FULL PAPER

Toxicology

Chronic low-dose exposure to imidacloprid potentiates high fat diet-mediated liver steatosis in C57BL/6J male mice

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ABSTRACT. Hepatic steatosis is known to precede a continuum of events that lead to hepatic metabolic dysfunction, inflammation and carcinogenesis. Recently, studies have linked xenobiotic exposures to hepatic steatogenesis and its associated metabolic disorders; however, the underlying mechanisms remain elusive. This study aimed to elucidate the mechanistic role of imidacloprid in the prevalence of high fat diet (HFD)-induced liver steatosis, using a C57BL/6J mice model. Mice (3 weeks old) were fed with HFD and treated with 0.6 mg/kg bw/day (one-tenth of the NOAEL) of imidacloprid through water or diet, for 24 weeks. In a controlled group, mice were fed with only HFD. At the end of the study, imidacloprid treatment significantly potentiated HFD-induced body weight gain in mice. Also, imidacloprid increased the liver weights of mice, with complimentary reductions in mesenteric and gonadal white adipose tissue weights. Histopathological analysis of liver revealed a drastic steatosis in imidacloprid treated mice. Following a real-time qPCR analysis, imidacloprid upregulated transcriptions of hepatic fatty acid biosynthesis-related transcription factors and genes. Imidacloprid also induced hepatic expression of the gene encoding pregnane X receptor; but had no significant effect on hepatic expressions of liver X receptor and aryl hydrocarbon receptor. The imidacloprid treatment further enhanced serum alanine aminotransferase levels but downregulated hepatic antioxidant mRNA expressions. Ultimately, this study suggested an imidacloprid-potentiation effects on prevalence of HFD-induced liver steatosis via transcriptional modulations of the hepatic FA biosynthesis pathway.

KEY WORDS: neonicotinoid insecticides, imidacloprid, liver steatosis, no observable adverse effect level (NOAEL)

Liver steatosis, commonly called fatty liver, is considered as the commonest liver disease in humans which when prolonged, leads to more complicated conditions such as steatohepatitis, fibrosis, cirrhosis, liver failure and hepatocellular carcinoma [15, 34]. The steatosis endpoint is highly employed in USEPA's IRIS assessment system for deciding on the safety integrity of many environmental chemicals [6]. Overconsumption of high fat diet is a well-known risk factor for liver steatogenesis [12, 34];

however, the interactive effects of environmental chemicals and high fat diet (HFD) on the prevalence of liver steatosis is not well reported. Induction of hepatic steatosis is mostly instigated by nonexclusive mechanisms such as (i) increased hepatic uptake of nonesterified fatty acids, derived from adipocyte lipolysis, (ii) increased hepatic *de novo* lipogenesis, (iii) reduced hepatic β-oxidation of fatty acids and, (iv) decreased export of hepatic triglycerides (TG) into blood as lipoprotein particles.

Environmental chemical exposures have been tipped to mediate the recent prevalence of liver steatosis in human populations. Previous studies using *in-vivo* models, suggested a link between exposures to perfluorooctanesulfonic acid (PFOS), Per- and Polyfluoroalkyl Substances (PFAS) and the development of liver steatosis in mammals [6]. Findings from recent studies [18, 45] have consistently predicted steatosis as a possible neonicotinoid endpoint; however, the exact molecular mechanistic basis for these predictions are still in obscurity.

Imidacloprid (*N*-{1-[(6-chloro-3-pyridyl) methyl]-4,5-dihydroimidazol-2-yl}-nitramide) is a systematic insecticide used in many pest management systems across the globe. Being a neonicotinoid, imidacloprid agonizes the insects' nicotinic acetylcholine receptor (nAChR); which is well expressed in the central nervous system of both vertebrates and invertebrates [31, 42]. It is heavily used in both agriculture and veterinary health care for various pest management routines [42]. Since its introduction in the1990's, the global sales and patronage of imidacloprid has been impressively high. In the United States alone, the market sales of imidacloprid surged to about U.S. $1.1 billion in 2009 [33, 35]. Globally, imidacloprid is considered as the most successful amongst the neonicotinoid class of insecticides, accounting for about 41.5% of the total neonicotinoid use [33]. In recent times however, the global preference for imidacloprid has instigated some public safety concerns; with numerous questions being raised about its potential infiltration into various environmental systems; and the rippling effects on ecosystem diversity. At the back of these concerns, several studies have been carried out to assess the environmental levels and safety integrity of imidacloprid; and most of these studies have detected residues of imidacloprid in many environmental matrices including soils, water, sediments etc.; and in food matrices such as vegetables, fruits, honey and tea [4, 10, 19, 23]. Also, some biomonitoring studies have consistently reported high detection frequencies of imidacloprid in human specimens and specimens from other non-target vertebrates [11, 14, 22, 36, 39–41, 46]. Despite these findings, little is known about the potential adverse health outcomes associated with chronic exposures to field realistic doses of imidacloprid in human populations. Moreover, mechanistic studies revealing the definitive associations existing between low dose exposures to imidacloprid and its adverse health effect outcomes in human populations are highly limited.

In a 3-month exposure study [30], mice co-exposed to a low dose of imidacloprid and HFD showed increased body weight gain, increased adiposity and increased plasma TG levels. These findings implicate that, imidacloprid exposures could disrupt physiological lipid homeostasis in the mammalian model. However, exact molecular pathway(s), clarifying imidacloprid effects in adipocyte and hepatic lipid homeostasis in mammalian systems, remain unknown.

The present study sought to determine whether chronic co-exposure to a low dose of imidacloprid (thus; less than the "no observable adverse effect level; NOAEL" of imidacloprid) and HFD could potentiate the development of liver steatosis in a C57BL6/J mice model; and to unravel the possible underlying mechanisms. We investigated the imidacloprid treatment effects on weights of whole body, liver and adipose tissues of mice. We also determined the imidacloprid effects on serum levels of nonesterified fatty acids (NEFA), TG and alanine aminotransferase (ALT); hepatic lipid content and hepatocyte morphology by histopathology; adipocyte lipid homeostasis by comparative mRNA expressions of genes such as hormone-sensitive lipase (*Hsl*), Adipose triglyceride lipase (*Atgl*), adenylyl cyclase (*Ac*), adipocyte protein 2 (*ap2*) and cluster of differentiation 36 (*Cd36*). Finally, we tested the potency of imidacloprid in triggering various molecular initiation events (MIEs) and key events (KEs) found within the steatosis adverse outcome pathway (AOP), proposed by Mellor *et al.* [21] and modified by Lichtenstein *et al.* [18].

To the best of our knowledge this study is the first to report on the interactive roles of imidacloprid and HFD in the development of liver steatosis.

MATERIALS AND METHODS

Materials

Imidacloprid (>98%) and imidacloprid-d4 (purity: >97.0%) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Male C57BL/6J mice, aged 3 weeks were obtained from Sankyo Labo Service Corp., Inc. (Tokyo, Japan). Open source powdered diet (D12451M, 45 Kcal % fat, with red dye, high fat diet) was purchased from Research Diets Inc. (New Brunswick, NJ, USA). Triglyceride assay kits were purchased from BioVision Inc. (Milpitas, CA, USA). NEFA assay kit was from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). The ALT assay kit was purchased from Fujifilm Corp. (Yokohama, Japan). All other reagents used in this study were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

Animals and diet

This animal study was conducted in accordance with the Institutional Animal Care and Use Committee of the Graduate school of Veterinary Medicine, Hokkaido University, Japan. The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, in conformity with the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC; approval number: 18–0061). The animal facility used for the present study was controlled for temperature (20–25°C), humidity (40–60°C) and 12-hr light/dark cycle. After 2 weeks of acclimatization, mice were divided into three groups (HFD_Only, IMI_Diet and IMI_Water groups; 4 mice per group). Mice in the HFD_Only group were treated with *ad libitum* access to 45% Kcal HFD only (control); mice in IMI_Diet group were provided with *ad libitum* access to 45% Kcal HFD fortified with 0.6 mg/kg bw/day of imidacloprid (imidacloprid exposure via diet); mice in IMI_Water group were provided
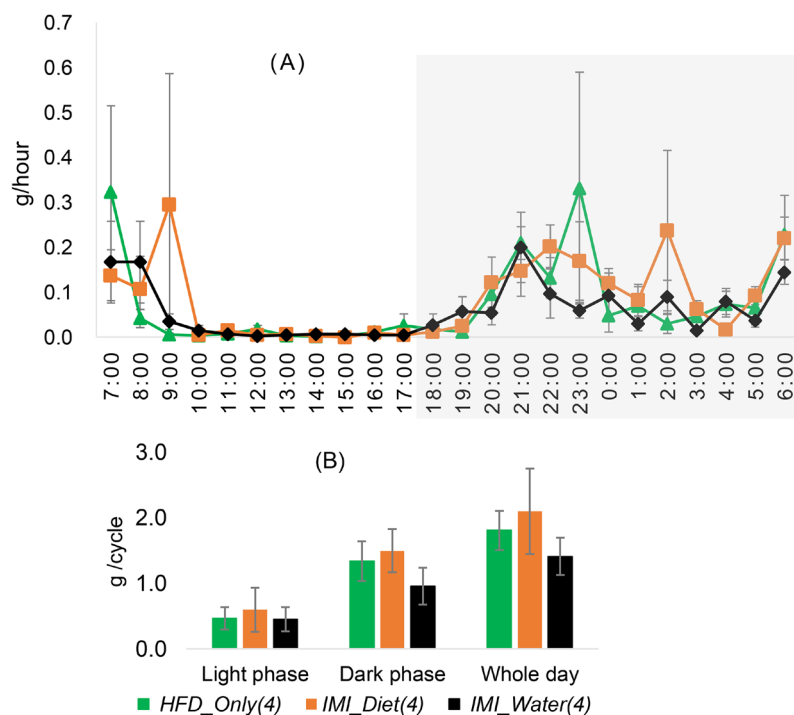
Fig. 1. (A) mean food consumption rate per hr, (B) mean food consumption rate per cycle. HFD_Only (High fat diet_Only) means mice exposed to only powdered high fat diet; IMI_Diet (Imidacloprid_Diet) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through powdered high fat diet; IMI_Water (Imidacloprid_Water) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through water.

with *ad libitum* access to water fortified with 0.6 mg/kg bw/day of imidacloprid; and a 45% Kcal HFD that does not contain imidacloprid (imidacloprid exposure via water). Diet and water were given to mice *ad libitum* throughout the experiment; and were provided fresh three times in a week. Composition of the high fat diet used for the present study has been shown in Supplementary Table 1. The entire imidacloprid treatment lasted for 24 weeks. Due to emergencies of workload, body weight gains of mice were monitored only for the first twelve weeks of the experiment. The dose of imidacloprid used in this study (0.6 mg/kg bw/day) was 10 times lower than the NOAEL dose of imidacloprid (5.7 mg/kg bw/day), set for mice [8]. The food consumption by mice were measured over a period of 48-hr (including a 24-hr acclimatization period), using a food consumption monitoring system (cFDM-300AS; Melquest, Toyama, Japan); the details of consumption measured for the mice have been presented in Fig. 1. In this study, the average daily intakes of imidacloprid by the mice were estimated to be 0.7 ± 0.02 mg/kg bw/day and 0.6 ± 0.02 mg/kg bw/day for IMI_Diet and IMI_Water groups, respectively. At the end of the imidacloprid exposure, mice were sacrificed by CO_2 asphyxiation. At necropsy, blood, liver, and adipose tissues (including inguinal, mesenteric and gonadal white adipose tissues; and interscapular brown adipose tissues) were collected and processed accordingly, for biochemical assays. Prior to processing and storage, liver and adipose tissue weights were duly measured.

Serum biochemical analysis

Serum levels of NEFA and TG were determined with commercial kits following the manufacturers' instructions. The serum ALT levels were measured by Automatic Analyzer 7180 (Hitachi High-Technologies, Tokyo, Japan).

Histopathological analysis

Livers of mice were histologically examined by hematoxylin and eosin (H&E) staining; and fatty degenerated hepatocytes (hepatocytes containing lipid droplets) were quantified using Image J software (version 1.44C). Liver tissues from all experimental groups were fixed in 10% neutral buffered formalin and embedded in paraffin. Three-micrometer sections of the paraffin embedded tissues were stained with H&E and analyzed by light microscopy for lipid droplets (LDs) in hepatocytes. Histological images were captured using Olympus FSX100 microscope (Olympus, Tokyo, Japan). LD in hepatocytes were quantified using the ImageJ software, as described previously by [7].

Chemical analysis

In this study, we evaluated the effectiveness of imidacloprid treatment by analyzing blood imidacloprid levels of all the mice, according to the method described previously by Ohno *et al.* [29], with slight modification. Briefly, a 20 µl aliquot of blood sample was fortified with 0.1 ng of imidacloprid-d4 standard (1 ppb; 100 µl). Further, a 0.4 ml of 1% formic acid in acetonitrile was added to the sample to precipitate blood protein. The sample was mixed thoroughly by vortex; and centrifuged at $10,000 \times g$ for 10 min.

The supernatant was separated as SP1 (supernatant 1). The residue was subsequently reconstituted in a 0.5 m*l* of methanol, mixed thoroughly by vortex and centrifuged at 10,000 × *g* for 10 min. Supernatant from the second extraction was carefully separated as SP2 (supernatant 2). Both SP1 and SP2 were combined, and the combined extract was purified by the solid phase extraction method, as described by [23]. Following the extraction and purification process, the sample was analyzed by an LC-ESI/MS/MS (Agilent 6495B, Agilent Co., Santa Clara, CA, USA) system equipped with Kinetex Biphenyl (2.1 mm ID × 150 mm, ø 1.7 μm; Phenomenex, Inc., Torrance, CA, USA). Solvents A and B used for the LC-ESI/MS/MS analysis were 0.1% formic acid + 10 mM ammonium acetate water solution and 0.1% formic acid + 10 mM ammonium acetate methanol solution, respectively. The gradient was programmed as follows: t=0–1 min. 5% B (isocratic), t=6 min: 95% B (gradient), t=6–8 min (gradient): 95% B (isocratic). The column oven temperature and flow rate were 60°C and 0.5 ml/min, respectively. Detection of target compounds was performed by multiple-reaction monitoring (MRM) in positive ionization mode with selected m/z ions: 256.1>175.1 for imidacloprid and 260.1>213.1 for imidacloprid-d4). Analytes were quantitated using internal standard method; and calibration curves were generated by mixing imidacloprid standards with blank blood specimens to final concentrations ranging from 0.0005 to 5 ng/ml (n=7). Extraction and purification of calibration standards were performed using the method described above; and linearity was found to exceed r^2=0.9 in the calibration curve. Reproducibility of the analysis was confirmed by multiple analysis, with a relative standard deviation (RSD) of 10% for all compounds. Limits of quantitation (LOQs) for imidacloprid was 0.003 ng/ml; and it was calculated as the lowest points on standard curves which had a with relative standard deviation of <15% (n=7) and signal-to-noise ratios of 5:1.

mRNA expression analysis

Total RNA in mice gonadal adipose tissues and liver tissues were extracted using TRI Reagent® (Sigma Life Science, St. Louis, MO, USA) and NucleoSpin® kit (Macherey-Nagel, Düren, Germany), and synthesized for cDNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Life Science Department, Osaka, Japan). The respective oligonucleotide primer pairs used for the mRNA expression analysis have been presented in Supplementary Table 2. The amplification efficiency of all the oligonucleotide primer pairs used in the current study were confirmed to be above 95%. The qRT-PCR (StepOnePlus Real-Time PCR system, Applied Biosystems, Foster City, CA, USA) was performed using a 10 μl PCR reaction mixture containing Fast SYBR Green Master Mix (Applied Biosystems), forward and reverse primers (Thermo Fisher Scientific, Life Technologies Japan Ltd., Tokyo, Japan) and cDNA of adipose tissue and liver samples. The qPCR condition was set 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Quantification of the transcripts was performed by the 2−ΔΔCT method, with GAPDH- and ACTB-assisted normalization.

Statistical analysis

Data from the present study were statistically analyzed using JMP Pro13 (SAS Institute, Cary, NC, USA). The data from all the experimental groups were tested for normality using the Shapiro-Wilk test; and for homogeneity of variance using the Levene's test. The student's *t*-test was used to evaluate the statistical differences in means of cumulative weight gains between the control and exposure groups. Moreover, the Dunnett's parametric and nonparametric multiple comparison tests were used to determine significance for each treatment relative to control, * represents *P*<0.05; non-parametric, ** represents *P*<0.001; non-parametric, #represents *P*<0.05; parametric.

RESULTS

Imidacloprid facilitated high fat diet-induced body weight gain

To validate the low dose effects of imidacloprid on HFD-induced body weight gain and adiposity, weekly body weight gains of mice were monitored and recorded for the first 12 weeks of imidacloprid exposure; and the results have been shown in Fig. 2. Mice that were treated with imidacloprid (IMI_Diet and IMI_Water groups) exhibited enhanced weekly body weight gains, compared to those that were fed with only high fat diet (HFD_Only group). Throughout the entire exposure period (24 weeks), mice in the IMI_Diet group exhibited higher margins of weekly body weight gains, compared to those in the IMI_Water group. Also, compared to the mice fed with only high fat diet (HFD_Only group), mice within the IMI_Diet group showed significant body weight gains; starting from week 3 until the 12th week of the experiment (Dunnett's parametric multiple comparison tests; # represents *P*<0.05). In case of the IMI_water group, significant differences in terms of body weight gains, were observed at week 3 and week 12 only. The total body weight gained by the IMI_Diet mice was found to be significantly higher than that of the HFD_Only mice by 9.9% (*P*<0.0001, *t*-test). Also, the cumulative body weight gained by mice in the IMI_Water group significantly exceeded that of the HFD_Only group by 4.9% (*P*<0.0001, *t*-test). The IMI_Diet group showed a significant differential weight gain just after 3 weeks of imidacloprid exposure.

In the present study, we also monitored the food consumption by mice over a 24 hr light/dark cycle are shown in Fig. 1. The mean food consumption rate per 24 hr was observed to be highest for mice in the IMI_Diet group, followed by those in the HFD_Only group; with the IMI_Water group consuming the least amount of diet per day. In all, the daily calorie intake by IMI_Diet group was observed to be 5.5% higher than that of the HFD_Only group. In contrast, mice in the IMI_Water group were found to consume 7.5% less calories per day, compared to that of the HFD_Only group. Nonetheless, the differences in daily calorie intakes observed amongst all the experimental groups were statistically insignificant.

Imidacloprid induced liver weight gain, with a complimentary reduction in adipose tissue weights

The weights of organs and tissues of mice measured in the present study, have been shown in Fig. 3. Mice in the IMI_Diet and
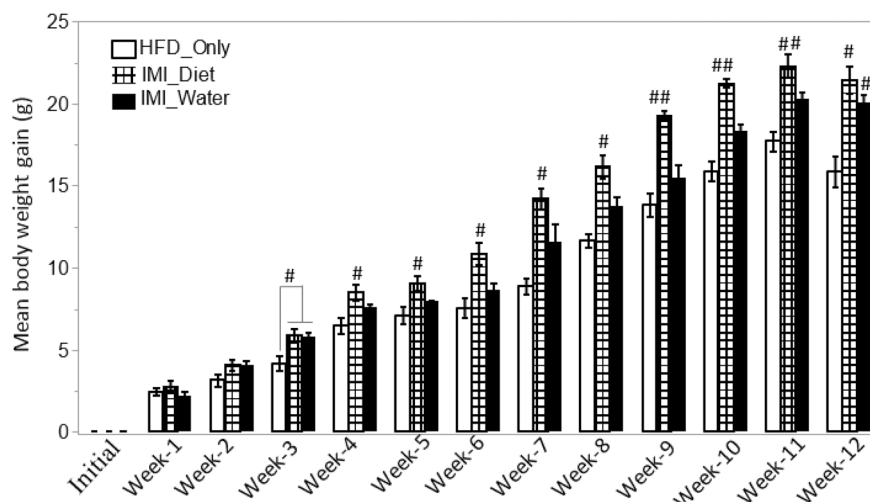
Fig. 2. Weekly body weight gains of mice measured for the first twelve (12) weeks of imidacloprid exposure. HFD_Only (High fat diet_Only) means mice exposed to only powdered high fat diet; IMI_Diet (Imidacloprid_Diet) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through powdered high fat diet; IMI_Water (Imidacloprid_Water) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through water. ANOVA with Dunnett's parametric multiple comparison tests were used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), # represents $P<0.05$, ## represents $P<0.01$.
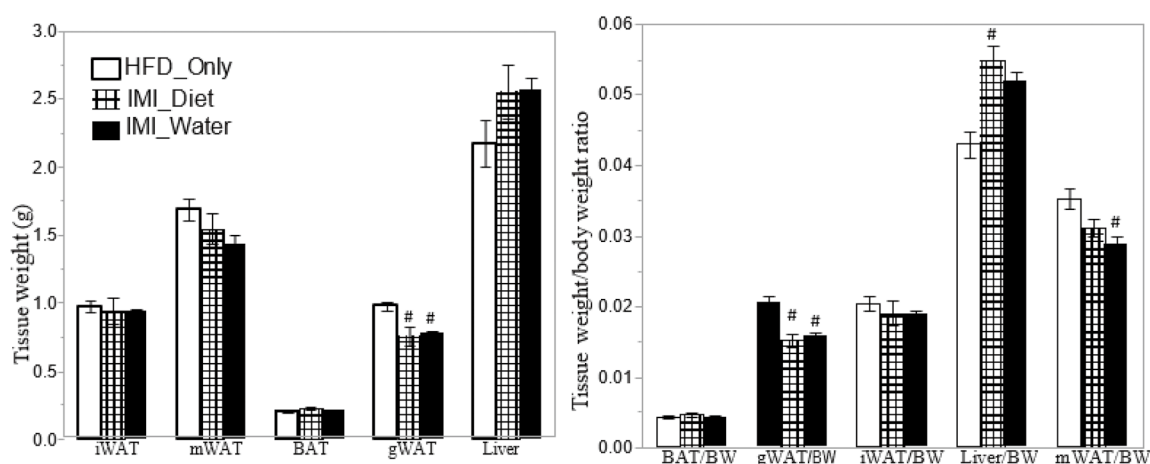
Fig. 3. Weight of tissues harvested from mice after 24 weeks of imidacloprid exposure. HFD_Only (High fat diet_Only) means mice exposed to only powdered high fat diet; IMI_Diet (Imidacloprid_Diet) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through powdered high fat diet; IMI_Water (Imidacloprid_Water) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through water; iWAT means inguinal adipose tissue, mWAT means mesenteric adipose tissue, BAT means brown adipose tissue, gWAT means gonadal adipose tissue. ANOVA with Dunnett's parametric multiple comparison tests were used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), # represents $P<0.05$.

IMI_Water groups exhibited reduced gonadal white adipose tissue (gWAT) and mesenteric white adipose tissue (mWAT) weights, compared to those in the HFD_Only group. The absolute gWAT weights of the imidacloprid treated mice (IMI_Diet and IMI_Water groups) were observed to be significantly lower, compared to the absolute gWAT weights of the HFD_Only mice (Dunnett's parametric multiple comparison tests; # represents $P<0.05$). Also, the gWAT to body weight ratio and the mWAT to body weight ratio of mice in the imidacloprid treated groups were found to be significantly lower compared to that of the HFD_Only mice (Dunnett's parametric multiple comparison tests; # represents $P<0.05$). However, weights of inguinal white adipose tissue (iWAT) and interscapular brown adipose tissue (BAT) were found to be unaltered by the imidacloprid treatments. Meanwhile, the imidacloprid treatments induced liver weight gains within the IMI_Diet and IMI_ Water groups. Particularly, the liver to body weight ratio of the IMI_Diet treated mice significantly exceeded the liver to body weight ratio of mice fed with only HFD fat diet (Dunnett's parametric multiple comparison tests; # represents $P<0.05$).

(A)

(B)
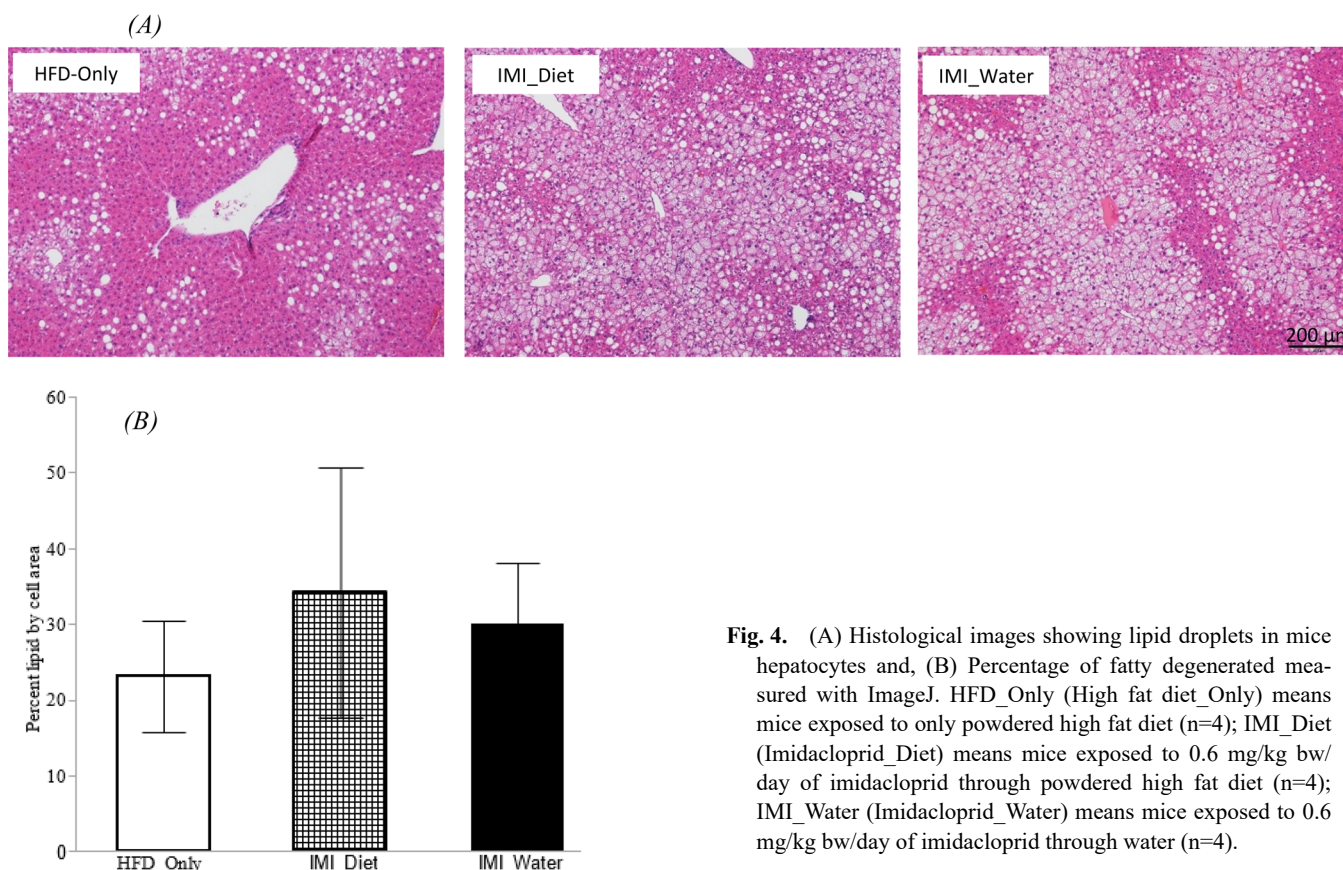
Fig. 4. (A) Histological images showing lipid droplets in mice hepatocytes and, (B) Percentage of fatty degenerated measured with ImageJ. HFD_Only (High fat diet_Only) means mice exposed to only powdered high fat diet (n=4); IMI_Diet (Imidacloprid_Diet) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through powdered high fat diet (n=4); IMI_Water (Imidacloprid_Water) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through water (n=4).

Table 1. Serum levels of nonesterified fatty acid (NEFA), triglycerides (TG) and alanine aminotransferase/glutamic pyruvic transaminase (ALT/GPT) recorded in mice

	NEFA (mequiv/l)		TG (mg/dl)		ALT/GPT (IU/dl)	
	Range	Mean ± STDEV	Range	Mean ± STDEV	Range	Mean ± STDEV
HFD_Only	0.14–0.16	0.15 ± 0.01	60.0–95.0	81 ± 15.1	67.0–121.0	89.4 ± 25.6
IMI_Diet	0.14–0.16	0.15 ± 0.01	71.0–156.0	120.5 ± 33.2	70.0–211.0	126.3 ± 53.2
IMI_Water	0.14–0.16	0.15 ± 0.01	85.0–128.0	111.5 ± 17.6	109.0–185.0	137.8 ± 29.0

STDEV means standard deviation; HFD_Only (High fat diet_Only) means mice exposed to only powdered high fat diet; IMI_Diet (Imidacloprid_Diet) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through powdered high fat diet; IMI_Water (Imidacloprid_Water) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through water.

*Imidacloprid treatment potentiated high fat diet-induce liver steatosis*

From the histological analysis (Fig. 4), livers of the IMI_Diet and IMI_Water treated mice were observed to contain more lipid droplets within the hepatocytes, compared to the liver tissues of mice within the HDF-Only group. Invariably, the imidacloprid treated mice (IMI_Diet and IMI_Water) showed a more drastic hepatic steatosis, compared to the HFD_Only mice (Fig. 4). Also, the hepatic morphometric analysis with Image J software revealed that, livers of mice that were treated with imidacloprid (IMI_Diet and IMI_Water) have high amount of fatty degenerated hepatocytes, compared to livers of the mice in HFD_Only group (Fig. 4).

Imidacloprid treatment increased serum triglyceride and alanine aminotransferase levels

Serum TG levels were found to be relatively higher in mice that were treated with imidacloprid (IMI_Diet; 120 ± 53.2 mg/dl, IMI-Water; 137.8 ± 29.0 mg/dl), compared to that of the mice fed with only HFD (81 ± 15.1 mg/dl) (Table 1). Mice that were treated with IMI_Diet and IMI_Water showed higher serum ALT levels (126.3 ± 53.2 and 137.8 ± 29.0 IU/dl respectively), compared to the serum ALT levels of mice treated with HFD_Only (89.4 ± 25.1 IU/dl). Imidacloprid treatment, either through diet or water, showed no significant effect on serum NEFA levels among all the experimental groups.

The dietary exposure showed higher imidacloprid treatment efficiency than the water exposure

The blood levels of imidacloprid detected in mice that were used in the current experimental model has been shown in Fig. 5. The blood-imidacloprid levels detected for mice in the HFD_Only group were all below LOQ. However, the blood-imidacloprid levels of
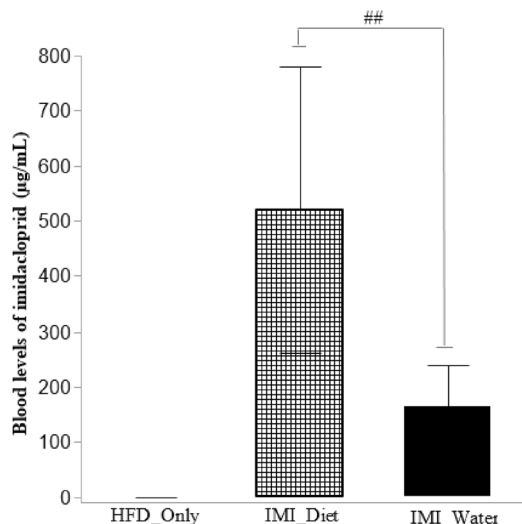
Fig. 5. Blood levels of imidacloprid detected in the mice model used in the present study. HFD_Only (High fat diet_Only) means mice exposed to only powdered high fat diet; IMI_Diet (Imidacloprid_Diet) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through powdered high fat diet; IMI_Water (Imidacloprid_Water) means mice exposed to 0.6 mg/kg bw/day of imidacloprid through water. One-way ANOVA with Dunnett's parametric multiple comparison tests (Steel with control; JMP Pro13) was used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), ## represents $P<0.01$.

Fig. 6. Effects of imidacloprid on mRNA expressions of genes which regulate lipolysis and fatty acid uptake in gonadal adipose tissue. One-way ANOVA with Dunnett's nonparametric multiple comparison tests (Steel with control; JMP Pro13) was used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4).
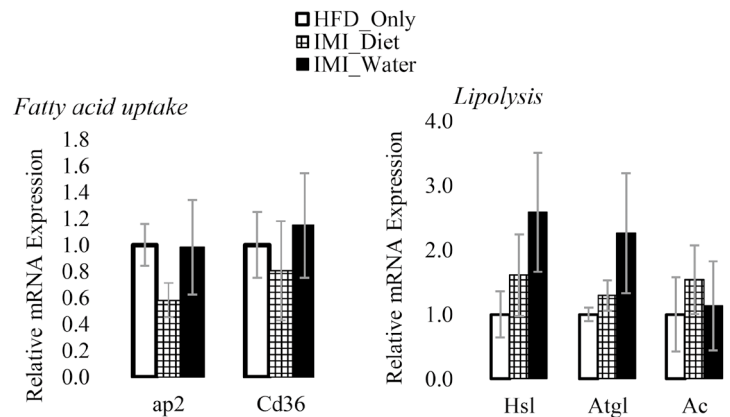
mice within the IMI_Diet group was found to be significantly higher than that of the mice in the IMI_Water group (Dunnett's parametric multiple comparison tests, ## represents $P<0.01$). Efficiency of the imidacloprid treatment routine adopted in the current experimental model was assumed to be dependent on the daily intakes of diet by IMI_Diet group or water by IMI_Water group. Daily intakes of IMI-water by the mice might be lower than expected, due changes in taste and/or smell of water presumably induced by the imidacloprid fortification. In a previous study, imidacloprid treatment through powdered diet was proven to be effective [35].

Imidacloprid treatment induced adipocyte lipolysis

The IMI_Diet treatment downregulated mRNA expressions of *ap2* and *Cd36* genes which are essential for adipocyte lipid uptake from plasma (Fig. 6). Meanwhile, imidacloprid treatments, either through diet or water, upregulated mRNA expressions of *Hsl* and *Atgl* genes which are key regulators of adipocytes lipolysis (Fig. 6). Transcription of the *Ac* gene was specifically upregulated in white adipose tissues of mice that were treated with imidacloprid through diet (Fig. 6).

Imidacloprid treatment induced hepatic expression of pregnane X receptor encoding gene

We tested the imidacloprid treatment effects on the mRNA expression of genes encoding the pregnane X receptor (*Pxr*), peroxisome proliferator-activated receptor γ (*Pparγ*), aryl hydrocarbon receptor (*Ahr*) and liver X receptor a and b (*Lxra*, *Lxrb*); and the results have been shown in Fig. 7. The
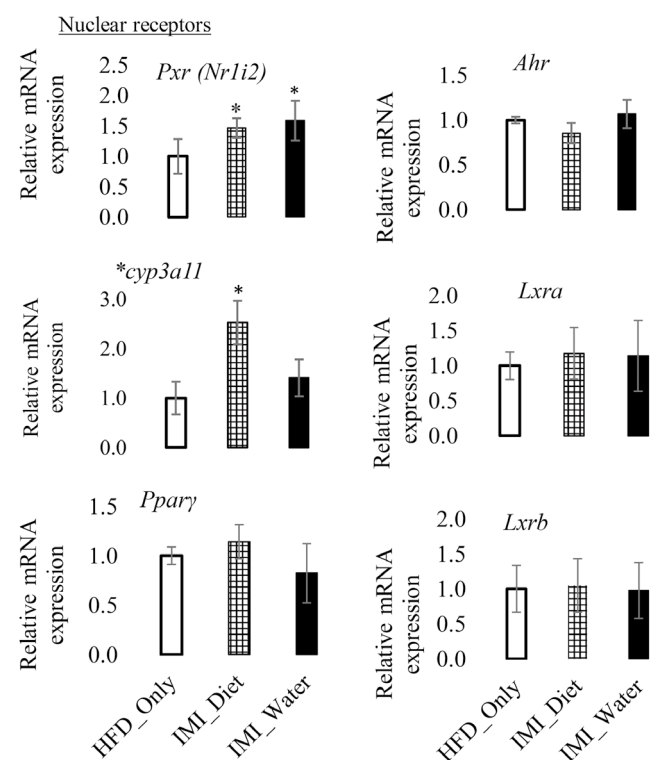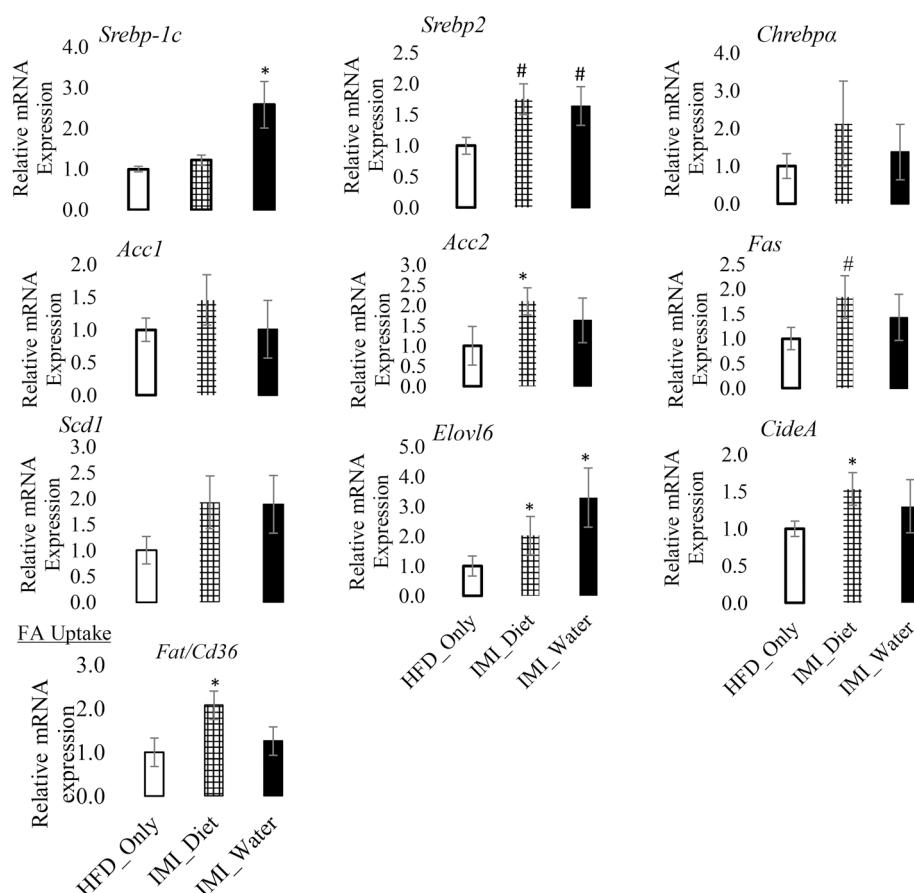

Fig. 7. Effects of imidacloprid on hepatic transcriptions of nuclear receptors associated with the steatosis adverse outcome pathway. *Cyp3a11 is a major *Pxr* target *Cyp450* isoform; hence its mRNA expression levels were analyzed to validate the imidacloprid-mediated *Pxr* transcription effects observed in this study. One-way ANOVA with Dunnett's nonparametric multiple comparison tests were used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), * represents $P<0.05$.

imidacloprid treatment through diet or water, significantly upregulated the hepatic expression of the *Pxr* encoding gene, *Nr1i2* (Dunnett's nonparametric multiple comparison tests, * represents $P<0.05$). In confirmation of the aforementioned observation, the imidacloprid dietary treatment significantly upregulated the mRNA expression of the main *Pxr*-target cytochrome P450 isoform, *Cyp3a11* (Dunnett's nonparametric multiple comparison tests, * represents $P<0.05$). However, the imidacloprid treatment showed no significant effects on hepatic transcriptions of genes encoding *Pparγ, Ahr, Lxra* and *Lxrb*.

*Imidacloprid treatment induced de novo lipogenesis (DNL) and fatty acid uptake in liver*

The imidacloprid treatments enhanced the hepatic mRNA expressions of all the DNL-related transcription factors such as; sterol regulatory element-binding protein 1c (*Srebp-1c*), sterol regulatory element-binding protein 2 (*Srebp2*) and carbohydrate-responsive element-binding protein-α (*Chrebpα*); Fig. 8. Specifically, the imidacloprid treatment through water and diet, significantly induced mRNA expression of *Srebp2* (Dunnett's parametric multiple comparison tests #represents $P<0.05$). In case of *Srebp-1c* however, the mRNA expression patterns observed was only significant for the IMI_Water treated mice (Dunnett's parametric multiple comparison tests #represents $P<0.05$). Nonetheless, the IMI_Diet treatment upregulate hepatic mRNA expressions of most of the *Srebp-1c*, *Srebp2* and *Chrebpα* target genes located along the DNL pathway, thus; fatty acid synthase (*Fas*), acetyl-CoA carboxylase 1 and 2 (*Acc1 and Acc2*), stearoyl-CoA desaturase-1 (*Scd1*) and elongation of very long chain fatty acids protein 6 (*Elovl6*). Especially, the IMI_Diet-induced hepatic transcriptions of *Fas, Acc2* and *Elovl6* were found to be statistically significant (Dunnett's parametric and nonparametric multiple comparison tests, # represents $P<0.05$; parametric, * represents $P<0.05$; nonparametric). Except for *Acc1*, the IMI_Water treatment also enhanced the hepatic mRNA expressions of all the *Srebp-1c, Srebp2* and *Chrebpα* target genes, considered in the present study (*Fas, Acc2, Scd1* and *Elovl6*). Again, the imidacloprid-diet treatment significantly induced hepatic mRNA expressions of cell death-inducing DNA fragmentation factor alpha-like effector A (*CideA;* Dunnett's nonparametric multiple comparison tests, * represents $P<0.05$). Also, the dietary imidacloprid treatment significantly upregulated hepatic mRNA expressions of fatty acid translocase (*Fat/Cd36*) which is mainly associated with hepatic FA uptake

Fig. 8.　Effects of imidacloprid on transcriptions of genes that regulate hepatic de novo lipogenesis and hepatic fatty acid uptake. One-way ANOVA with Dunnett's parametric and nonparametric multiple comparison tests were used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), * represents $P<0.05$; non-parametric, # represents $P<0.05$; parametric.

Fatty acid β-oxidation
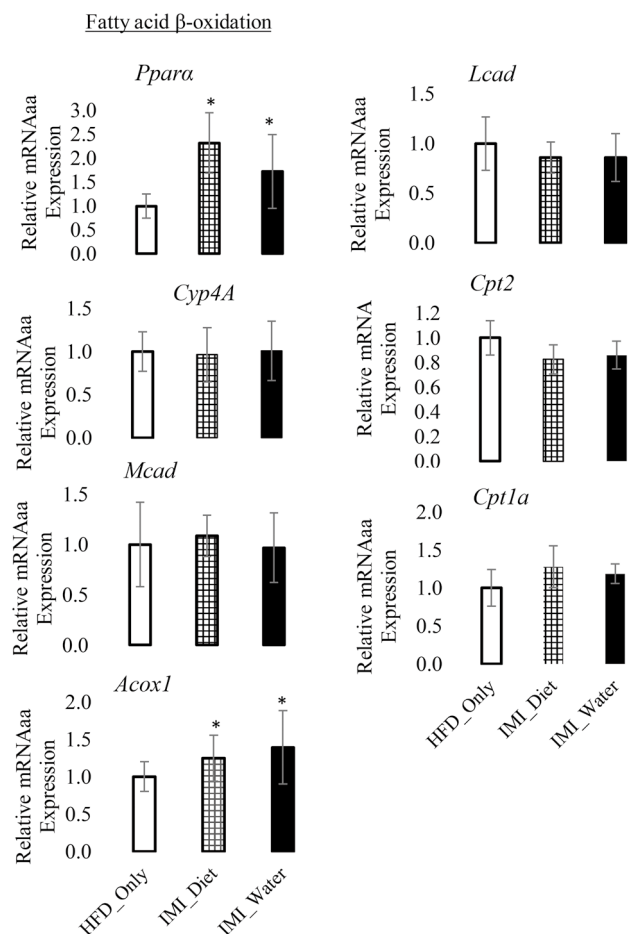
Fig. 9. Effects of imidacloprid on mRNA expressions of genes which regulate hepatic *de novo* lipogenesis and hepatic mitochondrial β-oxidation of fatty acid. One-way ANOVA with Dunnett's parametric and nonparametric multiple comparison tests were used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), * represents $P<0.05$; non-parametric.
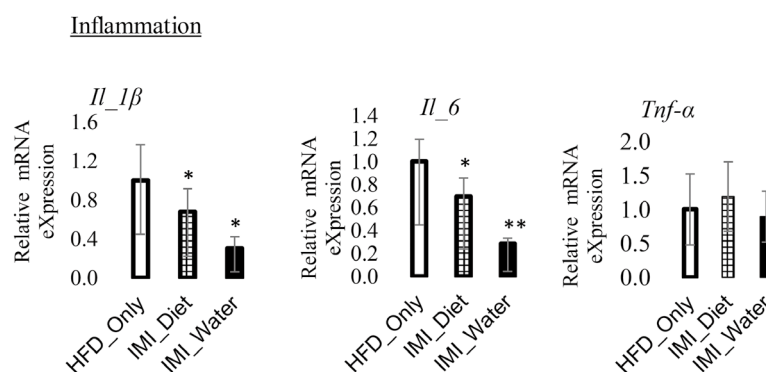
(Fig. 8; Dunnett's parametric and nonparametric multiple comparison tests, # represents $P<0.05$; parametric, * represents $P<0.05$).

Imidacloprid treatment showed a paradoxical effect on mitochondrial fatty acid β-oxidation

As shown in Fig. 9, the imidacloprid treatment, either through water or diet, significantly upregulated the mRNA expressions of peroxisome proliferator-activated receptor α (*Pparα*), a key transcription factor involved the transcriptional regulation of gene networks located along the hepatic mitochondrial FA β-oxidation pathway (Dunnett's nonparametric multiple comparison tests, * represents $P<0.05$; ** represents $P<0.01$). However, the imidacloprid treatment did not show any significant effects on mRNA expressions of most of the *Pparα* downstream target genes (medium-chain acyl-CoA dehydrogenase; *Mcad,* long-chain acyl-CoA dehydrogenase; *Lcad,* cytochrome P450 4A; *Cyp4A,* carnitine palmitoyltransferase 1A; *Cpt1a* and carnitine palmitoyltransferase 2; *Cpt2*) located within the hepatic mitochondrial FA β-oxidation cascade. Interestingly, the imidacloprid treatments upregulated the hepatic transcription of acyl-CoA oxidase 1 (*Acox1*), which is a known *Pparα* agonist (Fig. 9; Dunnett's nonparametric multiple comparison tests, * represents $P<0.05$).

Imidacloprid treatment repressed hepatic expression levels of some inflammatory cytokines

Hepatic mRNA expressions of inflammatory cytokines such as interleukin-1β (*Il-1β*), interleukin-6 (*Il-6*) and tumor necrosis factor-alpha (*Tnf-α*) were measured and compared between the HFD tread mice (HFD_Only) and the imidacloprid exposed mice (thus; IMI_Diet and IMI_Water groups) (Fig. 10). The imidacloprid treatment significantly repressed the hepatic transcriptions of *Il-1β* and *Il-6* (Dunnett's nonparametric multiple comparison tests, * represents $P<0.05$; ** represents $P<0.01$). However, no significant differences in hepatic mRNA expression levels of *Tnf-α* were observed between the HFD tread mice and the imidacloprid exposed mice.

Inflammation

Fig. 10. Effects of imidacloprid treatment on mRNA expressions of inflammation- and oxidative stress-related genes. One-way ANOVA with Dunnett's parametric and nonparametric multiple comparison tests were used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), * represents $P<0.05$; non-parametric, ** represents $P<0.001$; non-parametric.

Imidacloprid treatment downregulated the hepatic expressions of antioxidant enzymes

In the current experimental model, imidacloprid treatment effects on oxidative stress status of the liver were assessed by measuring hepatic mRNA expressions of stress-related genes (nuclear factor erythroid 2-related factor 2; *Nrf2,* inducible nitric oxide synthase; *Nos2*) and antioxidant enzymes (superoxide dismutase; *Sod,* glutathione reductase; *Gsr,* glutathione peroxidase; *Gpx,* catalase; *Cat* and glutathione S-transferases; *Gst*) (Fig. 11). The hepatic expression levels of *Nrf2* were suppressed by the imidacloprid treatment; especially, the difference in hepatic expression levels of *Nrf2* between the IMI_Water treated mice and the HFD_Only treated mice was found to be statistically significant (*P*<0.05, Dunnett's nonparametric multiple comparison tests). There were no imidacloprid treatment effects on hepatic mRNA expressions *Nos2*. With regards to antioxidant enzymes, the hepatic mRNA expressions of *Sod, Gsr* and *Gst* were observed to be significantly downregulated by imidacloprid treatment through diet (IMI_Diet; Dunnett's nonparametric multiple comparison tests, * represents *P*<0.05). Also, imidacloprid treatment through water (IMI_Water) significantly downregulated the mRNA expressions of *Gsr* and *Cat* in mice liver, after 24 weeks of exposure (Dunnett's nonparametric multiple comparison tests, * represents *P*<0.05).

DISCUSSION

The present study demonstrates that chronic low-dose exposures to the insecticide, imidacloprid, may facilitate HFD-induced liver steatosis and liver hypertrophy in the mammalian system. It was proven that, imidacloprid-potentiated liver steatosis may occur via transcriptional modulations of the hepatic *de novo* lipogenic pathway. This study further reveals that, occurrence of imidacloprid-mediated steatogenesis may be facilitated by activation of the *Pxr-Fat/Cd36* pathway. Overall, the imidacloprid-effects on hepatic lipid homeostasis may be paradoxical, as imidacloprid seemed to activate both FA accumulation and FA export pathways simultaneously. Imidacloprid induced steatosis probably because the rate of imidacloprid-mediated *de novo* FA biosynthesis outweighed the rates of imidacloprid-enhanced TG exports in liver.

The steatosis effects of imidacloprid observed in the current study is consistent with previous reports involving other neonicotinoid compounds. In a study by [45], low dose exposures to Dinotefuran, Nitenpyram and Acetamiprid induced hepatic steatosis and ballooning hepatocytes in ICR mice. In an *in vitro* study by Lichtenstein *et al.* [18], clothianidin and thiacloprid induced intracellular TG accumulation in HepaRG cells, in a concentration-dependent manner.

Also, the body weight gain effects of imidacloprid observed in the present study is consistent with a previous report [35]. Mice exposed to 0.6 mg/kg bw/day of imidacloprid through HFD showed significant weight gains after 7 weeks of exposure; and the trend continued until the end of the experiment (12 weeks; [35]).

The weight gain effect tendency of imidacloprid observed in livers of the current mice model, has never been reported in the past. In a 12-week exposure study, Sun *et al.* [35] observed no significant weight effects of imidacloprid on mouse liver. Since the dose of imidacloprid used in the present study is the same as that used in Sun *et al.*'s study, the contrasting liver-weight effects observed between the two studies may be due to; (i) differences in fat content of diet used for the two studies (present study; 45% w/w, Sun *et al.* [35]; 20% w/w fat), or (ii) the differences in imidacloprid exposure durations (present study; 24 weeks, Sun *et al.* [35]; 12 weeks).

Imidacloprid enhanced serum ALT levels in the current experimental mice; suggesting that imidacloprid-mediated steatosis could result into liver damage. ALT has been widely used as a specific indicator of liver damage in both humans and rodents, due to its high concentration within the liver [28, 37].

The serum TG levels detected in the current mice model is similar to what has been reported previously. Sun *et al.,* [35] also observed a significant increase in serum TG levels in mice, after a 12-week to imidacloprid. The consistency in these observations suggest that, imidacloprid may elicit inductive effects on hepatic TG secretion and export. However, further mechanistic studies may be necessary to elucidate the exact roles of imidacloprid in the hepatic TG export.
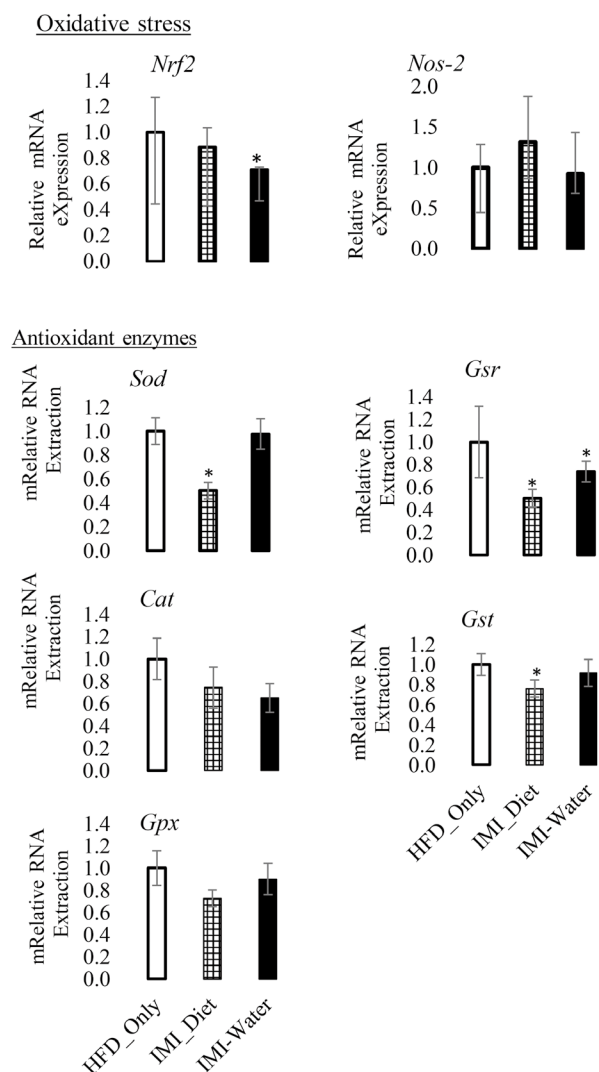
Fig. 11. Effects of imidacloprid on mRNA expressions of hepatic antioxidant enzymes. One-way ANOVA with Dunnett's nonparametric multiple comparison tests were used to determine significance for each treatment relative to control. Data represents Mean ± SE (n=4), * represents *P*<0.05.

Decreases in gWAT and mWAT weights observed in the current experimental model, might have been instigated by imidacloprid-mediated enhancement in adipocyte lipolysis. Accordingly, the imidacloprid treated mice showed enhanced adipocyte expressions of lipolysis-related genes such as *Atgl, Hsl* and *Ac*. Transcriptional activation of these genes are known to trigger key events that lead to sequential hydrolysis of triacylglycerol (TAG) to NEFA and glycerol [3]. The NEFA derived from adipocyte lipolysis is often released into the vasculature for use by other organs as energy substrates. In the current study, the measured serum NEFA levels did not vary among the various experimental models, suggesting that imidacloprid-potentiated steatogenesis might not be mediated by NEFA overflow from adipocyte lipolysis.

An AOP is a conceptual approach which provides a mechanistic representation of toxicological effects that extent over different levels of biological organization [18]. The steatosis AOP is characterized by many individual pathways which are regulated by nuclear receptors (NRs) such as *Ppara, Lxr, Pxr, Ahr,* etc. Transcriptional activations of these NRs (MIEs) may trigger gene transcriptions (KEs) which could lead to the steatosis endpoint [18, 21]. We reasoned that imidacloprid might have triggered the DNL and FA uptake pathways; thereby inducing steatosis in mice.

The DNL pathway is an important route for FA infiltration into the mammalian liver; and it is activated when dietary carbohydrate is abundant [34]. The MIEs of DNL involves *Lxr* (thus; *Lxra* and *Lxrb*) activation. Imidacloprid did not show any significant effects on transcriptional activations of these NRs, however it was able to enhance hepatic transcription of *Srebp-1c, Srebp2* and *Chrebpα*, as well as their target genes (KEs of the DNL pathway). By using an *in vitro* assay, Lichtenstein *et al.* [18] proved that activations of MIEs in AOPs do not always correspond to inductions of KEs and vice versa. The treatment-specific differences of imidacloprid observed in the transcriptions of *Srebp-1c* and *Chrebpα* might be due to variations in absorbed doses of imidacloprid existing within the experimental groups. This was confirmed by the differences in blood-imidacloprid levels detected among the various experimental groups. *Srebp2, Srebp-1c* and *Chrebpα* are important for initiation of the DNL pathway. Whereas *Srebp2* is known to regulate hepatic cholesterol synthesis, *Srebp-1c* and *Chrebpα* are actively involved in transcriptional activation of genes involved in DNL [6].

Imidacloprid-mediated inductions in transcriptions of *Acc, Fas, Scd1* and *Elovl6* confirmed its potency in inducing key events within the DNL pathway, which could result into steatosis. *Acc* (*Acc1* and *Acc2*) and *Fas* superintend over the initial carboxylation of acetyl-CoA to malonyl-CoA and the final conversion of malonyl-CoA to palmitate respectively, during DNL. *Scd1* plays an important catalytic role in hepatic conversion of stearoyl-CoA to oleoyl-CoA, during hepatic TG synthesis [24]. Also, the catalytic role of *Elovl6* in microsomal elongation of long chain FAs contributes greatly to increased FA stores in hepatocytes [26]. *CideA* has been reported to promote lipid droplet formation and triacylglycerol accumulation in the mammalian liver [47]. Imidacloprid might have facilitated lipid droplet formation and enlargement within the liver, through *CideA* activation. *CideA*-mediated hepatic steatosis may occur upon *Srebp-1c* activation of the *CideA* protein in mice liver [47]. *Fat/Cd36* is known to facilitate intracellular FA transport; and its overexpression is associated with enhanced hepatic FA uptake and accumulation [13, 32]. Hepatocyte-specific deletion of *Cd36* in mice, resulted into reduced hepatic lipid content, but mice that expressed high hepatic mRNA and protein levels of *Cd36* showed high hepatic FA levels [17, 43]. The imidacloprid-mediated upregulation in hepatic expression of *Fat/Cd36*, might have facilitated hepatic steatogenesis via enhancement of hepatic FA uptake from peripheral tissues. Meanwhile, transcriptional activation of *Fat/Cd36* is keenly regulated by *Pxr* [13, 32]. In this study, imidacloprid upregulated hepatic transcription of the *Pxr* (*Nr1i2*) its target *Cyp450* isoform, *Cyp3a11*. This proves imidacloprid as a potent activator of the *Pxr-Fat/Cd36* pathway within the steatosis AOP in mammalian species.

Ppara plays a crucial role in the transcriptional regulation of key genes involved in the β-oxidation of short-, medium- and long chain FAs within the liver [1, 6]. Hepatocyte-specific deletion of *Ppara* downregulated hepatic transcriptions of a large battery of genes involved in mitochondrial FA β-oxidation [25]. Surprisingly, the imidacloprid-mediated *Ppara* transcription, did not show any significant effects on mRNA expressions of *Ppara* target genes considered in the present study. This suggests that, though the imidacloprid treatment could mediate hepatic *Ppara* transcription, its overall impact on the mitochondrial FA β-oxidation pathway may be minimal. This observation generates interesting hypothetical questions which could be addressed in further studies. The *Acox1* gene and the *Fas*-dependent DNL process, have been marked as endogenous *Ppara* activators [30]. The imidacloprid-mediated inductions in both *Acox1* and *Fas* therefore, begs the question whether the *Ppara* transcription patterns observed in the current study was directly instigated by imidacloprid or through any of the endogenous pathways. Besides, hepatic activation of *Ppara* has been widely reported to induce variety of phenotypic responses; including peroxisomal proliferation leading to increased liver weight and liver hyperplasia [6]. It is possible that the liver weight gain effects of imidacloprid observed in the present study, might have occurred via *Ppara*-mediated peroxisomal proliferations in hepatocytes. Nonetheless, further studies may be required to validate the exact effects of imidacloprid on hepatic *Ppara* activation and/or hepatocyte peroxisomal proliferations.

Progression of obesity-mediated steatosis into steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma, is believed to be accompanied by higher expression levels of inflammatory cytokines such as *Tnf-α* and *Il-6* [15, 34, 44]. Previous studies found higher expression levels of *Tnf-α* and *Il-6* in patients living with non-alcoholic liver diseases [2]. In the present study, the occurrence of imidacloprid-mediated steatogenesis in the mice model, seemed to be independent of the hepatic inflammatory responses. Whereas imidacloprid induced steatosis in the current mice model, its effects on hepatic expressions of key inflammatory cytokines such as *Il-1β* and *IL-6* were repressive. Perhaps, the mechanisms underlying prevalence of IMI-mediated steatosis were discrete from the cytokine signaling pathways within the liver.

Imidacloprid treatment trough water was found to suppress the hepatic expression *Nrf2*. The *Nrf2* gene, directly affects the homeostasis of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) within the mammalian system [20]. Besides, the *Nrf2* gene is known to exhibit cytoprotective effects in the liver by regulating certain antioxidant genes [16]. In a

previous study, livers of *Nrf2*-null mice were found to be more vulnerable to many oxidative-stress pathologies compared to wild-type mice; and this was largely ascribed to the accumulation of ROS [16, 27]. By inference, the repressive effects of imidacloprid observed in the hepatic expressions of *Nrf2* suggest that, the imidacloprid exposure through water, increased the oxidative stress burden of the liver cells. The deference in *Nrf2* mRNA expression observed between the IMI_water and IMI_Diet treated mice may be explained by the differences in imidacloprid exposure levels detected within the two groups (Fig. 5).

Hepatic mRNA expression of *Nos-2* triggers the production of nitric oxide (NO) in liver cells. NO may combine with superoxide stoichiometrically to give rise to peroxynitrite, a highly reactive radical that can cause oxidative stress-mediated hepatic injuries [5]. In the present study, dietary exposure to imidacloprid upregulated the hepatic transcription of *Nos-2*; however, the margin of upregulation was not statistically significant. This suggests that the overall effects of imidacloprid treatment on hepatic NO synthesis was minimal

Meanwhile, the imidacloprid downregulated hepatic expressions of antioxidant enzyme expressions suggesting that, imidacloprid exposures may facilitate oxidative stress-mediated progression and severity of hepatic steatosis. A collapse in the antioxidant defense system of liver has been demonstrated to play a key role in the oxidative stress associated exacerbation of steatosis [38]. For instance, Hardwick *et al.* [9] observed that, a decreased hepatic *Gst* activity facilitates the severity of nonalcoholic steatohepatitis (advanced steatosis) in human subjects.

The present study provides an *in vivo* evidence suggesting that, the mechanisms of xenobiotic-mediated hepatic steatogenesis may not strictly follow the continuum of events defined within the steatosis AOP. Although AOP framework is highly essential for including mechanistic data into risk assessment processes, determining xenobiotic effects on specific MIEs and KEs within the AOP framework is hardly conceivable in real-life situations. This is because most xenobiotics have the potential to activate various MIEs and KEs within the AOP framework, independently [18].

In summary, the present study highlights the interactive effects of imidacloprid and high fat diet on the induction of steatosis within the livers of C57BL/6J male mice. We hypothesize that low dose exposures to imidacloprid may exacerbate HFD-mediated liver steatosis by increasing hepatic fatty acid accumulation through transcriptional activation of the DNL pathway. The occurrence of imidacloprid-mediated liver steatosis may be facilitated by increased hepatic FA uptake from peripheral tissues. This study further pointed out imidacloprid as a possible *Pxr* activator within the steatosis AOP. Imidacloprid showed significant effects on DNL-related key events within the steatosis AOP, but its impact on the DNL molecular initiation events was minimal. Although this study is the first to report on imidacloprid-HFD effects on mammalian liver, we acknowledge that our study has several limitations that need to be addressed in future studies. The number of mice per group (4) used in the current study was quiet small; a larger number of mice per group (n=8) might have been more appropriate. The stability and concentrations of imidacloprid in diet and water were not measured; this measurement might be necessary for clarifying the treatment-specific effects of imidacloprid observed in this study. In addition, the current study did not consider a negative control (normal diet) in the experiment, inclusion of a negative control might be important for assessing the degree of hepatic steatosis appropriately. Despite these elaborated limitations, lessons drawn from the current study will be indispensable, in that, the dynamics of imidacloprid-HFD interactions showcased in this study, will be of great essence in understanding the mechanistic roles of insecticides in the global prevalence of metabolic syndrome.

POTENTIAL CONFLICTS OF INTEREST. The authors declare no conflict of interest in the present study.

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