

Original Contribution

Oltipraz chemoprevention trial in Qidong, People's Republic of China: Unaltered oxidative biomarkers

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Abstract

Aflatoxin, which leads to formation of carcinogen-DNA adducts as well as oxidized DNA, is a well-known risk factor for development of hepatocellular carcinoma. The aim of the present study was to investigate if the chemopreventive agent oltipraz had an effect on DNA oxidation measured as oxidized guanine derivatives in urine among healthy individuals living in a region of China at high risk of exposure to aflatoxin and development of hepatocellular carcinoma. Two hundred thirty-three healthy residents of Qidong, PRC, were randomized to 8 weeks treatment with placebo, oltipraz 125 mg daily, or oltipraz 500 mg weekly, with a subsequent 8-week follow-up period. Urine samples were collected as overnight voids. Samples collected 4 weeks into the treatment period and 6 weeks into the follow-up period were analyzed for oxidized guanine derivatives with a HPLC-MS/MS method. A repeated-measures analysis of variance showed no significant differences between the randomization groups regarding changes in oxidized guanine derivatives. In the present double-blind, randomized, placebo-controlled trial performed among healthy individuals, oltipraz had no major effect on oxidative DNA damage. Mechanisms other than prevention of oxidative DNA damage may be of higher importance when oltipraz is used as a chemopreventive agent in humans.

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Keywords: Chemoprevention; Randomized clinical trial; Oltipraz; Aflatoxin; Free radicals

Introduction

Hepatocellular carcinoma (HCC) is the primary cause of death in the People's Republic of China with estimated 300,000 deaths annually [1]. Chronic infection with hepatitis B virus (HBV) and dietary exposure to aflatoxins are well-recognized risk factors for the development of HCC [2,3]. Hepatitis B virus vaccination programs have been implemented to reduce HCC and beneficial results in Taiwan are documented [4]. But as HBV infection often happens before the age of 3 years and as it is difficult to reduce the dietary exposure to aflatoxins, chemoprevention may be an alternative approach [3,5].

The aim of cancer chemoprevention is inhibition, retardation, or reversal of carcinogenesis at a premalignant stage [6].

Oltipraz (4-methyl-5-(2-pyraziny)-1,2-dithiole-3-thione) was initially investigated as an antischistosomal agent but has been proven to be an effective chemopreventive agent in experimental studies in animal models (reviewed in [7]). Oltipraz modulates aflatoxin metabolism by inhibiting formation of the reactive epoxide and induction of phase 2 conjugation and detoxification pathways [8–10]. Oltipraz could hence be a potential chemopreventive agent in aflatoxin-exposed populations [11].

Aflatoxins probably act as carcinogens by mechanisms that include the formation of epoxide-mediated covalent adducts with DNA and the formation of free reactive oxygen species and subsequent oxidative changes in DNA [12,13]. The oxidized nucleoside 8-oxo-2'-deoxyguanosine (8-oxodG) is a known biomarker of oxidative stress [14] with mutagenic potential in human cells. The formation of 8-oxodG may lead to transversion mutations and genetic alterations and has been found in human HCC cells [15]. Excreted repair products in the form of 8-oxoGua or 8-oxodG can be

Abbreviations: 8-oxoGua, 8-oxo-guanine; 8-oxoGuo, 8-oxo-guanosine; 8-oxodG, 8-oxo-2'-deoxyguanosine.

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measured in urine as indicators of oxidative stress and DNA damage [14,16–18].

Phase I trials have been performed using oltipraz in humans [19–21] proving the tolerability and safety of this chemopreventive agent. Recently, a Phase IIa chemoprevention placebo-controlled trial with oltipraz was performed in Qidong City, an area with one of the highest incidences of HCC in the People's Republic of China [10,11,22,23]. We here present the results from urine analysis measuring the oxidatively modified nucleobase 8-oxoGua and the nucleosides 8-oxodG and 8-oxoGuo in study participants.

Methods

Recruitment of participants and overall study design

The methodology has been thoroughly described in [11]. Study participants were recruited among healthy inhabitants of Daxin Township, Qidong, Jiangsu Province, People's Republic of China. A medical history, physical examination, liver ultrasound, electrocardiogram, chemstrip urinalysis, and blood samples were performed on 1006 individuals between the ages 25 and 65 years. Ultimately, 378 people remained eligible.

Aflatoxin-albumin adducts measured in serum were used as a biomarker of aflatoxin exposure; among the 362 individuals tested 244 had values ranging from 1.25 to 10 pmol/mg. Finally, a total of 233 individuals were randomized and included in the study.

The participants were randomized into three intervention arms: placebo, 125 mg oltipraz administered daily, or 500 mg oltipraz administered weekly. In all intervention arms the participants received identical capsules containing oltipraz or placebo as appropriate on each day of the 8-week intervention to preserve double masking. The oltipraz doses and the dosing schedule were chosen in accordance with previous Phase I chemoprevention trials [19,21].

Urine samples and blood samples were collected throughout the 8-week intervention and the 8-week follow-up period. Urine samples were collected at three consecutive overnight voids every 2 weeks in the study period and subsequently frozen at -80°C .

Subgroup analysis

On the basis of available urine samples from two time points, 64 participants treated with placebo, 47 participants treated with oltipraz 125 mg daily, and 54 participants treated with oltipraz 500 mg weekly were included in the present subgroup analysis ($n=165$).

A total of 43 participants reported to smoke according to questionnaires administered 2 weeks before study entry (14 smokers in the oltipraz 125 mg daily, 11 smokers in the oltipraz 500 mg weekly, and 17 smokers in the placebo group). No stratification for smoking status was made.

We measured three oxidized guanine forms in urine samples collected from study participants 4 weeks into the intervention period and 6 weeks into the follow-up period. Measurements

were performed on urine collected 12–24 h after oltipraz/placebo administration. The Clinical Pharmacological Department, Rigshospitalet, Denmark, carried out the analysis using high-performance liquid chromatography electrospray tandem mass spectrometry (HPLC-MS/MS) [24,25]. HPLC-MS/MS is a validated method for the analysis of oxidized guanine derivatives which in our laboratory has shown a detection limit at 2 nmol/L for nucleobases and 0.5 nmol/L for nucleosides measured in urine [24]. Urine creatinine was measured spectrophotometrically (Sigma Diagnostics Kit) and used to correct for variations in urine concentration.

Results

For further information on the demographics of the study participants see [11]. The mean concentration of creatinine in urine was 9.13 mmol/L (SD 5.20 mmol/L) during treatment and 7.87 mmol/L (SD 3.75 mmol/L) during follow-up.

Table 1 shows the numbers of successful analyses in the different randomization groups. Inconclusive analyses were due to not fully resolved peaks during chromatography or missing urine creatinine values.

Table 2 shows measurements of oxidized guanine derivatives in urine in the three randomization groups during intervention and during follow-up.

A repeated-measures analysis of variance showed no significant differences between the randomization groups regarding changes in 8-oxodG ($p=0.25$), 8-oxoGuo ($p=0.75$), or 8-oxoGua ($p=0.44$). One-way analysis of variance showed no significant differences in measured oxidized guanine derivatives between randomization groups during neither treatment nor follow-up (all $p>0.05$).

Smoking was a potential confounder and a two-way analysis of variance including randomization group and smoking status (smoking yes/no) was performed on changes in 8-oxodG, 8-oxoGuo, and 8-oxoGua. In the confounder-adjusted analyses smoking status significantly affected measured changes in 8-oxoGuo ($p=0.02$) and 8-oxodG ($p=0.03$) but not 8-oxoGua ($p=0.37$). However, there were still no significant differences between randomization groups (all $p>0.05$).

Table 1
Numbers of conclusive analyses during intervention and follow-up in the various randomization groups

Period	Analysis	Number of successful analyses by randomization group		
		Placebo	125 mg oltipraz daily	500 mg oltipraz weekly
Follow-up	8-oxoGua	58	39	50
	8-oxoGuo	56	44	45
	8-oxodG	63	46	54
Intervention	8-oxoGua	62	45	51
	8-oxoGuo	59	42	41
	8-oxodG	63	45	51
Total number of included participants		64	47	54

Table 2
Oxidized guanine derivatives measured in urine during follow-up and intervention

Randomization group	Oxidized guanine derivative	Intervention period		Follow-up period		Difference between measurements during intervention and follow-up		
		Mean	SD	Mean	SD	Mean difference	Two-sample paired <i>t</i> test for means (<i>p</i> value)	95% CI for difference between means
125 mg oltipraz daily	8-oxoGua	51.8	59.07	66.3	122.63	-13.8	0.49	-53.6–26.0
	8-oxoGuo	3.5	3.38	2.8	3.24	0.59	0.33	-0.62–1.80
	8-oxodG	1.9	0.91	1.8	0.90	0.15	0.39	-0.51–0.20
500 mg oltipraz weekly	8-oxoGua	49.7	86.56	54.5	101.85	-5.88	0.75	-42.6–30.8
	8-oxoGuo	4.0	2.71	3.9	3.12	0.084	0.82	-0.68–0.85
	8-oxodG	2.5	1.50	1.9	1.19	0.51	0.0089	0.13–0.88
Placebo	8-oxoGua	41.4	59.96	28.2	43.56	13.2	0.17	-6.0–32.5
	8-oxoGuo	3.1	2.35	2.6	2.50	0.49	0.24	-0.33–1.31
	8-oxodG	2.1	1.24	1.7	0.84	0.44	0.0049	0.14–0.79

Two-sample paired *t* tests for means. Guanine derivatives were measured in nmol/mmol creatinine.

A paired *t* test showed significantly higher values of 8-oxodG during intervention in the placebo group ($p=0.0049$) and the intervention group taking 500 mg oltipraz weekly ($p=0.0089$) compared to values measured during follow up (Table 2). This significant result indicates a period effect. However, because one-way analysis of variance with and without adjustment for the follow-up period was without statistical significance the outcome of the trial is concluded to be without effect.

Discussion

The aim of the present study was to establish if treatment with the chemopreventive agent oltipraz had an effect on cancer-related oxidative stress among aflatoxin-exposed individuals measured as the excretion of oxidatively modified nucleobases and nucleosides in urine. This hypothesis could not be confirmed and we found no significant differences between intervention groups.

Aflatoxin is a well-known risk factor for the development of hepatocellular carcinoma. The formation of the reactive oxygen species 8-oxodG and increased hepatic oxidative damage may be important contributors to the cytotoxic and carcinogenic effects of aflatoxin [13,15]. Aflatoxin injected in rats induces the formation of 8-oxodG in liver, perhaps with hydroxyl radicals as intermediates. The formation is reduced by the antioxidants selenium and deferoxamine [12]. In rat hepatocytes aflatoxin exposure increases the formation of reactive oxygen species in a dose-dependent manner and again antioxidants (catalase, desferrioxamine, dimethyl sulfoxide) decrease their formation [13]. The formation of both reactive aflatoxin metabolites and reactive oxygen species may be closely linked to the P450 system in the liver [13].

Oltipraz is a chemopreventive agent, which has shown beneficial modulative effects in aflatoxin metabolism and hence decreased formation and/or increased degradation of the AFB1-8,9-oxide [8,10,23,26–30]. Additionally, oltipraz reduces formation of 8-oxo-dG in bronchoalveolar cells and tracheal epithelium in rats exposed to the carcinogens in cigarette smoke [31]. Other studies support the finding that oltipraz and oltipraz

analogues increase endogenous levels of antioxidant enzymes and diminish the production of reactive oxygen perhaps through gene alterations [32–36].

Human studies on oltipraz's potential ability to modulate oxidative stress are limited. But based on the findings in the aforementioned studies, we investigated whether the treatment with oltipraz in aflatoxin-exposed healthy human individuals included antioxidative properties measured as effects on oxidized nucleobases and nucleosides excreted in urine.

As the present study was performed among a larger group of healthy human volunteers, it was not practically possible to obtain cellular DNA for the analyses of oxidatively modified nucleobases from target tissue. Instead we carried out measurements on urine samples—urine may act as a surrogate for the target organ [37,38]. As the repair products from oxidative DNA damage are poor substrates for nucleotide synthesis, they are generally excreted unmetabolized into the urine [39]. It is believed that when the rate of DNA damage/oxidation is changed, a steady state is achieved at which the activity of repair enzymes is increased due to linear enzyme kinetics and hence the excretion of oxidized nucleosides will correspond to the formation [39,40]. This adaptation may occur within hours [41,42]. Therefore, if treatment with oltipraz was able to change/reduce the formation rate of oxidatively modified nucleobases, this should be measurable in urine when steady-state conditions were obtained. The excretion of 8-oxodG and 8-oxoGuo is believed to be relatively unaffected by the diet [17,18,40].

Previous studies showed that oltipraz-induced alterations in aflatoxin metabolism were present after 4 weeks of treatment [10]; hence we chose urine samples collected at this time point for the analysis of oltipraz-induced effects. In Phase I studies oltipraz had a half-life of less than 6 h [20]; hence we expected urine analysis performed after 6 weeks of follow-up to illustrate conditions without oltipraz influence.

However, the use of urine as a surrogate for oxidative damage in tissue is not without problems. The oxidation of human DNA occurs as a consequence of free radicals arising endogenously and exogenously [38]. Perhaps continuous whole-body oxidation of the guanine nucleotide pool makes

too much background noise and prevents the detection of smaller changes in treatment groups [43]. Renal impairment and urinary creatinine are important factors that affect urinary levels of oxidative lesions [17]. As it was not possible to achieve 24-h urine collections in the present study population, urine samples were collected as overnight voids. Measurements of urine creatinine and calculation of corrected values of oxidized/nonoxidized biomarkers were done to meet this aspect. This method has proven sufficient in previous trials, with strong correlation between values measured in 24-h and in overnight urine [38]. The method is valid because the excretion of creatinine is believed to be unaffected by treatment with oltipraz. Furthermore, no differences in creatinine levels were expected between study groups because of the random allocation of males/females and different body weights between intervention groups, and all participants were healthy and had normal plasma levels of creatinine [40,42,44].

We have earlier shown that smoking is associated with increased DNA oxidation and elevated levels of 8-oxodG in urine [45,46]. In the present study participants were not randomized according to smoking habits and the inclusion of only a limited number of smokers gave the risk of an unbalanced distribution between intervention groups. Indeed smoking status was a significant confounder according to the statistical analysis, but the overall lack of oltipraz treatment effect was unaltered. Our study has not been powered to detect minor differences between intervention groups. It is still unsettled how large differences in biomarkers should be in order to have potential clinical importance. In a previous study smoking cessation decreased urinary excretion of 8-oxodG by 23% [45]. In the present study the reduction in oxidative damage was of the same magnitude but greatest in the placebo group. The two studies were on similar numbers of subjects; therefore we can conclude that if we have missed an effect of oltipraz due to type II error, it is of a small magnitude.

Oltipraz is still a promising chemopreventive agent in aflatoxin-exposed populations [10,23]. The induction of phase 2 glutathione conjugation systems and hence beneficial effects on aflatoxin metabolism is a more likely effective mechanism. Indeed, longer term treatment with oltipraz is now being investigated in Phase IIb trials. However, the exact mechanisms of action and the efficacy of oltipraz in preventing HCC in humans remain to be established.

Conclusion

The present randomized double-blind placebo-controlled study performed among aflatoxin-exposed healthy individuals showed no effects of oltipraz on DNA oxidation.

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