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

Ayumi Eguchi
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Sayaka Mizukami
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Misato Nakamura
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Sousuke Masuda
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Hirotada Murayama
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Masashi Kawashima
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Mari Inohana
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Rei Nagahara
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Mio Kobayashi
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Risako Yamashita
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Suzuka Uomoto
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Emi Makino
Institute of Environmental Toxicology
Ryoichi Ohtsuka
Institute of Environmental Toxicology
Naofumi Takahashi
Institute of Environmental Toxicology
Shim-Mo Hayashi
San-Ei Gen FFI, Inc

Robert R Maronpot
Manropot Consulting
Makoto Shibutani
Tokyo University of Agriculture and Technology: Tokyo Noko Daigaku
Toshinori Yoshida (yoshida7@cc.tuat.ac.jp)
Tokyo University of Agriculture and Technology https://orcid.org/0000-0003-2476-7794

Research Article

Keywords: γ-H2AX, LC3, metronidazole, NAFLD, p62, PPARγ

DOI: https://doi.org/10.21203/rs.3.rs-160304/v1

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 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.

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 et al. 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. 1979; Mohn et al. 1979); however, data in human cells remain controversial (Menéndez et al. 2001). While no DNA strand breaks were reported in an *in vivo* genotoxicity study in isolated peripheral lymphocytes after the MNZ therapy (Fahrig and Engelke 1997), inducible DNA damage was described in human lymphocytes following MNZ treatment (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). Furthermore, 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. We recently developed a steatosis-related early hepatocarcinogenicity rat model based on a medium-term liver bioassay (Ito et al. 2013) 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 apoptotosis (Czarny et al. 2014). 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. 2014).

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-hour 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-weeks-old rats. All rats were injected DEN (200 mg/kg body weight) intraperitoneally at the beginning of the experiment. The control (CTL) group (n = 11) 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=12, HFD+MNZ group) during weeks 2 and 9. All rats had a two-thirds partial hepatectomy at week 3. 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 around bilateral spermatic cords were 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). 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 were also counted. The numbers 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) were 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.

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[a]	310.0 ± 16.9[b]
Food intake (g/rat/day)	18 ± 0.8	12.7 ± 0.6[a]	13.3 ± 1.9[a]
Water intake (g/rat/day)	23.3 ± 1.3	18.7 ± 0.2[a]	17.7 ± 0.3[a]
Absolute adipose tissue weight (g)	7.8 ± 0.9	12.4 ± 1.5[a]	10.5 ± 1.4[ab]
Relative adipose tissue weight (%BW)	2.6 ± 0.2	3.7 ± 0.3[a]	3.4 ± 0.3
Absolute liver weight (g)	7.9 ± 0.7	6.8 ± 0.6[a]	7.3 ± 0.9
Relative liver weight (%BW)	2.6 ± 0.1	2.0 ± 0.1[a]	2.4 ± 0.2[ab]
Absolute cecum weight (g)	5.0 ± 0.6	3.5 ± 0.6[a]	11.7 ± 1.3[ab]
Relative cecum weight (%BW)	1.7 ± 0.2	1.0 ± 0.2[a]	3.8 ± 0.4[ab]
Abbreviations: 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.			
Data are shown as the mean ± standard deviation.			
a: $p < 0.05$ vs CTL (Tukey's or Steel-Dwass test).			
b: $p < 0.05$ vs HFD (Tukey's or Steel-Dwass test).			

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. 1A). 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. 1A). 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. 1B). No significant changes were detected in autophagy-related genes (Fig. 1C).

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. 2A-C). Lipid droplet membranes stained positive for adipophilin and the immunoreactivity increased in the HFD groups with or without MNZ (Fig. 2D-F). NAS was significantly increased in both HFD groups (with or without MNZ) compared with that in the CTL group (Fig. 2J). Steatosis was significantly increased in a same way (Fig. 2K) and hepatocyte ballooning measurements (data not shown). 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. 2G-I). Furthermore, the number of PPARγ-positive nuclei was significantly increased in the MNZ group compared with that in the other groups (Fig. 2L). PPARγ was not expressed in the liver foci.

MNZ administration significantly increased the number and area of GST-P-positive liver foci compared with those in the other two groups (Fig. 3A-C, G and 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. 3D-F, I and J). The levels of the autophagy markers LC3 and p62 in the liver foci were also elevated (Fig. 4A-G and I). Significant changes were not detected in the number of LC3-positive cells surrounding hepatocytes (Fig. 4H), whereas the number of p62-positive cells was significantly lower in the HFD group compared with that in the other groups (Fig. 4J). 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 early hepatocarcinogenesis in rats (Yoshida et al. 2017; Murayama et al. 2018); 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).

HFD causes obesity, as demonstrated by increased body weight and intraabdominal adipose tissue weight. We 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. Alternatively, overnight fasting reduced glycogen deposition in the liver, and lesions were apparent when compared with the non-fasting liver (Murayama et al. 2018). HFD feeding 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. Here, MNZ reduced obesity and affected the cecum weight. Lower body weight could also be associated with the reduced food intake, since MNZ has an extremely bitter taste (Yamazaki et al. 2017). It is also possible that 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). 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.

In the liver, MNZ did not affect HFD-mediated increased NAS, based on the scores of steatotic or ballooning cells, and inflammation. MNZ administration increased the severity of steatosis as confirmed by immunohistochemistry staining for adipophilin, the protein expressed on lipid droplet membranes (Obert et al. 2007). MNZ also 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 synthetizing 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). HFD might have inhibited fatty acid synthesis by reducing the expression of *Fasn* in response to excessive lipid deposition in the present study. 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 Naar 2014). 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).

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 *Acox1*, the PPARα-dependent gene, in our study.

GST-P-positive foci are one of the most reliable markers of early hepatocarcinogenicity (Ito et al. 2003). Therefore, 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. 2016; 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 (Ré 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 ducal 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). 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). 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 can increase oxidative stress, which may also contribute to increased DNA damage (Czarny et al. 2014). As autophagy acts as a tumor repressor prior to cellular transformation (Czaja et al. 2013), the pre-transformed cells in the preneoplastic foci could be promoted by MNZ-mediated inhibition of autophagy.

The results of our study suggest that steatosis could be one of the outcomes following the use of MNZ in humans infected with anaerobic bacteria and/or intestinal parasites, since the MNZ-mediated DNA damage and modulation of autophagy together with the altered oxidative stress and lipid metabolism may increase preneoplastic lesions. Here, we did not assess the effect of MNZ on the basal diet group, and this is one of the limitations of our study. 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.

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.

Availability of data and materials: All data generated and analyzed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was supported by a Grand-in-Aid for Scientific Research © (Grant No. 981039) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Japan Food Chemical Research Foundation.

Authors' contributions: AE and TY conceived the project, designed and conducted animal experiments, analyzed and interpreted the data, and wrote the manuscripts. SaM, MN, HM, MK, MI, and RN conducted animal experiments, SoM, MK, RY and SU performed immunohistochemistry, and EM, RO, and NT analysed blood samples. SMH and RRM reviewed the manuscripts, and MS intiated the project and interpreted the results. All authors read and approved the manuscript.

Acknowledgements: We would like to thank Editage (www.editage.com) for English language editing.

References

Abdel-Magied AA, El-Kholya EI, Abou El-Khair SM, Abdelmegeed ES, Hamoudaa MM, Mohamed SA, El-Tantawy NL (2017) The genetic diversity of metronidazole susceptibility in Trichomonas vaginalis clinical isolates in an Egyptian population. Parasitol Res 116:3125-3130.

Al-Muzafar HM, Amin KA (2018) Thiazolidinedione induces a therapeutic effect on hepatosteatosis by regulating stearoyl-CoA desaturase-1, lipase activity, leptin and resistin. Exp Ther Med 16:2938-2948.

Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB (2007) Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Investig 117:326–336.

APVMA (Australian Pesticides and Veterinary Medicines Authority) (2007) Final Review Report and Regulatory Decision: The Reconsideration of Registrations of Products Containing Dimetridazole and their Associated Approval Labels. Kingston, Australian Capital Territory, Australia:APVMA.Available: http://www.apvma.gov.au/products/review/docs/dimetridazole_final_report.pdf [accessed 31 May 2013].

Basaranoglu M, Neuschwander-Tetri BA (2006) Nonalcoholic fatty liver disease: clinical features and pathogenesis. Gastroenterol. Hepatol (NY) 2:282-291.

Cantelli-Forti G, Aicardi G, Guerra MC, Barbaro AM, Biagi GL (1983) Mutagenicity of a series of 25 nitroimidazoles and two nitrothiazoles in Salmonella typhimurium. Teratog Carcinog Mutagen 3:51-63.

Cavaliere A, Bacci M, Amorosi A, Del Gaudio M, Vitali R (1983) Induction of lung tumors and lymphomas in BALB/c mice by metronidazole. Tumori 69:379-382.

Cave MC, Clair HB, Hardesty JE, Falkner KC, Feng W, Clark BJ, Sidey J, Shi H, Aqel BA, McClain CJ, Prough RA (2016) Nuclear receptors and nonalcoholic fatty liver disease. Biochim Biophys Acta 1859:1083-1099.

Charrez B, Qiao L, Hebbard L (2016) Hepatocellular carcinoma and non-alcoholic steatohepatitis: The state of play. World J Gastroenterol 22:2494-2502.

Czaja MJ, Ding WX, Donohue TM Jr, Friedman SL, Kim JS, Komatsu M, Lemasters JJ, Lemoine A, Lin JD, Ou JH, Perlmutter DH, Randall G, Ray RB, Tsung A, Yin XM (2013)
Functions of autophagy in normal and diseased liver. Autophagy 9:1131-1158.

Czarny P, Pawlowska E, Bialkowska-Warzecha J, Kaarniranta K, Blasiak J (2015) Autophagy in DNA damage response. Int J Mol Sci 16:2641-2662.

Drenick EJ, Fisler J, Johnson D (1982) Hepatic steatosis after intestinal bypass--prevention and reversal by metronidazole, irrespective of protein-calorie malnutrition. Gastroenterology 82:535-548.

EU (European Union) (2010) Commission Regulation (No 37/2010) on Pharmacologically Active Substances and their Classification Regarding Maximum Residue Limits in Foodstuffs of Animal Origin. Brussels:Off J Eur Union.Available: http://ec.europa.eu/health/files/eudralex/vol-5/reg_2010_37/reg_2010_37_en.pdf [accessed 31 May 2013].

Fahrig R, Engelke M (1997) Reinvestigation of in vivo genotoxicity studies in man. I. No induction of DNA strand breaks in peripheral lymphocytes after metronidazole therapy. Mutat Res 395:215-221.

Foley J, Ton T, Maronpot R, Butterworth B, Goldsworthy TL (1993) Comparison of proliferating cell nuclear antigen to tritiated thymidine as a marker of proliferating hepatocytes in rats. Environ Health Perspect 101 Suppl 5:199-205.

Freeman CD, Klutman NE, Lamp KC (1997) Metronidazole. A therapeutic review and update. Drugs 54:679-708.

Galati S, Boni C, Gerra MC, Lazzaretti M, Buschini A (2019) Autophagy: A Player in response to oxidative stress and DNA damage. Oxid Med Cell Longev 2019:5692958.

González-Rodríguez A, Mayoral R, Agra N, Valdecantos MP, Pardo V, Miquilena-Colina ME, Vargas-Castrillón J, Lo Iacono O, Corazzari M, Fimia GM, Piacentini M, Muntané J, Boscá L, García-Monzón C, Martín-Sanz P, Valverde ÁM (2014) Impaired autophagic flux is associated with increased endoplasmic reticulum stress during the development of NAFLD. Cell Death Dis 5:e1179.

Griffin MJ, Sul HS (2004) Insulin regulation of fatty acid synthase gene transcription: roles of USF and SREBP-1c. IUBMB Life 56:595-600.

Hall AP, Elcombe CR, Foster JR, Harada T, Kaufmann W, Knippel A, Küttler K, Malarkey DE, Maronpot RR, Nishikawa A, Nolte T, Schulte A, Strauss V, York MJ (2012) Liver hypertrophy: a review of adaptive (adverse and non-adverse) changes--conclusions from the 3rd International ESTP Expert Workshop. Toxicol Pathol 40:971-994.

Hanafi MY, Zaher ELM, El-Adely SEM, Sakr A, Ghobashi AHM, Hemly MH, Kazem AH, Kamel MA (2018) The therapeutic effects of bee venom on some metabolic and antioxidant parameters associated with HFD-induced non-alcoholic fatty liver in rats. Exp Ther Med 15:5091-5099.

IARC (1987) IARC Monographs on the evaluation of carcinogenic risks to humans, Supplement 7, Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42, IARC, Lyon.

Igarashi H, Maeda S, Ohno K, Horigome A, Odamaki T, Tsujimoto H (2014) Effect of oral administration of metronidazole or prednisolone on fecal microbiota in dogs. PLoS One 9: e107909.

Ito N, Tamano S, Shirai T (2003) A medium-term rat liver bioassay for rapid in vivo detection of carcinogenic potential of chemicals. Cancer Sci 94:3-8.

Jo HK, Kim GW, Jeong KJ, Kim DY, Chung SH (2014) Eugenol ameliorates hepatic steatosis and fibrosis by down-regulating SREBP1 gene expression via AMPK-mTOR-p70S6K signaling pathway. Biol Pharm Bull 37:1341-1351.

Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ (2005) Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease, Hepatology 41:1313-1321.

Kobori M, Masumoto S, Akimoto Y, Oike H (2011) Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice. Mol Nutr Food Res 55:530-540.

Lavallard VJ, Gual P (2014) Autophagy and non-alcoholic fatty liver disease. Biomed Res Int 2014:120179.

Leitsch D (2017) Drug susceptibility testing in microaerophilic parasites: Cysteine strongly affects the effectivities of metronidazole and auranofin, a novel and promising antimicrobial. Int J Parasitol Drugs Drug Resist 7:321-327.

Leung HW, Jin L, Wei S, Tsui MM, Zhou B, Jiao L, Cheung PC, Chun YK, Murphy MB, Lam PK (2013) Pharmaceuticals in tap water: human health risk assessment and proposed monitoring framework in China. Environ Health Perspect 121:839-846.

Levy JR, Clore JN, Stevens W (2004) Dietary n-3 polyunsaturated fatty acids decrease hepatic triglycerides in Fischer 344 rats. Hepatology 39:608-616.

Ligha AE, Paul CW (2011) Oxidative effect of metronidazole on the testes of Wistar rats. Aust J Basic Appl Sci 5:1339-1344.

Marotta F, Barreto R, Wu CC, Naito Y, Gelosa F, Lorenzetti A, Yoshioka M, Fesce E (2005) Experimental acute alcohol pancreatitis-related liver damage and endotoxemia: synbiotics but not metronidazole have a protective effect. Chin J Dig Dis 6:193-197.

Masuda S, Mizukami S, Eguchi A, Ichikawa R, Nakamura M, Nakamura K, Okada R, Tanaka T, Shibutani M, Yoshida T (2019) Immunohistochemical expression of autophagosome markers LC3 and p62 in preneoplastic liver foci in high fat diet-fed rats. J Toxicol Sci 44:565-574.

Matsuda Y, Wakai T, Kubota M, Osawa M, Takamura M, Yamagiwa S, Aoyagi Y, Sanpei A, Fujimaki S (2013) DNA damage sensor γ -H2AX is increased in preneoplastic lesions of hepatocellular carcinoma. Sci World J 2013:597095.

Menéndez D, Rojas E, Herrera LA. López MC, Sordo M, Elizondo, G, Ostrosky-Wegman P (2001) DNA breakage due to metronidazole treatment. Mutat Res 478:153-158.

Mitsuyoshi H, Yasui K, Harano Y, Endo M, Tsuji K, Minami M, Itoh Y, Okanoue T, Yoshikawa T (2009) Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease. Hepatol Res 39:366-373.

Mizushima N, Komatsu M (2011) Autophagy: renovation of cells and tissues. Cell 147:728-741.

Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. Cell 140:313-326.

Mohn GR, Ong TM, Callen DF, Kramers PG, Aaron CS (1979) Comparison of the genetic activity of 5-nitroimidazole derivatives in Escherichia coli, Neurospora crassa, Saccharomyces cerevisiae and Drosophila melanogaster. J Environ Pathol Toxicol 2:657-670.

Murayama H, Eguchi A, Nakamura M, Kawashima M, Nagahara R, Mizukami S, Kimura M, Makino E, Takahashi N, Ohtsuka R, Koyanagi M, Hayashi SM, Maronpot RR, Shibutani M, Yoshida T (2018) Spironolactone in combination with α-glycosyl Isoquercitrin prevents steatosis-related early hepatocarcinogenesis in rats through the observed NADPH oxidase modulation. Toxicol Pathol 46:530-539.

Nseir W, Mahamid M (2013) Statins in nonalcoholic fatty liver disease and steatohepatitis: updated review. Curr Atheroscler Rep 15:305.

Obert LA, Sobocinski GP, Bobrowski WF, Metz AL, Rolsma MD, Altrogge DM, Dunstan RW (2007) An immunohistochemical approach to differentiate hepatic lipidosis from hepatic phospholipidosis in rats. Toxicol Pathol 35:728-734.

Palla VV, Karaolanis G, Katafigiotis I, Anastasiou I, Patapis P, Dimitroulis D, Perrea D (2017) gamma-H2AX: Can it be established as a classical cancer prognostic factor? Tumour Biol 39:1010428317695931.

Ré JL, De Méo MP, Laget M, Guiraud H, Castegnaro M, Vanelle P, Duménil G (1997) Evaluation of the genotoxic activity of metronidazole and dimetridazole in human lymphocytes by the comet assay. Mutat Res 375:147-155.

Reitz M, Rumpf M, Knitza R (1991) Metronidazole induces DNA strand-breaks in cultures of human lymphocytes and phytohemagglutinin-stimulated human lymphocytes. Arzneimittelforschung 41:65-69.

Rey S, Quintavalle C, Burmeister K, Calabrese D, Schlageter M, Quagliata L, Cathomas G, Diebold J, Molinolo A, Heim MH, Terracciano LM, Matter MS (2017) Liver damage and senescence increases in patients developing hepatocellular carcinoma. J Gastroenterol Hepatol 32:1480-1486.

Rottiers V, Näär AM (2012) MicroRNAs in metabolism and metabolic disorders. Nat Rev Mol Cell Biol 13:239-250.

Rustia M, Shubik P (1979) Experimental induction of hepatomas, mammary tumors, and other tumors with metronidazole in noninbred Sas:MRC(WI)BR rats. J Natl Cancer Inst 63:863-868.

Sanchez-Lazo L, Brisard D, Elis S, Maillard V, Uzbekov R, Labas V, Desmarchais A, Papillier P, Monget P, Uzbekova S (2014) Fatty acid synthesis and oxidation in cumulus cells support oocyte maturation in bovine. Mol Endocrinol 28:1502-1521.

Shamkhani K, Azarpira M, Akbar MH (2003) An open label crossover trial of effects of metronidazol on hyperlipidaemia. Int. J Cardiol 90:141-145.

Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ (2009) Autophagy regulates lipid metabolism. Nature 458:1131-1135.

Sisson G, Jeong JY, Goodwin A, Bryden L, Rossler N, Lim-Morrison S, Raudonikiene A, Berg DE, Hoffman PS (2000) Metronidazole activation is mutagenic and causes DNA fragmentation in Helicobacter pylori and in Escherichia coli containing a cloned H. pylori RdxA(+) (Nitroreductase) gene. J Bacteriol 182:5091-5096.

Starley BQ, Calcagno CJ, Harrison SA (2010) Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. Hepatology 51:1820-1832.

Takahashi Y, Soejima Y, Fukusato T (2012) Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol 18:2300-2308.

Tanaka N, Aoyama T, Kimura S, Gonzalez FJ (2017) Targeting nuclear receptors for the treatment of fatty liver disease. Pharmacol Ther 179:142-157.

Wada T, Kenmochi H, Miyashita Y, Sasaki M, Ojima M, Sasahara M, Koya D, Tsuneki H, Sasaoka T (2010) Spironolactone improves glucose and lipid metabolism by ameliorating hepatic steatosis and

inflammation and suppressing enhanced gluconeogenesis induced by high-fat and high-fructose diet. Endocrinology 151:2040-2049.

Wang W, Xu MJ, Cai Y, Zhou Z, Cao H, Mukhopadhyay P, Pacher P, Zheng S, Gonzalez FJ, Gao B (2017) Inflammation is independent of steatosis in a murine model of steatohepatitis. Hepatology 66:108-123.

Xiao H, Tong R, Ding C, Lv Z, Du C, Peng C, Cheng S, Xie H, Zhou L, Wu J, Zheng S (2015) γ-H2AX promotes hepatocellular carcinoma angiogenesis via EGFR/HIF-1α/VEGF pathways under hypoxic condition. Oncotarget 6:2180-2192.

Yamazaki Y, Kawarai S, Morita H, Kikusui T, Iriki A (2017) Faecal transplantation for the treatment of Clostridium difficile infection in a marmoset. BMC Vet Res 13:150.

Yu J, Shen B, Chu ES, Teoh N, Cheung KF, Wu CW, Wang S, Lam CN, Feng H, Zhao J, Cheng AS, To KF, Chan HL, Sung JJ (2010) Inhibitory role of peroxisome proliferator-activated receptor gamma in hepatocarcinogenesis in mice and in vitro. Hepatology 51:2008-2019.

Yoshida T, Murayama H, Kawashima M, Nagahara R, Kangawa Y, Mizukami S, Kimura M, Abe H, Hayashi SM, Shibutani M (2017) Apocynin and enzymatically modified isoquercitrin suppress the expression of a NADPH oxidase subunit p22phox in steatosis-related preneoplastic liver foci of rats. Exp Toxicol Pathol 69:9-16.

Zhang R, Kang KA, Piao MJ, Kim KC, Kim AD, Chae S, Park JS, Youn UJ, Hyun JW (2010) Cytoprotective effect of the fruits of Lycium chinense Miller against oxidative stress-induced hepatotoxicity. J Ethnopharmacol 130:299-306.

Zhang XJ, Chen S, Huang KX, Le WD (2013) Why should autophagic flux be assessed? Acta Pharmacol Sin 34:595-599.