Metronidazole enhances steatosis-related early-stage hepatocarcinogenesis in high fat diet-fed rats through DNA double-strand breaks and modulation of autophagy

Ayumi Eguchi 1 · Sayaka Mizukami 1,2 · Misato Nakamura 1 · Sousuke Masuda 1 · Hirotada Murayama 1 · Masashi Kawashima 1 · Mari Inohana 1 · Rei Nagahara 1 · Mio Kobayashi 1 · Risako Yamashita 1 · Suzuki Uomo 1 · Emi Makino 3 · Ryoichi Ohtsuka 3 · Naofumi Takahashi 3 · Shim-Mo Hayashi 4 · Robert R. Maronpot 5 · Makoto Shibutani 1 · Toshinori Yoshida 1

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Abstract
Nonalcoholic fatty liver disease is a hepatic disorder with deposition of fat droplets and has a high risk of progression to steatosis-related hepatitis and irreversible hepatic cancer. Metronidazole (MNZ) is an antiprotozoal and antimicrobial agent widely used to treat patients infected with anaerobic bacteria and intestinal parasites; however, MNZ has also been shown to induce liver tumors in rodents. To investigate the effects of MNZ on steatosis-related early-stage hepatocarcinogenesis, male rats treated with N-nitrosodiethylamine following 2/3 hepatectomy at week 3 were received a control basal diet, high fat diet (HFD), or HFD containing 0.5% MNZ. The HFD induced obesity and steatosis in the liver, accompanied by altered expression of Pparg and Fasn, genes related to lipid metabolism. MNZ increased nuclear translocation of lipid metabolism-related transcription factor peroxisome proliferator-activated receptor gamma in hepatocytes, together with altered liver expression of lipid metabolism genes (Srebf1, Srebf2, Pnpla2). Furthermore, MNZ significantly increased the number of preneoplastic liver foci, accompanied by DNA double-strand breaks and late-stage autophagy inhibition, as reflected by increased levels of γ-H2AX, LC3, and p62. Therefore, MNZ could induce steatosis-related hepatocarcinogenesis by inducing DNA double-strand breaks and modulating autophagy in HFD-fed rats.

Keywords γ-H2AX · LC3 · Metronidazole · NAFLD · p62 · PPARγ

Introduction
Metronidazole (2-(2-methyl-5-nitroimidazol-1-yl) ethanol, MNZ) is an anti-bacterial or anti-protozoal drug widely used in humans and animals. MNZ is usually administered either alone or together with other antibiotics to treat dracunculiasis, giardiasis, trichomoniasis, amebiasis, and other anaerobic bacterial and parasitic infections (Abdel-Magied et al. 2017; Freeman et al. 1997; Leitsch 2017). After oral administration, MNZ is taken up by the mucosal epithelia via diffusion and is then selectively absorbed by the anaerobic bacteria and sensitive protozoa as an inactive pro-drug (Ligha and Paul 2011). In these pathogens, the nitro group of the drug is partially reduced to produce cytotoxic and mutagenic metabolites, facilitating their antibacterial action through single- or double-strand breakage and destabilization of DNA (Sisson et al. 2000).
MNZ is a potent mutagen in bacteria (Cantelli-Forti et al. 1983; Mohn et al. 1979); however, data in human cells remain controversial (Menéndez et al. 2001). While no DNA strand breaks were reported in peripheral lymphocytes isolated from patients after the MNZ therapy (Fahrig and Engelke 1997), inducible DNA damage was described in peripheral lymphocytes isolated from healthy volunteers following MNZ treatment at selected dose levels (Ré et al. 1997; Reitz et al. 1991). Oxidative stress could be one of the factors involved in genotoxicity, since supplementation with vitamin C, catalase, and superoxide dismutase elicited protective responses against DNA damage induced by MNZ in human lymphocytes (Ré et al. 1997). Earlier reports on carcinogenicity demonstrated that MNZ significantly increased the incidence of lymphomas and lung tumors in mice (Cavaliere et al. 1983) as well as pituitary, testicular, and hepatic tumors in rats (Rustia and Shubik 1979). Therefore, the International Agency for Research on Cancer classified MNZ as a Group 2B agent, identifying it as possibly carcinogenic to humans (IARC 1987). In the USA, MNZ administration is limited to animals not intended for human consumption, and the European Union also prohibits administration of MNZ in animals (APVMA 2007; EU 2010). However, MNZ has been reported to pollute aquatic environment worldwide; it was detected in tap water, creating an unpredictable route for human exposure (Leung et al. 2013).

Hepatocellular carcinoma (HCC) with increasing incidence rates is the second leading cause of cancer death (Charrez et al. 2016; Starley et al. 2010). Nonalcoholic fatty liver disease (NAFLD) refers to a group of hepatic disorders characterized by deposition of excess fat droplets in the hepatocyte without significant alcohol intake (Basaranoglu and Neuschwander-Tetri 2006; Nseir and Mahamid 2013). In most patients, the liver disease is benign; however, some patients may advance to nonalcoholic steatohepatitis (NASH), the more progressive form of the disease (Basaranoglu and Neuschwander-Tetri 2006). NASH increases the risk of progression to irreversible hepatic disorders, including HCC (Starley et al. 2010; Charrez et al. 2016). To study the molecular mechanisms of NAFLD, many genetic and/or dietary rodent models, such as leptin-deficient mice, and high fat diet (HFD)-received mice and rats have been established (Takahashi et al. 2012). However, studies on steatosis-related hepatocarcinogenesis have been limited (Wu 2016). We recently developed a steatosis-related early hepatocarcinogenicity rat model based on a medium-term liver bioassay (Ito et al. 2003) that allowed us to evaluate the effects of carcinogenic chemicals on the interconnection between steatosis and precancerous foci (Yoshida et al. 2017; Murayama et al. 2018). Apart from having antibacterial activity, MNZ has also been reported to suppress hepatic steatosis or hyperlipidemia in patients with intestinal bypass surgery (Drenick et al. 1982) and in human volunteers (Shamkhani et al. 2003), respectively, and worsen alcohol pancreatitis-related liver damage in rats (Marotta et al. 2005). Currently, the precise mechanisms underlying how MNZ induces hepatic tumors and modulates steatosis remain unknown.

Autophagy plays a key role in the pathogenesis of steatosis (Lavallard and Gual 2014). Suppression of lipophagy, a type of autophagy, was shown to enhance steatosis in Atg5 knockdown models (Singh et al. 2009). In NASH patients and HFD-fed mice, microtubule-associated protein 1A/1B light chain 3 (LC3), the autophagosome marker, and p62, the autophagic flux marker that binds to LC3, were highly expressed in hepatic samples (González-Rodríguez et al. 2014; Zhang et al. 2016). We also confirmed the specific expression of both autophagosome markers in preneoplastic hepatic foci in HFD-fed rats (Masuda et al. 2019). In addition to steatosis, autophagy also plays a role in DNA damage response (DDR). A variety of chemicals induce DNA damage and can trigger DDR, including DNA repair, regulation of cell cycle checkpoint, and apoptosis (Czarny et al. 2015). Autophagy can also protect cancer cells against anti-cancer drug-mediated DNA damage. Inhibition of autophagy can sensitize cancer cells to drugs; however, this can lead to chromosomal instability, which in turn may activate oncogenes and induce tumor progression (Amaravadi et al. 2007; Czarny et al. 2015).

Here, we examined the effects of MNZ on steatosis and precancerous lesions in HFD-fed rats and determined the underlying mechanisms of MNZ-induced tumor development and its connection to DNA damage and autophagy.

Materials and methods

Chemicals

N-Nitrosodiethylamine (DEN; CAS No. 55-18-5, purity >99%) and MNZ (CAS No. 443-481) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Sigma-Aldrich Co. LLC. (St. Louis, MO, USA), respectively.

Experimental condition

Fifty-two male F344/N rats were purchased from Japan SLC, Inc. (Shizuoka, Japan), and then acclimatized and maintained in an air-conditioned room (room temperature, 22 ± 3 °C, relative humidity, 50 ± 20%; 12 h light/dark cycle). The animals received a basal diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water freely. A medium-term liver carcinogenesis bioassay (Ito et al. 2003) was conducted in 6-week-old male rats. Male rats were chosen in this model, as male rats were more susceptible to hepatocarcinogens than female rats (Tamano et al. 1983). All rats were injected DEN (200 mg/kg body weight) intraperitoneally at the beginning of the experiment. The control (CTL) group (n = 13) was fed a basal diet (Oriental MF) for 11 weeks. The other groups
were fed a HFD (D12451; Research Diets, Lane, NJ, USA) mixed with 0% (n = 13, HFD group) or 0.5% MNZ (n = 13, HFD + MNZ group) during weeks 2 and 9. All rats had a two-thirds partial hepatectomy at week 3. Initial numbers of rats were 14 in each group; however, one rat each died, caused by partial hepatectomy. The MNZ dose was selected during the preliminary study using 0%, 0.5%, and 1% MNZ in the basal diet. The rats in the 1% MNZ group displayed weight loss and tremors; however, the 0.5% dose did not cause any obvious side effects. Daily clinical observations were conducted during the study, and body weight and intake of water and food were measured weekly. The overnight-fasted rats were sacrificed under isoflurane anesthesia at week 11. Blood was withdrawn from abdominal large vein, and serum was obtained by centrifugation. Liver, cecum, and intraabdominal adipose tissue was ablated, and liver and intraabdominal adipose tissues were weighed at necropsy. For histopathology and immunohistochemistry experiments, liver slices were fixed with 4% paraformaldehyde (dissolved in 0.1 M phosphate-buffered saline, pH 7.4). For RT-PCR, the pieces were stored at −80 °C. Clinical, operative, and experimental procedures were performed according to the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and the study protocol authorized by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Blood biochemistry

Alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP), bilirubin (total, T.BIL; indirect, I.BIL; direct, D.BIL), total cholesterol (T.CHOL), and triglyceride (TG) were measured in serum samples using the JCA-BM1250 automatic analyzer (JEOL, Ltd., Tokyo, Japan).

RT-PCR

Expression levels of mRNA were analyzed in the liver tissues (six animals per group) using real-time RT-PCR (Murayama et al. 2018). The results were normalized to β-actin (Actb). Sequences of PCR primers used in these experiments are summarized in Supplemental Table 1.

Histopathology and immunohistochemistry

Histopathological examination was conducted on hematoxylin and eosin (H&E)-stained liver sections. NAFLD activity score (NAS) including steatotic or ballooning cells and lobular inflammation was evaluated (Kleiner et al. 2005; Nakamura et al. 2018). For immunohistochemistry, the deparaffinized sections were stained (Murayama et al. 2018) (antibodies and experimental conditions are summarized in Supplemental Table 2). The area and number of glutathione S-transferase placental form (GST-P)-expressed foci (>200 μm diameter) per area of the liver sections were quantified as previously reported (Murayama et al. 2018). Ki-67-, PCNA-, active caspase-3-, p22phox-, and gamma-H2A histone family member X (γ-H2AX)-positive hepatocytes were counted in a total of over 1000 cells within GST-P-expressed foci. The number of p62 or LC3 and GST-P double positive foci per total liver areas was also counted. The number of Ki-67-, active caspase-3-, p22phox-, γ-H2AX, LC3-, p62-, and peroxisome proliferator activated-receptor-gamma (PPARγ)-expressed hepatocytes in non-GST-P-expressed foci (outside foci) was measured in ten fields at 400× magnification (10 high power fields, 10HPFs). Cells in each cell cycle, G1, S, G2, or M phases or all phases, were counted in the PCNA-stained sections (Foley et al. 1993).

Statistical analyses

Data are presented as mean and standard deviation. The statistically significant differences between each group were analyzed using multiple comparison tests, Tukey, or Steel-Dwass method at a p value of less than 0.05.

Results

Treatment of metronidazole decreases body weight and adipose tissue weight in HFD-fed rats

Treatment-related clinical symptoms were not observed in any group during the course of the in-life study. The HFD feeding significantly increased final body weight, as well as the absolute and relative weights of the adipose tissue, and significantly decreased absolute and relative weights of the cecum compared with CTL (Table 1). The HFD feeding with or without MNZ also significantly reduced the intake of food and water compared with that in the CTL group. MNZ administration significantly decreased HFD-mediated body weight and adipose tissue weight gain; however, it significantly increased cecum weight. Some significant changes in the liver weights were secondary to the body weight changes (Fig. 1).

Metronidazole treatment does not alter hyperbilirubinemia in HFD-fed rats

The HFD feeding without MNZ significantly reduced ALP and TG and increased bilirubin parameters (T.BIL, I.BIL, D.BIL) compared with those in the CTL group (Supplemental Table 3). MNZ administration alleviated HFD-mediated reduction of TG and increase of D.BIL and significantly decreased ALT levels compared with those in the other groups.
Metronidazole treatment alters mRNA expression levels of fat metabolism- and oxidative stress-related genes

First, we assessed the expression of lipid metabolism-related genes. The HFD feeding significantly increased expression levels of *Pparg* and decreased expression of *Fasn*, compared with that in the CTL group. However, the MNZ treatment lowered HFD-induced *Pparg* expression but had no effect on *Fasn* (Fig. 2A). MNZ administration, in turn, significantly decreased the expression levels of *Pnpla2* and *Srebf2* compared with those in the CTL group, whereas *Srebf1* level was significantly increased compared with that in the HFD group (Fig. 2A). Next, we assessed the expression of the oxidative stress-related genes. MNZ administration significantly decreased the expression levels of *p22phox* relative to CTL and increased the levels of *Catalase* and *Cyp2e1* when compared with those in the CTL and CTL and/or HFD groups, respectively (Fig. 2B). No significant changes were detected in autophagy-related genes (Fig. 2C).

Metronidazole treatment increases preneoplastic liver lesions in HFD-fed rats via induction of DNA double-strand breaks and modulation of autophagy, without altering NAS

Minimal to moderate steatosis, ballooning, and inflammation were detected in liver tissues in all the groups (Fig. 3A–C). Lipid droplet membranes stained positive for adipophilin and the immunoreactivity increased in the HFD groups with or without MNZ (Fig. 3D–F). NAS was significantly increased in both HFD groups (with or without MNZ) compared with that in the CTL group (Fig. 3J). Steatosis (Fig. 3K) and hepatocyte ballooning (data not shown) measurements were significantly increased in a same way. No significant changes were detected when evaluating lobular inflammation in any group (data not shown). PPARγ was expressed both in the cytoplasm and the nuclei of hepatocytes (Fig. 3G–I). Furthermore, the number of PPARγ-positive nuclei was significantly increased in the MNZ group compared with that in the other groups (Fig. 3L). PPARγ was not expressed in the liver foci.

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Table 1  Final body weight, food intake, water intake, and organ weight in rats

| Group      | CTL       | HFD       | HFD+MNZ‡  |
|------------|-----------|-----------|-----------|
| No. of animals | 13        | 13        | 13        |
| Final body weight (g) | 299.8±13.1 | 329.5±13.6 serif | 310.0±16.9 serif |
| Food intake (g/rat/day) | 18±0.8 | 12.7±0.6 serif | 13.3±1.9 serif |
| Water intake (g/rat/day) | 23.3±1.3 | 18.7±0.2 serif | 17.7±0.3 serif |
| Absolute adipose tissue weight (g) | 7.8±0.9 | 12.4±1.5 serif | 10.5±1.4 serif |
| Relative adipose tissue weight (%BW) | 2.6±0.2 | 3.7±0.3 serif | 3.4±0.3 serif |
| Absolute cecum weight (g) | 5.0±0.6 | 3.5±0.6 serif | 11.7±1.3 serif |
| Relative cecum weight (%BW) | 1.7±0.2 | 1.0±0.2 serif | 3.8±0.4 serif |

Data are shown as the mean ± standard deviation

BW body weight, CTL control (basal diet), HFD high fat diet, MNZ metronidazole

§All animals were subjected to a medium-term liver carcinogenesis bioassay

‡Rats were given HFD mixed with MNZ during weeks 2 and 11

* p<0.05 vs CTL (Tukey’s or Steel-Dwass test)

† p<0.05 vs HFD (Tukey’s or Steel-Dwass test)
Fig. 2  RT-PCR analysis of gene expression in liver samples from the CTL, HFD, and HFD + MNZ groups. The mRNA levels of selected genes were normalized to that of \textit{Actb}. A Lipid metabolism-associated genes. B Oxidative stress-associated genes. C Autophagy-associated genes. Gene symbols are listed in Supplemental Table 1. Data are presented as mean ± standard deviation. *, † \( p < 0.05 \) compared with the CTL or HFD group, respectively (multiple comparison tests: Tukey or Steel-Dwass test).

Fig. 3  Steatosis and NAFLD activity score in rat livers. A–I Representative liver changes in the CTL (A, D, G), HFD (B, E, H), and HFD + MNZ (C, F, I) groups. A Normal liver. B Mild steatosis and inflammatory foci. C Moderate steatosis with inflammatory cell infiltration. Bar = 25 μm. A–C H&E-stained sections. Immunohistochemistry of adipophilin (D–F) and PPARγ (G–I). J The NAFLD activity score (NAS). K Steatosis score. L Quantifications of PPARγ-positive nuclei in the hepatocytes outside foci (non-foci) are presented as % of total cell number. Data are presented as mean ± standard deviation. *, † \( p < 0.05 \) compared with the CTL or HFD group, respectively (multiple comparison tests: Tukey or Steel-Dwass test). Bar = 12.5 μm.
MNZ administration significantly increased the number and area of GST-P-positive liver foci compared with those in the other two groups (Fig. 4A–C, G, H). These changes were related to a significant increase in the number of γ-H2AX-positive cells (the DNA damage marker), both in the liver foci and in the surrounding hepatocytes (outside foci) (Fig. 4D–F, I, J). The levels of the autophagy markers LC3 and p62 in the liver foci were also elevated (Fig. 5A–G, I). Significant changes were not detected in the number of LC3-positive cells surrounding hepatocytes (Fig. 5H), whereas the number of p62-positive cells was significantly lower in the HFD group compared with that in the other groups (Fig. 5J). The PCNA labeling index was not significantly different between the groups (Supplemental Fig. 1). The percentage of hepatocytes in the G1 phase was significantly higher in the HFD + MNZ group compared with that in the CTL group. The oxidative stress marker p22phox was expressed in hepatocellular cytoplasm in the liver foci as well as in the surrounding hepatocytes. The p22phox labeling index in the liver foci was significantly higher in the HFD group compared with that in the CTL group; similar results were obtained by measuring the surrounding hepatocytes (outside foci) (Supplemental Table 4). HFD and MNZ did not have any effect on labeling indices of Ki-67 and active caspase-3 in the liver foci, as well as in the surrounding hepatocytes in any of the groups.

### Discussion

MNZ is one of the most effective antibacterial and antiprotozoal medications used to treat animals and humans infected with anaerobic bacteria or parasites (Abdel-Magied et al. 2017; Freeman et al. 1997; Leitsch 2017). However, MNZ is categorized by IARC as a Group 2B agent due to its genotoxicity and carcinogenicity (IARC 1987), thus limiting its use. Data from established carcinogenicity studies in rodents showed that MNZ has the potential to induce tumors in rat livers (Rustia and Shubik 1979). However, a report by Drenick et al. (Drenick et al. 1982) suggested that MNZ can alleviate steatosis in patients. Therefore, we decided to focus on the effects of MNZ on steatosis-related hepatocarcinogenesis in rats; this is particularly important, since the incidence of NAFLD is increasing worldwide and is known to progress to HCC (Charrez et al. 2016; Starley et al. 2010). NAFLD-associated HCC models have been developed in rats and mice (Wu 2016), in which it may take a long time (6 month to 1 year) to observe HCC in the liver. Notably, GST-P-positive preneoplastic foci are one of the most reliable markers of early hepatocarcinogenicity in rats (Ito et al. 2003) and useful to evaluate the effects of HFD in combination with drugs in several rat models, including HFD-fed, carbon tetrachloride-treated male SHRSP.Z-Leprfa1/ IzmDmer rats (Kochi et al. 2014), and HFD-fed, DEN-treated male Sprague–Dawley rats (Sumi et al. 2013; Wang
et al. 2009). Quantification of GST-P-positive foci was originally proposed in a well-established two-stage hepatocarcinogenesis model of male F344 rats, and 313 compounds were tested to possess carcinogenic potentials in the liver (Ito et al. 2003). We previously reported that HFD induced GST-P-positive preneoplastic foci in male F344 rats, along with increases of NAS (Murayama et al. 2018; Nakamura et al. 2018), which is a histopathological score system addressing the spectrum of NAFLD (Kleiner et al. 2005). Therefore, we determined the effects of MNZ treatment in combination with HFD feeding in a steatosis-related early hepatocarcinogenicity rat model based on a medium-term liver bioassay and mainly demonstrated the effects of MNZ on steatosis, DNA damage, and autophagy in the preneoplastic lesions in the liver.

In this model, HFD causes obesity, as demonstrated by increased body weight and intraabdominal adipose tissue weight, as previously reported (Al-Muzafar and Amin 2018; Levy et al. 2004; Murayama et al. 2018; Yoshida et al. 2017). MNZ reduced obesity in HFD-fed rats: lower body weight could also be associated with the reduced food intake, since MNZ has an extremely bitter taste (Yamazaki et al. 2017). We also previously reported that HFD induced hyperlipidemia in rats without overnight fasting before the animals were sacrificed (Murayama et al. 2018). In the present study, overnight fasting reduced hyperlipidemia, suggesting that it was fully reversible in this model. Although a previous study reported that MNZ reduced hyperlipidemia in volunteers (Shamkhani et al. 2003), we did not observe any effects of MNZ on plasma TG and T.CHOL in rats in our present study. HFD feeding with or without MNZ decreased ALT and AST levels, possibly by altering hepatic metabolism, including reducing the levels of pyridoxal 5 phosphate, a cofactor of hepatic enzymes (Hall et al. 2012; Murayama et al. 2018). The observed hyperbilirubinemia was consistent with the findings of HFD-fed rat-based studies (Al-Muzafar and Amin 2018; Hanafi et al. 2018). However, the effects of MNZ on hyperbilirubinemia were not detected in the present study. HFD reduced the cecum weight which might be related to the reduced food intake. It is also possible that MNZ increased cecum enlargement was caused by altered microbiota beneficial for gastrointestinal health, as shown by the MNZ-mediated increased levels of Bifidobacterium, Lactobacillales, and Enterobacteriales relative to levels of Bacteroides, Turicibacter, Clostridiales, and Fusobacteriaceae in healthy dogs (Igarashi et al. 2014).

In the liver, HFD induced NAS, based on the scores of steatotic or ballooning cells, and inflammation in the hepatocytes surrounding preneoplastic foci, as previously reported (Kochi et al. 2014; Murayama et al. 2018; Nakamura et al. 2018; Sumi et al. 2013; Wang et al. 2009). We confirmed that only a limited effect of MNZ in steatosis, as MNZ did not affect HFD-mediated increased NAS. However, MNZ altered expression levels of genes involved in hepatic lipid metabolism, such as Srebf1 and Fasn (fatty acid synthesis), Pnpla2 (lipolysis), and Srebf2 (cholesterol pool). FAS mediates de
** novo** lipogenesis by synthesizing free fatty acids, which can subsequently be esterified to TG, whereas SREBF1 regulates Fasn transcription (Griffin and Sul 2004). According to previous reports, the study conditions could also have an effect on Fasn and Srebf1 expression in liver tissues, both in animal models and in human patients. Higher expression levels of Fasn and Srebf1 were observed in mouse models fed a HFD (Kobori et al. 2011; Jo et al. 2014), whereas increased expression levels of Fasn, but not Srebf1, were detected in another mouse study (Wada et al. 2010). In the present study and our previous study (Masuda et al. 2019), HFD might have inhibited fatty acid synthesis by reducing the expression of Fasn in response to excessive lipid deposition. This interpretation is supported by the finding in rat models in which lard-containing food protected the gene expression response to re-feeding compared with control diets (Levy et al. 2004). In humans, NASH patients showed higher expression levels of Fasn and Srebf1 compared with simple steatosis patients; surprisingly, these levels decreased during the disease progression (Mitsuyoshi et al. 2009). A higher expression of Srebf1 in the MNZ-treated group indicated that the drug enhanced fatty acid synthesis, leading to lipotoxicity; however, a lower expression of Srebf2 suggested that MNZ suppressed cholesterol synthesis (Rottiers and Näär 2012). Increased expression of Pnpla2 in the MNZ-treated group might be associated with the lower β-oxidation, since Pnpla2 is responsible for conversion of TGs to free fatty acids, leading to β-oxidation (Sanchez-Lazo et al. 2014). Further studies including western blotting of these steatosis regulators are required to understand the significant contribution of MNZ in diet-induced steatosis.

Activation of PPARα alleviates steatosis in NAFLD (Cave et al. 2016). Compared with those of PPARα, functions of the PPARγ isoform are not characterized, except for that in adipocytes, where it plays a key role in cell growth and adipogenesis (Cave et al. 2016). Expression levels of PPARγ in the normal liver are relatively low; however, they are increased in NAFLD livers (Tanaka et al. 2017). PPARγ induces steatosis, and disruption of PPARγ alleviates fatty liver (Yu et al. 2010). Therefore, the increased Pparg expression in the HFD group might be associated with the induction of steatosis in our experiments. Although the Pparg expression levels in the HFD + MNZ group was comparable with those in the CTL group, PPARγ nuclear translocation was evident in the HFD + MNZ group compared with that in all other groups. Therefore, MNZ-activated PPARγ could be responsible for steatosis induction in our study. The precise roles of PPARγ in NASH livers, however, remain controversial. PPARγ was shown to reduce activation of nuclear factor-kappa B, followed by increased expression of CXCL1 and neutrophil infiltration in livers (Wang et al. 2017). In contrast, PPARα upregulated β-oxidation and cholesterol elimination during the fasted state. Expression levels of PPARα are low during NAFLD; however, they increase as the disease progression is attenuated by diet and exercise (Cave et al. 2016). We did not detect any clear treatment-related differences in gene expression of either Ppara or Acosl, the PPARα-dependent gene, in our study.

The combined analysis of GST-P with immunohistochemical markers of cell responses could be a useful tool to explore the carcinogenic mechanisms involved in responses to test substances. Here, we showed that MNZ-induced GST-P-positive foci were accompanied by higher γ-H2AX labeling index in hepatocytes, both within and outside the liver foci. γ-H2AX is a reliable marker of DNA double-strand breaks, the indicator of genomic instability, potentially playing an important role in tumor growth (Palla et al. 2017). Therefore, monitoring γ-H2AX expression can be used as a sensitive method to detect the development of cancer, including HCC (Rey et al. 2017; Xiao et al. 2015). γ-H2AX levels were increased in preneoplastic lesions of HCC (Matsuda et al. 2013). Interestingly, γ-H2AX levels were also increased in oxidative stress-mediated DNA damage in vitro (Zhang et al. 2010). Indeed, the expression of hepatic Catalase was significantly increased by MNZ treatment, which may induce genotoxicity in an oxidative stress-dependent manner (Rê et al. 1997).

Negative expression of PPARγ in preneoplastic liver foci could also be related to tumor induction. Previous studies have implicated Pparg as a tumor suppressor gene, since it suppressed cancer development by decreasing cell proliferation and increasing cell cycle arrest and apoptosis in hepatoma cell lines. Moreover, Pparg-deficient mice were prone to developing HCC when treated with DEN (Yu et al. 2010).

On a western blot, LC3 separates into LC3-I and LC3-II at higher and lower molecular weights, respectively; LC3-I is distributed in the cytoplasm, whereas LC3-II is localized in inner and outer sides of autophagosome membranes (Mizushima and Komatsu 2011; Zhang et al. 2013). Therefore, LC3-II is widely accepted as an autophagy marker; however, monitoring the autophagic process in preneoplastic foci within the whole liver containing numerous surrounding (non-preneoplastic) hepatocytes and other cells, such as Kupffer cells, endothelial cells, and bile ductal cells, using western blotting is challenging. Furthermore, p62 interacts with LC3 in the inner membrane of the autophagosomes which trapped dispensable organelles. Subsequently, p62 and trapped organelles incorporated into the autolysosome are degraded (Mizushima and Komatsu 2011). Therefore, a decreased level of p62 corresponds to induction of autophagy, and an increased level of p62 indicates inhibition of autophagy (Zhang et al. 2013). In NASH models, wild type mice did not increase p62 expression in liver samples when fed HFD alone, while the mice increased p62 expression when treated with DEN followed by HFD (Umemura et al. 2016). A decreased expression of p62 in the neighboring hepatocytes in the HFD group suggested that overnight fasting induced autophagy. Furthermore, MNZ treatment increased expression levels of both LC3 and p62 in the preneoplastic foci, suggesting that
MNZ induces late-step inhibition of autophagy; increased levels of LC3 indicate induction or late-step inhibition of autophagic flux, whereas decreased levels of LC3 indicate early step inhibition of autophagic flux (Mizushima and Komatsu 2011; Zhang et al. 2013). A previous immunohistochemistry study demonstrated p62 was highly expressed in HCC compared with non-cancerous cirrhotic livers (Chava et al. 2017). NASH-prone (major urinary protein-urokinase-type plasminogen activator) mice increased p62 expression which was closely related to the development of NASH and HCC, when fed HFD; in a mechanism study, ectopic expression of p62, along with LC3, induced autophagy, while mTORC1 and ER stress might induce accumulation of p62 by inhibition of autophagy, leading to cell proliferation (Umemura et al. 2016). By supplying molecules that induce oxidative stress and DNA damage (i.e., bleomycin and menadione), the inhibition of autophagy through the co-treatment with rapamycin induces pronounced cytotoxicity (Galati et al. 2019). Expression of p62 (inhibition of autophagy) can increase oxidative stress, which may also contribute to increased DNA damage (Czarny et al. 2015). As autophagy acts as a tumor repressor prior to cellular transformation (Czaja et al. 2013), the pretransformed cells in the preneoplastic foci could be promoted by MNZ-mediated inhibition of autophagy.

The results of our study suggest that the MNZ-mediated DNA damage and modulation of autophagy together with the altered oxidative stress and lipid metabolism may increase preneoplastic lesions in the present model. A major limitation in our study is that we did not assess the effects of MNZ on the basal diet group and compare those with the HFD group. Therefore, further studies using both basal diet and HFD are required to determine how MNZ modulates autophagy in gradual stages of steatosis and steatosis-related hepatitis, leading to the development of precancerous lesions and HCC.

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Author contribution  AE and TY conceived the project, designed and conducted animal experiments, analyzed and interpreted the data, and wrote the manuscripts. SaM, MN, HM, MaK, MI, and RN conducted animal experiments. SoM, MiK, KY, and SU performed the immunohistochemistry, and EM, RO, and NT analyzed the blood samples. SMH and RRM reviewed the manuscripts, and MS initiated the project and interpreted the results. All authors read and approved the manuscript.

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Data availability  All data generated and analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate  Clinical, operative, and experimental procedures were performed according to the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and the study protocol authorized by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Consent for publication  Not applicable.

Competing interests  The authors declare no competing interests.

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