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Genomic Stability Role of Folic Acid on the Mutagenicity of Phytoestrogens in *Drosophila Melanogaster*

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Abstract

In this study, the potential genotoxic effects of three phyto-estrogens (genistein, quercetin and glycitein) whose biological effects bear similarities in natural and cultured plants which are used in feeding animals and human beings, and antimutagenic effects of folic acid were researched via using *Drosophila* wing SMART. We worked on four concentrations of each of the three phyto-estrogens whose genotoxic effects were researched. In our study also we applied by far the highest dosage of these phyto-estrogens, which is 10 µM, together with the 10 µM dosage of folic acid on the larva in the same feeding environment. The wing preparations of the obtained individuals were prepared for normal wing and serrate wing phenotype. As a result of the applications glycitein yielded in all concentrations either negative or inconclusive genotoxicity. However the other two phytoestrogens on the whole showed positive genotoxicity depending on the increase in concentration. Total mutation frequency for normal wing phenotype the order of the genotoxicity was: quercetin 0.89 %, genistein 0.58 % and glycitein 0.19 %. When these phytoestrogens were applied together with the folic acid, total mutation frequency decreased, respectively for quercetin 0.46 %, genistein 0.28 % and glycitein 0.16 %. The negative or positive genotoxic effects of the plant estrogens used in this study were determined and it was found out that folic acid is anti-genotoxic against these effects.

Keywords: *Drosophila melanogaster*, folic acid, genomic stability, phytoestrogens, SMART.

Introduction

Phyto-estrogens (PEs) are obtained from about 300 different plants and show impact attaching on estrogen receptors. They are found abundantly in fresh vegetables (broccoli and spinach) and fruit (apple, kiwi, bananas, etc.), barley, wheat, corn, soya bean, linseeds (Colborn et al. 1996). When they are taken long, negative effects have been observed on several organisms. Although there is increasing popularity of dietary phytoestrogen supplementation and of vegetarian and vegan diets among adolescents and adults, concerns about potential detrimental or other genotoxic effects persist. A different variety of *Ferula communisin*, *Brevifolia*, have showed poisonous and anticoagulant effect on the sheep (Lamnaouer 1999). Furthermore, oestrogen like chemicals caused infertility on the sheep (Setchell et al. 1987), gender changes on the fish and decreased the number of sperms on human beings (Andreas et al. 1995). In the developmental toxicity study it was noted that in animals that received the top dose of phytoestrogen (especially genistein), cyclicity was disturbed and the animals remained in persistent estrus (Lewis et al. 2003).
Numerous in vitro cell culture studies have reported phytoesterogens to be clastogenic (chromosome breaking), DNA damaging, proapoptotic, carcinogenic and even mutagenic (Stopper et al. 2005; McClain et al. 2006). For example, at high concentrations, 50-150 µM (3 h) genistein was found to induce micronucleus (MN) formation, indicative of chromosome breakage in Chinese hamster V79 cells (Snyder and Gillies 2003). In other micronucleus studies in V79 cells, genistein in longer exposures over a lower dose range of 5-25 µM (18 h) generated dose-dependent increases in micronucleated binucleated cells, and the micronuclei induced (Virgilio et al. 2004). PEs also generate

DNA fragmentation, gaps and interchanges. For example, coumestrol at 50 and 75 µM or genistein at 25 µM are clastogenic (Leclercq and Cremoux 2011).
Folic acid (folate, FA) is a water-soluble vitamin whose biologically active form is tetrahydrofolic acid (THF), which participates in the transfer of one-carbon units (such as methyl, methylene, and formyl groups) to the essential substrates involved in the synthesis of DNA, RNA, and proteins, crucial for metabolical function (Kronenberg et al. 2009; Fenech 2010). FA is at present in plants (spinach, lettuce, parsley, and rosehip) just like phyto-esterogens (Güler and Liman 2005). This essential vitamin B can be produced by bacteria in the intestines of animals. Since human beings can not synthesize it, folic acid must be taken externally. FA is a necessary vitamin in organism for DNA repairing and synthesizing, amino acid metabolism, cell growth and regeneration (Teo and Fenech 2008). Chromosomal breakage, micronucleus and DNA damage may appear in the deficiency of folic acid (Fenech 2001). In addition, the protective effect of folic acid is known to be many types of cancer, especially colon cancer (Duthie 2011). Moreover, this vitamin also has antioxidant properties (Tousson 2012). In addition, it has been showed that folic acid has an important role on DNA stability maintenance, preventing uracil misincorporation into DNA, single and double-strand DNA breaks and micronucleus formation (Tousson 2012).
In this study, we investigated possible mutagenic effects of genistein (GN), quercetin (QU) and glycitein (GL) from pyhto-esterogens on *Drosophila melanogaster* and tried to find out whether folic acid had protective effect on it or not.

Materials and methods

Strains

To investigate the possible genotoxic effects of PEs, two *Drosophila* strains were used, the multiple wing hairs strain (*mwh/mwh)* and the *flr*^{3} strain (*flr3/In(3LR)TM3*, *ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S}*)*. In the *mwh* phenotype, the wing cells contain three or more hairs instead of one hair per cell as in wild type; the *flr*^{3} phenotype exhibits quite a variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes balloon like extrusions of melanotic chitinous material. More detailed information on the genetic symbols and markers can be found in Lindsley and Zimm (1992).
Preparation of genistein, quercetin and glycitein stock solutions for experiments
Genistein, quercetin and glycitein were dissolved in 0.1 % (v/v) DMSO to make 25 µM PEs stock solutions from which dilutions were made for experiments. To test any effect of DMSO in the experiments, DMSO solution was added to the cultures at the final concentration of 1 %, which was the highest concentration of DMSO used in the phytoestrogens, treated medium.

Drosophila SMART test

The SMART assay in *D. melanogaster* monitors loss of heterozygosis induced by point mutation, deletion, unbalanced half-translocation and mitotic recombination, as described by Graf and his colleagues (Graf et al. 1984). Two different genotypes of larvae were used in the tests: *mwh* and *flr*^{3}, which are wing markers located on the left arm of chromosome 3. Virgin females of *flr*^{3}*/TM3, Bds* were crossed with *mwh* males. From the crosses, eggs were collected during 8-h periods in culture bottles containing standard medium. Three-day old larvae were washed out of the bottles with tap water, and 50-100 larvae were transferred to plastic vials with 4.5 g of Drosophila instant medium re-hydrated with 9 ml of the PEs at 10 µM dose. The larvae were fed on this medium until pupation of the surviving larvae. All experiments were carried out at 25±C° and 60 % relative humidity. All surviving flies were collected and stored in 70 % ethanol. EMS was used as a positive control at 1 mM. 1 % DMSO served as negative control.

Statistical analyses

For the statistical calculations, the conditional binominal test according to Kastenbaum and Bowman (1970) was used with p = 0.05 significance levels. The frequency of clone formation was calculated (Frei and Würgler 1996).

Results

Findings belonging at normal (*mwh/flr*^{3}*)* and serrate (*mwh/TM3*) wings phenotype obtained from PEs (genistein, quercetin and glycitein) and PEs + FA application groups are given in table 1, figure 1 and figure 2. As shown in Table 1, 1 mM DMSO showed insignificant difference on both normal and serrate

wing phenotype, and 1 mM EMS showed positive difference on both two groups. When 10 µM GN and 10 µM QU application groups were compared with DMSO, a significant difference was observed in all clones and but inconclusively at 10 µM GL. In 10 µM GN and QU applications, CIF was 2.05 and 3.28, respectively, and again in GL application group, this value was measured to be 0.77. On the other hand, once again positive results were observed when total mutant clone frequency was compared with DMSO group in individuals of *mwh/TM3* genotype in GN and QU application groups (Figure 2). But, this value was measured as an inconclusive difference at GL. In the second part of our study, FA, a member of vitamin B family, was applied with phytoestrogens (FA + GN, FA + QU and FA + GL) to 72±4 hour's trans-heterozygote larvae. As the results obtained from 10 µM FA + 10 µM GN application were compared with 10 µM GN application, a decrease in all clone types in *mwh/flr*^{3} genotype was observed (Figure 1). The result statistically turned to be inconclusive from positive effective. CIF dropped to 1.02 from 2.05 (Table 1). Also in serrate wing phenotype, a decrease both at the number of clone and the value of CIF were determined (Table 1).

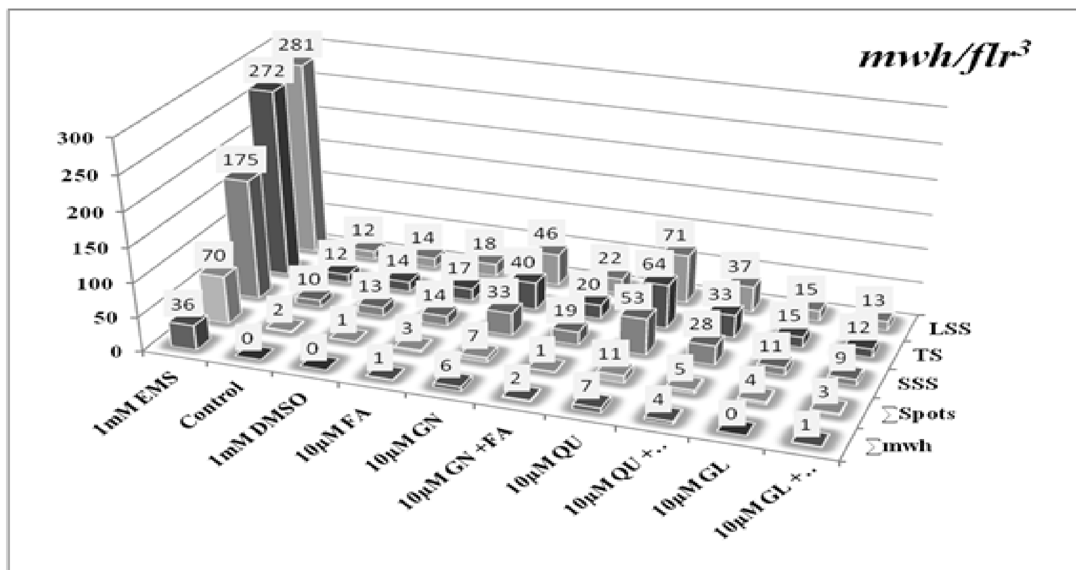

Figure 1 Comparison of the effects of FA and PEs on the *mwh/flr3* (normal wing phenotype) genotype

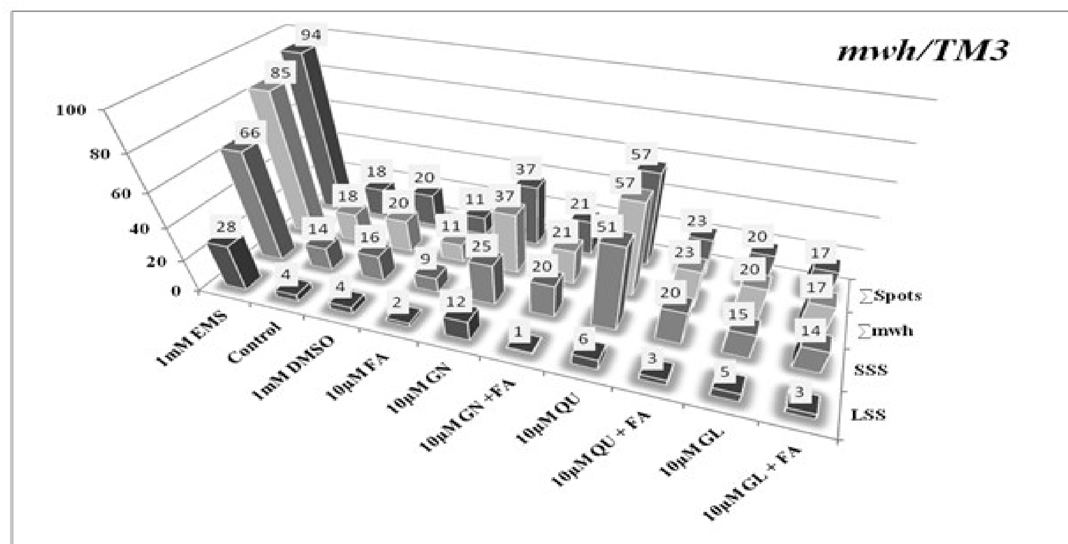

Figure 2 Comparison of the effects of FA and PEs on the *mwh/TM3*(serrate wing phenotype) genotype

Table 1 Summary of results obtained with the *Drosophila* Somatic Mutation and Recombination Test (SMART) after the chronic treatment of larvae from MH and BH individuals with GN, QU, GL and FA.

Treatment	Number of wings (N)	Small single spots (1-2 cells) (m=2)			Large single spots (>2 cells) (m=5)			Twin spots (m=5)			Total mwh spots (m=2)			Total spots (m=2)			
		No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	CIF
MH (*mwh/flr³*)																	
Control	80	10	(0.13)		2	(0.03)		0	(0.00)		12	(0.15)		12	(0.15)		0.61
1 mM EMS	80	175	(2.19)	+	70	(1.14)	+	36	(2.22)	+	272	(3.40)	+	281	(3.51)	+	13.93
1mM DMSO	80	13	(0.16)	i	1	(0.01)	i	0	(0.00)	i	14	(0.18)	i	14	(0.18)	i	0.72
10µM FA	80	14	(0.18)	i	3	(0.04)	i	1	(0.01)	i	17	(0.21)	i	18	(0.23)	i	0.87
10µM GN	80	33	(0.41)	+	7	(0.09)	i	6	(0.08)	+	40	(0.50)	+	46	(0.58)	+	2.05
10µM GN +FA	80	19	(0.24)	i	1	(0.01)	i	2	(0.03)	i	20	(0.25)	i	22	(0.28)	i	1.02
10µM QU	80	53	(0.66)	+	11	(0.14)	+	7	(0.09)	+	64	(0.80)	+	71	(0.89)	+	3.28
10µM QU + FA	80	28	(0.35)	+	5	(0.06)	i	4	(0.05)	i	33	(0.41)	+	37	(0.46)	+	1.69
10µM GL	80	11	(0.14)	-	4	(0.05)	i	0	(0.00)	i	15	(0.19)	i	15	(0.19)	i	0.77
10µM GL + FA	80	9	(0.11)	-	3	(0.04)	i	1	(0.01)	i	12	(0.15)	i	13	(0.16)	i	0.61
BH (*mwh/TM3*)																	
Control	80	14	(0.18)		4	(0.05)		b			18	(0.23)		18	(0.23)		0.92
1 mM EMS	80	66	(0.83)	+	28	(0.35)	+				85	(1.06)	+	94	(1.18)	+	4.35
1mM DMSO	80	16	(0.20)	i	4	(0.05)	-				20	(0.25)	i	20	(0.25)	i	1.02
10µM FA	80	9	(0.11)	-	2	(0.03)	-				11	(0.14)	-	11	(0.14)	-	0.56
10µM GN	80	25	(0.31)	i	12	(0.15)	+				37	(0.46)	+	37	(0.46)	+	1.84
10µM GN + FA	80	20	(0.25)	i	1	(0.01)	-				21	(0.26)	i	21	(0.26)	i	1.08
10µM QU	80	51	(0.64)	+	6	(0.08)	i				57	(0.71)	+	57	(0.71)	+	2.92
10µM QU + FA	80	20	(0.25)	i	3	(0.04)	-				23	(0.29)	i	23	(0.29)	i	1.18
10µM GL	80	15	(0.19)	i	5	(0.06)	i				20	(0.25)	i	20	(0.25)	i	1.02
10µM GL + FA	80	14	(0.18)	-	3	(0.04)	-				17	(0.21)	-	17	(0.21)	-	0.87

[a]Statistical diagnoses according to Frei and Würgler [1988, 1995] for comparison of response with corresponding negative control: -, negative; i, inconclusive; +, positive ($P < 0.05$); m, minimal risk multiplication factor for the assessment of negative results; MH, marker-heterozygous; BH, balancer heterozygous.; [b]Balancer chromosome *TM3* does not carry the *flr³* mutation.

Quercetin showed the most genotoxic effect on phytoesterogens used in our experiments. But, the number of clones both in normal and serrate wing phenotype decreased in QU + FA application. Positive difference turned to be inconclusive/negative difference. CIF reduced from 3.28 to 1.69 for normal wing and from 2.92 to 1.18 for serrate wing (Table 1). But, values for all clone types in 10 µM GL application group were obtained less than GN and QU (Table1). These values were even more decrease in GL + FA application group. CIF in normal wing phenotype was determined as 0.61 whereas it had to be 0.77. On the other hand, in the serrate wing phenotype it was found to be 0.87 from 1.02. QU, GN and GL were used in our experiments as PE. QU and GN are genotoxic and recombinogenic as shown in Table1. This effect was observed as inconclusive in GL.

FA was used in the second part of our study to eliminate mutagenic and recombinogenic effects of PEs. As shown in Table 1, the positive difference belonging to QU and GN became insignificant, and inconclusive difference belonging to all doses of GL became negative difference ($P<0.05$).

Discussion

In the previous studies, QU had caused sister chromatid exchanges in human lymphocytes and ovarian cells of Chine Hamster (Popp and Schimmer 1991), and genotoxic damage in mice bone marrow (da Silva et al. 2002). GN from PHs has formed micronucleus at 1.5-100 nM dose range in mice lymphoma cells (Stopper et al. 2005) and mice V79 cells (Virgilio et al. 2004). Again this PE has caused tumor formation at humans (Newbold et al., 2001), sister chromatid exchange in mice (Giri and Lu 1995) and genotoxicity in human sperm and lymphocytes at 10-400 µM application doses (Anderson et al. 1997). GN has reduced fertility in female mice and malfunction has been observed at ovarian (Jefferson et al. 2007). Furthermore, it has also inhibited oocyte maturation in pigs (Jung et al. 1993) and mice (Cauwenberge and Alexandre 2000).
In our experiments, mutagenic and genotoxic effects observed to be depending on dose increasing and chronic feeding in *D. melanogaster* were found to be compatible with literature.
The probable reason of these effects depending on PE is the inhibiting synthesis of functional protein or synthesises of enzymes at different activity and properties with modified protein structure (IPCS 1985). Moreover, reactive oxygen derivatives that formed depending mutagenic PEs may also cause oxidative damage in DNA (Collins et al. 1996). Depending on this damage, chain breakages in DNA cause micronucleus and cell death (Mazur and Blawat 1999). According to Ji and his colleagues (1999), genistein inhibits cell proliferation by means of preventing DNA topoisomerase and protein tyrosine kinase activity. Hence, cytotoxic effect occurs.
Generally, the processing mechanism of antigenotoxic / antimutagenic is based on scavenging free radicals, DNA repair and protecting endogen antioxidant (Nebel et al. 2002). The antimutagenic features of folic acid were shown with several studies *in vitro* and *in vivo*. It is reported that folic acid deficiency increases chromosomal aberrations in human lymphocytes and when this deficiency is removed, reduction occurs on the rate of chromosomal aberrations (Silva et al. 1992). Protective role of folic acid against AFBı (Bhattacharya 1987), arsenic (Ruan et al. 2000) and phenol application (Aşkın et al. 2007) were reported.
According to some researchers, folic acid is corrector at cellular state by manipulating timidilate synthetase activity which is correlated with DNA synthesis (Glover 1982), and composition of cellular nucleotide (Kunz 1988). It is also well known that FA is necessary for epigenetic change occur by DNA methylation, protein methylation, DNA synthesis and maintenance of the overall integrity of DNA (Molloy 2006).
FA supplementation can protect various organisms and humans (Nazki et al. 2014). Epidemiological studies have shown that FA supplementation can significantly reduce not only the risk of cardiovascular but also haematological diseases (Verhaar et al. 2002), neurological and neuropsychiatry disorders (Alpert and Fava 2003), neural tube defects (Olney and Mulinare 2002), Type 2 Diabetes (Ramos et al. 2012). FA on its own and in multivitamins has showed protective effect against various illnesses. This vitamin especially is known as preventive against spina bifida, which increases new born fatality (Czeizel and Dudas 1992). It was proposed that the presumed protective effects of FA in the pathogenesis of these degenerative diseases could be associated with its antioxidant activity (Nakano et al. 2001).

Conclusion

As a mutagenic / genotoxic and recombinogenic substance, PEs cause somatic mutations by methylation DNA in especially *D. melanogaster*. Fortunately, vitamins like FA used in our study have prevented occurrence of this type of mutations.

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