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ORIGINAL ARTICLE

Male Health

Preventive effects of β-cryptoxanthin against cadmium-induced oxidative stress in the rat testis

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β-cryptoxanthin (CRY), a major carotenoid of potential interest for health, is obtained naturally from orange vegetables and fruits. A few research studies have reported that CRY could decrease oxidative stress and germ cell apoptosis. The purpose of this study was to examine the effects of CRY on acute cadmium chloride ($CdCl_2$)-induced oxidative damage in rat testes. For this study, 24 rats were divided into four groups, one of which serves as a control group that received intraperitoneal (i.p.) injections of corn oil and physiological saline. The other rats were i.p. injected with CRY (10 μg kg^{-1}) every 8 h, beginning 8 h before $CdCl_2$ (2.0 mg kg^{-1}) treatment. The pathological and TUNEL findings revealed that CRY ameliorated the Cd-induced testicular histological changes and germ cell apoptosis in the rats. Furthermore, the Cd-induced decrease in the testicular testosterone (T) level was attenuated after CRY administration ($P < 0.05$). The administration of CRY significantly reversed the Cd-induced increases in the lipid peroxide (LPO) and malondialdehyde (MDA) levels ($P < 0.01$). The testicular antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) were decreased by treatment with Cd alone but were restored by CRY co-treatment. These results demonstrated that the application of CRY can enhance the tolerance of rats to Cd-induced oxidative damage and suggest that it has promised as a pharmacological agent to protect against Cd-induced testicular toxicity.

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INTRODUCTION

Cadmium (Cd) is a major industrial and environmental pollutant that is primarily produced from mining, smelting, electroplating, battery manufacturing, pigments and plastics. The main route of exposure to Cd is by the inhalation of Cd particles or fumes from occupational exposure[1] or via passive smoking by the general population.[2] Toxic heavy metals have been found to produce both acute and chronic pathological conditions such as hepatic and renal dysfunction, testicular damage and respiratory and nervous system disorders.[3–5] Cd is also regarded as a potentially dangerous carcinogen by the International Register of Potentially Toxic Chemicals of the United Nations Environment Program.[6] It can easily accumulate in many organs, especially following the intake of contaminated food and water. It has a long half-life of ~20–30 years in the human body with a low rate of excretion (<~1–2 μg day^{-1}).[7,8] Several studies have revealed the deterioration and dysfunction of different tissues due to Cd exposure. For instance, morphological and pathological changes have been noticed in the testes of mouse models at different stages of growth and maturity.[9–11] The testicular pathogenicity of Cd is associated with severe hemorrhage, edema and atrophy, as well as spermatogonial apoptosis, a reduction in the number and motility of sperm and decreased testosterone (T) concentrations in the plasma and testes.[10,11] The pathogenesis of testicular damage and spermatotoxicity following Cd have been shown to result from oxidative damage, which induces the formation of reactive oxygen species (ROS) such as superoxide radical, hydroxyl ion, and hydrogen peroxide.[12,13] The generation of ROS could lead to damage and oxidation-mediated toxicity to the cellular membranes in the testes, which are susceptible to peroxidation injury.[14] However, the precise mechanism(s) by which Cd induces testicular toxicity remain unclear.[9] To eliminate oxidative stress, cells are protected by antioxidant defense systems in the body that include superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH).[15]

β-cryptoxanthin (CRY, 3-hydroxy-b-carotene) has shown promise as a chemo-preventive agent against lung cancer.[16] Recent studies revealed that CRY could inhibit chemically induced skin tumorigenesis[17] and rat colon carcinogenesis with moderate CRY intake.[18] Like other carotenoids, CRY is considered to be an important antioxidant capable of scavenging various types of ROS and nitrogen species-induced oxidative stress in experimental animals.[19] The scavenging oxygen radicals have been considered the first line of defense against lipid peroxide (LPO) incorporation into biological membranes.[20,21]

Based on the previous studies, we hypothesized that absorbed CRY may have a protective effect against oxidative stress in several tissues. However, there has been scant research on the impact of CRY on Cd-induced oxidative stress in rats. Therefore, this study aimed to evaluate the protective effects of CRY against $CdCl_2$-induced oxidative damage in the testes of rats.

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MATERIALS AND METHODS

$CdCl_2$ and CRY were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Corn oil was purchase from Aladdin Industrial Co., Ltd., (Shenzhen, China). $CdCl_2$ was dissolved in sterile physiological saline and CRY was dissolved in corn oil. The kits used for the measurement of the oxidant status and antioxidant enzyme activities were purchased from Nanjing Jiancheng Bio-engineering Institute (Nanjing, China). All other reagents were of analytical grade.

Twenty-four adult male Sprague-Dawley rats (8-week-old and 200–220 g in weight) were purchased from Hubei Provincial Center for Disease Control and Prevention, Wuhan, China. The animals were kept on 12 h light: 12 h dark cycles with controlled humidity and temperature (20–22°C). All of the animals were housed in individual polypropylene cages and given standard diet and water *ad libitum*. All animal treatment procedures were approved by the Ethics Committee on Animal Experimentation of Huazhong Agricultural University.

The rats were divided into four groups, each consisting of six animals. The groups were treated as follows: the control rats were treated intraperitoneally (i.p.) with 100 μl of corn oil every 8 h, beginning 8 h before they received 0.1 ml isotonic saline via i.p. injection; in the CRY group, the rats were treated i.p. with CRY (10 μg kg^{-1}) in 100 μl corn oil every 8 h, beginning 8 h before they received 0.1 ml i.p. isotonic saline; in the Cd group, the rats were treated i.p. with 100 μL corn oil every 8 h, beginning 8 h before they received a single i.p. dose of 2 mg Cd kg^{-1} body weight in 0.1 ml isotonic saline; in the CRY + Cd group, the rats were treated i.p. with CRY (10 μg kg^{-1}) in 100 μL corn oil every 8 h, beginning 8 h before they received a single i.p. the dose of 2 mg Cd kg^{-1} body weight in 0.1 ml isotonic saline. The rats were sacrificed under diethyl ether anesthesia 24 h after Cd treatment, and the testes were immediately removed and dissected as described previously.[22]

Rats were weighed before sample preparation; the testes were immediately excised and weighed after the rats were sacrificed using diethyl ether anesthesia at the end of the 24 h experimental period. The right testis tissue was minced and homogenized (10%, *w/v*) in 0.1 M phosphate buffer (pH 7.4) with an ice-bath electric homogenizer (MP Biomedicals, Santa Ana, USA). The homogenate was centrifuged at 1500 g for 15 min at 4°C to pellet the nuclei and cell debris, and the resulting supernatant fraction was frozen at −80°C for further measurements.

The left testes were fixed in 4% paraformaldehyde and then embedded in paraffin to make serial sections of approximately 5 μm thick using a microtome. At least two nonserial sections were stained with hematoxylin and eosin (H and E) using standard procedures for morphological analyses. To detect apoptosis, paraffin-embedded sections were stained with the TUNEL technique using an *in situ* apoptosis detection kit (Promega, Mannheim, Germany) according to the manufacturer's protocols.

The testicular T level was quantified employing an ELISA method using a commercially available kit (CSB-E05100r kit, CUSABIO, Wuhan, China). The detection limit of the assay was 0.06 ng ml^{-1} and the average intra- and inter-assay coefficients of variation were <10% and 15%, respectively.

The LPO in the testes was determined based on the formation of MDA as an end-product of the peroxidation of lipids.[23] Briefly, each sample was mixed with 20% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA), and the mixture was incubated at 100°C for 15 min. The precipitate that resulted upon cooling was removed by centrifugation (1500 g, 15 min). The fluorescence was measured at 535 nm in the organic phase using a fluorescence spectrophotometer, and the results were expressed as the nmol MDA per mg protein.

The total SOD activity was measured in the supernatant according to the epinephrine method.[24] This method determined the rate of inhibition of epinephrine autoxidation by SOD, which was monitored at 480 nm in a reaction medium containing the testes samples in glycine/NaOH (pH 10.2). The activity of SOD was expressed as U mg^{-1} protein. The tissue CAT activity was assayed as the rate of degradation of hydrogen peroxide by CAT, which was measured at 230 nm in a reaction medium containing the examined samples in 5 mmol l^{-1} EDTA and 1 M Tris–HCl solution (pH 8.0).[25] The enzyme activity was expressed as UCAT mg^{-1} protein. The activity of GSH-Px was measured according to the method described by Beutler.[26] in 1963. The method is based on the rate of NADPH oxidation observed at 340 nm using hydrogen peroxide as the substrate in a coupled assay with glutathione reductase. The activity was expressed as the amount of NADPH (U) that disappeared per min per mg protein. The protein concentration in testes was determined by the method described by Bradford using bovine serum albumin as the standard.[27]

The data were presented as the means ± s.d. The statistical significance of differences was analyzed by means of a one-way analysis of variance (ANOVA), followed by a *post-hoc* Duncan's multiple range test, using SAS (version 9.1; SAS Institute Inc., Cary, NC, USA). The results were considered statistically significant at $P < 0.05$.

RESULTS

Effects of CRY on Cd-induced testicular injury

A histological assessment was performed to evaluate the effects of CRY on Cd-induced injury using H and E staining (**Figure 1**). Normal histological features were observed in the control and CRY groups (**Figure 1a** and **1b**). In the $CdCl_2$ group, H and E staining showed severe vacuolation, exudative germ cells and atrophy of spermatogenic cells in the seminiferous tubules (**Figure 1c**). However, pretreatment with 10 μg kg^{-1} CRY inhibited the $CdCl_2$-induced testicular edema completely (**Figure 1d**) Furthermore, there was a decrease of almost 20% in the testis weight and a 13% decrease in the testis/body weight ratio in the $CdCl_2$ group compared with the control group ($P < 0.05$). CRY therapy was effective in preventing the loss of testicular weight in the $CdCl_2$-treated animals (**Figure 2a** and **2b**). To further assess the effects of CRY on Cd-induced injury, the testicular T level was quantified by employing an ELISA method (**Figure 3a**). The testicular T levels were significantly (23%) reduced after Cd exposure; however, CRY administration attenuated this decrease ($P < 0.05$).

CRY inhibited Cd-induced germ cell apoptosis

Many recent findings have revealed that oxidative stress can induce apoptosis, so the effect of CRY on Cd-induced testicular germ cell apoptosis was analyzed by TUNEL staining (**Figure 4**). As shown in the control and CRY groups, the spermatogenic cells and interstitial connective tissue between the seminiferous tubules presented normal histological structures (**Figure 4a** and **4b**). In the Cd-treated group, there were many more TUNEL-positive germ cells in the seminiferous tubules compared with the control group (**Figure 4c**). However, CRY treatment dramatically reduced the number TUNEL-positive cells compared with the Cd group (**Figure 4d**).

Oxidant status

MDA is an end-product of LPO and an indicator of oxidative stress induced by ROS. In the current study, the LPO production in the Cd-treated group was higher ($P < 0.01$) than that in the control group (**Figure 3b**). However, CRY treatment alleviated the increase in

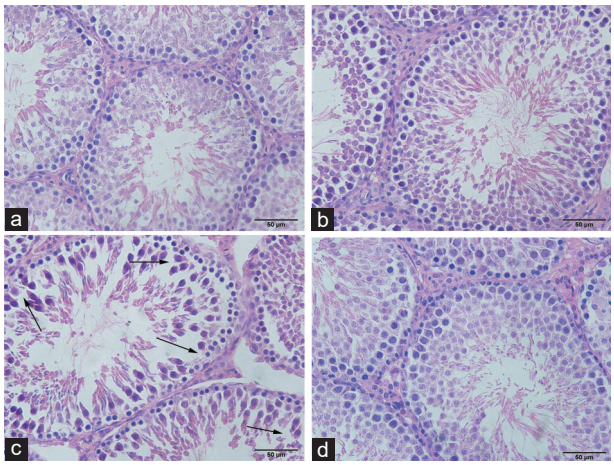

Figure 1: Effects of CRY on Cd-induced histopathological change in testes. Testicular cross sections were stained with Hematoxylin and eosin (H and E). Original magnification: ×400. Male rats were injected with physiological saline and oil as control rats (**a**), or CRY (**b**), or $CdCl_2$ (2.0 mg kg^{-1}) (**c**) and $CdCl_2$ (2.0 mg kg^{-1}) pretreated with CRY (**d**) as described in materials and methods. Black arrows showed marked vacuolation in the spermatic epithelium. Scale bar = 50 μm.

LPO production induced by Cd. A significant increase in MDA activity was observed in the testes of Cd-treated rats compared with control rats ($P < 0.01$, **Figure 3c**). CRY treatment partly, but significantly, prevented the increase in MDA induced by Cd ($P < 0.01$).

Antioxidant status

The activities of antioxidant enzymes including SOD (**Figure 3d**), CAT (**Figure 3e**) and GSH (**Figure 3f**) were evaluated after different treatments. The SOD activity decreased approximately 50% following Cd exposure compared with the control group ($P < 0.01$). CRY therapy partially rescued this decrease in testicular enzyme activity induced by Cd treatment. Cd administration also led to significant ($P < 0.01$) inhibition of the GSH and CAT activities in the testes homogenates. However, CRY treatment partially restored the activities of GSH and CAT in the testes compared with the Cd group ($P < 0.01$). These results indicate that CRY has preventive effects against Cd-induced oxidative stress.

DISCUSSION

Cd is a toxic metal that is hazardous for human and animal reproductive health due to its accumulation primarily in the testes.[28–30] In addition, Cd has been shown to induce testicular injury and germ cell apoptosis in rodents.[22,31] In the current study, we revealed that $CdCl_2$ exposure induced significant testicular injury, not only decreasing the weight of the testes but also causing severe cellular damage, edema and atrophy of spermatogenic cells in the seminiferous tubules. Importantly, acute Cd exposure led to obvious apoptosis in germ cells. Histopathological studies revealed that Cd-induced severe hydropic degeneration in the centrilobular zones, which led to necrosis.

Like other carotenoids, CRY was speculated to be an important antioxidant due to the presence of a hydroxyl group in its structure. In this study, microscopic examinations showed that pretreatment with CRY (10 μg kg^{-1} BW) led to almost complete inhibition of the testicular edema induced by $CdCl_2$ and rescued the Cd-induced reduction in the absolute and relative testes weights. Surprisingly, CRY dramatically reduced the number of TUNEL-positive cells induced by Cd. These findings demonstrated that CRY could protect against Cd-induced germ cell apoptosis in rat testes. Of note, the Cd-induced decrease in the testicular testosterone level was also attenuated after CRY administration.

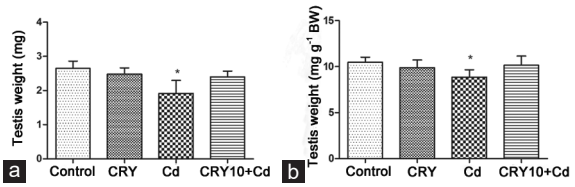

Figure 2: CRY rescued Cd-induced decrease of the weight of testis. Male rats were injected with $CdCl_2$ (2.0 mg kg^{-1}). In the CRY + Cd group, rats were pretreated with CRY as described in Material and Methods. Testes were collected at 24 h after Cd injection. (**a**) testis weight (**b**) testis/body weight. All data were expressed as means ± s.d. ($n = 6$). *$P < 0.05$ indicates significance between groups.

A recent study showed that subchronic exposure to Cd decreases the activity of antioxidant enzymes and increases the lipid peroxidation and DNA oxidation in rat testis.[32] The most important mechanism underlying the reproductive damage induced by Cd toxicity was considered to be that it produces oxidative damage in the testes by enhancing the peroxidation of membrane lipids, a deleterious process solely carried out by free radicals.[33] These free radicals damage the mitochondria and affect cellular respiration,[34] activate xanthine oxidase and heme-oxygenase[35] and reduce the activities of antioxidant enzymes.[36] Our results demonstrated that the LPO and MDA levels were markedly elevated in the rat testes after Cd treatment, which is in agreement with the other reports on Cd-intoxicated rats.[10] Previous studies reported that LPO was one of the main indicators of oxidative injury in the testes.[32,37,38] The changes in LPO might be due to inhibition of the activity of antioxidants, which results from the increased production of free radicals and/or a decrease in the antioxidant status.[39]

Furthermore, the current results revealed that $CdCl_2$ exposure induced decreases in the levels of antioxidant enzymes, including SOD, CAT and GSH, in the testicular tissue. SOD participates in the biological defense against oxidative stress processes by catalyzing the dis-mutation of endogenous cytotoxic superoxide radicals to hydrogen peroxide and molecular oxygen. The GSH enzyme is involved in detoxifying xenobiotics and carcinogens and thus protects cells against redox cycling and oxidative stress. It has been reported that GSH was able to keep the cellular redox state at a low level, decrease the oxidative stress.[40] This supported our observation that there was decreased GSH and increased LPO. It is generally known that Cd can induce tissue injury and can affect the physiology of any cell in an animal's body by passing through biological membranes, such as the blood-testis barrier.

Until date, only a few studies have been conducted to assess the impact of CRY on oxidant stress, both of which were performed in mice.[41,42] In the present study, we evaluated the efficacy of CRY in preventing Cd-induced oxidative stress in rats. We found that the administration of CRY to rats before Cd intoxication could prevent the increase in the LPO and MDA levels at 24 h after treatment compared with the group treated with Cd alone. Interestingly, our data suggested that the CRY-treated rats showed the ability to significantly counteract the enhancement of LPO, whereas rats were still susceptible to an increase in LPO in the absence of CRY. This finding is in agreement with the previous reports showing that CRY can help resist oxidative stress and showing protective effects against Cd-induced testicular tissue damage in experimental rats.[28] Consistent with these findings, CRY treatment induced a significant increase in the activities of SOD, GSH and CAT in the testes compared with the Cd group ($P < 0.01$).

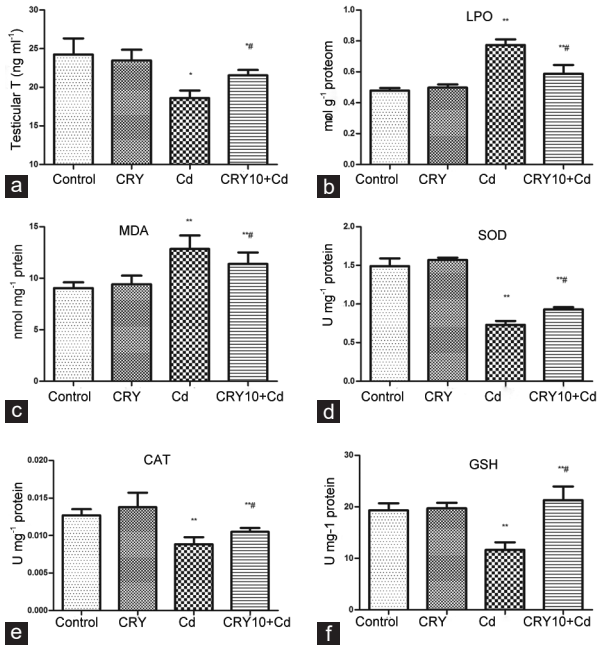

Figure 3: Effect of CRY on testicular T, oxidative stature and anti-oxidative statue of Cd treated rats. (**a**) T level; (**b**) LPO activity; (**c**) MDA activity; (**d**) SOD activity; (**e**) CAT activity; (**f**) GSH activity. Results are expressed as means ± s.d. $n = 6$ for each treatment group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control and CRY group; $^{\#}P < 0.05$, compared to Cd group. Scale bar = 50 μm.

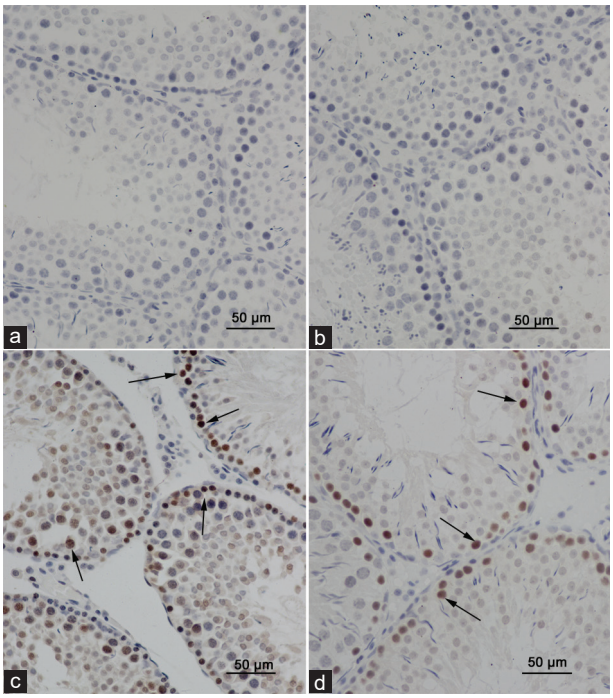

Figure 4: Effects of CRY on Cd-induced testicular germ cell apoptosis (×400). Male rats were injected with physiological saline and oil as control rats (**a**), or with CRY (**b**), or $CdCl_2$ (2.0 mg kg^{-1}) (**c**) and $CdCl_2$ (2.0 mg kg^{-1}) pretreated with CRY (**d**) as described in Materials and methods. Testes were collected 24 h after Cd treatment. Apoptotic germ cell was showed by TUNEL staining (brown). Nuclei were stained with Hematoxylin (blue). Arrow showed apoptotic germ cells in seminiferous tubules.

However, the exact mechanism(s) by which CRY can improve the Cd-induced tissue damage and cell apoptosis remain unclear. In the last decade, CRY was considered to be an important antioxidant that stimulates the repair of oxidative DNA damage[41] and eliminates singlet molecular oxygen and free radicals,[43] making it a more potent ROS scavenger than β-carotene.[44] The SOD activity was increased in the Cd-treated rats but was normalized by treatment with CRY. With regard to the structure of CRY, it is enzymatically converted to retinol and is involved in cell differentiation, similar to β-carotene. Dietary administration of CRY (25 ppm) was previously shown to significantly reduce the cyclin D1-positive cell ratios in bladder lesions and decreased the incidence of urinary bladder carcinomas as well as bladder dysplasia.[45]

Furthermore, CRY was reported to be involved in inhibiting mouse skin tumorigenesis[18] and to decrease the incidence of rat colon tumors,[46] at least partly by reducing inflammation[46] and optimizing the immune response. CRY can suppress gastric BSG823 cell proliferation and growth and may function as a cancer preventive agent in the early promotion stage.[47] Bertram and Bortkiewicz concluded that the possible anti-carcinogenic mechanisms associated with CRY treatment are due to the presence of a hydroxyl residue leading to antioxidant properties, which were related to decreased DNA damage, membrane lipid peroxidation, and inhibited malignant transformation *in vitro*.[48]

Other studies indicate that CRY participates in different physiological activities. For example, it participated in anabolic activities in bone components of aged female rats *in vivo* and *in vitro*,[49] improving the serum adipocytokine status and alleviating the progression of metabolic syndrome.[50] Sugiura *et al*. stated that CRY might be a most useful micronutrient for the prevention of age-related oxidative damage in the brain and cognitive dysfunction among the various subtypes of carotenoids.[51] Similarly, several previous studies suggested that carotenoids (including β-carotene, lycopene, lutein, and CRY) showed antioxidant activity and the ability to protect the rat liver, HepG2 human liver cells and multilamellar liposomes against lipid peroxidation.[13,52,53] Interestingly, CRY can accumulate at a high concentration in the spleen, suggesting that it may be transported and accumulated in the spleen by old erythrocytes.[51] However, little is known about the potential physiological role of α-CRY.

The results of this study demonstrated that $CdCl_2$ causes oxidative stress in rat testes and that CRY administration prevents the testicular damage induced by this oxidative stress.

AUTHOR CONTRIBUTIONS

XRL contributed to the conception and design of this study, carry out the search for the articles and drafted the manuscript. YYW were responsible for analysis and interpretation of data and helped to draft the manuscript. HRF and CJW contributed to the acquisition and coordination. AK participated in the article screening and critically revising the manuscript. LGY were responsible for the initial idea and design of this study together with XRL, and provided many proposals for the manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

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