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Research Article

Inhibitory Effect of Hydroxymethylfurfural in Viability of BALB/C Mice Splenocytes

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ABSTRACT

Background: This study was designed to discover if hydroxymethylfurfural (HMF) exposure modifies cell proliferation and DNA damage in BALB/c mice splenocytes.
Methods: Mitogenesis in T cells and B cells was induced by Concanavalin A (Con A) and lipopolysaccharide (LPS). The colorimetric tetrazolium assay was used to evaluate cell proliferation. DNA damaging consequences were evaluated via measurement of 8-hydroxy-2-deoxyguanosine (8-OHdG) level in BALB/c mice splenocytes.
Results: Spleen cells proliferation elicited by ConA, was dramatically suppressed by 25, 50 and 100 mM of HMF. However, there was not any significant difference between various concentrations of HMF. The same result was observed following treatment with LPS and HMF in different concentrations. Eight-OHdG concentration was elevated significantly in HMF treated groups compared with untreated control and mitogens.
Conclusion: HMF was found to have immunosuppressing and DNA damaging properties in mM concentrations in mice splenocytes.

Introduction

Malignant diseases are considered as a leading cause of mortality.[1] Cells of the human body are continuously exposed to oxidative stress. Eight-hydroxy-2-deoxyguanosine (8-OHdG), a nucleoside form of 8-oxoGua, is a substantial biomarker of oxidation associated with DNA damage products. Carcinogenesis has been correlated with 8-OHdG formation based on reports of various studies.[2]

Hydroxymethyl furfural (HMF), a furan derivative,[3] is formed under high temperature food environments by the degradation of carbohydrates through Maillard reaction or caramelization.[4] The concentration of HMF in some heat-treated foods including bread,[5] dried fruits,[6] honey,[7] breakfast cereals,[8] coffee and instant coffee[9] is high. Depend on the country and its culture, consumption of these food items and therefore exposure to HMF is different. For instance, consumption of bread, which is baked traditionally under direct heating, and subsequently HMF exposure, is high in Iran.[5] Hence, in vitro and in vivo studies focusing on toxicity and metabolism of HMF is necessarily recommended. Some studies have reported it's cytotoxic, mutagenic and DNA damaging effects;[10-13] Evaluation of DNA damage in various cell lines with different sulfotransferase (SULT) activity showed that 100 mM HMF led to a significant DNA damage after 3 hours incubation independent of SULT activity.[14] Sulfoxymethylfurfural (SMF) is a bioactive compound which is found in blood of mice through sulfonation of hydroxymethyl functional group of HMF by SULT. Treatment of multiple intestinal neoplasia (Min/+) mice with HMF or SMF influenced the number of small intestinal adenoma and flat aberrant crypt foci (ACF) in large intestine without having any effect on the size of the adenomas.[15] However, there was no evidence of ACF induction by HMF or its metabolite SMF in extensive studies in mice.[13]

Intraperitoneal treatment of FVB/N mice with SMF caused renal tubules damage, serositis of peritoneal tissues and hepatoxic effects. Evaluating 40 tissues of rats and mice after treating with HMF in water for about 2 years revealed a clear increase of hepatomas at dosage of 188 and 375 mg/kg body mass/5 times a week, in female B6C3F, mice but not in Fischer 344N rats of both sexes and in male B6C3F1 mice.[16] However, a 2 year animal study declared that different doses of HMF had no neoplastic effect,[17] Interestingly, in recent studies this compound has been characterized to exert antioxidant properties with promising potential in cancer chemoprevention,[18] decrease of oxidative stress elicited by sugar,[19] anti-hypoxia[20] and anti- allergic effects.[21]

Until now, there is neither no data showing correlation between exposure to HMF and risk of cancer, nor experimental evidence available regarding HMF-mediated cell proliferation and DNA damage in model cells. In this study, we decided to examine the effect of HMF in different concentrations on cell proliferation and DNA damaging consequences via measurement of 8-

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OHdG level as in BALB/c mice splenocytes.

Methods and Materials

Preparation of spleen cell suspension

The study was approved by the local ethic committee (Ethic committee approval number. Ir.tbzmed.rec.1393.226). BALB/c mice at 6 weeks of age, weighing 15-20 g were anesthetized using nembutal and blood was collected from inferior vena cava. Then, mice were killed and spleen cell suspensions were obtained following the procedure described by Alizadeh, et al. with some modifications.[22] Briefly, spleens were removed and smashed by a syringe and passed through a mesh screen in RPMI-1640 (Sigma, USA)/Antibiotic-Antimycotic medium (GIBCO BRL Life., USA). They were then washed by centrifugation at 4°C. Elimination of red blood cells in the cell suspensions was carried out using lysis buffer (5 ml of 0.83% NH4Cl in Tris-HCl (pH 7.4)) at 37°C for 5 minutes and 3 subsequent washing with medium was done. All the procedures were followed meticulously to observe aseptic condition.

Cell proliferation and cell viability assays

Viability of the cells was evaluated via mixing 100 µl of the resultant solution with equal volume of a solution of 0.4% trypan blue. Finally, a suspension of spleen cells (5 × 10^6 cells/well) in a 96-well plate was prepared in RPMI-1640/antibiotic-antimycotic medium and 10% fetal bovine serum. Two µg/dl of concanavalin A (Con A) as T cell mitogen or 10 µg/dl of lipopolysaccharide (LPS) as B cell mitogen was used under air containing 5% carbon dioxide. Cell growth in absence and presence of HMF (0, 25, 50 and 100 mM) for 72 h was determined by the tetrazolium MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. These concentrations were used based on Durling et al., study.[14] MTT incorporated wells were kept in 95/5 ratio of CO2 to air environment at 37ºC for 3 hours. Then, the media were removed and 200 µL of DMSO were added to each well to dissolve the formazan product by vigorous mixing. Finally, the absorbance was determined at 570 nm. All of the treatments were in triplicate. Treatments are shown in Table 1.

DNA damage evaluation

Extracted mice splenocytes were cultured in 96-well culture plates. After the cells were doubled, one ml of fresh medium and HMF in different concentrations (0, 25, 50 and 100 mM) were added to the culture wells. Absolute medium was served as control.[14] The treatments were similar to the above-mentioned ones. Also, there were three additional treatments with HMF in various concentrations without adding Con A/LPS. After 72 h, the supernatants of cultures were collected to measure 8-OHdG concentration as oxidative DNA damage indicator via enzyme-linked immunosorbent assay (ELISA) analysis (Glory science, China) following the instructions made by manufacturers. Briefly, cells were transferred into two ml microtubes and centrifuged for 20 minutes at 2000-3000 g. The supernatants were removed and the cells were diluted with PBS to 1*10^6 cells after counting. Freeze-defreeze cycle was repeated four times to help release of 8-OHdG. Finally, cells were centrifuged for 20 minutes at 2000-3000 g and clear supernatants were collected to measure 8-OHdG level by using the kit.

Statistical analysis

The normality of distribution of data was tested by the Kolmogorov-Smirnov test. The experiments were performed in triplicates and the results were presented as mean ± standard deviation. One-way analysis of variance (ANOVA), together with Tukey model was employed to assess differences among groups. P value less than 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS software version 20.0 (SPSS Inc, Chicago, IL, USA).

Table 1. Different kinds of treatments.

Treatment	Cells	Medium	Con A[a]	LPS[b]	HMF[c]
1	+	+	-	-	-
2	+	+	2 µg/dl	-	-
3	+	+	-	10 µg/dl	-
4	+	+	2 µg/dl	-	25 mM
5	+	+	-	10 µg/dl	25 mM
6	+	+	2 µg/dl	-	50 mM
7	+	+	-	10 µg/dl	50 mM
8	+	+	2 µg/dl	-	100mM
9	+	+	-	10 µg/dl	100mM

[a]Concanavalin A
[b]Lipopolysaccharide
[c]Hydroxymethylfurfural

Results

Lymphocytes are considered as an essential component of mammalian immune system cells and our study showed that the proliferation of different subpopulations of lymphocytes were stopped by HMF at varying levels. As shown in Figure 1, lymphocyte viability was significantly suppressed by HMF at the concentrations of 25, 50 and 100 mM compared to LPS ($p < 0.05$). However, there was not statistically significant difference between groups treated by HMF in different levels. Cytotoxicity effect of HMF on mice splenocytes in comparison to Con A is presented in Figure 2. There was a significant reduction in cell viability after treatment with HMF at the concentrations of 25, 50 and 100 mM compared to Con A ($p < 0.05$).

Eight-OHdG concentrations following treatment with HMF are displayed in Figure 3. There was a statistically significant difference between groups ($p < 0.05$). DNA damage percent was significantly higher in HMF treated groups, also Con A/ LPS + HMF treated groups than control group; However, it was not significantly different in cells treated with LPS or Con A compared with control (cells + medium). The highest 8- OHdG level was observed in cells treated with LPS + 100 mM HMF, followed by Con A + 100 mM HMF and the lowest concentration of 8- OHdG was in LPS treated cells, followed by Con A compared to control group.

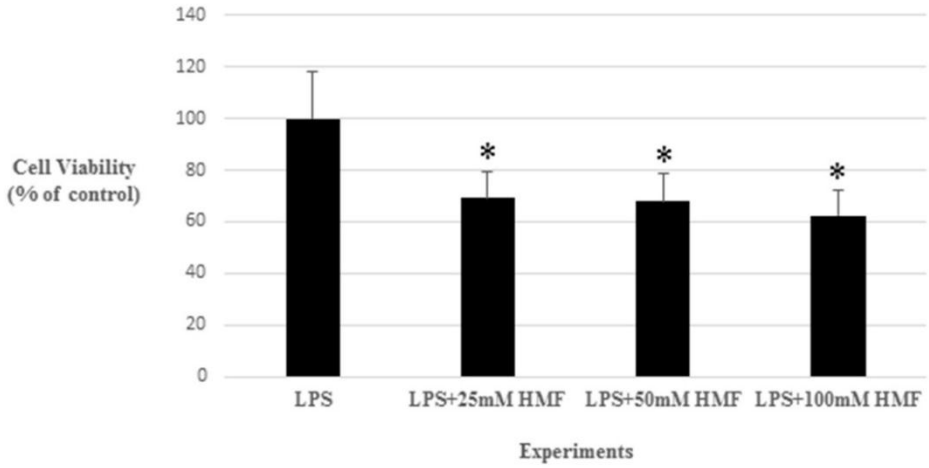

Figure 1. Cytotoxicity effect of HMF on mice splenocytes by MTT assay. The calculation of cell viability percent was based on the untreated control. * Significantly different from the LPS control (*: $p < 0.05$, ANOVA-test).

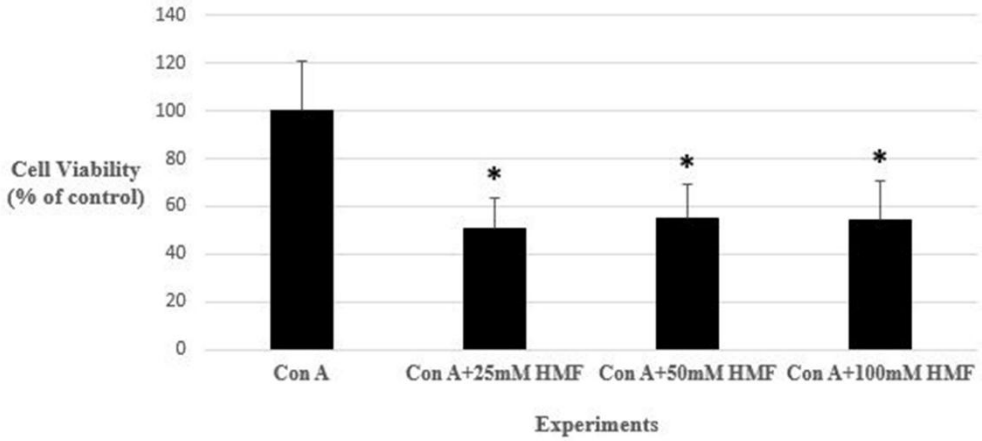

Figure 2. Cytotoxicity effect of HMF on mice splenocytes by MTT assay. The calculation of cell viability percent was based on the untreated control. * Significantly different from the Con A control (*: $p < 0.05$, ANOVA-test).

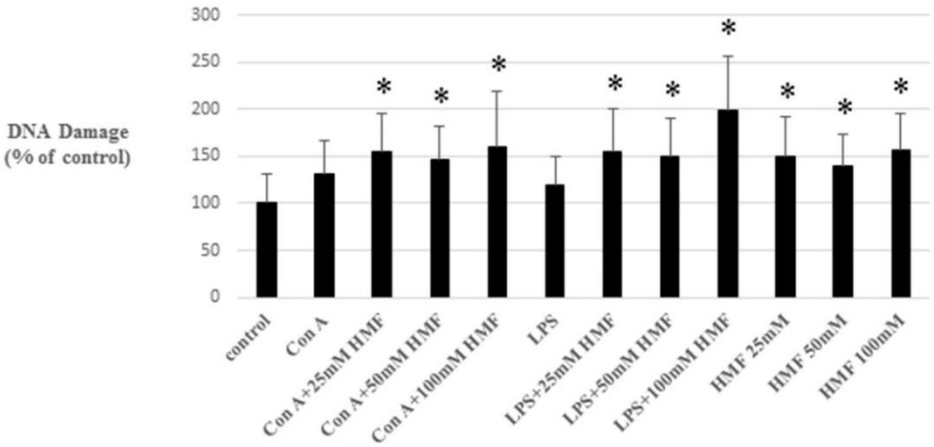

Figure 3. DNA damaging effect of HMF on mice splenocytes via measurement of 8-OHdG concentration. The calculation of percent DNA damage was based on the untreated control. * Significantly different from the control (*: $p < 0.05$, ANOVA-test).

Discussion

In this study, harmful actions of HMF on mice splenocytes were evaluated in vitro. It has been established in clinical trials that defects in immunoregulatory cells might produce disease. Thus, inbalance in immunoregulatory action as well as effector cells sensitivity or insensitivity to control mechanisms are the main causes of disease development in immune system disfunction. Based on data, some malignances are developed due to increased cell proliferation via an external substance. However, it is not general and high doses of some chemicals may decrease cell viability and

result in cell death.[23] In this study, MTT assay was applied to determine HMF effect on cell proliferation and mitogenicity and to explore whether mitogenecity is correlated with genotoxicity. To our knowledge, toxic effect of HMF has not been explored in mice splenocytes so far. In addition, DNA damaging effect of this compound has been evaluated in different cell lines via methods other than measurement of 8-OHDG. Lymphocytes proliferation is an indicator of immunopotentiation.[24] Proliferation of spleen lymphocyte was induced by ConA in vitro to evaluate T lymphocyte activity, while B lymphocyte activity was examined after induction by LPS.[25] In vitro lymphocyte proliferation assay, HMF had directly antimitogenic effect on stimulated splenocytes via conA and LPS. These data indicated HMF was a potent immunosuppressing substance. Moreover, it showed that HMF in different concentrations induced a significant oxidative DNA damage to splenocytes in presence or absence of mitogens.

Several studies have examined the effect of HMF in various cell lines. LO_2 cell viability was increased following treatment with HMF. Therefore, it could inhibit induction of hepatocyte-originated LO_2 cell line injury by H_2O_2.[26,27] Hypoxic injury in ECV304 cells was attenuated with HMF.[20] Similarly, ameliorative effects of HMF was observed on alcoholic liver oxidative injury in mice.[28] HMF had a protective effect in HUVEC treated with high glucose. Since, it increased the viability of injured cells with glucose. Moreover, it inhibited expression of JNK1 and JNK2/3 and high activity of IL-8 induced by glucose, a downstream activator of P-Akt.[19] In a dose dependent manner, HMF at the concentrations lower than 10.0 µg/mL did not show any cytotoxicity, although KU812 cells viability was reduced remarkably at the dose of 10.0 µg/mL.[21] Similar to this study, Li et al showed that HMF at different concentrations had no cytotoxic effects on RAW 264.7, HL-60, and MRC-5 cells, while the highest concentration (100 µM) could influence cell viability.[18] In the present study, the decreased cell viability in HMF treated cells could be due to its extremely high concentrations compared to previous studies.

Production of 8-OHdG, one of the main oxidative stress biomarkers, was statistically significant between groups in the present study; although the involved mechanism was not evaluated. Overall antioxidant and antiproliferative activities against 2 type colon cancer cells were not altered after treatment with increasing concentrations of HMF (from 1 to 13 µg/mL) and olive oil, a source of HMF. A higher concentration of HMF (300 µg/mL), 300 times greater than the amount found in olive leaves, inhibited proliferation in both of the cell lines.[29] Evaluation of antiproliferative effect of HMF in different cell lines indicated that proliferation of human melanoma A375 cells was influenced more than other cell lines after treatment with HMF by activation of apoptosis in A375 cell and G_0/G_1 cell cycle arrest.[30]

Generally, different cells show contradictory response to cytotoxic agents.[14] Humans are more sensitive to toxic effects of HMF than rats, when exposed orally.[31] However, dried fruits with different HMF levels showed a negative association with changes of 8-OHdG in a sample of healthy volunteers.[32] Spleen cells of mice immunized with OVA following exposure to HMF orally, led to inhibition of IL-4 production without influencing IFN-γ, which could be considered as a promising complementary substance in preventing (Th2) cytokine-dominant disease.[33]

Based on the controversial results in previous studies on the biological effects of HMF, the cytoprotective effects of the compound are not established. HMF exerts its toxic effect to live cells by causing damage to proteins, nucleic acids and cell organs as reactive oxygen species.[34] The HMF induced DNA damage in different cell lines with different SULT activities.[14] It was considered a weak genotoxic in Hep-G2 cell line.[11] A significant incidence of chromosome aberrations and a significant lowering of mitotic activity was observed after exposure to furfural and HMF in cultured Chinese hamster V79 cells.[35] Similar results were observed with animal studies. HMF can initiate and promote the growth of ACF in rat colons in a dose-dependent manner.[12] Similarly, growth of ACF in rats after administration of caramelized sugar was higher than the control group.[36] The Min/+mice treated with HMF or SMF did not have any effect on the adenomas size, although HMF increased the number of small intestinal adenomas and SMF increased the flat ACF number in the large intestine.[15] High doses of HMF induced nephrotoxic and hepatotoxic effects in wild-type mice and transgenic mice expressing human SULT.[16] Conversely, any adverse effects were revealed following treatment by HMF (400 mg, injected subcutaneously, twice per day for one week) on several parameters including weight, hemoglobin, leucocytes, platelets, serum- protein, serum-alanine-aminotransferase, alkaline phosphatase, liver cell necrosis and hepatic steatosis as compared with the control group.[37] The different degree of receptors expression, metabolism, chemical structure, and enzyme activity of the HMF might be responsible for the variation in the susceptibility of cells to the compound.

It is not clear whether HMF acts as a hazardous substance for human health. Based on estimations, dietary intake of HMF (1.6 mg/person/day) is above the threshold of concern (540 µg/person/day).[38] Exposure to HMF is inevitable for humans. Because this chemical compound has been known one of the prominent heat derived contaminants. Its concentration in foods exceeds 1g/kg in caramels and dried fruits.[39] On the other hand, cellulose and hemicelluloses are known as main precursors of HMF which serves as a sustainable feedstock for medicines, liquid fuels and chemicals, such as the precursors of transportation fuels, liquid alkanes, the building-block of polyester (2, 5-furandicarboxylic acid), and anti-sickling agent.[40] Considering toxic effect of HMF, several strategies have been applied to mitigate HMF content of foods some of which are recipe based such as replacing reducing sugars (glucose and fructose) with non-reducing

sugars (sucrose).[38] Consumption of protein with HMF-rich foods could act as a detoxifier through forming an adduct between free amino acids upon digestion and HMF.[40]

Conclusion

In conclusion, HMF was found to have immunosuppressing properties in mice splenocytes. We also discovered that HMF induces DNA damage in mM concentrations. HMF is found in high levels in heat-treated foods. Considering a high bioavailability of HMF via food intake, it is speculated that the compound might have a substantial effect in induction of oxidative DNA damage and possibly further mutagenic or carcinogenic effects. This warrants future in depth studies addressing undetecting mechanism. As well, further studies are needed to understand the mechanism involved in the immunosuppressing effect of HMF.

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Conflict of interests

The authors claim that there is no conflict of interest.

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