Research

Effects of 5-Hydroxymethylfurfural on Pubertal Development of Female Wistar Rats

Short Title: 5-hydroxymethylfurfural and Puberty

Selin Elmaogullari1 ORCID 0000-0003-4879-7859, Elcin Kadan2 ORCID 0000-0002-8006-0972, Elvan Anadol3 ORCID 0000-0002-6029-7358, Ayris Gokceoglu4 ORCID 0000-0003-4050-429X, Seyit Ahmet Ucakturk5 ORCID 0000-0001-8666-4454, Zehra Aycan1 ORCID 0000-0003-4584-2976

1Dr. Sami Ulus Children Training and Research Hospital, Department of Pediatric Endocrinology, Ankara, Turkey
2Gulhane Training and Research Hospital, Department of Pathology, Ankara, Turkey
3Gazi University, Laboratory Animal Breeding and Experimental Researches Center, Ankara, Turkey
4Ondokuz Mayis University, Faculty of Veterinary Medicine, Department of Biochemistry, Samsun, Turkey
5Ankara Children Hematology and Oncology Training and Research Hospital, Department of Pediatric Endocrinology, Ankara, Turkey

What is already known on this topic?
5-Hydroxymethylfurfural (HMF) is an organic compound that is present at high amounts in processed foods and food stuffs as a result of heating, roasting, frying and toasting. There is conflicting data on potential genotoxic, mutagenic, carcinogenic, DNA-damaging, organotoxic and enzyme inhibitory effects of HMF and its metabolites. To the best of our knowledge there is not any published data about effects of HMF on pubertal development.

What this study adds?
This is the first study about effects of HMF on pubertal development and the results of the study indicate that peripubertal exposure to HMF in high doses result in precocious puberty and decreased AMH levels in female Wistar rats.

Abstract

Background: 5-Hydroxymethylfurfural (HMF) is formed when sugars are heated in the presence of amino acids. HMF is naturally present in many foods.

Objective: We aimed to investigate the toxic effects of HMF on reproductive system in peripubertal rats.

Method: In the study, 24 immature female Wistar rat were divided into control (CT) and HMF groups fed with 750 mg/kg/day and 1500 mg/kg/day for 3 weeks from postnatal day 21. The vaginal opening (VO) was monitored daily and euthanasia occurred on postnatal day 44. Gonadotropins, estradiol (E2), progesterone and anti-Mullerian hormone (AMH) levels were measured. Reproductive organ weight and ovarian follicle counts were compared.

Results: High dose (HD) HMF group had earlier VO with higher luteinising hormone (LH) (2.9 ± 1.2 mIU/ml in HD and 1.3 ± 0.3 mIU/ml in CT group) and E2 (34.7 ± 8.8 pg/ml in HD and 21.2 ± 3.9 pg/ml in CT group) levels. HD group also had increased number of secondary atrophic follicles and decreased AMH (2.7 ± 0.5 ng/ml in HD and 4.7 ± 0.7 in CT group) levels.

Conclusion: These results indicate that peripubertal exposure to HMF in high doses result in precocious puberty and decreased AMH levels in female Wistar rats.

Key Words: Hydroxymethylfurfural; puberty; vaginal opening; anti-Mullerian hormone; rat

Corresponding Author
Selin Elmaogullari, MD
Dr. Sami Ulus Children Training and Research Hospital, Department of Pediatric Endocrinology, Ankara, Turkey
Caddesi No:44 (06080) Altındag, Ankara, Turkey
Email: ekerbicercelin@yahoo.com
Tel: +905325808862, +903123056509
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1. Introduction

5-Hydroxymethylfurfural (HMF) is an organic compound produced by dehydration of fructose and glucose, through a nonenzymatic chemical reaction in the presence of amino acids(1). HMF presence reduces protein digestibility, decreases the nutrition quality of foods and its concentration is widely used as a parameter to assess honey freshness and appropriate storage conditions(2). It is also ubiquitous in human diet and is present at high amounts in processed foods and food stuffs as a result of heating, roasting, frying and toasting(3). HMF concentration is greater than 1 g/kg in dried fruits, caramel products and some fruit juices and up to 6.2 g/kg in instant coffee(4). It is also determined in cigarette smoke, beer and medical products like parenteral solutions containing glucose and pharmaceutical syrups containing fructose(5-8). Additionally HMF is used industrially in production of polymers, surfactants, solvents, pharmaceuticals, and plant protection agents(9).

Daily consumption of HMF from diet is estimated to be between 30-150 mg and safe levels of HMF consumption is not well clarified(7,10). The effect of HMF on human health has long been the subject of research however yet it is not clear if HMF represents a potential health risk for humans by dietary exposure. There are conflicting data on potential genotoxic, mutagenic, carcinogenic, DNA-damaging, organotoxic and enzyme inhibitory effects of HMF and its metabolites(11-14). As a carcinogenic effect, HMF derivatives were found to cause hepatocarcinoma and increase skin tumor initiating activity in mouses(15). Zhang et al. also showed that orally administered HMF in thermolyzed sucrose initiates intestinal aberrant crypt foci formation and causes an increase in both number and size of them in a dose dependent manner in rats(16). However, another study in mice remarked no evidence of intestinal aberrant cript formation with HMF or its deriavate(17). US National Toxicology Program (NTP) on toxicology and carcinogenesis of HMF on rats and mice, the most comprehensive study on toxic effects of HMF to date, revealed increased incidences of lesions of the olfactory and respiratory epithelium of the nose in rat and mice, and increased incidence of liver cancer in female mice after two years administration of oral HMF(8).

Exposure of children to HMF has increased with changing eating habits in last decades. Any data about toxic effects of HMF on pubertal development has not been reported so far. So, the aim of this study was to evaluate whether peripubertal exposure to high levels of HMF has effects on pubertal timing, reproductive organ growth, hormone levels and ovarian follicle development.

2. Materials and Methods

This study was conducted in Gazi University Laboratory Animal Breeding and Experimental Researches Center (GÜDAM) and approved by Gazi University local ethics committee for animal experiments (approval code:17.025).

2.1. Animals and experimental design

Twenty four Wistar albino rats, weaned on postnatal day (PND) 21, were divided into 3 groups (n = 8 / group). Control group (CT) was given 5 ml/kg/day of tap water, low dosage group (LD) was given 750 mg/kg/day and high dosage group (HD) was given 1500 mg/kg/day of HMF (Sigma W501808-25G-K)(4). The treatments were performed orally (gavage), once daily (6 days/week), at the same hour (between 9:00 and 10:00 AM), until PND 44. The groups were kept in different cages under same conditions (22-24°C, 25-30% humidity, 12 hour light-dark cycle with free access to water and food). Each rat was weighed on PND 21, 26, 33, 40 and 44 just prior to feeding.

2.2. Analysis of Vaginal Opening

They were examined for vaginal opening (VO) for the assesment of sexual maturity every morning (between 9:00 and 10:00 AM). The procedure was performed visually without using a surgical loupe. VO was scaled as: those never had VO (0 points), VO between PND 39-44 (1 point) and VO between PND 33-38 (2 points), so that puberty times could be compared including the rats those did not have VO on termination day. The scale steps were set by dividing the time period (PND 33-44) that rats had VO into two.

2.3. Euthanasia

The animals were anesthetized by intramuscular xylazine and ketamine (5 and 45 mg/kg, respectively) and then euthanized by cardiac puncture on PND 44, 24 hours after the last dosage of HMF. Blood samples were collected with cardiac puncture on termination day. After centrifugation, serum samples were stored at -80°C until the study for follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (P) and anti-Müllerian hormone (AMH).

2.4. Measurement of uterus length, organ weight and assessment of follicular score

After euthanasia, uterus and ovaries were dissected with a limited gross necropsy focused on reproductive organs. Ovaries and uterus were weighed to the nearest 0.001 g with an electric scale (Sartorius Research R200D Electronic Semi-Microbalance). Organ weight per 100 mg of final body weight were presented as relative organ weight. In macroscopic analysis, cervix lengths and both of the uterine corns were measured from fundus to cervix of uterus individually, and the results were recorded. Afterward, length of the longer corn and the cervical length were gathered for the measurement of uterus length. Ovaries and uterus were fixed in %10 buffered formaline, serial
sections of 5 µm were made from the mid part of the ovaries and they were stained with haematoxylin and eosin. Four sections were evaluated for each ovary. Follicular quantitative analysis was performed in equidistant sections. Number of follicles at different stages was counted and grouped as healthy secondary, atrophic secondary, healthy tertiary and atrophic tertiary follicles. The follicle was defined as: ‘primary’, if the follicle has one layer of follicular cells; ‘secondary’, if the follicle has two or more layers of follicular cells and is larger from primary follicles; ‘tertiary’, if the follicle has a fluid filled antrum and ‘atretic’, if the follicle has degenerated oocyte and/or destructed layers of the membrana granulosa(18,19). The follicular growth phases were shown in Figure 1. All the microscopic analysis was performed with x4, x10, x20, and x40 magnification as blind test.

2.5. Hormonal Assays

The serum concentration of FSH was determined using a commercially ELISA kit specific for rat (Elabscience, E-EL-R0391, Memorial Drive, Suite 216, Houston, Texas, USA) according to the manufacturer’s instruction. The sensitivity of the assay was 1.88 ng/ml. The serum concentration of LH was measured using a commercially available ELISA kit specific for rat (Elabscience, E-EL-R0026, Memorial Drive, Suite 216, Houston, Texas, USA) according to the manufacturer’s instruction. The sensitivity of the assay was 0.94 mlU/ml. The serum concentration of E2 was measured using a commercially available ELISA kit specific for rat (LSBio, LS-F13008, 2401 Fourth Avenue Suite 900, Seattle WA, USA) according to the manufacturer’s instruction. The sensitivity of the assay was 15.6 pg/ml. The serum concentration of P was measured using a commercially available ELISA kit specific for rat (MBSP762170, MyBioSource Inc., San Diego, CA, USA) according to the manufacturer’s instruction. The sensitivity of the assay was <0.188 ng/ml. The serum concentration of AMH was measured using a commercially available ELISA kit specific for rat (Elabscience, E-EL-R0640, Memorial Drive, Suite 216, Houston, Texas, USA) according to the manufacturer’s instruction. The sensitivity of the assay was 0.1 ng/ml. All of these assays were performed concurrently in duplicate and a standard curve was established for assay. Inter- and intra-assay variations were <10%.

2.6. Statistical Analysis

Statistical analysis of the data was made with “The Statistical Package for the Social Sciences 20” (SPSS, Inc. Chicago IL, USA, Microsoft) programme. Values were provided as mean ± standard deviation (minimum-maximum). Statistical significance was determined with Kruskal–Wallis one-way analysis of variance for multiple group comparisons and with the Mann–Whitney U-test for two-group comparisons. Significance level was accepted as p<0.05.

3. Results

Study was completed with 23 rats, CT group (n=8), LD group (n=8) and HD group (n=7) as one rat from HD group died during the experiment from an unknown reason. The mean body weight of the rats was 42.5 ± 1.7 g on PND 21, at the beginning of the experiment. The mean body weight of CT, LD and HD on PND 26, 33, 40 and 44 is given in Table 1. Although mean body weight differed among groups throughout the experiment, the difference was not statistically apparent on final body weight.

Mean age at VO was PND 40 ± 3.2 (range 34-43) in CT and 35.7 ± 2.7 (range 33-40) in HD group. Three rats from LD group did not have VO on termination day. The difference in time of VO was significant (p=0.025). HD group had VO earlier than CT (p=0.023) and LD group (p=0.018). According to the scale, VO seemed to be slightly delayed in LD group compared to CT group, however the difference was not statistically apparent (Table 2). Serum FSH and P levels were not different among study groups. Serum LH levels were increased in HD group compared to CT group (p=0.001). But there was not any difference between serum LH levels of LD group and CT group or HD group and LD group. Serum E2 levels were increased in HD group compared to LD group (p=0.04) and CT group (p=0.01). Serum AMH levels were decreased in HD group compared to LD group (p=0.03) and CT group (p=0.01) (Table 3).

The mean absolute and relative weight of ovaries and uterus lengths were not different among groups. The mean absolute and relative uterine weight was increased in HD group when compared to CT group. The mean number of healthy follicles also was not different among groups but number of atrophic secondary follicles was increased in LD and HD groups (p=0.02). Measurements of reproductive organs, numbers of follicles and hormone levels are given in Table 3 and ovarian photomicrographs of each experimental group are given in Figure 2.

4. Discussion

The only study on reproductive and developmental toxicity of HMF was done by NTP on toxicology and carcinogenesis of HMF on rats and mice. The study revealed that estrous cycle was elongated and regular cycles were fewer in rats which were given 750 mg/kg/day or 1500 mg/kg/day of oral HMF for 3 months starting from PND 42. These data showed HMF’s potential to produce adverse effects in reproductive system and fertility(4). In this study, 750 mg/kg/day or 1500 mg/kg/day of HMF was given orally to female rats from PND 21 for three weeks.
Rats become sexually mature at age 6 weeks(20). To the best of our knowledge this is the first study searching effects of HMF on reproductive system in sexually immature rats. Although HMF is mostly present in high calorie food, its isolated effect on body weight and energy metabolism is controversial. In physiological analyses, redox metabolism is severely affected by HMF, while the effects on the energetics is less evident(21). We found no difference on mean final body weight between the control and HMF groups. Zaitzev et al. reported no change in final body weight of rats receiving 40 mg/kg or 80 mg/kg of HMF for 11 months(22). NTP study reported different results for different groups, loss in body weight of rats receiving HMF for 3 weeks or 3 months exceeding dose of 750 mg/kg/day and no change in body weight of rats receiving HMF for 2 years of any dose(4). Heaton and Robinson reported acceleration in body weight gain with 75-225 mg/kg of HMF for an unmentioned duration, with unmentioned nutrition conditions(23). But it is not approtiate to compare these studies because of the different doses and durations of HMF consumption.

VO, marker of pubertal onset in rodents, is caused by an apoptotic process in vaginal epithelial cells triggered by increased levels of estrogen. VO of rats of same strain from different laboratories or rats of same strain and laboratory but different litters varies hugely. Mean VO in Wistar rats was reported to range between 33.4 ± 1.98 and 41.6 ± 3.7 days, compatible with mean VO of CT group(24). VO in HD group was still within the range of literature but it was earlier than CT and LD group and also E2 levels were higher in HD group which may be interpreted as high doses of HMF causes precocious puberty in female rats.

Uterine weight increases along with the puberty in rodents and this increase is associated with estradiol levels. However, there are studies reporting that estrogenenous environment or increased E2 causes decrease or no change in uterine weight of immature rats(25-27). These unexpected results were explained by altered sensitivity of estrogen receptors on uterus due to high E2 level or the substance used in experiment(27). Absolute and relative uterine weight was increased in HD group and both LH and E2 levels were higher in HD group compared to CT and LD group in this study. HMF may somehow activated the hypothalamo-pituitary system resulting in rise in E2, early VO and may also caused an uterotrophic effect. Detailed physiological studies are needed to understand and explain the mechanism fully.

Intense maturational changes in hypothalamic-pituitary system is accompanied by an increase in gonadotropin response of the ovaries resulting in development of gonadotropin related follicles. Measuring ovarian weight and microscopic examination are indispensable steps for female reproductive toxicology studies(28). In this study, absolute or relative ovarian weight did not vary within groups but number of atrophic secondary follicles was increased in HMF groups compared to CT group. Numerous atrophic follicles may be present in normal peripubertal stage, before ovulation. As the rat matures and cyclicity is set after several cycles, number of atrophic follicles decrease. Atrophic follicles may be prominent in rats that are euthanized around PND 42(28). However, ovarian toxicity studies had shown that increased number of atrophic follicles was among the most common histopathologic features pointing ovarian detriment even in rats at 6 weeks of age(29). So, increased number of atrophic follicles in LD group may be attributed to their immaturity, as 3/8 of LD rats did not have vaginal opening on necropsy day and 2/8 of them had VO the day before necropsy, but ovaries of HD group seem to be affected from HMF toxicity.

AMH is produced by growing ovarian follicles and reflect the antral follicle count(30). Rodent studies have shown that AMH has critical role in initial follicle recruitment and selection of dominant follicle(31). Decrease in serum AMH correlates directly with the decrease in the number of growing follicles(32). In this study, AMH levels were also decreased in HD group, which may be pointing decreased ovarian reserve and HMF related ovarian damage. Although the role of E2 on AMH expression is not clear, another possible cause for the decline in AMH levels may be increased levels of E2. Increased E2 is shown to reduce the activation of AMH promoter in some in vitro studies(33,34). However, there are also studies supporting the opposite or denying that E2 has a direct effect on AMH(35,36).

5. Conclusion

HMF is present in numerous foodstuffs at high levels and peripubertal children have an increasing exposure to this potential toxic metabolite with the changing diet habits. This is the first study on toxic effects of HMF in peripubertal rats and we showed that high doses of HMF given orally for 3 weeks caused early VO, increased number of secondary atrophic follicles and decreased AMH levels. But it is hard to estimate the translation of toxic effects of HMF on reproductive system of human beings with the dosage and duration we applied on rats. Further studies should be done to show the mechanisms causing these findings.

Acknowledgement

This work was supported by the Turkish Pediatric Endocrinology and Diabetes Society.

References
4. NTP toxicology and carcinogenesis studies of 5-(Hydroxymethyl)-2-furfural (CAS No. 67-47-0) in F344/N rats and B6C3F1 mice (gavage studies). Natl Toxicol Program Tech Rep Ser 2010;7:13, 5-9, 21-31 passim.

### Table 1: Mean body weight (g) of each experimental group on different postnatal days (PND)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PND</th>
<th>CT (n=8)</th>
<th>LD (n=8)</th>
<th>HD (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>62.0 ± 8.0</td>
<td>55.3 ± 5.8*</td>
<td>58.1 ± 8.6*, **</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>80.0 ± 10.2</td>
<td>74.6 ± 7.8*</td>
<td>76.2 ± 11.2*, **</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>88.7 ± 11.2</td>
<td>87.4 ± 9.2*</td>
<td>91.7 ± 13.5*, **</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>94.7 ± 12.1</td>
<td>96.8 ± 10.2</td>
<td>105.3 ± 15.6</td>
<td></td>
</tr>
</tbody>
</table>

PND, postnatal day; CT, control group; LD, low dosage group; HD, high dosage group;
* Significantly different (p≤0.05) from mean body weight of the CT
** Significantly different (p≤0.05) from mean body weight of the LD

### Table 2 Vaginal opening time (in days) in different experimental groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CT</th>
<th>LD</th>
<th>HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>PND</td>
<td>34</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>VO scale*</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

uncorrected proof
* 0: VO until the end of the experimental period, 1: VO between PND 39-44, 2: VO between PND 33-38

Table 3: Measurements of serum hormone levels, weight/length of reproductive organs and follicle counts of the study groups

<table>
<thead>
<tr>
<th>Parameters (mean ± SD)</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT (n=8)</td>
</tr>
<tr>
<td>FSH level (ng/ml)</td>
<td>9.4 ± 1.9</td>
</tr>
<tr>
<td>LH level (mIU/ml)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>E2 level (pg/ml)</td>
<td>21.2 ± 3.9</td>
</tr>
<tr>
<td>P level (ng/ml)</td>
<td>10.1 ± 1.8</td>
</tr>
<tr>
<td>AMH level (ng/ml)</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>Absolute weight of ovaries (mg)</td>
<td>58.6 ± 17.6</td>
</tr>
<tr>
<td>Relative weight of ovaries (mg/%)</td>
<td>59.4 ± 12.0</td>
</tr>
<tr>
<td>Absolute weight of uterus (mg)</td>
<td>208.5 ± 84.4</td>
</tr>
<tr>
<td>Relative weight of uterus (mg/%)</td>
<td>214.0 ± 72.4</td>
</tr>
<tr>
<td>Uterus length (mm)</td>
<td>27.6 ± 4.9</td>
</tr>
<tr>
<td>Healthy secondary follicles (n)</td>
<td>53.0 ± 16.4</td>
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<tr>
<td>Atrophic secondary follicles (n)</td>
<td>4 ± 1.6</td>
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<tr>
<td>Healthy tertiary follicles (n)</td>
<td>6.6 ± 2.6</td>
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<tr>
<td>Atrophic tertiary follicles (n)</td>
<td>2.1 ± 1.8</td>
</tr>
<tr>
<td>Atrophic/total follicle (%)</td>
<td>10.3 ± 7.0</td>
</tr>
</tbody>
</table>

*Significantly different (p≤ 0.05) from the control group

* Significantly different (p≤ 0.05) from LD group

Figure 1.
Figure 2A.
Figure 2C.