The Seoul Journal of Medicine
Vol. 34, No. 2: 117-125, June 1993

Effect of Red Pepper and Bracken Fern on Gastroduodenal Carcinogenesis in Wistar Rats Induced by N-Methyl-N′-nitro-N-nitrosoguanidine

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=Abstract= **Epidemiologic and experimental studies have shown that gastric carcinogenesis is strongly associated with dietary habits. This experiment was performed to analyze the effects of red pepper and bracken fern on gastroduodenal carcinogenesis by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) in vivo. Male Wistar rats were divided into 6 groups: control group (A), red pepper only treated group (B), bracken fern only treated group (C), MNNG only treated group (D), MNNG and red pepper treated group (E) and MNNG and bracken fern treated group (F). The control group was supplied with a normal diet and distilled water. MNNG was given at a concentration of 100 μg/ml in distilled water for D, E and F groups, supplemented with 3% red pepper for E group and supplemented with 5% bracken fern for F group. Three percent red pepper in diet was given to B group and five percent bracken fern in diet was given to C group. These diet regimens were given ad libitum continuously for 40 weeks. Histopathological examination was performed after 40 weeks of experiment. Tumors occurred only in MNNG administered groups. There were no differences in the occurrence rates of adenocarcinoma of the glandular stomach between D, E and F group (78.3%, 70.8%, 73.9%). There was a significantly higher occurrence rate of adenocarcinoma of the duodenum in E and F groups (41.7%, 30.4%) than in the D group (4.3%) ($P<0.05$). These results suggest that red pepper and bracken fern have a promoting effect on duodenal carcinogenesis by MNNG.**

Key Words: *Chemical carcinogenesis, MNNG, Red pepper, Bracken fern*

INTRODUCTION

Stomach cancer is the most common malignant tumor in Korea. In Korean men, its relative frequency is 29.2% of all malignant tumors and it is the most frequent one. In Korean

Received March 1993, and in final form June 1993.
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women, its relative frequency is 18.1% of all malignant tumors and it is the most frequent after uterine cervix cancer. The stomach cancer death rate among Korean people is reported as 32.7 per 100,000 persons (EPB, Economic Planning Board 1987). But in the western countries, its occurrence rate is in decreasing tendency (Silverberg 1984).

There have been many studies to elucidate the relationship between the occurrence of stomach cancer and several kinds of carcinogenic agents, but there are many limitations to find the causes of stomach cancer because its occurrence is affected by many factors and we need long periods of observation. The relationship between stomach cancer occurrence and environmental factors was evaluated by regional differences and epidemiologic investigations (Haenszel *et al.* 1972). These epidemiologic investigations revealed that food has the greatest effect on stomach carcinogenesis.

It has been believed that there are different kinds of carcinogenic agents in different races but many authors think that nitrosamides are the most important carcinogenic agents in the stomach (Haenszel *et al.* 1975). Nitrates which are rich in the food are reduced to nitrites by intestinal flora and these nitrites combine with amines or amides and become nitroso compound like nitrosamines or nitrosamides. These nitroso compounds were noted to have a carcinogenic effect and especially nitrosamides were confirmed to cause stomach cancer in experimental animals (Hill 1981). The epidemiological study also showed the importance of nitroso compound in stomach carcinogenesis (Doll 1956; Hartman 1983).

Several studies were tried to compare certain factors in animal models of experimental stomach cancer. 20-Methylcholanthrene was the first agent to produce adenocarcinoma in the stomach, and N,N′-2,7- fluorenylene bis-acetamide, 4-nitroquinoline-1-oxide, N-methyl-N-nitrourethane and N-alkyl-N-nitrourethane followed. Especially it was found N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) could easily produce adenocarcinoma in the animal intestine (Shoental 1966). Sugimura *et al.* (1967, 1969, 1970) fed MNNG as solution to experimental animals and produced stomach cancer easily. Now we regard this as a standard method for experimental stomach carcinogenesis (Bralow *et al.* 1970).

Nitroso compounds cause cancer cells by way of initiation and promotion process (Becker 1981). Reactive moieties released from carcinogenic agents have electrophilic properties and act on DNA, RNA, protein, glutathione and polysaccharides then cause mutation on genes which we call the initiation process. The promoter is the substance which promotes cancer or tumor occurrence in the tissue that has been exposed to carcinogenic agent and has changes in DNA. It is also very important in cancer occurrence and growth (Farber 1981).

It is believed that nitroso compound can not reach mucosal cells due to antioxidants in food or the mucous barrier in the mucosa. This barrier could be broken by hard cereals, salty food and surfactants. Once the first mutation occurs, the glandular gastric epithelium is gradually changed to intestinal type epithelium, the mucous barrier altered, and the pH elevated. Under these conditions, bacteria proliferate in the gastric cavity and facilitate the conversion of nitrates to nitrites, thereby increasing the nitrite pool and the probability of formation of mutagenic-carcinogenic nitroso compounds. In this stage, nitroso compounds could easily approach gastric mucosa (Correa *et al.* 1975).

Several studies were done using MNNG on cancer promotion or suppression by foods, red pepper (Park *et al.* 1980) and salty foods (Lee *et al.* 1985) on stomach cancer promotion, Maejoo (Korean fermented soy bean cake) (Lee *et al.* 1982), Ginseng extract (Han *et al.* 1983) and milk (Han *et al.* 1985) on stomach cancer suppression.

In our experiment, we used two kinds of foods, one was red pepper and the other

bracken fern. Koreans consume a lot of red pepper in their diet. There have been reports that long term administration of red pepper on rabbits caused multiplication of gastric epithelium, infiltration of inflammatory cells, edematous change, mucosal sloughing and ulcers (Han 1961; Lee 1963). It has been known that bracken fern is a kind of toxic plant since the end of the 19th century. It was reported that cows fed with bracken fern developed high fever, bleeding diathesis and critical illness. Bladder cancer was also found in the cows with large amounts of bracken fern ingestion (Pamukcu 1963). Its carcinogenicity was confirmed in other experimental animals and its primary involving sites were the bladder and small intestine (Price and Pamukcu 1968). It is known that two kinds of toxic materials exist in it. One is carcinogenic substance and the other is thiamine dissolving factor. Still we don't know exactly which component could produce cancer (Pamukcu *et al.* 1970).

Our experiment was conducted to evaluate the enhancement of red pepper and bracken fern on gastroduodenal carcinogenesis induced by MNNG in male Wistar rats.

MATERIALS AND METHODS

Six-week-old male Wistar rats weighing about 130 to 150 gm (Seoul National University Laboratory Animals) were housed in plastic cages, 4 rats/cage. MNNG (Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in dimethylsulfoxide (DMSO) at 100 mg/ml for storage and everyday it was dissolved in distilled water at 100 μg/ml. It was given to rats ad libitum. The diet was standard rat feeding formula and was purchased from Samyang Oil and Food Co. Ltd., Korea. In the red pepper feeding groups, it was given as 3% mixture in weight with standard diet. In the bracken fern feeding groups, it was given as 5% mixture in weight with standard diet. These experimental diets were given ad libitum.

One hundred and fifty-five rats were divided into 6 experimental groups. A group was given a standard diet and distilled water, B group was given a standard diet with 3% red pepper and distilled water, C group was given a standard diet with 5% bracken fern and distilled water, D group was given a standard diet and MNNG, E group was given a standard diet with 3% red pepper and MNNG, F group was given a standard diet with 5% bracken fern and MNNG. The rats were distributed as 18, 28, 27, 27, 27 and 28, respectively.

MNNG and the diets were given continuously for 40 weeks and the rats were sacrificed at the 41st week. The sacrificed rats were autopsied to find any cancer occurrences, especially in the upper gastrointestinal tracts. The stomach and upper intestine were opened along the greater curvature, pinned flat on a cork board and fixed with 10% neutralized formalin. The fixed stomach and upper intestine were photographed and cut along the lesser curvature into strips (4 mm), embedded in paraffin, and cut into 5-6 μm thickness. The sections were stained with hematoxylin and eosin, alcian blue-PAS and Masson trichrome.

The results were analyzed by means of chi-square and ANOVA tests of the SPSS program on a personal computer.

RESULTS

Non-MNNG treated groups had a statistically significant weight gain compared to MNNG treated groups. But in each MNNG treated group, there were no statistically significant differences in weight gain.

During the whole experimental period, 21 rats died; in the A, B, C, D, E, F groups, 3, 3, 3, 4, 3, 5 rats, respectively. Survival rates after 40 weeks of experiment were 83.3%, 89.3%, 88.9%, 85.2%, 88.9%, 82.1%, respectively. There were no statistical differences in survival rate among them.

We could find many tumors in the MNNG treated groups but none in the non-MNNG treated groups. All the tumors were located in

the glandular stomach and duodenum. Tumors in the glandular stomach were located at the lesser curvature side (Fig. 1). Average tumor size in each group was from 0.56 cm to 0.76 cm (Table 3). The shape of the tumors was variable, such as polypoid protrusion, excavation and elevated tumor with central ulceration. All the tumors in the glandular stomach occurred singly, but more than two lesions were found in two cases of duodenal tumors (Fig. 2).

All the tumors were evaluated under the microscope after cutting and staining process. The occurrence pattern of adenocarcinoma and sarcoma is shown in Table 1. The occurrence

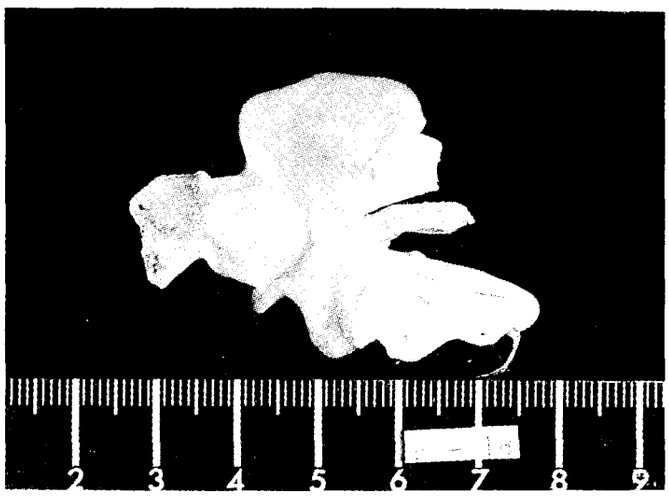

Fig. 1. Gross finding of adenocarcinoma in the glandular stomach.

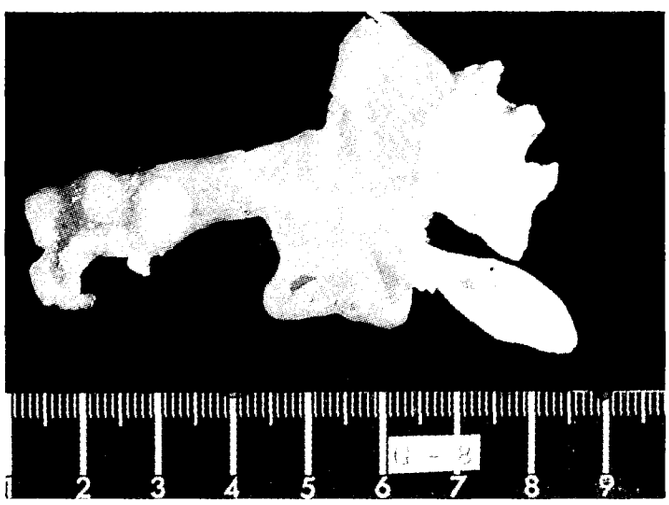

Fig. 2. Gross finding of double duodenal adenocarcinoma.

rates of adenocarcinoma in the glandular stomach were MNNG only treated group (D), 18/23 (78.3%); MNNG with red pepper treated group (E), 17/24 (70.8%); MNNG with bracken fern treated group (F), 17/23 (73.9%). These findings revealed similar cancer occurrence in the D, E and F groups. The average size of adenocarcinoma of the glandular stomach in the D, E and F groups was 0.67 ± 0.27 cm, 0.56 ± 0.22 cm, 0.76 ± 0.33 cm, respectively. Cases of more than propria muscle involvement in the glandular stomach in the the D, E and F groups were 11/18 (61.1%), 13/17 (76.5%), 13/17 (76.5%), respectively. These showed slightly increased cancer penetration in the E and F groups than that of the D group, but they were not statistically significant ($P>0.05$)(Table 2).

Occurrences of adenocarcinoma in the duodenum in the D, E and F groups were 1/23 (4.3%), 10/24 (41.7%), 7/23 (30.4%), respectively. These showed statistically significant differences between D and E group and between D and F group ($P<0.05$). In the E group, there was one case of 3 lesions of duodenal adenocarcinoma and another case of 2 lesions of that. Counting all duodenal cancer lesions in the D, E and F groups, there were 1, 13, 7 lesions, respectively. The size of duodenal adenocarcinoma in the D, E and F groups was 1.5 cm, 0.72 ± 0.42 cm, 0.76 ± 0.14 cm, respectively. Cases of more than propria muscle involvement were 1/1, 11/13, 6/7, respectively (Table 3). Counting all stomach and duodenal carcinomas in the D, E and F groups, there were 19, 27, 24 lesions, respectively. These showed higher occurrence of adenocarcinoma in the E and F groups than that of the D group but showed no statistical significance ($p>0.05$).

Pathologic findings in the glandular stomach were variable from well differentiated to poorly differentiated adenocarcinoma but moderately differentiated was the most frequent (Fig. 3, 4). In the well differentiated adenocarcinoma, there were well organized glands without mucus formation or necrosis. In poorly differentiated adenocarcinoma, it showed ill defined, deformed

Table 1. Occurrence of gastroduodenal malignancy in each group

Group	Total No.	Glandular stomach		Duodenum		Total	
		Carcinoma	Sarcoma	Carcinoma	Sarcoma	Carcinoma	Sarcoma
Control	15	0	0	0	0	0	0
Red pepper	25	0	0	0	0	0	0
Fern	24	0	0	0	0	0	0
MNNG	23	18	1	1	1	19	2
MNNG+ red pepper	24	17	1	10(13)	0	27	1
MNNG+ fern	23	17	1	7	0	24	1

*( ): No. of sites

Table 2. Profiles of adenocarcinoma of glandular stomach

Group	Total No.	Case of cancer(%)	Size mean (cm)	S.D.# (cm)	Cancer..depth over P.M.a(%)
Control	15	0			
Red pepper	25	0			
Fern	24	0			
MNNG	23	18(78.3)	0.67	±0.27	11(61.1)
MNNG+ red pepper	24	17(70.8)	0.56	±0.22	13(76.5)
MNNG+ fern	23	17(73.9)	0.76	±0.33	13(76.5)

#S.D.: Standard deviation of size

aP.M.: *Propria muscle*

Table 3. Profiles of adenocarcinoma of duodenum

Grooup	Total No.	Case of cancer(%)	No. of lesions	Size mean (cm)	S.D.# (cm)	Cancer..depth over P.M.a(%)
Control	15	0				
Red pepper	25	0				
Fern	24	0				
MNNG	23	1(4.3)	1	1.5		1(100)
MNNG+ red pepper	24	10(41.7)	13	0.72	±0.42	11(84.6)
MNNG+ fern	23	7(30.4)	7	0.76	±0.14	6(85.7)

#S.D.: Standard deviation of size

aP.M.: Propria muscle

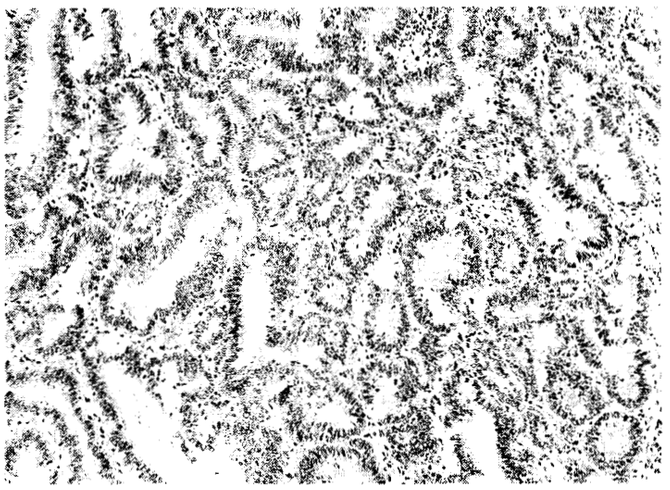

Fig. 3. Well differentiated adenocarcinoma of the stomach. The neoplastic glands are lined by uniform columnar cells. Each gland is separated by its own basement membrane (HE, × 100).

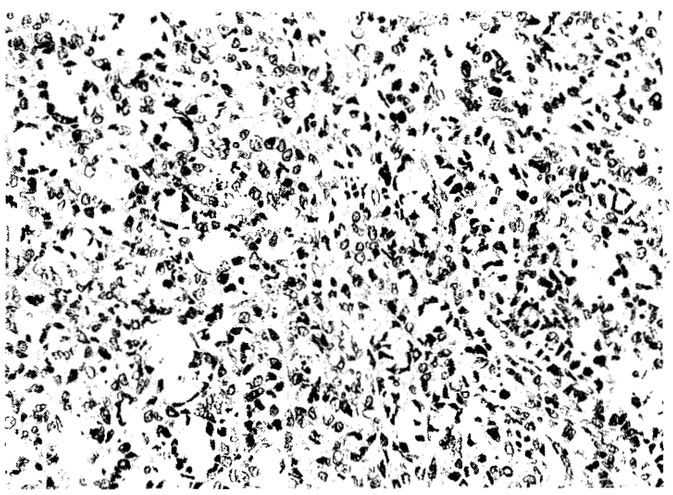

Fig. 4. Poorly differentiated adenocarcinoma of the stomach with the feature of signet ring cell carcinoma. The neoplastic cells have abundant intracellular mucin and are seldom arranged in glandular pattern (HE, × 400).

gland structure, profuse connective tissue, occasional mucus lakes, scattered necrotic areas and occasional signet ring cells. Around small cancer growth, it was surrounded by lymphocytes and stromal cells. There were many inflammatory cells inside the tumors. There were 7 cases of calcifications, all were in the glandular stomach.

All of the cancers in the duodenum were adenocarcinoma histologically. They infiltrated deeply the propria muscle and serosa. The deeper they infiltrated, the larger the mucoid amount was.

Sarcoma occurred both in the glandular stomach and duodenum. There were no histological differences between them. Under microscope, sarcoma was composed of spindle cells with hyperchromatic nuclei and numerous mitotic features.

DISCUSSION

The experiments were conducted using Wistar rats to evaluate the effect of red pepper and bracken fern on MNNG induced gastroduodenal adenocarcinoma. It was reported that MNNG responses were different among different species. Adenocarcinoma occurred in 12% of Wistar-Mai-Furth rats, but sarcoma occurred only in 4% of Buffalo-Mai rats after 52 weeks of MNNG administration. In Wistar rats, gastric cancers occurred 70-80% of the rats with 83 mg/L of MNNG given for 25-52 weeks duration after 6 weeks of birth (Bralow *et al.* 1971). Thus Wistar rats were recommended for the experimental model of gastric cancer. It was reported that the younger the rats were, the more occurrences of gastric cancer by MNNG were (Kimura *et al.* 1975; Saito and Inokuchi 1975). We used 6 week old rats and distributed randomly to each group. There were no statistical differences in the average weight of each group. MNNG dissolved in drinking water was used for the experiment of gastroduodenal carcinogenesis at a concentration between 83 μg/ml and 163 μg/ml. Adenocarcinoma occurred more than 30 weeks after MNNG administration (Sugimura *et al.* 1969; Saito *et al.* 1970). We did our experiments using 100 μg/ml of MNNG concentration and a 40 weeks observation period.

Park *et al.* (1980) experimented with MNNG and red pepper. They used 1% red pepper in a regular diet for an initial 20 weeks and 3% for another 17 weeks. We used 3% red pepper for

the whole experimental period. But there was no feeding problem or difficulty in growth. Bracken fern was fed solely for carcinogenic experiments at a concentration of 25 to 33% of regular diet (Pamukcu *et al.* 1970; Woo and Hahn 1975). In our experiments, we used 5% bracken fern in a regular diet because we used MNNG and bracken fern simultaneously.

During the 40 weeks of the experiment, 21 rats died; 3, 3, 3, 4, 3, 5 rats in A, B, C, D, E and F group, respectively. We found that the mortality rate in each group was about the same and MNNG was effective for carcinogenic experiments without influencing the survival of the rats for the 40 weeks of the experiment.

In our experiment, we found more than 70% of adenocarcinomas in the glandular stomach in D, E and F groups. We thought that the effect of MNNG was so strong that red pepper and bracken fern could not influence the stomach. In the duodenum, we found statistically significant increases of adenocarcinomas in E and F groups compared to the D group, this suggested that there were cancer promoting effects in red pepper and bracken fern.

In 1775, Dr. Pott in England reported testicular malignancy in chimney sweepers and then there was interest in the chemical carcinogenesis. Several kinds of chemical agents were reported to have carcinogenic effects, such as polyaromatic hydrocarbon derived from coal tar, benzo(a)pyrene, anthracene, azo dye, 2- acetylaminofluorene, 2-naphthylamine, 4-aminobiphenyl and ethyl carbamate. Especially nitrosamines or nitrosamides of the dialkylnitrosamine group were confirmed to have carcinogenic effects in the gastrointestinal tract.

Hot tasting element in the red pepper was purified and named capsaicin by Thresh. The molecular formula of it was proved to $C_{18}H_{28}NO_3$. Several elements are contained in the red pepper other than the capsaicin, such as carotenoid dye, sterol, fatty acid and wax. In the red pepper, the most important characteristics come from the capsaicin. Several authors reported that the red pepper could cause gastric mucosal edema, hyperemia, atrophy of glands and hypertrophy of mucosa in the animal experiments. The capsaicin has been extensively studied on the physiological influence on the stomach. The capsaicin increased acid output in the stomach by way of increasing mucosal blood flow (Kang *et al.* 1992). The mechanism of increased gastric mucosal blood flow was explained by the stimulation of capsaicin-sensitive sensory neuron in the stomach (Takeuchi *et al.* 1992). There were several reports of its mucosal damage. The administration of capsaicin markedly enhanced gastric mucosal damage by intragastric indomethacin (Holzer *et al.* 1987) or ethanol (Peskar *et al.* 1992). The state of increased gastric mucosal blood flow might be vulnerable to gastric irritants. This mechanism could explain possible co-carcinogenic effect of the capsaicin.

In our experiment, the red pepper didn't show any cancer promoting effect in the glandular stomach, but did show promoting effect in the duodenum. This suggested red pepper could have a role as a promoter. We think that the capsaicin in red pepper irritates stomach and duodenal mucosa and increases cancer occurrence as a promoter.

From the ancient times, bracken fern has been known to cause beriberi. Several studies confirmed that bracken fern has a thiamine reducing enzyme. Bladder cancer was found in cows fed with bracken fern and several studies had been performed on carcinogenesis of bracken fern. Experiments until now proved bladder cancer occurred in cows, rats and guinea pigs and intestinal adenocarcinoma in rats. It has been known that it contains astragalin, isoquercitrin, rutin, catecholtannins, pteraquilin, sugar, starch, aliphatic nondrying oil, pectose mucin and so forth, but still we don't know which is carcinogenic. Until now, animal experiments with bracken fern for elucidating its carcinogenic property had been performed in more than 25% of a regular diet and more than 10 months of feeding. We fed a low dose (5%) of

bracken fern in MNNG given rats to see its cancer promoting effect in the the stomach and duodenum. We didn't find any cancer promoting effect in the stomach but did find increased cancer occurrences in the duodenum. This suggests that bracken fern could promote cancer in chemical carcinogenesis.

In our experiment, we had some difficulties in evaluating cancer promoting effect because a lot of gastric adenocarcinomas occurred in the MNNG only administered comparison group. Fujimura *et al.* (1970) reported that many adenocarcinomas occurred in the small intestine and mesentery after 7 months of MNNG administration at a concentration of 83 μg/ml in drinking water. In our experiments, more than 70% of gastric adenocarcinomas occurred after 10 months of 100 μg/ml of MNNG administration, and we think that many duodenal adenocarcinomas occurred by the promoting effect of red pepper and bracken fern.

Our experiment used crude forms of red pepper and bracken fern but if we experimented using purified components, we could find which components could initiate or promote, or which components could enhance damage in the DNA damaged by MNNG or could suppress DNA repair. Then we could make a step forward to find the cause of the high occurrence of gastric cancer in the Korean people.

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