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Modifying Effects of Flumequine on Dimethylnitrosamine-Induced Hepatocarcinogenesis in Heterozygous p53 Deficient CBA Mice

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Abstract: Flumequine (FL), a quinolone-antibiotic used for veterinary treatment of infections, was found to elicit hepatocellular tumors in a conventional 18-month carcinogenicity study in mice, and hepatocellular necrosis-regeneration cycle was considered to be a possible underlying mechanism. In order to clarify whether FL has any modifying effects on development of hepatocellular proliferation, groups of heterozygous p53 deficient CBA mice [p53(+/-) mice], sensitive to genotoxic carcinogen, of both sexes and their wild-type littermates [p53(+/+) mice] were fed diet containing 4,000 or 0 ppm FL for 26 weeks after an intraperitoneal injection of 5 or 0 mg/kg body weight of dimethylnitrosamine (DMN). Higher incidences of hepatocellular foci were observed in animals receiving FL, with or without DMN-initiation, than in the corresponding control groups in both p53(+/-) and p53(+/+) mice. Incidences and multiplicities of foci were generally similar in p53(+/-) and p53(+/+) mice, but, in the DMN+FL group, multiplicity of foci and their PCNA labeling indices were greater in p53(+/-) mice. There were also small numbers of hepatocellular adenomas and carcinomas in the DMN+FL group of p53(+/-) mice, hepatocellular adenoma in the FL alone group of p53(+/-) mice, and hepatocellular adenomas in the DMN+FL group of p53(+/+) mice. Induction of hepatocellular tumors in these mice within a relatively short period strongly suggests that mechanisms such as direct or indirect damage to DNA might be responsible for the hepatocarcinogenesis of FL. (J Toxicol Pathol 2001; 14: 135–143)

Key words: flumequine, hepatocarcinogenesis, heterozygous p53 deficient mice

Introduction

Flumequine is a fluoroquinolone-antimicrobial agent which is predominantly active against Gram-negative bacteria1. It has been used by veterinarians for the treatment of urinary tract infections, and the parent drug and/or its metabolites are suspected to persist in edible tissues. It has been reported that flumequine induced hepatocellular tumors in a 18-month carcinogenicity study in mice2. In 1997, safety assessment of the drug was performed by the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA)3. The following results of toxicological studies of flumequine were taken into account: i) Hepatocellular tumors were associated with hepatic damage such as hepatoxic and hepatic drug-metabolizing enzyme induction or peroxisomal proliferation were not observed; and iii) there was no evidence of genotoxicity of flumequine in in vitro bacterial or mammalian cells gene mutation assays and in vivo chromosomal aberration test in bone-marrow cells of rats. The JECFA thus considered that the induction of hepatocellular necrosis-regeneration cycles due to hepatotoxicity was the relevant mechanism for the hepatocarcinogenicity of flumequine, and an acceptable daily intake (ADI) was calculated based on the no-observed-effect-level (NOEL) of hepatotoxicity. However, the bacterial gene mutation assay is not always reliable for detection of genotoxicity of the chemicals with anti-microbial activity such as flumequine, because the concentrations examined are usually limited to low levels. Moreover, there is no information concerning the damage to the genes in hepatocytes that proved to be target cells in the long-term carcinogenicity study. Furthermore, Yoshida et al.4 reported that flumequine not only enhanced a development of altered foci and adenomas of the hepatocytes in CD-1 mice by dietary administration at 4,000 ppm for 30 weeks after an initiation of diethylnitrosamine, but also induced small numbers of hepatocellular foci at the same
dose without any initiation. Moreover, 8-hydroxy-2'-deoxyguanosine (8OH-dG), a product of oxidative stress on DNA\(^5\), was found in hepatocytes of these treated mice. The results suggest that direct or indirect genetic damage as well as liver tumor promoting effects may participate in the hepatocellular carcinogenicity of flumequine.

It has been reported that heterozygous \(p53\) deficient mice, \(p53\)(+/-) mice, in which the lateral allele of the \(p53\) gene is inactivated, provide a useful model to detect the carcinogenic potential of genotoxic carcinogens within short-term treatment period of less than 6 months\(^6\)-\(^10\). They were therefore employed in the present study in order to clarify whether flumequine has any promoting or tumorigenic activity for the liver under dimethylnitrosamine-initiated or non-initiated condition.

### Materials and methods

#### Test substance

Flumequine, \(p53\) (FL) was obtained as a white fine crystalline powder from Kyowa Hakko Kogyo (Tokyo, Japan) and admixed into basal diet (CRF-1\(^\circ\), Oriental Yeast Co., Tokyo, Japan) at 4,000 ppm. The diet was freshly prepared every week. Dimethylnitrosamine (DMN) obtained from Nacarai Tesque Inc. (Kyoto, Japan) was dissolved in saline and passed through a membrane filter obtained from Nacarai Tesque Inc. (Kyoto, Japan) just before usage.

#### Experimental animals

The mice used in the present study were heterozygous \(p53\) deficient CBA mice [\(p53\)(+/-) mice] in which exon 2 of the lateral \(p53\) allele was inactivated, being F1 offspring of heterozygous \(p53\) deficient C57BL/6J male mice back-crossed with CBA female mice\(^11\). Forty-one male and 27 female \(p53\)(+/-) mice, and 42 male and 32 female wild-type littermates [\(p53\)(+/+) mice] were purchased from Oriental Yeast Co. and used after an acclimatization period of 1-week. The animals used were 9-10 weeks of age in males and 12-13 weeks of age in females, at the start of the study. They were housed at a maximum of 5 per cage in plastic cages with soft chip bedding in a room that was controlled for light-dark cycle (12-12 hours, lights on 7:00–19:00), ventilation (air-exchange rate of 18 times per hour), temperature (21–25\(^\circ\)C) and relative humidity (50–60%) during the study. The cages and the chip bedding were exchanged for new ones twice a week. Each animal had free access to powdered diet (CRF-1\(^\circ\), Oriental Yeast Co.) and tap water.

#### Experimental design

Both \(p53\)(+/-) and \(p53\)(+/+) mice were randomly allocated into four experimental groups based on their body weights. Two groups of \(p53\)(+/-) mice of both sexes received a single intraperitoneal injection of DMN at 5 mg/kg body weight. One of their groups was fed the diet containing 4,000 ppm FL for 26 weeks, commencing 1-week after DMN injection, and the other group, serving as a control, was fed basal diet for the same period. The remaining two groups of both sexes were maintained with the FL-admixed diet or basal diet for 26 weeks without DMN initiation. The four groups of \(p53\)(+/+) mice of both sexes were also subjected to the same treatment as their \(p53\)(+/-) counterparts. During the study period, the animals were observed once a day for their general condition, and weighed once weekly.

#### Histopathological examination

At the end of treatment period, surviving animals were sacrificed under ether anesthesia and necropsied. After measuring the liver weights, a wide variety of organs and tissues, including the liver, were fixed in 10% neutral buffered formalin for routine histopathological processing. Liver tissue specimens were trimmed from the designated left, median and caudate lobes. Then, paraffin-embedded sections with 4–5 \(\mu\)m thick were stained with hematoxylin and eosin. Histopathological examination was performed on these sections of each animal. In addition, tissue sections were immunohistochemically stained for proliferating cell nuclear antigen (PCNA) using an anti-PCNA mouse monoclonal antibody (DAKO, Glostrup, Denmark) at dilutions of 1:100 and avidin-biotin peroxidase complex kits (Burlingame, CA, USA) with a chromogen of 3, 3’-diaminobenzidine followed by counterstaining with hematoxylin. Moreover, in order to detect apoptotic cells, in situ detection kit for terminal deoxynucleobonucleotidyltransferase-mediated deoxyuridine triphosphate-dioxygenin nick end labeling (TUNEL) was employed according to the manufacturer’s instructions. Labeling of cells forming each proliferative lesion was examined for up to 1000 cells from different areas and indices for PCNA or TUNEL positivity were calculated as a percentage.

#### Statistical analysis

The two-tailed Student’s t-test was used to test for significant differences between groups in the PCNA and TUNEL labeling indices as well as body and liver weights. Incidences of hepatic proliferative lesions observed were analyzed with Fisher’s exact test. Significance was inferred at either 5% or 1% level.

### Results

Three male and two female \(p53\)(+/-) mice receiving FL after DMN-initiation (DMN+FL), 3 male \(p53\)(+/-) mice receiving FL without DMN-initiation (FL alone) and one male \(p53\)(+/+) mouse each in the DMN+FL and FL groups died due to the treatment (Table 1). A few \(p53\)(+/-) mice in other groups also died due to lymphomas or lung adenocarcinomas. Male \(p53\)(+/-) and \(p53\)(+/+) mice in the DMN+FL and FL alone groups showed depression of body weight gain, when compared to the corresponding basal diet groups with DMN-initiation (DMN alone) or without initiation (Basal diet) (Figs. 1 and 2). In females,
were increased with statistical significance as compared to
altered foci per animal in the DMN+FL and FL alone groups
adenoma in one male
without DMN initiation also induced a hepatocellular
p53

DMN alone group, but their growth curve was similar to that
mice became evident after week-20 in comparison with the
DMN+FL and FL alone groups of male
control groups.  Relative liver weights were increased in the
p53
absolute liver weights in the FL alone group of female
basal diet groups, respectively, associated with an increase in
DMN initiation was associated with small numbers of
(data not shown).  In addition, the administration of FL after
difference in phenotypes of the foci was seen among groups
sometimes mixed in several clear cell foci (Fig. 3).  No
predominantly clear cell type, and eosinophilic cell type was
DMN alone (10 to 67%) and basal diet (0 to 17%) groups,
initiation, their incidences being higher than those in the
(93 to 100% incidence) receiving FL with or without DMN-
Terminal body weights in the DMN+FL group of
(+/-) mice and the DMN+FL group of female
DMN FL rate a weights (g)b Absolute (g)b Relative (g/100g BW)b
(+/-) Male + + 12/15 33.6 ± 3.3 1.50 ± 0.25 4.46 ± 0.45
+ - 6/6 39.5 ± 6.6 1.50 ± 0.18 3.92 ± 0.99
- + 12/15 33.3 ± 3.0 1.46 ± 0.18 3.47 ± 0.29
- - 4/5 40.2 ± 6.8 1.74 ± 0.36 4.31 ± 0.45
Female + + 8/10 27.6 ± 1.7** 1.56 ± 0.22 5.64 ± 0.53**
+ - 6/7 36.0 ± 4.4 1.40 ± 0.13 3.91 ± 0.53
- + 5/5 25.3 ± 2.1 1.44 ± 0.18** 5.68 ± 0.33**
- - 4/5 25.9 ± 3.3 1.06 ± 0.08 4.13 ± 0.36

a: No. of surviving mice/No. of mice examined.  b: Each value shows mean ± SD.
*, **: statistical significance between the FL-treated group and the corresponding untreated group at p<0.05 and p<0.01.
DMN: dimethylnitrosamine, FL: flumequine.

suppression of body weight gain in the DMN+FL p53(+/-)
mice became evident after week-20 in comparison with the
DMN alone group, but their growth curve was similar to that
in the basal diet group (Fig. 1).

Terminal body weights in the DMN+FL group of
p53(+/-) and p53(+/-) females and the FL-treated groups of
p53(+/-) males were significantly lower than those in the
control groups. Relative liver weights were increased in the
DMN+FL and FL alone groups of male p53(+/-) and female
p53(+/-) and p53(+/-) mice compared to the DMN alone and
basal diet groups, respectively, associated with an increase in
absolute liver weights in the FL alone group of female
p53(+/-) mice and the DMN+FL group of female p53(+/-)
mice.

In histopathological examination, hepatocellular altered
foci were observed in almost all surviving males and females
(93 to 100% incidence) receiving FL with or without DMN-
initiation, their incidences being higher than those in the
DMN alone (10 to 67%) and basal diet (0 to 17%) groups,
respectively (Table 2). Phenotypes of the foci were
predominantly clear cell type, and eosinophilic cell type was
sometimes mixed in several clear cell foci (Fig. 3). No
difference in phenotypes of the foci was seen among groups
(data not shown). In addition, the administration of FL after
DMN initiation was associated with small numbers of
hepatocellular adenomas and/or carcinomas in males and
females of p53(+/-) mice and p53(+/-) males. FL treatment
without DMN initiation also induced a hepatocellular
adenoma in one male p53(+/-) mouse. The numbers of the
altered foci per animal in the DMN+FL and FL alone groups
were increased with statistical significance as compared to
respective control groups in both p53(+/-) and p53(+/-) mice
(Table 2), without any difference noted between the
DMN+FL and the FL alone groups. Foci in the DMN+FL
and FL alone groups were more frequent in males than in
females. Their multiplicity in female p53(+/-) mice of the
DMN+FL group was greater than in their p53(+/-)
counterparts.

PCNA labeling indices of hepatocellular foci in the
DMN+FL and FL alone groups were also increased in
p53(+/-) mice compared to p53(+/-) mice, with statistical
significance in the DMN+FL group of both sexes (Fig. 4).
PCNA labeling indices in nonproliferative areas of the liver
did not differ between p53(+/-) and p53(+/-) mice, but were
higher in the DMN+FL and FL alone groups than in the
respective control groups in both p53(+/-) and p53(+/-) mice
(Table 2). With respect to modification of proliferative hepatocyte
lesions in mice of CBA-origin in the present study,
administration of FL following DMN initiation increased the
incidences as well as multiplicities of hepatocellular altered
foci in both p53(+/-) and p53(+/-) mice, and induced
hepatocellular adenomas and carcinomas in p53(+/-) mice

Discussion

With respect to modification of proliferative hepatocyte
lesions in mice of CBA-origin in the present study,
Fig. 1. Body weight changes in p53(+/−) mice fed diet containing flumequine for 26 weeks with/without DMN-initiation treatment.

Fig. 2. Body weight changes in p53(+/+) mice fed diet containing flumequine for 26 weeks with/without DMN-initiation treatment.
and hepatocellular adenomas in p53(+/-) mice. Interestingly, FL-treatment without DMN-initiation also induced hepatocellular altered foci in almost all surviving males and females of p53(+/-) and p53(+/+) mice and an adenoma in one male p53(+/-) mouse. Since no such proliferative lesions were not observed in the animals fed basal diet in the present study in line with our previous 26-week study, the induction can be considered due to the FL-treatment.

Yoshida et al. also reported proliferative hepatocyte lesions in CD-1 (ICR) mice after administration of FL, with or without prior DEN-initiation, for a maximum of 30 weeks, but the lesions induced by FL alone treatment were infrequent and much lower than those induced by FL-treatment after DEN-initiation. In contrast, in our study, proliferative lesions were numerous, and enhanced development of proliferative lesions was not remarkable in the DMN+FL group in comparison with those in the FL alone group. The carcinogen used for the initiation treatment and the mice used were different in these two studies, but the susceptibility of males.

Regarding mechanisms of induction of hepatic proliferative lesions by FL, a number of possibilities can be considered. Firstly, the percentage of spontaneous gene mutations may be increased due to recurring hepatocellular-death and regeneration. Microscopic examination in our study revealed frequent single cell necroses and anisonucleosis of hepatocytes. The higher PCNA labeling index and thymidine uptake in hepatocytes were high in p53(+/-) mice compared to their wild counterparts, while Delker et al. demonstrated that loss of p53 gene was not associated with the increased number of PCNA positive hepatocytes. Moreover, spontaneous and chemically-induced apoptosis was not controlled by status of p53 gene in these studies. Since our data demonstrated higher expression of PCNA labeling index only in the DMN+FL group but not in other groups including the FL alone group, the involvement of p53 gene in PCNA expression, if any, is limited. Our results suggest a higher susceptibility of CBA mice to FL-induced hepatic tumorigenesis. Moreover, multiplicities of hepatocellular foci were higher in males than in females in both the DMN+FL and FL alone groups of p53(+/-) and p53(+/+) CBA mice, suggesting high susceptibility of males.

Table 2. Incidences and Multiplicities of Hepatic Proliferative Lesions in p53(+/-) and p53(+/+) Mice Fed Diet Containing Flumequine for 26 Weeks with/without DMN-initiation Treatment

<table>
<thead>
<tr>
<th>Mice</th>
<th>Sex</th>
<th>Treatment</th>
<th>No. of animals examined</th>
<th>No. of animals with lesions</th>
<th>No. of foci per mouse ± SD</th>
<th>DMN</th>
<th>FL</th>
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<tr>
<td>p53 (+/-)</td>
<td>Male</td>
<td>+ +</td>
<td>12</td>
<td>12* 1 0</td>
<td>45.3 ± 16.1*</td>
<td>+</td>
<td>+</td>
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<td>+ -</td>
<td>6</td>
<td>2 0 0</td>
<td>0.3 ± 0.5</td>
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<td></td>
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<td>- +</td>
<td>12</td>
<td>12* 1 0</td>
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<td>0 0 0</td>
<td>0.0 ± 0.0</td>
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<tr>
<td></td>
<td>Female</td>
<td>+ +</td>
<td>8</td>
<td>8 1 1</td>
<td>7.4 ± 4.7*$</td>
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<td>+</td>
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<td></td>
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<td>+ -</td>
<td>6</td>
<td>3 0 0</td>
<td>0.7 ± 0.8</td>
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<td>5</td>
<td>5* 0 0</td>
<td>4.6 ± 1.1*$</td>
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<td>4</td>
<td>0 0 0</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>p53 (++)</td>
<td>Male</td>
<td>+ +</td>
<td>14</td>
<td>13 2 0</td>
<td>41.2 ± 19.0*</td>
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<td>+</td>
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<td>+ -</td>
<td>6</td>
<td>4 0 0</td>
<td>1.0 ± 1.1</td>
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<td>14</td>
<td>14* 0 0</td>
<td>27.5 ± 12.7*</td>
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<td>6</td>
<td>1 0 0</td>
<td>0.2 ± 0.4</td>
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<tr>
<td></td>
<td>Female</td>
<td>+ +</td>
<td>12</td>
<td>12* 1 0</td>
<td>3.2 ± 1.4*</td>
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<td>+</td>
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<td>+ -</td>
<td>10</td>
<td>1 0 0</td>
<td>0.1 ± 0.3</td>
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<td>0 0 0</td>
<td>0.0 ± 0.0</td>
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</table>

a: Each value shows mean ± SD. DMN: dimethylnitrosamine, FL: flumequine.
*: p<0.05 between the FL-treated group and the corresponding untreated group.
$: p<0.05 between p53(+/-) mice and p53(+/+) mice.
Flumequine-Induced Liver Tumors can be set based on the NOEL for the hepatotoxicity. However, there remained unsolved points concerning the occurrence of spontaneous gene mutation by FL treatment. The administration of FL without prior initiation induced hepatocellular proliferative lesions within a short latent period in the present study. We can not demonstrate the enhanced carcinogenic susceptibility of \( p53(+/-) \) mice in contrast to the previous data for distinguishing genotoxic carcinogens from noncarcinogens\(^6\text{–}^{10}\). A lack of enhancement of hepatocellular proliferation has been also proved in the TSG-\( p53(+/+) \) mouse, which is another type of \( p53 \)-deficient mouse derived from C57BL/6 background.

Fig. 3. Hepatocellular foci in FL-treated male mice. a and b) \( p53(+/-) \) mouse in the DMN+FL group, c and d) \( p53(+/+) \) mouse in the DMN+FL group, and e and f) \( p53(+/+) \) mouse in the FL alone group. Single cell necroses were observed around the foci. a, c, e; HE. ×15. b, d, f; HE. ×75.
Takizawa, Mitsumori, Takagi et al.

TSG-p53(+/−) mice did not show higher susceptibility to DMN-induced hepatocellular proliferation as compared with their wild type mice, while they showed higher susceptibility in terms of 5-bromo-2'-deoxyuridine labeling index to proliferation of epithelial cells of the urinary bladder induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine17. Phenobarbital, a potent hepatocellular tumor promoter in rodents18, and pentachlorophenol, a hepatocarcinogen with hepatotoxic activity in mice19, failed to induce hepatocellular tumors in TSG-p53(+/+) mice20,21. Therefore, the role of p53 gene in hepatocellular carcinogenesis and the advantages of p53(+/-) mice for detecting hepatocellular carcinogens are issues of future investigations. Regarding the genotoxicity of FL, the 48th JECFA concluded no apparent evidence of genotoxicity of FL, assays using hepatocytes, target cells of the FL-induced tumorigenesis, for in vivo UDS test or for

![Diagram](image)

**Fig. 4.** PCNA labeling indices of hepatocellular foci in p53(+/-) and p53(+/+) mice.
$: p<0.05$ between p53(+/-) mice and p53(+/+) mice. $n$: number of lesions examined, NE: not examined, DMN: dimethylnitrosamine, FL: flumequine.

![Diagram](image)

**Fig. 5.** TUNEL labeling indices of hepatocellular foci in p53(+/-) and p53(+/+) mice.
$: p<0.05$ between p53(+/-) mice and p53(+/+) mice. $n$: number of lesions examined, NE: not examined, DMN: dimethylnitrosamine, FL: flumequine.
DNA damage or DNA adduct were not performed in FL. Therefore, the possibility that FL exerts a direct genotoxic effect on hepatocytes can not be ruled out. However, since a relatively higher dose of FL was selected in the present study according to the results of previous study, FL-induced hepatotoxic changes make the evaluation of the hepatocarcinogenesis difficult. Further experiments addressing on genetic alteration will be necessary to elucidate the precise mechanisms of hepatocarcinogenicity of FL. A third possible mechanism is that indirect genotoxicity may occur. Yoshida et al. showed that 8OH-dG was present in periportal hepatocytes and duct epithelial cells. They thus considered that the oxidative stress to DNA might be an important factor for the induction of proliferative lesions. Although the mechanism for the induction of 8-OHdG after administration of FL remains uncertain, the indirect damage of DNA might participate the tumorigenesis of FL.

In conclusion, in contrast to most short-term carcinogenicity studies of non-genotoxic carcinogens or promoters in p53(+/-) mice, FL induced proliferative lesions of hepatocytes in p53(+/-) as well as p53(+/+) CBA mice after 26-week treatment independent of prior DMN initiation. Therefore, it is strongly suggested that unverified mechanisms such as direct/indirect damage to hepatocellular DNA are responsible for the tumorigenesis of FL in addition to proliferation-dependent tumor promoting effects.

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### Table 3. Hepatic Non-proliferative Lesions and PCNA Labeling Indices of Non-proliferative Areas in p53(+/-) and p53(+/+) Mice Fed Diet Containing Flumequine for 26 Weeks with/without DMN-initiation Treatment

<table>
<thead>
<tr>
<th>Mice</th>
<th>Sex</th>
<th>Treatment</th>
<th>N</th>
<th>Single cell necrosis</th>
<th>Anisonucleosis</th>
<th>PCNA labeling index</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DMN</td>
<td>FL</td>
<td>-</td>
<td>+</td>
<td>++</td>
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<tr>
<td>p53 (+/-)</td>
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<td>+</td>
<td>12</td>
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<td>9</td>
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<td></td>
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<td>+</td>
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<td>6</td>
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<td>0</td>
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<td>-</td>
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<td>4</td>
<td>4</td>
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<tr>
<td></td>
<td>Female</td>
<td>+</td>
<td>+</td>
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<td></td>
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<td>+</td>
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<td>0</td>
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<tr>
<td>p53 (+/)</td>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>14</td>
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</tbody>
</table>

a; -: normal, +: slight, ++: moderate. b: Each value shows mean ± SD.
DMN: dimethylnitrosamine, FL: flumequine, N: number of animals.
**: p<0.01 between the FL-treated group and the corresponding untreated group.


