrence, with a variation between 0.02±0.02% and 0.03± 0.02%.

Caffeine together with other methyl purines strongly inhibits the enzymatic dark repair of UV irradiation damage to DNA. This inhibition also is readily reversed by moderate dilution of the caffeine solution and led Sauerbier (1964) to suggest that since it does not inhibit photoreactivation, caffeine suppresses the dark repair reaction by specific enzymatic inhibition rather than by combination with the substrate (DNA). It is not known which of the several steps postulated in dark repair is inhibited by caffeine. It is perhaps pertinent that the concentrations of caffeine effective as inhibitor of purine phosphorylases (Koch and Lamond, 1956), dark repair and stationary phase mutations are similar.

The accumulation of mutants in the absence of cell division has been reported in cells of a number of other organisms including stored seeds, *Drosophila* sperm and *Serratia* (Stubbe, 1935, 1936; Blakeslee, 1954) suggesting that stationary phase mutation is not limited to *Escherichia coli.*

Novick and Szilard (1952) demonstrated an antimutagenic effect of certain natural purine ribonucleosides (adenosine, guanosine and inosine) on the mutagenic effect of some N-methylxanthines (including caffeine) on *Escherichia coli.* In view of these data, it seems possible that the high concentration of RNA and nucleotides in the yeast contained in the normal *Drosophila* food medium might overlay the mutagenicity of caffeine. On the basis of this consideration, the adult feeding treatment was repeated with a standard medium from which yeast was omitted.

Experiment II. Effect of caffeine on undernourished adults. The design of the experiment was the same as in Experiment I, with the following modifications. Prior to mating, female adults were aged

TABLE I

EFFECT OF CAFFEINE ON PATERNAL X AND Y CHROMOSOME LOSS IN *Drosophila melanogaster*

Concentration of Caffeine	*Days After Treatment* 0-3	3-5	5-7	7-9	9-12	12-14	*Total* %±S.E.	*P*
Control	5/1,353	7/1,084	4/908	2/864	2/764	7/989	27/5,962	
	0.37	0.65	0.44	0.23	0.26	0.71	0.45±0.09	
0.04	3/833	4/857	2/773	4/693	3/680	6/850	22/4,686	
	0.36	0.47	0.26	0.58	0.44	0.71	0.47±0.10	>0.80
0.08	8/1,206	8/1,286	8/1,111	6/972	3/726	6/842	39/6,143	
	0.66	0.70	0.72	0.62	0.41	0.71	0.63±0.10	>0.02
0.10	4/944	6/883	5/986	6/588	8/860	8/1,074	37/5,335	
	0.42	0.68	0.51	1.02	0.93	0.74	0.69±0.11	<0.01
0.20	10/1,285	12/966	4/785	3/721	4/911	5/1,093	38/5,761	
	0.78	1.24	0.51	0.41	0.44	0.46	0.66±0.11	<0.02

TABLE II

EFFECT OF CAFFEINE ON PATERNAL X AND Y CHROMOSOME LOSS IN UNDERNOURISHED *Drosophila melanogaster*

Type of Nutrition	*Days After Treatment* 0-3	3-7	7-9	9-11	11-14	14-17	17-19	*Total* %+S.E.	*P*
Normal	6/2,488	3/2,120	4/1,157	9/1,051	10/1,189	4/851	4/891	40/9,747	
Nutrient	0.24	0.14	0.34	0.86	0.84	0.47	0.45	0.41±0.06	
Undernourished	3/705	10/1,125	13/1,082	12/1,152	9/1,155	3/869	3/793	53/6,881	
	0.42	0.89	1.20	1.04	0.78	0.35	0.38	0.77±0.11	<0.001
Undernourished	4/722	4/936	3/617	3/601	7/648	5/634	3/736	29/4,894	
+0.05% caffeine	0.55	0.43	0.48	0.50	1.08	0.79	0.41	0.59±0.11	<0.05
Undernourished	1/237	2/987	1/1,076	4/1,145	1/1,146	1/1,148	1/1,084	11/6,823	
+0.10% caffeine	0.42	0.20	0.09	0.35	0.09	0.09	0.09	0.16±0.05	<0.001
Undernourished	0/221	0/853	3/607	1/647	0/661	6/664	0/596	10/4,249	
+0.20% caffeine	0.00	0.00	0.49	0.15	0.00	0.90	0.00	0.23±0.07	<0.001

for six days in culture flasks containing standard medium without yeast to which caffeine was added at various concentrations. After mating these females with six day old normally fed males, the females were transferred to new mass culture bottles containing normal nutrient. Thereafter, periodic transfers were made to fresh food bottles in order to obtain new broods every two or three day for a period of 19 days.

The results of this experiment are shown in Table II. There is a significant increase in the frequency of X or Y paternal chromosome loss in the undernourished group as compared with the normally fed group. ($X^2 = 30.88$, $P < 0.001$). However, the overall rate of paternal X or Y chromosome loss in the undernourished flies treated with caffeine is lower than that obtained in the undernourished flies nurtured without caffeine, an effect which is more noticeable at higher concentrations where P values of X^2 are highly significant (caffeine at the concentrations of 0.10 and 0.20%; $P < 0.001$). As in Experiment I, no noticeable difference was found among the normally fed and undernourished groups, with or without caffeine, when the frequencies of paternal and maternal non-disjunction and maternal X chromosome loss were compared. *Experiment III. Non-disjunction among the progeny of undernourished adults crossed with normally fed adults.* In this experiment newly eclosed males and virgin females were undernourished in separate cultures for 11 days and crossed with adults reared on a standard medium (with or without caffeine) for 24 hours. Tables III and IV contain summarized results obtained from the two combinations of adult treatments. As seen in Table IV, up to 0.20% caffeine in the complete medium does not increase the frequency of non-disjunction in males crossed with undernourished females. However, there is a two-fold increase in non-disjunction in males at caffeine concentration of 0.40% which has a X^2 value whose P value is significant at the level of 0.05.

TABLE III

FREQUENCY OF NON-DISJUNCTION AMONG THE PROGENY OF UNDERNOURISHED MALES CROSSED WITH FEMALES FED ON NORMAL NUTRIENT WITH CAFFEINE ADDED

% caffeine in female nutrient	*non-disjunction in males ± S. E.*	*P*	*non-disjunction in females ± S. E.*	*P*
0.00	5/4,734 0.11±0.05		2/4,734 0.04±0.03	
0.05	1/4,553 0.02±0.02	>0.05	0/4,553	—
0.10	2/4,234 0.05±0.03	>0.20	0/4,234	—
0.20	3/2,033 0.15±0.09	>0.30	0/2,033	—

TABLE IV

FREQUENCY OF NON-DISJUNCTION AMONG THE PROGENY OF UNDERNOURISHED FEMALES CROSSED WITH MALES FED ON NORMAL NUTRIENT WITH CAFFEINE ADDED

% caffeine in male nutrient	*non-disjunction in males ± S. E.*	*P*	*non-disjunction in females ± S. E.*	*P*
0.00	2/4,205 0.05±0.03		1/4,205 0.02±0.02	
0.05	3/4,683 0.06±0.03	>0.50	0/4,683	—
0.10	1/4,660 0.02±0.02	>0.20	0/4,660	—
0.20	1/4,228 0.02±0.02	>0.30	0/4,228	—
0.40	5/4,440 0.11±0.05	<0.05	0/4,440	—

DISCUSSION

The feeding of newly emerged adults with normal nutrient and caffeine added at concentrations higher than 0.08% for a period of six days before mating, gave a significant P value for the frequency of paternal ring X or Y chromosomes in the progenies obtained from 0 to 14 days after the treatment. No significant effect was recorded for the maternal X chromosome loss, or for the non-disjunction of sex chromosome in both parents.

Undernourishment of females before they were mated to normally nourished males significantly increased the rate of paternal sex chromosome loss. The rate of this maternal effect diminished when caffeine was given to the undernourished adults.

In the crossing of undernourished females with males nourished with normal nutrient, with or without caffeine, as well as in the reciprocal mating, a non-significant diminution was recorded in the proportion of non-disjunctional progeny recovered from males reared in low concentrations of caffeine or in an undernourished medium.

The spontaneous frequency of non-disjunction obtained in males goes from 0.05±0.03% to 0.11±0.05%. In females the same parameter had a variation between 0.02±0.02% and 0.04±0.03%. Non disjunctional progeny were not recovered from any of the treated females in any experiment.

These results, which were not expected because they are contradictory to the data derived from experiments I and II, suggest differences among the genetic and nutrient factors which influence non-disjunction and chromosome loss.

Herzkowitz (1963) demonstrated that maternal undernourishment increases the rate of paternal chromosome loss. Since

the joining of the broken ends of sperm chromosomes is normally delayed until after fertilization, undernourishment of the oocyte seems to inhibit, destroy or limit some factor required for the joining of the broken ends.

Grigg and Stuckey (1965) reported the antimutagenic effect of caffeine which reduced the spontaneous mutation rate during the stationary phase of auxotrophic *Escherichia coli*. As the nucleotide turnover associated with the repair system might also be the basis of spontaenous stationary phase mutation in *Escherichia coli*, they tested the idea of inhibiting the repair process with caffeine, thus decreasing the mutation rate, probably by the enzymic inhibition which this compound exhibits during dark repair.

While in microorganisms, some gene-controlled systems are known which are capable of repairing DNA damage induced by radiation of chemicals, in higher organisms the mechanisms connected with the formation of chromosome aberrations are not yet characterized in detail. For such studies, whose aim is to find gene-controlled repair mechanisms, *Drosophila melanogaster* with its well-known genetics is a suitable test system.

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