International Food Research Journal 27(2): 208 - 216 (April 2020)
Journal homepage: *http://www.ifrj.upm.edu.my*

Artificial food sweetener aspartame induces stress response in model organism *Schizosaccharomyces pombe*

[1]Bayrak, B., [2]Yilmazer, M. and [2]*Palabiyik, B.

[1]*Institute of Graduate Studies in Sciences, Department of Molecular Biology and Genetics Istanbul University, Istanbul 34116, Turkey*
[2]*Faculty of Science, Department of Molecular Biology and Genetics, Istanbul University, Istanbul 34134, Turkey*

Article history

Received: 30 October 2019
Received in revised form: 13 February 2020
Accepted: 2 March 2020

Keywords

aspartame, cancer, Schizosaccharomyces pombe, Swi6, stress response

Abstract

Aspartame (APM) is a non-nutritive artificial sweetener that has been widely used in many products since 1981. Molecular studies have found that it alters the expression of tumour suppressor genes and oncogenes, forms DNA-DNA and DNA-protein crosslinks, and sister chromatid exchanges. While these results confirm that aspartame is a carcinogenic substance, other studies have failed to detect any negative effect. The present work was aimed to reveal the molecular mechanisms of APM's effects in the simpler model organism, *Schizosaccharomyces pombe,* which has cellular processes similar to those of mammals. The human HP1 (heterochromatin protein 1) family ortholog *swi6* was selected for the evaluation because *swi6* expression is downregulated in cancer cells. Swi6 is a telomere, centromere, and mating-type locus binding protein which regulates the structure of heterochromatin. To verify whether the carcinogenic effects of APM are linked with Swi6, *S. pombe* parental and *swi6*Δ strains were analysed through a number of tests, including cell viability, intracellular oxidation, glucose consumption, nucleus DAPI (4',6-diamidino-2-phenylindole) staining, and quantitative real time polymerase chain reaction (qRT-PCR) methods. Based on the results, the *S. pombe* parental strain adapts to APM effects by activating the stress response pathway, while *swi6*Δ did not show a meaningful response. Thus, it is proposed that there is a relationship between APM and *Swi6*, and that APM may be carrying out its effects through Swi6. Nevertheless, it is understood that aspartame is not an effective carcinogenic agent since its effects are weak when compared with the *swi6*Δ phenotype.

Introduction

Additives are substances generally used to improve the storage qualities, appearance, taste, and texture of foods (Lok *et al.,* 2010). Preservatives, sweeteners, colour additives, flavour enhancers, and emulsifiers are some major categories of the over 3,000 additives approved for worldwide use (Whitehouse *et al.,* 2008). Artificial sweeteners remain a significant class of additives, which were used mainly in the areas of pharmaceuticals and foods for diabetic patients until the 1970s (Talbot and Fisher, 1978). Today, a group of new artificial sweeteners has been developed for the food industry, where they are used in an arsenal of foods and drinks marketed as diet, low calorie, and light. One of these artificial sweeteners, aspartame, has become a market leader since its approval by FDA in 1981. Aspartame is a methyl ester of a dipeptide composed of aspartic acid and phenylalanine. It has two different forms (α and β), and its α form was found to be 200 times sweeter than sucrose (Kirkland and Gatehouse, 2015). The synthetic nature of the compound has driven researchers to assess its safety in many model organisms and, while numerous studies have found carcinogenic effects (Soffritti *et al.,* 2006; Gombos *et al.,* 2007), there also exist neutral results (Magnuson *et al.,* 2007).

Epigenetics investigates the heritable changes in gene functions that are not dependent upon DNA sequence variations. Among these changes, histone modifications such as methylation, acetylation, and phosphorylation play an important role by configuring chromatin structure. While highly compact chromatin is classified as heterochromatin and contains inactive genes, euchromatin displays a looser structure with active genes. The regulation of facultative heterochromatic regions depends on developmental or environmental signals (Trojer and Reinberg, 2007). Recent studies show that constitutive heterochromatin is also dynamically regulated and responsive to stimuli.

*Corresponding author.
Email: bediag@istanbul.edu.tr

While these changes could potentially help organisms to adapt to new environments, in some cases they may also lead to human diseases (Wang *et al.,* 2016).

The development and progression of cancer is accompanied by changes in gene expression. These changes are often affected on a global scale and can be caused by both genetic and epigenetic alterations. Tumour initiation and progression alters the epigenome via DNA hypomethylation, aberrant nucleosome occupancy, and histone modification. Most transcription factors are not gene-specific; a defect in one factor can have consequences on the expression of many target genes. Defects in a chromatin packaging protein or histone modifying enzyme can alter the chromatin status of multiple genomic regions, simultaneously affecting the expression of hundreds of genes (Dialynas *et al.,* 2008). One such protein, known as heterochromatin protein 1 (HP1), which localises to centromeres, telomeres, and specific sites within euchromatin, was found to have downregulated gene expression in several types of cancer (Kirschmann *et al.,* 2000; Maloney *et al.,* 2007). This is likely due to the role HP1 plays in chromosome integrity by securing centromere stability, telomere capping, and the regulation of euchromatic and heterochromatic gene expression (Dialynas *et al.,* 2008).

In the fission yeast *Schizosaccharomyces pombe*, the major structural heterochromatin protein is Swi6. Like all mammalian HP1 homologues, Swi6 is composed of a chromo-domain (CD) and a chromo-shadow domain (CSD), separated by a hinge region (Wang *et al.,* 2000). Swi6 binds to three transcriptionally silent heterochromatic regions, the mating type loci, telomeres, and centromeres (Grewal, 2000; Huang, 2002); all of which have a high concentration of H3-K9 methylation (Nakayama *et al.,* 2001). Swi6, which is a highly conserved protein from fission yeast to mammals, is also involved in centromere function and recruitment of cohesin to heterochromatin regions and is required for proper cohesion of centromeres during mitosis (Nonaka *et al.,* 2002; Cheutin *et al.,* 2004). In the absence of Swi6, fission yeast cells are defective in silencing and in centro¬mere cohesion (Ekwall *et al.,* 1995; Li *et al.,* 2011).

Experiments are frequently carried out to understand the risks of food additives, and model organisms are used to investigate the toxicity and carcinogenicity features of chemical agents. However, these animal experiments require a huge amount of resources and time. While it is hard to determine the toxicity of chemicals at low doses in animal experiments, the risks of these chemical agents at lower doses can be determined through *in vitro* studies (Horio *et al.,* 2014). Single celled eukaryotic organisms such as yeast are frequently used in studies to determine the risks of chemical agents.

Aspartame exhibits unfavourable results in genotoxicity tests and it is thought that aspartame has a carcinogenic potential (Huff and LaDou, 2007). Moreover, some studies on the carcinogenic risks of aspartame carried out on rodents, and the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) assert that the findings of carcinogenic experiments on rodents can be used for humans (Huff, 1999). However, the results of aspartame studies on rodents are inconsistent; some experiments show that aspartame leads to cancer, while others show that it does not (Ishii *et al.,* 1981; Mallikarjun and Sieburth, 2015).

Fission yeast is a good model organism for heterochromatin analysis because of its well-conserved but simple heterochromatin. The present work aimed to explore whether the heterochromatin protein Swi6 plays a role in the effects of aspartame in the simple model organism *S. pombe*. Since fission yeast is similar to higher eukaryotes in stress response and energy metabolism regulation, the effect of aspartame was assessed with methods that indicate the changes in these mechanisms.

Materials and methods

Strains and growth conditions

To test this hypothesis, parental EDD666 (ade6-M210 ura4-D18 leu1-32) and *swi6*Δ (EDD666 *swi6*::KANMX4) strains were used, obtained from *S. pombe* Genome-wide Deletion Mutant Library (Bioneer). To culture the cells, Minimal Medium Agar (MMA), the standard rich media Yeast Extract Liquid (YEL) and Yeast Extract Agar (YEA) containing 0.5% yeast extract (Merck), and 3% glucose (Sigma-Aldrich) were used throughout the study. All cultures were grown at 30°C. At the beginning, colonies were selected with geneticin (200 mg/L) and auxotrophy markers on MMA, then a single colony was grown on YEA slants for storage at 4°C. Growth rates were monitored by spectrophotometric measurements at 600 nm, taken every 2 h in 96-well microplates until a stationary phase was observed.

Cell viability

The initial assessment of any chemical toxicity study is to determine the inhibitory concentration of the chemical concerned. Generally, a mild inhibitory effect of 20% is sufficient and necessary for

managing subsequent experiments as well as drawing healthy conclusions. The colony forming units (CFU) method was used to assess APM toxicity and to determine the mild inhibitory concentration. APM solution was prepared beforehand in distilled water as a 10 mg/mL stock solution, then filter sterilised and added to the autoclaved media. Prior to using this method, parental and *swi6*Δ cells from overnight YEL culture were counted on Malassez slide and treated with APM concentrations ranging from 0.05 to 3 g/L for 24 h in YEL. Following treatment, control and treated cell cultures were diluted 10,000-fold and plated on YEA for an incubation period of 3 d. Then, the resulting colonies of given concentrations were scored for viability (number of colonies counted on treated plates / number of colonies counted on control plates × 100%).

Glucose consumption assay

Known as the Warburg effect, one of the most prominent characteristics of cancer cells is their utilisation of excess amounts of glucose via fermentation. In order to determine whether APM displays such an effect in fission yeast cells and whether Swi6 plays a distinctive role in this metabolic transition, a glucose consumption assay was conducted on overnight cell cultures of parental and *swi6*Δ strains freshly treated with 1 mg/mL APM in YEL. 500 µL of control and treated cell culture samples were taken from each culture every 30 min for 3 h and centrifuged for 10 min at 4,700 *g*. The resulting supernatants were transferred to new tubes and analysed according to the instructions provided with Colorimetric Glucose Assay Kit (Cayman Chemicals, Item No: 10009582). Absorbance values were measured at 520 nm with BioTek Eon Microplate Spectrophotometer.

Fluorescence microscopy

Morphological changes in cell shape and loss of nuclear integrity are symptoms observed in cells affected by external stressors. In pursuance of these alterations and to determine whether Swi6 absence triggers a different response to APM, the nucleus of parental and *swi6*Δ strains were stained with fluorescent probe DAPI. The strains were first with 1 mg/mL APM for 24 h in YEL. Subsequently, 1 mL culture of control and treated cells were washed with water for 10 min at 4,700 *g*, then resuspended in 1 mL cold 70% EtOH for 15 min at -20°C. Following incubation, EtOH was removed with a second water wash, and cells were resuspended in 200 µL cold 70% EtOH. At this point, DAPI was added at a final concentration of 1 µg/mL, and cells were incubated for 2 h at 37°C. Excess stain and EtOH were removed with several PBS washes at 2,400 *g* for 5 min. Observations were made with Olympus BX53 fluorescence microscope with UNA filter and DP73 digital camera.

Intracellular oxidation assay

The apoptotic and cell death pathways increase intracellular oxidation levels by inducing mitochondrial membrane permeabilisation. To analyse this event in APM treated cells and to assess if these pathways include the action of Swi6, free radical sensor fluorescent dye DCFH-DA was applied to the fission yeast. Equal amounts of cells from the parental and *swi6*Δ cultures treated with 1 mg/mL APM for 24 h in YEL were sampled, centrifuged for 5 min at 2,400 *g*, then resuspended in 1 mL fresh YEL. After transferring the samples to a 24-well microplate, DCFH-DA was added at a final concentration of 10 µM, and the aluminium foil covered plate was incubated for 1 h in a rotary shaker at 30°C. Following incubation, samples were transferred again to microcentrifuge tubes and washed with water for 5 min at 2,400 *g*. Pellets were resuspended in 1× PBS and the kinetic reading at 37°C of the samples were performed in a 96-well black microplate by Biotek FLx800 spectrofluorometer at 485/20 excitation and 530/20 emission wavelengths. Fluorescence intensity was stated for every 10^6 cells.

Total RNA isolation and cDNA synthesis

As part of detecting APM-induced adjustments in several *S. pombe* cellular processes with qRT-PCR, the total RNA from control and treated cells were isolated, and cDNA was synthesised for use as a template. At the start of this process, 10 mL of culture from control and treated cells were collected by centrifugation for 10 min at 4,700 *g*, then resuspended in 500 µL lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM DTT). The cells were mechanically homogenised with 0.3 g acid-washed glass beads (0.45 - 0.55 mm) for 5 min for three times by Sartorius Micro-Dismembrator S. The lysate was then centrifuged for 5 min at 2,600 *g* to eliminate any residue. RNA isolation from these samples was conducted following the instructions provided with Pure Link® RNA Mini Kit (Ambion by Life Technologies). Isolate confirmation was made with agarose gel electrophoresis (1% agarose gel, 70 V, 30 min). Concentration was measured with NanoDrop 2000c. cDNA synthesis from the RNA samples was conducted following the instructions provided with High Capacity cDNA Reverse Transcription Kit (Thermo Fisher) and performed using a Bio-RAD

T100 Thermal Cycler. Both RNA and cDNA samples were stored at -20°C.

Gene expression analysis with real-time PCR

It is known that cancerous cells have specifically remodelled chromatin structures. The protein that was selected to investigate the carcinogenic effects of APM in *S. pombe* was Swi6, which has a human ortholog protein of HP1 acting as a chromatin silencer. Swi6 also acts as a heterochromatin regulator and its gene homology with a human protein allowed for similar results in the fission yeast affected by APM. The main goal was to explore whether APM downregulates *swi6* expression through carcinogenic effects on *S. pombe*, as is the case of HP1 in cancerous cells.

S. pombe also has cellular processes and pathways, such as nutrient sensing, signalling, energy metabolism, and stress response which are simpler than but similar to those of higher eukaryotes. These processes are again known to be involved in the phenotype of cancerous cells. Thus, five key genes in energy metabolism, hexokinase 2 (*hxk2*), fructose 1,2-biphosphatase 1 (*fbp1*), adenylate cyclase 1 (*cyr1*), cytochrome c oxidase 4 (*cox4*), and transcriptional corepressor *tup11*; as well as three key genes in stress response, transcription factor *atf1*, catalase 1 (*ctt1*), and superoxide dismutase 1 (*sod1*), were selected for expression analysis, supplementing the study by investigating the effect of APM and its relationship with Swi6.

For positive control, glyceraldehyde 3-phosphate dehydrogenase 3 (*gpd3*) gene was used. Designed with Primer 3 software, commercially synthesised primers were used for the chain reaction (Table 1). The experiment was conducted following the instructions provided with the Thermo Scientific Power SYBR Green PCR Master Mix Kit (Thermo Fisher) and performed by Roche 480 II Light Cycler. Threshold cycle (Ct) value normalisation was calculated according to *gdp3* values with Pfaffl equation.

Statistical analysis

Statistical analysis was carried out in GraphPad Prism 6 software by one-way analysis of variance (ANOVA) and two-way ANOVA tests coupled with Tukey and Holm-Sidak *post-hoc* tests. Data were considered significant when $p < 0.05$.

Results

The effect of aspartame on cell viability

The Colony Forming Units (CFU) method was used to assess cell viability and determine the mild inhibitory concentration of APM. *S. pombe* parental and *swi6*Δ cells were cultured in a range of 0.05 - 3 mg/mL APM concentrations for 24 h, then plated on YEA. Based on the results, the effect of 1 mg/mL APM concentration on cell viability was 84.81 and 83.66% for parental and *swi6*Δ cells, respectively, which is approximately 20% inhibitory concentration. On the other hand, statistical analysis showed that viability difference was significant between parental and *swi6*Δ, the latter growing less. At this point, 1 mg/mL APM concentration was selected to conduct the rest of the experiments as it was the 20% inhibitory concentration.

Table 1. Quantitative real time polymerase chain reaction primers (F = Forward, R = Reverse).

Gene	Primer	Primer sequence
swi6	*swi6F*	5′-TTTTTGGACTGGCTAAACCAT-3′
	swi6R	5′-GCCGAGAGGGGACTGATGTC-3′
hxk2	*hxk2F*	5′-CAACAAGGACTTTGCCCAAT-3′
	hxk2R	5′-AAGGTGTCGCTCTCCTTTGA-3′
fbp1	*fbp1F*	5′-GTATGGTGCTTCGGCTCATT-3′
	fbp1R	5′-TTCATGTTTCGATGGGTCAA-3′
tup11	*tup11F*	5′- AAATCCTCAATTGCCTGGTG -3′
	tup11R	5′- CCGAAGGAGTTGCTTTGGTA-3′
atf1	*atf1F*	5′-ACCCCTACTGGAGCTGGATT-3′
	atf1R	5′-ACCATCCCTTGGGGTAAAAC-3′
cox4	*cox4F*	5′-AAGAGCAGCGGTCAGGAGTA-3′
	cox4R	5′-GCGTTGGCTAATTCTTGAGC-3′
ctt1	*ctt1F*	5′-ATCCTCAATCCGACCACTTG-3′
	ctt1R	5′-AACGTCGGTAATTTCGTCCA-3′
sod1	*sod1F*	5′-ATTGGCCGTACCATTGTCAT-3′
	sod1R	5′-GACACCACAAGCGTTACGTG-3′
cyr1	*cyr1F*	5′-CCGCTATACTGCATGGGTTT-3′
	cyr1R	5′-TCGATACGCAAAGTCAGACG-3′
gpd3	*gpd3F*	5′-GGTGACAACCACTCCTCCAT-3′
	gpd3R	5′-TCAACAACACGGTGGGAGTA-3′

The growth rates

To trace the logarithmic phase of parental and *swi6*Δ growth in liquid culture, control and APM-treated cultures were monitored for 34 h, and the graph of OD_{600} absorbance values were plotted in a time dependent manner. Considering the absorbance values, it was detected that 1 mg/mL APM did not alter the growth rate dynamics of parental and *swi6*Δ cultures, but that *swi6*Δ culture had a slower growth rate than parental culture.

Glucose consumption

To detect whether there was a relationship between the effect of APM and Swi6 regarding glucose uptake, the parental and *swi6*Δ strains were cultured in YEL containing 1 mg/mL APM. Glucose consumption assay results showed that, with the same trend as in the growth curve, APM treatment did not affect the glucose uptake rate of either strain. Additionally, it was found that *swi6*Δ cells consumed 2.5 times less glucose than parental cells (Figure 1). This result coincides with the slow growth of *swi6*Δ cells. It was concluded that there was no relation between the effect of APM and Swi6 in terms of glucose consumption.

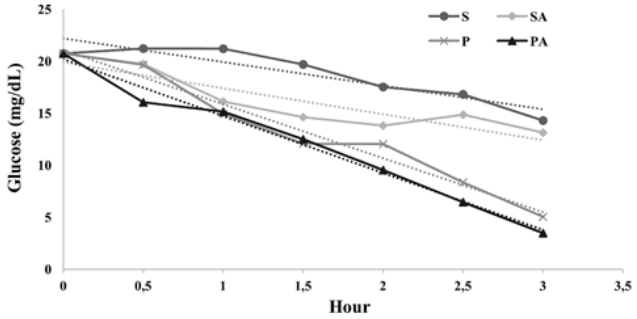

Figure 1. Glucose consumption rate of 1 mg/mL APM-treated *S. pombe* cells. Growth was maintained in an incubator with 180 rpm orbital shaking at 30°C. Glucose concentration of cultures sampled every 30 min from each group were measured in a spectrophotometer at 530 nm. P = Parental; PA = Parental+APM; S = *swi6*Δ; and SA = *swi6*Δ+APM.

Cell morphology and nuclear structure

On the question of whether APM affected cell morphology and nuclear structure through the action of Swi6, DAPI staining of the nucleus was performed, and the control and APM-treated cells were observed with both visible light and fluorescence filter. APM caused a significant increase in the cell size of parental cells, while no effect was observed in *swi6*Δ cell size (Figure 2). Additionally, *swi6*Δ cells were significantly longer than parental cells. Taken together, these data demonstrated that there was a significant relationship between APM and Swi6. According to fluorescent microscopy images, aspartame did not affect nuclear integrity in either strain (Figure 3).

Intracellular oxidation

To detect whether Swi6 was involved in the mechanism of reactive oxygen species (ROS) production when cells were treated with APM, the intracellular oxidation was measured with

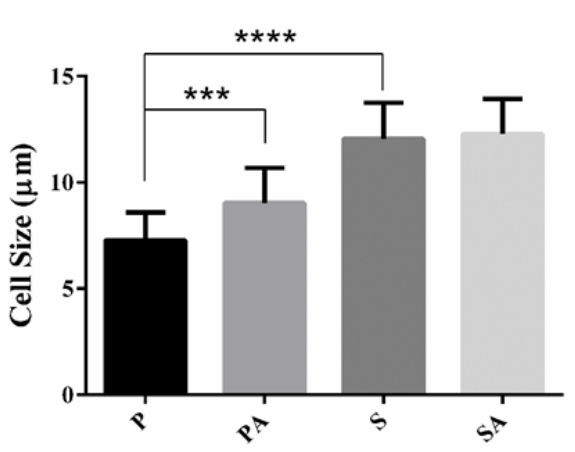

Figure 2. Cell size of 1 mg/mL APM-treated *S. pombe*. Cell size difference between control and treated groups were analysed with One-Way ANOVA and Tukey *post-hoc* test. Significance was found between P and S (****$p < 0.0001$), P and PA (***$p = 0.0002$). A two-way ANOVA test revealed a significant relationship between APM and Swi6 (*$p = 0.0151$). The experiment was repeated three times. Means and SD (error bars) are shown. P = Parental; PA = Parental+APM; S = *swi6*Δ; and SA = *swi6*Δ+APM.

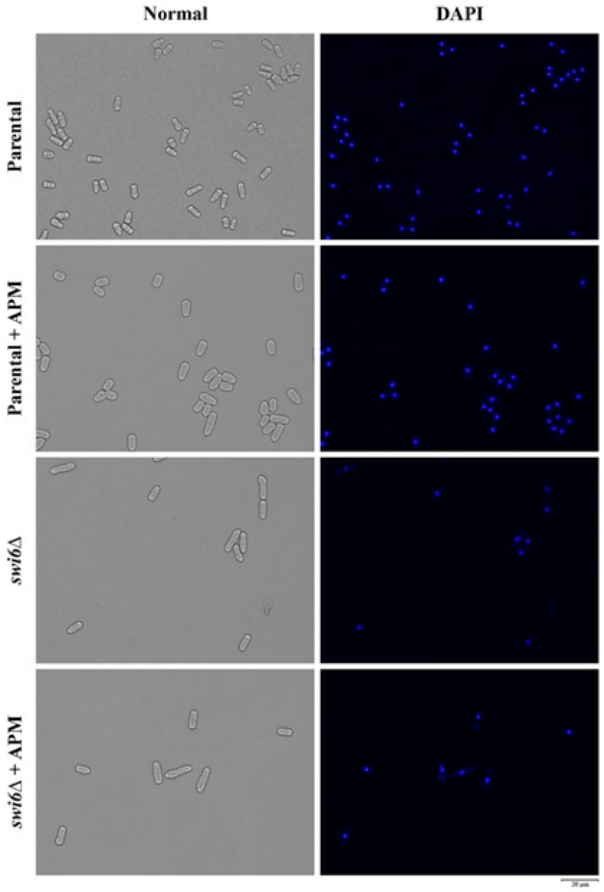

Figure 3. Fluorescence microscope images of cell morphology and nuclear structure of 1 mg/mL APM-treated *S. pombe*. After 24-h incubation, cells were fixed with ethanol and dyed with DAPI.

DCFH-DA in *S. pombe* parental and *swi6*Δ strains. The results in Figure 4 show that aspartame did not cause any significant ROS production in treated groups. However, *swi6*Δ cells did contain a higher amount of ROS than parental cells. Collectively, a significant correlation between APM and Swi6 in terms of ROS production was not detected.

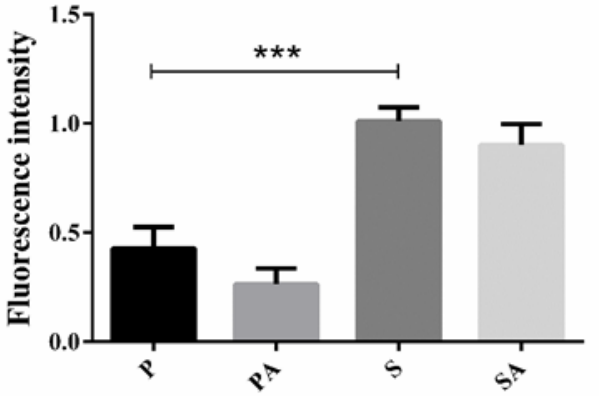

Figure 4. Fluorescence intensity of DCFH-DA indicating ROS in 1 mg/mL APM-treated *S. pombe*. ROS differences between control and treated groups were analysed with One-Way ANOVA and Tukey *post-hoc* test. Significance was found between P and S (***p = 0.0001). The experiment was repeated three times. Means and SD (error bars) are shown. P = Parental; PA = Parental+APM; S = *swi6*Δ; and SA = *swi6*Δ+APM.

Gene regulation in cellular processes

To evaluate the effect of APM and its relationship with Swi6 on chromatin structure, energy metabolism, and stress response, several genes that take part in these cellular processes were selected to examine the gene expression profiles using qRT-PCR. First, RNA isolation and cDNA synthesis were conducted on *S. pombe* parental and *swi6*Δ cells that were grown for 24 h in YEL with or without APM. Then, qRT-PCR was performed, and the changes in gene expression was calculated by comparing the C_t values of control and treated groups.

Based on the results (Figure 5), the expression of the *swi6* gene, which is responsible for silencing chromatin, was significantly downregulated in the parental strain under the condition of 1 mg/mL APM. For the expression of energy metabolism genes, respirative *fbp1* and *cox4* were upregulated, while fermentative *hxk2*, *tup11*, and *cyr1* were downregulated, all significantly. The stress response transcription factor, *atf1*, did not display a significant change in its expression but the ROS scavengers *sod1* and *ctt1* were significantly upregulated. In the *swi6*Δ strain, however, *sod1* and ctt1 were significantly downregulated.

Discussion

Aspartame is an artificial sweetener which has been tested in many model organisms, resulting in both positive and negative effects (Gebara *et al.,* 2003; Gombos *et al.,* 2007; Schernhammer *et al.,* 2012). Cancer progression is an epigenetic as well as genetic process due to the changes in expression it induces in various genes. This transcriptional control of cellular responses to environmental stresses at the epigenetic level is associated with the dynamic nature of chromatin. The proteins that provide for this structure may act as silencers by maintaining a compact inactive state (heterochromatin) or by loosening to permit gene expression (euchromatin). The downregulated state of chromatin regulator HP1 in the highly metastatic MDA-MB-231 breast carcinoma cell line and the discovery that its metastatic character declines after transforming the cells with an expression vector containing the HP1 gene, support the epigenetic importance of heterochromatin structure in cancer progression (Kirschmann *et al.,* 2000). Also, HP1 protein silences the expression of genes that are required for invasion and metastasis by forming heterochromatin, as another study suggests (Norwood *et al.,* 2004).

The *S. pombe* ortholog of HP1 and Swi6 regulates the formation of heterochromatin structures in the centromere, telomere, and mating-type loci (Cam *et al.,* 2005). The presence of Swi6 is especially important for the stabilisation of centromeric regions; it has been observed that chromosomal segregation anomalies arise in the absence of the gene (Ekwall *et al.,* 1995). Chromosome number differences (aneuploidy), which are the result of aberrant chromosome segregation, are also known to be found in neoplastic cells (Yona *et al.,* 2012). Swi6 plays a role in replication initiation by participating in the preRC complex with Cdc18 and the absence of Swi6 postpones replication (Li *et al.,* 2011). This delay is caused by S phase checkpoint proteins Rad3 and Cds1 that prevent the progression of the cell cycle before the completion of replication (Martinho *et al.,* 1998). One fission yeast study found that ROS production increases not only because there is a mutation in replication initiation genes, but also the application of hydroxyurea (HU) stalls replication forks (Burhans *et al.,* 2003). In the present work, despite not using HU, growing the adenine and uracil auxotrophic *swi6*Δ strain in unsupplemented rich media (YEL) may have caused replication stress and consequent ROS production, because growing the *swi6*Δ cells in supplemented rich media did not cause any proliferation problems.

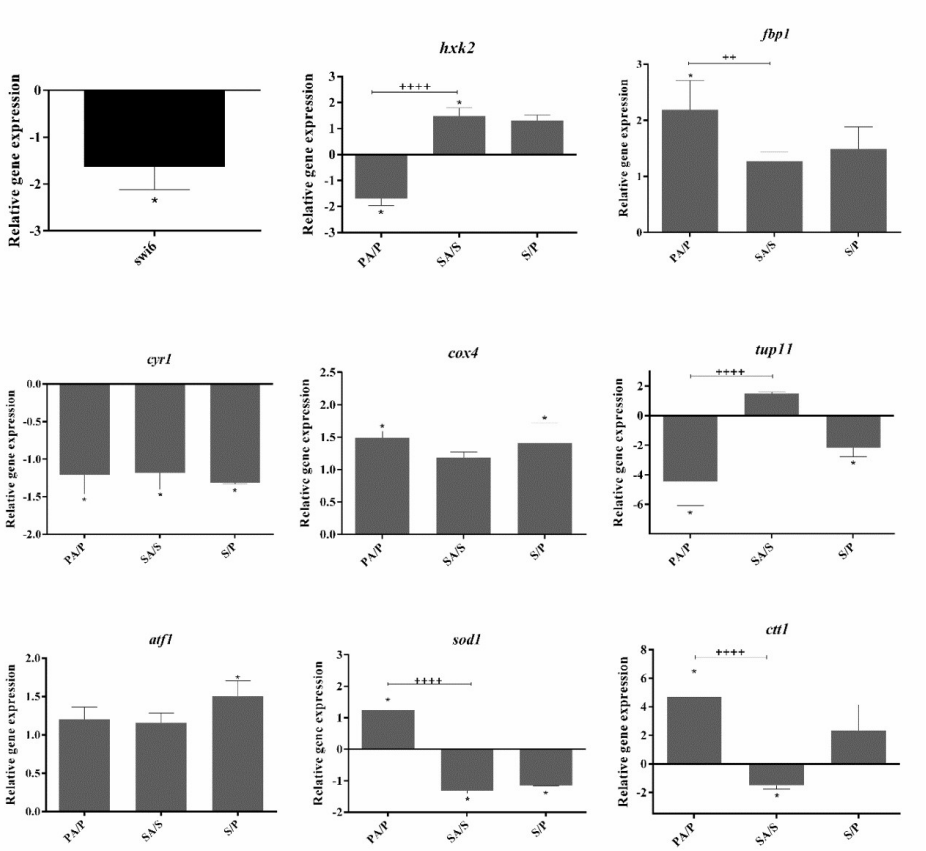

Figure 5. Gene expression analysis of APM-treated *S. pombe* parental and *swi6*Δ strains. Chromatin silencer *swi6* expression was significantly downregulated with a $*p < 0.05$ value in the parental strain. The significant relationship of APM and Swi6 in genes that take part in energy metabolism was $++p < 0.01$ for *fbp1,* $++++p < 0.0001$ for *hxk2* and *tup11*. The significant relationship of APM and Swi6 in genes that take part in stress response was $++++p < 0.0001$ for *sod1* and *ctt1*. Experiment was repeated three times. Means and SD (error bars) are shown. P = Parental; PA = Parental+APM; S = *swi6*Δ; and SA = *swi6*Δ+APM.

In the present work, it was found that treating the parental cells with the artificial sweetener aspartame, which has controversial carcinogenic effects, caused the downregulation of *swi6*. Based on qRT-PCR analysis, despite the glucose repressive conditions, cells upregulated the gluconeogenetic enzyme *fbp1* and downregulated the glycolytic enzyme *hxk2* (Walker, 1998). The downregulation of *tup11*, which represses *fbp1* expression, also supports this finding (Asada *et al.,* 2015). Under these conditions, a preference for respiration over fermentation is understood by both the upregulation of electron transport system protein *cox4* and the consequent ROS scavengers *sod1* and *ctt1* (Plakunov and Shelemekh, 2009). In addition, the gene that encodes the enzyme that catalyses intracellular signalling metabolite cAMP under high glucose concentrations, *cyr1*, is also downregulated (Hoffman and Winston, 1991). Thus, as explained by Neely and Hoffman (2000), the cells have activated the stress response pathway against the effects of APM and adapted successfully to the stress conditions at the transcriptional level. Unaffected cell viability, as was seen in growth rate and CFU results, also supports this idea. The fact that APM did not significantly affect glucose consumption suggests that, via the action of stress-induced mitochondrial respiration, the small amount of transported glucose is being used in energy metabolism with high efficiency, and the rest is utilised for catalysing various molecules to respond to stress (Weeks *et al.,* 2006).

Owing to replication stress, *swi6*Δ cells displayed a significant lethality when compared with parental cells in unsupplemented rich media. Their elongated cell size points to a delay in the cell cycle and its decreased growth, and glucose consumption rates support this phenotype (Auth *et al.,* 2016). Additionally, increased production of ROS (a marker for cell death) was detected in *swi6*Δ cells (Munoz *et al.,* 2012). The downregulation of *sod1* expression coincides with this result. Beyond this, APM treatment did not cause a meaningful change in transcriptional regulation in *swi6*Δ cells. These findings suggest that either the effect of APM was concealed due to cell death, or the effect was Swi6-dependent. In addition, the statistical analysis on cell size difference presented a relationship between APM and Swi6.

Conclusion

It is known that HP1 expression is downregulated in several types of cancer. Additionally, since APM is thought to have a carcinogenic effect, the present work investigated the relationship between APM and Swi6, which is the ortholog of HP1 in *S. pombe*. Taken together, it is obvious that the absence of Swi6 caused severe consequences for *S. pombe*. But the amount of Swi6 downregulation when parental cells were treated with 1 mg/mL APM was not sufficient to present any of the serious changes mentioned above for the *swi6*Δ strain. Thus, under the given conditions, it is suggested that APM may contribute to cancer progression but is not enough on its own to generate cancer. Lastly, due to the gene expression regulation implications, it is also proposed that APM may carry out its effects through Swi6 in energy metabolism and stress response pathways.

Acknowledgement

The present work was financially supported by Scientific Research Project Coordination Unit of Istanbul University (project number: FYL-2018-30854).

References

Asada, R., Takemata, N., Hoffman., C. S., Ohta, K. and Hirota, K. 2015. Antagonistic controls of chromatin and mRNA start site selection by Tup family corepressors and the CCAAT-binding factor. Molecular and Cellular Biology 35(5): 847-855.

Auth, T., Kunkel, E. and Grummt, F. 2016. Interaction between HP1α and replication proteins in mammalian cells. Experimental Cell Research 312(17): 3349-3359.

Burhans, W. C., Weinberger, M., Marchetti, M. A., Ramachandran, L., D'Urso, G. and Huberman, J. A. 2003. Apoptosis-like yeast cell death in response to DNA damage and replication defects. Mutation Research 532(1-2): 227-243.

Cam, H. P., Sugiyama, T., Chen, E. S., Chen, X., Fitzgerald, P. C. and Grewal, S. I. 2005. Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nature Genetics 37(8): 809-819.

Cheutin, T., Gorski, S. A., May, K. M., Singh, P. B. and Misteli, T. 2004. *In vivo* dynamics of Swi6 in yeast: evidence for a stochastic model of heterochromatin. Molecular and Cellular Biology 24(8): 3157-3167.

Dialynas, G. K., Vitalini, M. W. and Wallrath, L. L. 2008. Linking Heterochromatin Protein 1 (HP1) to cancer progression. Mutation Research 647(1-2): 13-20.

Ekwall, K., Javerzat, J. P., Lorentz, A., Schmidt, H., Cranston, G. and Allshire, R. 1995. The chromodomain protein SWi6: a key component at fission yeast centromeres. Science 269(5229): 1429-1431.

Gebara, J. S., Querol, C. B., Gebara, M. and Castro-Prado, M. A. A. 2003. Mitotic segregation induced by edulcorant l-aspartyl-l-phenylalanine-methyl-ester (aspartame) in diploid cells of *Aspergillus nidulans*. Acta Scientiarum: Biological Sciences 25(1): 203-206.

Gombos, K., Varjas, T., Orsós, Z., Polyák, E., Peredi, J., Varga, Z., … and Ember, I. 2007. The effect of aspartame administration on oncogene and suppressor gene expressions. In Vivo 21(1): 89-92.

Grewal, S. I. 2000. Transcriptional silencing in fission yeast. Journal of Cellular Physiology 184(3): 311-318.

Hoffman, C. S. and Winston, F. 1991. Glucose repression of transcription of the *Schizosaccharomyces pombe fbp1* gene occurs by a cAMP signaling pathway. Genes and Development 5(4): 561-571.

Horio, Y., Sun, Y., Liu, C., Saito, T. and Kurasaki, M. 2014. Aspartame-induced apoptosis in PC12 cells. Environmental Toxicology and Pharmacology 37(1): 158-165.

Huang, Y. 2002. Transcriptional silencing in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Nucleic Acids Research 30(7): 1465-1482.

Huff, J. 1999. Long-term chemical carcinogenesis bioassays predict human cancer hazards. Issues, controversies, and uncertainties. Annals of the New York Academy of Sciences 895: 56-79.

Huff, J. and LaDou, J. 2007. Aspartame bioassay findings portend human cancer hazards. International Journal of Occupational and Environmental Health 13(4): 446-448.

Ishii, H., Koshimizu, T., Usami, S. and Fujimoto, T. 1981. Toxicity of aspartame and its diketopiperazine for Wistar rats by dietary administration for 104 weeks. Toxicology 21(2): 91-94.

Kirkland, D. and Gatehouse, D. 2015. Aspartame: a review of genotoxicity data. Food and Chemical Toxicology 84: 161-168.

Kirschmann, D. A., Lininger, R. A., Gardner, L. M., Seftor, E. A., Odero, V. A., Ainsztein, A. M., ... and Hendrix, M. J. 2000. Down-regulation of

HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer. Cancer Research 60(13): 3359-3363.

Li, P. C., Chretien, L., Côté, J., Kelly, T. J. and Forsburg, S. L. 2011. *S. pombe* replication protein Cdc18 (Cdc6) interacts with Swi6 (HP1) heterochromatin protein. Cell Cycle 10(2): 323-336.

Lok, K. Y. W., Chung, W.-Y., Benzie, I. F. and Woo, J. 2010. Colour additives in snack foods consumed by primary school children in Hong Kong. Food Additives and Contaminants Part B - Surveillance 3(3): 148-155.

Magnuson, B. A., Burdock, G. A., Doull, J., Kroes, R. M., Marsh, G. M., Pariza, M. W., … and Williams, G. M. 2007. Aspartame: a safety evaluation based on current use levels, regulations, and toxicological and epidemiological studies. Critical Reviews in Toxicology 37(8): 629-727.

Mallikarjun, S. and Sieburth, R. M. 2015. Aspartame and risk of cancer: a meta-analytic review. Archives of Environmental and Occupational Health 70(3): 133-141.

Maloney, A., Clarke, P. A., Naaby-Hansen, S., Stein, R., Koopman, J. O., Akpan, A., … and Workmann, P. 2007. Gene and protein expression profiling of human ovarian cancer cells treated with the heat shock protein 90 inhibitor 17-allylamino-17-demetoxygeldanamycin. Cancer Research 67(7): 3239-3253.

Martinho, R. G., Lindsay, H. D., Flaggs, G., DeMaggio, A. J., Hoekstra, M. F., Carr, A. M. and Bentley, N. J. 1998. Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses. The EMBO Journal 17(24): 7239-7249.

Munoz, A. J., Wanichthanarak, K., Meza, E. and Petranovic, D. 2012. Systems biology of yeast cell death. FEMS Yeast Research 12(2): 249-265.

Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D. and Grewal, S. I. 2001. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science 292(5514): 110-113.

Neely, L. A. and Hoffman, C. S. 2000. Protein kinase A and mitogen-activated protein kinase pathways antagonistically regulate fission yeast *fbp1* transcription by employing different modes of action at two upstream activation sites. Molecular and Cellular Biology 20(17): 6426-6434.

Nonaka, N., Kitajima, T., Yokobayashi, S., Xiao, G., Yamamoto, M., Grewal, S. I. and Watanabe, Y. 2002. Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. Nature Cell Biology 4(1): 89-93.

Norwood, L. E., Grade, S. K., Cryderman, D. E., Hines, K. A., Furiasse, N., Toro, R., … and Wallrath, L. L. 2004. Conserved properties of $HP1^{Hs\alpha}$. Gene 336(1): 37-46.

Plakunov, V. K. and Shelemekh, O. V. 2009. Mechanisms of oxygen regulation in microorganisms. Microbiology 78(5): 535-546.

Schernhammer, E. S., Bertrand, K. A., Birmann, B. M., Sampson, L., Willett, W. C. and Feskanich, D. 2012. Consumption of artificial sweetener and sugar-containing soda and risk of lymphoma and leukemia in men and women. The American Journal of Clinical Nutrition 96(6): 1419-1428.

Soffritti, M., Belpoggi, F., Degli Esposti, D., Lambertini, L., Tibaldi, E. and Rigano, A. 2006. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. Environmental Health Perspectives 114(3): 379-385.

Talbot, J. M., and Fisher, K. D. 1978. The need for special foods and sugar substitutes by individuals with diabetes mellitus. Diabetes Care 1(4): 231-240.

Trojer, P. and Reinberg, D. 2007 Facultative heterochromatin: is there a distinctive molecular signature? Molecular Cell 28(1): 1-13.

Walker, G. M. 1998. Yeast physiology and biotechnology. 1st ed. United States: John Wiley and Sons.

Wang, G., Ma, A., Chow, C. M., Horsley, D., Brown, N. R., Cowell, I. G. and Singh, P. B. 2000. Conservation of heterochromatin protein 1 function. Molecular and Cellular Biology 20(18): 6970-6983.

Wang, J., Jia, S. T. and Jia, S. 2016. New insights into the regulation of heterochromatin. Trends in Genetics 32(5): 284-294.

Weeks, M. E., Sinclair, J., Butt, A., Chung, Y. L., Worthington, J. L., Wilkinson, C. R., … and Timms, J. F. 2006. A parallel proteomic and metabolomic analysis of the hydrogen peroxide- and Sty1p-dependent stress response in *Schizosaccharomyces pombe*. Proteomics 6(9): 2772-2796.

Whitehouse, C. R., Boullata, J. and McCauley, L. A. 2008. The potential toxicity of artificial sweeteners. American Association of Occupational Health Nurses Journal 56(6): 251-259.

Yona, A. H., Manor, Y. S., Herbst, R. H., Romano, G. H., Mitchell, A., Kupiec, M., … and Dahan, O. 2012. Chromosomal duplication is a transient evolutionary solution to stress. Proceedings of the National Academy of Sciences of the United States of America 109(51): 21010-21015.