

Immunohistochemical Demonstration of Nitrosative Tissue Damage in Copper Induced Liver Toxicity in Japanese Quails

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Summary

Excess copper may cause liver toxicosis in various species. In this study, nitrosative tissue damage was investigated in an experimental chronic copper toxicity model in Japanese quails by immuno-histopathological means. Four groups (control, 1st, 2nd and 3rd), each comprised of 10 one-day-old chicks, were fed for 6 weeks with rations containing none, 100, 250 and 500 ppm copper sulfate, respectively. At the end of the feeding period, animals were euthanized, liver tissues were collected and processed routinely for histopathologic applications. Immunohistochemical staining was performed for inducible nitric oxide synthase (iNOS) and nitrotyrosine antibodies with indirect avidin-biotin peroxidase technique. Apoptotic cell death was investigated with *in situ* TUNEL staining. Microscopically, there were only mild and moderate degenerative changes in the liver of the chicks of 2nd and 3rd groups, respectively. In immunohistochemical staining, little immunoreactivity was detected in the control group for both antibodies. Immunoreactivities were gradually stronger with the increasing copper concentration. A few apoptotic hepatocytes were present only in the 3rd group. These findings suggest that nitric oxide play a role in the oxidative tissue damage of copper induced liver toxicity.

Keywords: *Copper toxicity, Inducible nitric oxide synthase, Nitrotyrosine, Apoptosis*

Bıldırıcınlarda Bakır İle Oluşturulan Karaciğer Toksikasyonunda Nitrozatif Doku Hasarının İmmunohistokimyasal Yöntemlerle Araştırılması

Özet

Bakırın fazlası çeşitli türlerde karaciğer toksikasyonuna neden olabilir. Bu çalışmada, bıldırıcınlarda deneysel kronik bakır toksikasyonunda, karaciğerde oluşan nitrozatif doku hasarı ve apoptotik hücre ölümü immunohistopatolojik metodlarla incelendi. Her birinde 10 adet bir günlük bıldırıcın bulunan kontrol, 1, 2 ve 3. gruplar, 6 hafta süre ile sırasıyla 0, 100, 250 ve 500 ppm düzeylerinde bakır sülfat içeren yemle beslendiler. Beslenme dönemi sonrasında, hayvanlara ötanazi uygulanarak karaciğerleri alındı ve histopatolojik yöntemler için rutin şekilde işlendi. İndüklenebilir nitrik oksit sentaz (iNOS) ve nitrotirozin için indirekt avidin-biotin peroksidaz yöntemi uygulandı. Apoptotik hücre ölümü *in situ* TUNEL boyama yöntemi uygulanarak araştırıldı. Mikroskobik düzeyde karaciğerde sadece 2. grupta hafif, 3. grupta orta derecede dejeneratif değişiklikler tespit edildi. İmmunohistokimyasal boyamalarda her iki antikor için de kontrol grubunda hafif derecede immun tepkime tespit edildi. Artan bakır konsantrasyonuna bağlı olarak immun tepkimeler de artma gözlemlendi. Sadece 3. grupta ve az sayıda olmak üzere apoptotik hepatositler mevcuttu. Elde edilen bu bulgular, bakır ile oluşturulan karaciğer toksikasyonunda, nitrik oksitin oksidatif doku hasarında rol oynadığını göstermektedir.

Anahtar sözcükler: *Bakır toksikasyonu, İndüklenebilir nitrik oksit sentaz, Nitrotirozin, Apoptozis*

INTRODUCTION

Copper is an essential trace element for health. It is required for synthesis of many molecules and enzymes including catechol oxidase, ceruloplasmin, cytochrome-c oxidase, superoxide dismutase, lysyl oxidase, amine

oxidase, dopamine beta-monooxygenase and peptidylglycine monooxygenase^{1,2}. Therefore, copper plays important roles in melanin synthesis, normal iron metabolism, regulation of cellular respiration,



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free radical defense, connective tissue biosynthesis, neurotransmitter signaling, reproduction, and effective immune response. Although copper is imperative for life, its excess intake can cause toxicity both in mammals and poultry^{3,4}. There also seems to be differences in tolerance to copper toxicity among different species^{3,5}. Copper toxicity can be either acute or chronic. Acute toxicity is mostly due to accidental intake of excess amount of copper contaminated substances or water⁶. Chronic copper toxicity is the more likely seen form and mostly due to a genetic defect in the copper metabolism⁷. However, consumption of a diet containing high levels of copper or copper pipe drained water over a long period of time can also result in toxicity⁸⁻¹⁰.

In poultry industry, high levels of copper are added to daily rations to serve as a growth stimulant and anti-fungal agent. Although poultry are known to be the most tolerant to high levels of copper, ingestion of copper over a long period of time can be a danger⁴. Copper is normally absorbed from the small intestine, and then transported through portal vein to liver via carrier proteins such as albumin and transcuprein. In the liver, copper is stored in a form bound to metallothionein or similar proteins in hepatocytes, and then excreted in bile¹¹.

How excess copper results in cellular toxicity has not been well defined. High levels of copper in liver tissue have been shown to cause oxidative tissue damage^{12,13}. This oxidative tissue damage has been proposed to occur through the copper ions catalyzed formation of hydroxyl radicals via Haber-Weiss and Fenton reactions^{14,15}.

The effect of nitric oxide (NO) and its metabolites in copper associated liver disease and the molecules involved in the oxidative liver damage have not been previously investigated, especially in poultry. Therefore, the purpose of the current research is first; to provide a model of copper induced toxicosis in Japanese quails, second; to investigate the extent of copper induced tissue damage and last; to search the extent of copper induced nitrosative tissue injury and to have a better understanding of the molecules involved in the oxidative stress response.

MATERIAL and METHODS

Experimental design

Four groups (Control, 1st, 2nd and 3rd) of one-day-old

Japanese quails, each containing 10 animals, were fed *ad libitum* for 6 weeks with a ration containing 2900 kcal/kg metabolisable energy and 22% crude protein. Additionally, 1st, 2nd and 3rd groups were offered 100, 250, and 500 ppm copper sulfate pentahydrate (CuSO₄·5H₂O), respectively. At the end of the feeding period, the animals were weighted and then euthanized with cervical decapitation. Livers were collected, weighted and relative organ weights were calculated. The differences were considered significant if the P value was less than 0.05 by Tukey's multiple comparison test. The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), version 10.0, was used for statistical analysis.

Histopathology

The formalin-fixed and paraffin-embedded tissue samples were processed routinely for hematoxylin and eosin staining for further investigation.

Immunohistochemistry

Immunohistochemical staining for inducible nitric oxide synthase (iNOS) and nitrotyrosine was performed in sections of liver tissues. For this purpose, 4-5 µm sections were cut from the formalin-fixed and paraffin-embedded tissue samples, deparaffinized with xylene, and rehydrated with alcohol series. After blocking the endogenous peroxidase activity with 0.3% H₂O₂ for 20 min, antigen retrieval was performed by microwave treatment with 10 mM citrate solution, pH 6.0 for 15 min. After washing the sections with phosphate buffered saline (PBS, 0.1 M, pH 7.4), a pretreatment with normal goat serum for 30 min to block non-specific antibody binding was performed. Then, in a humidified chamber primary antibodies against iNOS (rabbit anti-iNOS polyclonal antibody, Lab Vision, Fremont, CA, USA) or nitrotyrosine (rabbit anti-nitrotyrosine polyclonal antibody, Calbiochem, Darmstadt, Germany) were applied to tissue sections for 1 h at room temperature in 10 µg/ml and 6.5 µg/ml concentrations, respectively. Biotinylated rabbit antibody and streptavidin-biotin immunoperoxidase (Lab Vision, Fremont, CA, USA) were consecutively applied onto sections with 3 times PBS washes between the two applications. 3,3-diaminobenzidine-H₂O₂ solution was used for the visualization of antigenic localizations of both molecules. Finally, the sections were rinsed in distilled H₂O, counterstained with hematoxylin,

rinsed again under running tap water and coverslipped.

In situ TUNEL staining

DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) was used to visualize apoptotic cells. Briefly, 4-5 µm sections of liver samples were cut and processed through xylene and alcohol series. The sections were rinsed in PBS and placed in PBS buffered Proteinase K solution for 30 min. Following rinses in PBS, the sections were placed in an equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM DTT, 2.5 mM cobalt chloride, and 0.25 mg/ml BSA) for 10 min and then incubated with reaction buffer (biotinylated nucleotide mix and terminal deoxynucleotidyl transferase) for 1 h in a humidified chamber at 37°C. To stop the reaction, the samples were incubated in sodium citrate solution at room temperature for 15 min. The sections were then rinsed twice in PBS and the endogenous peroxidase activity was blocked by 3% H₂O₂ for 5 min. Then, the sections were incubated with a streptavidin horse-radish peroxidase solution for 30 min. After rinsing with PBS, peroxidase activity in the samples was revealed with a solution of 3,3-diaminobenzidine-H₂O₂. Finally, the sections were rinsed in distilled H₂O and counterstained with 0.1% methyl green for 10 min. Following rinses in distilled H₂O and butanol, the sections were placed in two changes of xylene for 2 min each, and coverslipped.

RESULTS

Statistical comparison of relative liver weights did not reveal any significant variation among the groups ($P>0.05$) (data not shown). In gross examination of liver tissues, there were no recognizable changes in any of the groups except the 3rd one, which had some degrees of paleness.

In microscopic view, normal liver architecture was observed in the control and the 1st group (*Fig 1a-b*). There was only mild hydropic degeneration in the 2nd group (*Fig 1c*). In the 3rd group, moderate hydropic degeneration that was characterized by the partial loss of liver cords and cloudy appearance of some hepatocytes throughout the liver zones were observed (*Fig 1d*). In addition, spaces of Disse were slightly distended, and there was a mild increase in the numbers of Kupffer cells.

Immunoreactivity against iNOS antibody was observed on the hepatocytes with varying intensities among the groups. There was little immunoreactivity in the control group. Immunostaining was diffuse throughout the liver zones, and there was little variation in the intensity of the staining among the cells (*Fig 2a*). In the 1st group, a mild immunoreactivity with the same pattern as described in the control group was observed (*Fig 2b*). A moderate immuno-

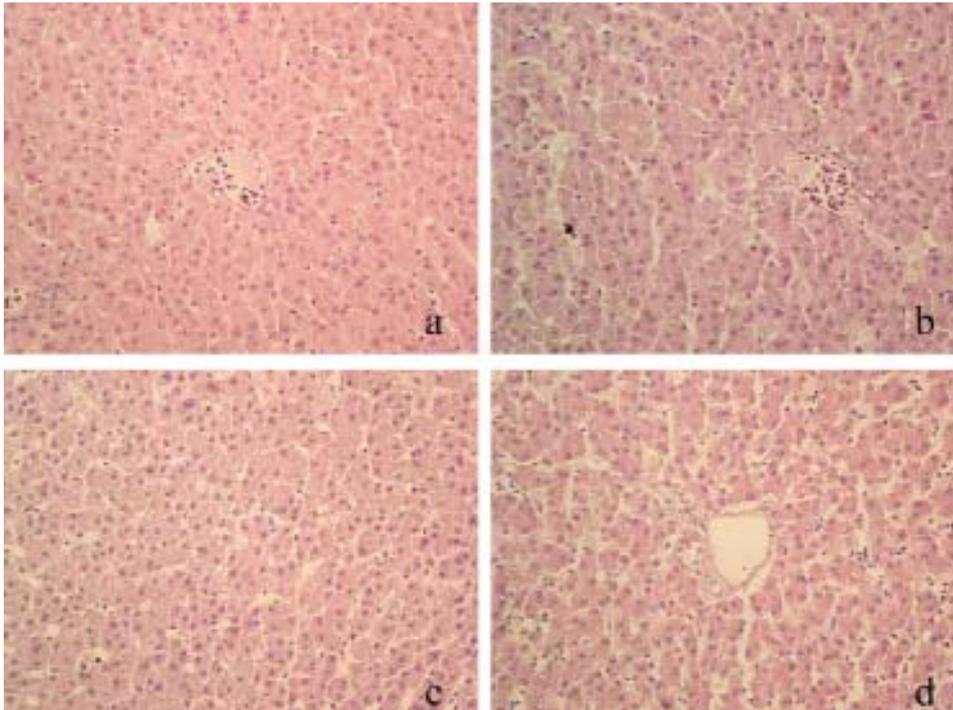


Fig 1. Representative micrographs of livers from the control (a), 1st (b), 2nd (c) and 3rd (d) groups. Mild and moderate hydropic degeneration is present in the 2nd and 3rd groups, respectively. Hematoxylin and Eosin. x370

Şekil 1. Kontrol (a), 1 (b), 2 (c) ve 3. (d) grupların karaciğer örneklerini gösteren resimler. İkinci grupta hafif ve 3. grupta orta derecede hidropik dejenerasyon. Hematoksilen ve Eozin. x370

reactivity with more variation in the staining intensity among hepatocytes was determined in the 2nd group. That is, some hepatocytes were stained more intensely than others in this group (Fig 2c). There was strong immunoreactivity against iNOS antibody in the 3rd group. Immunostaining in this group was diffuse and did not show any variation among hepatocytes (Fig 2d).

Immunoreactivity against nitrotyrosine antibody generally showed the same pattern of immunostaining observed for iNOS in the groups. In general, there was very little immunostaining in the control group (Fig 3a) whereas little to mild immunostaining was present in the 1st group (Fig 3b). Moderate and strong immunoreactivity were detected in the 2nd (Fig 3c) and 3rd (Fig 3d) groups, respectively.

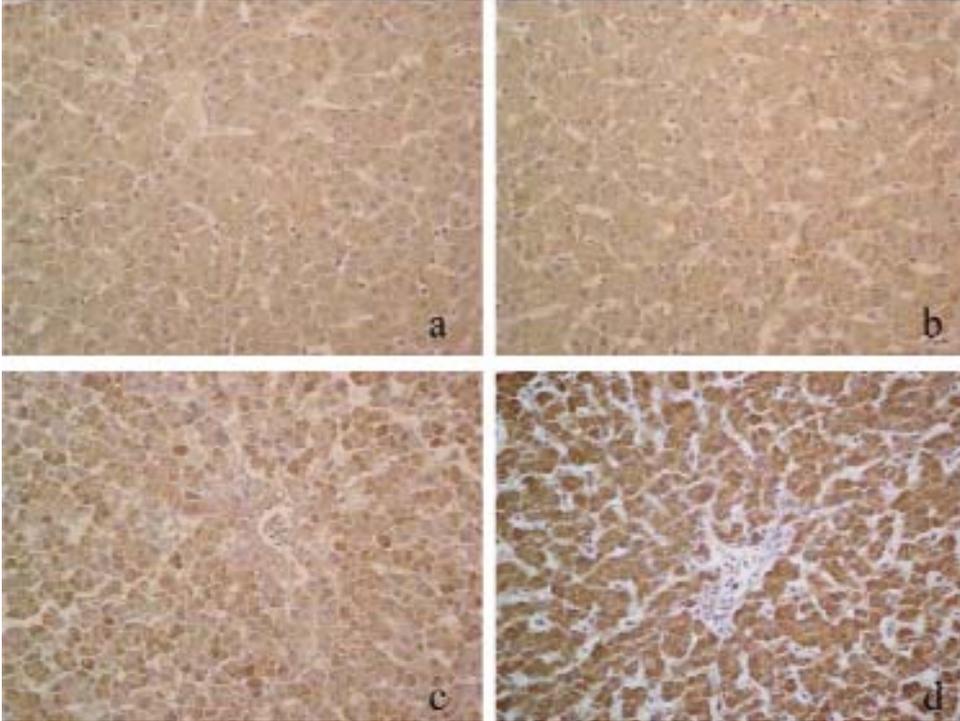
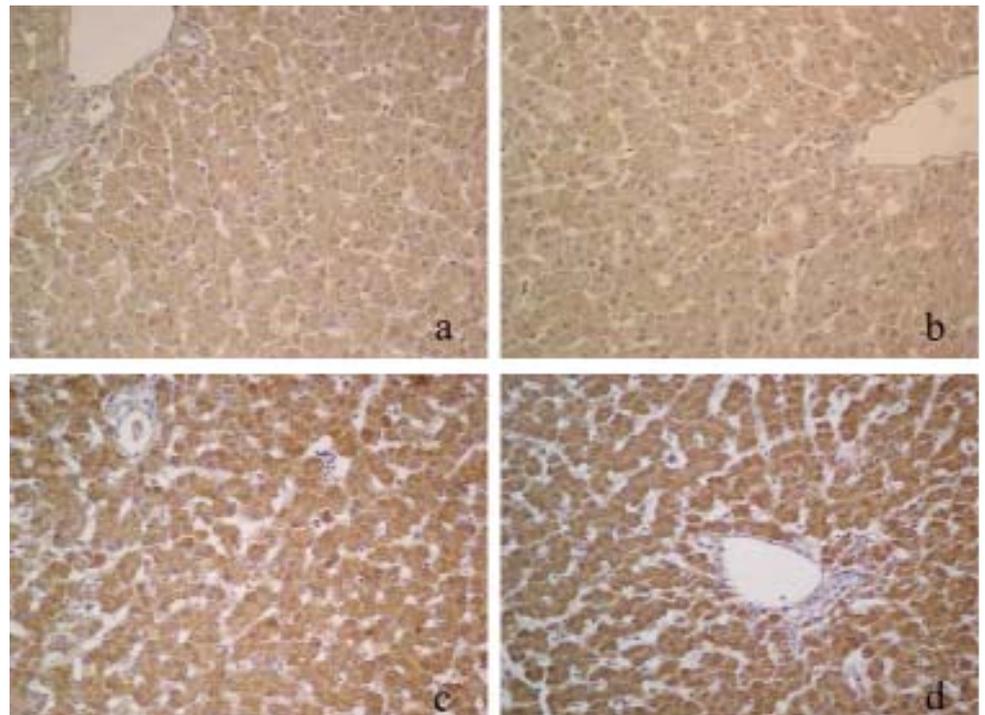


Fig 2. Immunostaining for inducible nitric oxide synthase (iNOS). Note that while there is little immunoreactivity in the control group (a) a gradual increase in the immunoreactivity is present in the 1st (b), 2nd (c), and 3rd (d) groups. x370

Şekil 2. İndüklenebilir nitrik oksit sentaz (iNOS) immunboyaması. Kontrol (a) grubundaki hafif immun reaktiviteye karşılık 1 (b), 2 (c) ve 3. (d) gruplarda artan immun reaktivite. x370

Fig 3. Immunostaining for nitrotyrosine. While there is little immunoreactivity in the control (a) and 1st (b) groups, moderate and strong immunoreactivity is present in the 2nd (c) and 3rd (d) groups, respectively. x370

Şekil 3. Nitrotirozin immun boyaması. Kontrol (a) ve 1. (b) grupta hafif, 2. (c) grupta orta ve 3. (d) grupta şiddetli immun reaktivite. x370



In *in situ* TUNEL staining, few cells with positive reactivity were observed in the liver sections of only the 3rd group (Fig 4). These stained cells showed no specific location throughout the sections. There were no recognizable apoptotic cells in the other groups.

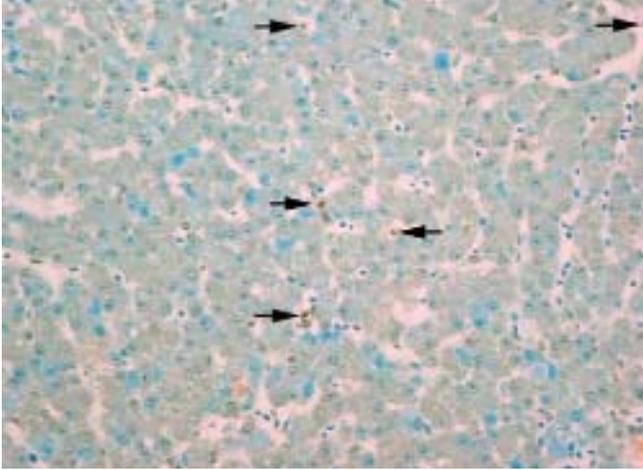


Fig 4. Few apoptotic hepatocytes (arrows) detected by *in situ* TUNEL staining in the 3rd group. x370

Şekil 4. *In situ* TUNEL boyaması ile 3. grupta belirlenen az sayıdaki apoptotik hepatositler (oklar). x370

DISCUSSION

Copper is an imperative mineral for health, but its excess might cause toxicity. However, there are differences in tolerance to copper toxicity among various species. Ruminants are the most susceptible species to copper toxicity. Among them, sheep are the most susceptible since as low as 25 ppm of copper can cause toxicity in these animals³. Comparably, chronic copper toxicity can occur in cattle fed with a ration containing 4 or 5 fold of the required amount^{16,17}. Other ruminants also seem to be highly susceptible to copper toxicity since llamas that had a diet containing 36 ppm of copper had suffered from toxicity¹⁸. Non-ruminants are more resistant to copper toxicity. Pigs can easily tolerate as high as 250 ppm of copper in diet¹⁹. The differences in tolerance to copper toxicity between ruminants and non-ruminants were proposed to be due to the differences in sulfur metabolism⁵.

Copper is routinely added in the amounts of 100 to 250 ppm to poultry rations to improve growth rate and as an anti-fungal agent. It has been shown that copper sulfate toxicity, which is accompanied by oral ulcers, reduced feed intake and a drop in egg

production in commercial laying hens could be produced with as high as 1437 ppm of copper in the ration⁴. Therefore, poultry animals seem to be the most resistant to copper toxicity. On the other hand, copper requirement for Japanese quails was reported to be 5 mg/kg/day and this requirement can be easily provided from daily nutrition²⁰. Knowing that copper is added to poultry diets routinely, we wanted to test if toxic effects occur in these animals.

In the present study, copper induced toxicity in Japanese quails was investigated immunohisto-pathologically. Three different copper doses, 100, 250 and 500 ppm, were investigated for the potential pathological changes in the liver of these chicks since it has been known that after intake liver is the main deposition site of copper²¹. It was found that 250 and 500 ppm of copper in diet cause mild and moderate hepatic degeneration, respectively. In contrary to many reports in mammalian species, a centrilobular pattern of degeneration was not observed in the current investigation even with the highest tested dose of copper^{7,22,23}. Although degenerative changes were observed in the 2nd and 3rd groups, necrosis was not seen in any of the animals in these groups. The reason for this might be that the doses, even the highest one, were not strong enough to cause necrosis and only able to produce reactive oxygen species that lead to mild and moderate degenerative changes.

Apoptosis was previously reported to occur during copper induced toxicity²²⁻²⁴. We also showed apoptotic hepatocytes in chicks fed with 500 ppm of copper sulfate. However, there were only few of these cells indicating that Japanese quails, and most probably other poultry animals, are more resistant to copper toxicity.

Nitric oxide-dependent pathology in liver was investigated in the copper toxicity using antibodies against iNOS and nitrotyrosine. A dose dependent immunoreactivity against both antibodies was observed. In the 3rd group, strong immunoreactivity against both antibodies, contrary to moderate degeneration observed by hematoxylin and eosin stain, is an indication to estimate the level of tissue damage. NO was previously reported to be generated from L-arginine by the catalytic action of iNOS²⁵. NO could react with some oxygen free radicals such as superoxide and produce peroxynitrite, which is highly cytotoxic²⁶. Peroxynitrite could either cause tyrosine nitration and hence loss of function of the proteins or be parted to yield NO₂ or NO₃ that might further cause DNA

damage²⁷. Nitrated tyrosine residues can be detected by anti-nitrotyrosine antibodies and hence can show NO generation.

As previously proposed, copper associated tissue damage had most probably occurred through reactive oxygen species in these animals^{12,15,28}. Hence, oxidative stress mediated DNA strand breaks and oxidation of bases resulted in apoptosis though less severely. Since the oxidative stress was not strong enough to cause severe tissue damage routine histopathological investigation of the liver sections was not enough to estimate the extent of the tissue injury, especially in the lower doses of copper. Therefore, markers of oxidative tissue stress had to be tested.

In conclusion, we have shown that NO play an important role in the copper induced toxicity of Japanese quails. Oxidative stress induced by the high level of copper results in production of NO that can be indirectly detected by antibodies against iNOS and nitrotyrosine. Degenerative changes, as well as apoptotic cell death, in the liver of these chicks are mainly dependent on the dosage of copper. Moderate hepatic degeneration observed with even the highest dose of 500 ppm tested is a confirmation that poultry animals are highly resistant to copper toxicity.

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