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10 This study investigated the effect of exposure to flutriafol based on residues in pigs.
11 Pigs were exposed to different concentrations (0.313, 0.625, 3.125, 6.25, and 12.5
12 mg/kg bw/d, n = 20) for 4 wks in different treatment groups. Serum biochemical
13 analysis, residue levels, and histological analysis were conducted using the VetTest
14 chemistry analyzer, liquid chromatography mass spectrometry, and Masson's trichrome
15 staining, respectively. The body weight (initial and final) was not significantly different
16 between groups. Parameters such as creatinine, blood urea nitrogen, alanine
17 aminotransferase, and lipase levels were significantly different as compared to the
18 control group. Flutriafol increased the residue limits in individual tissue of the pigs in a
19 dose dependent manner. Flutriafol exposures indicated the presence of fibrosis, as
20 confirmed from Masson's trichrome staining. These results suggest that flutriafol affects
21 the morphology and serum levels in pigs. The dietary flutriafol levels can provide a
22 basis for maximum residue limits and food safety for pig meat and related products.

23

24 Keywords: pig, flutriafol, fibrosis

25

26 **Introduction**

27 Pesticides are chemical or biological substances that inhibit the growth of living
28 organisms or prevent and destroy pests for improving product yields. The effects of
29 pesticide exposure include respiratory, neurological, gastrointestinal, and skin problems
30 (David, 2012). Pesticides cause biochemical changes, leading to clinical health signs
31 (Balani et al., 2011; Jonnalagadda et al., 2010). These biochemical changes result from
32 the destructive and degenerative effects of pesticides on the organs (Khan et al., 2013;

33 Mossalam et al., 2011). Farm workers exposed to pesticides were found to have
34 significantly increased serum concentrations of urea and creatinine (Haghighizadeh et
35 al., 2015; Ritu et al., 2013). Pesticides are being used globally for improving yields and
36 the quality of agricultural products (Eddleston et al., 2008; Songa and Okonkwo, 2016;
37 Yan et al., 2018). However, the abuse of pesticides has led to food and environmental
38 contamination (Carvalho, 2017; Li et al., 2018; Liu et al., 2016).

39 Pesticides are effective to increasing agricultural yields, but there are not easy to
40 management and monitoring (Peshin et al., 2009; Peshin and Zhang, 2014). For
41 increasing crop yield and quality, pesticides are classified according to their purpose,
42 such as herbicides, pesticides, and fungicides. Pesticides can also be classified
43 according to the pest's origin or structure or activity site, such as fungicides, fumigants,
44 herbicides, and insecticides (EPA, 2020). Therefore, global regions (e.g., Codex, EU, US,
45 Canada, India, Australia) have established policies on maximum residual limits in food
46 and feedstuff to limit pesticide residues in human and animals (Handford et al., 2015).

47 Triazole fungicides (e.g., flutriafol, propiconazole, tebuconazole, and tetraconazole)
48 are used on different types of plants to protect against different fungal diseases (Lass-
49 Flörl, 2011). Among these fungicides, flutriafol ((R,S)-1-(2-fluorophenyl)-1-(4-
50 fluorophenyl)-2-(1H-1,2,4-triazol-1-yl) ethanol) is commonly used to control leaf and
51 ear diseases in cereal crop and in seed treatment (FAO, 2011). It is a chiral triazole
52 fungicide employed to control plant pathogens. The fungicidal mechanism of such
53 pesticides inhibits ergosterol biosynthesis and cell wall synthesis (Song et al., 2019;
54 Yang et al., 2020).

55 Several studies have found that fibrosis is caused by pesticide exposure. Exposure to
56 ethylated dialkylphosphates, which are known to have immunomodulatory potential,

57 can induce long-term damage to the heart, leading to fibrosis (Medina-Buelvas et al.,
58 2019). Exposure to residual pesticides increased liver fibrosis and nonalcoholic fatty
59 liver disease in HepG2 cells in rat liver (Kwon et al., 2021a; Kwon et al., 2021b).
60 Furthermore, meta-analysis revealed that the risk of idiopathic pulmonary fibrosis
61 increased in agricultural workers exposed to pesticides (Park et al., 2021). Pesticide
62 residues have a substantial influence on growth and health of livestock and humans.
63 Although flutriafol has been detected in cells (e.g., HepG2, Neuro2A, NIH/3T3, SH-
64 SY5Y, VERO), humans, and laboratory animals, studies on pig meat and related
65 products are scarce. Therefore, this study investigated the potential effects of flutriafol
66 residues in pig meat and related products.

67

68 **Materials and Methods**

69 **Ethics statement**

70 All experimental procedures were reviewed and approved by the Institutional Animal
71 Care and Use Committee of the National Institute of Animal Science, Korea (No. 2019-
72 1576).

73

74 **Animal care and experimental design**

75 Pigs were purchased from the Darby breeding company (Anseong, Republic of
76 Korea). Twenty castrated male pigs (Landrace × Yorkshire, 72.0 ± 2.2 kg) were housed
77 in individual pens (2.1×1.4 m). For the experimental period including acclimatization,
78 the housing conditions were: a light-dark cycle of 12:12 h and a constant temperature
79 (22 ± 2 °C) and relative humidity ($55 \pm 5\%$). The pigs were divided into six groups
80 according to acceptable daily intake on OECD test guideline 505: control (n = 3), T1

81 (0.313 mg/kg bw/d; n =3), T2 (0.625 mg/kg bw/d; n =3), T3 (3.125 mg/kg bw/d; n =3),
82 T4 (6.25 mg/kg bw/d; n =4), and T5 (12.5 mg/kg bw/d; n =4). Animals were fed
83 according to the Korean feeding standards for pig (2017). Flutriafol (NH chemical,
84 Republic of Korea) was thoroughly mixed into the feed according to the concentrations
85 per body weight. Animals were treated a diet exposed to flutriafol twice daily for 28 d.
86 At the end of the experimental period, all pigs were anesthetized with the T61 agent.
87 After exsanguination, the blood, liver, kidney, ileum, muscle, and fat tissues were
88 quickly removed. These tissues were immediately frozen in liquid nitrogen for residue
89 analysis and then stored at -80°C . Some tissues were fixed with 10% neutral buffered
90 formalin (NBF; Sigma-Aldrich) for histological analysis. Average daily weight gain
91 (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were
92 calculated as follows: $\text{ADG} = (\text{finish weight} - \text{start weight}) / \text{age (days)}$, $\text{ADFI} =$
93 $\text{provide feed amount} - \text{residual feed amount}$, $\text{FCR} = \text{feed intake} / \text{average daily gain}$.

94 **Biochemical analysis**

95 Blood samples were collected with a suitable vacutainer tube containing no
96 anticoagulants. The serum was extracted using centrifugation (700 g for 15 min at 4°C)
97 and then kept at -80°C . A total of 15 parameters, consisting of glucose, creatinine,
98 blood urea nitrogen, phosphate, calcium, total protein, albumin globulin, alanine
99 aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, total bilirubin,
100 cholesterol, amylase, and lipase were determined using the VetTest chemistry analyzer
101 (IDEXX, USA), following the manufacturer's procedure.

102

103 **Pesticide residue analysis**

104 To quantify flutriafol, the collected samples (2.0 g tissue or 2.0 ml blood) were mixed

105 with distilled water (10 mL), set for 10 min, and mixed with acetonitrile and sodium
106 chloride (20 mL and 5 g, respectively). The samples were stirred using a vortex for 10 s
107 and shaken for 60 min. The extract was centrifuged at 3,500 g for 5 min. Primary-
108 secondary amine (PSA) and octadecylsilane (C18) were used for analyzing the samples.
109 The filtered samples were injected and the peak area was compared to estimate the
110 residue levels. The samples (5 uL each of plasma, liver, kidney, muscle, and fat) were
111 injected into a liquid chromatography-tandem mass spectrometer (LC-MS/MS). The
112 quantitative limit of the assay was 0.01 mg/kg. Residue analysis was conducted by an
113 ExionLC system with a QTRAP 4500 mass spectrometer (SCIEX, Framingham, MA,
114 USA). The conditions were: columns (100 × 2.0 mm, 3.0 μm) maintained at 40 °C,
115 mobile phase composition of 10 mM ammonium acetate and methanol, linear gradient
116 mode from 20% to 90% methanol, and flow rate of 0.1 mL/min.

117

118 **Histological analysis**

119 The tissue (i.e., liver, kidney, muscle, fat, and ileum) samples were fixed with 10%
120 neutral buffered formalin and then dehydrated (from 70% to 100% EtOH), embedded,
121 cut (5 μm-thick), mounted, and heated (40 °C) for 1 h on a hot plate. For staining, the
122 sections were dewaxed with xylene, rehydrated (from 100% to 70% EtOH), and washed
123 with distilled water. The sections were stained using Masson's trichrome (MT) staining
124 reagents following the manufacturer's protocol. The slices were observed under 100x
125 magnification in an optical microscope.

126

127 **Statistical Analysis**

128 All results including growth performance and biochemical analysis were analyzed

129 using one-way ANOVA (Prism 5.01, San Diego, CA, USA), followed by Tukey's
130 multiple comparison post-hoc test. Results are showed as mean and standard error of the
131 mean. A p-value of less than 0.05 between the control and treatment groups was
132 considered to be significant.

133

134 **Results and Discussion**

135 **Growth performance of flutriafol-treated pig**

136 The growth performances of flutriafol-exposed pigs were not significantly different
137 between control and treatment groups (data not shown). Briefly, the difference in initial
138 (72.0 ± 2.36 kg) and final (96.9 ± 4.48 kg) body weights was not statistically significant.
139 Furthermore, no significant differences were detected in average daily weight gain,
140 average daily feed intake, and feed conversion ratio of flutriafol treated pigs as
141 compared to control, despite its acute toxicity. Body weight of flutriafol treated rats was
142 increased compared to control at 1 and 2 wks. However, the body weights at 3 wks
143 were not significantly different (Kwon et al., 2021). No significant effects of
144 tebuconazole treatment were observed on body condition, growth, and sex ratio of
145 chicks (Lopez-Antia et al., 2021). In this study, flutriafol exposure did not affect the
146 growth performances in pigs, similar to the observations of previous studies.

147

148 **Blood biochemical analysis**

149 Table 1 presents the effect of pesticide exposure on biochemical properties of pig
150 serum. The parameters are as follows: glucose (GLU), creatinine (CREA), blood urea
151 nitrogen (BUN), phosphate (PHOS), calcium (CA), total protein (TP = ALB+GLOB),
152 albumin globulin (ALB), alanine aminotransferase (ALT), alkalinephosphatase (ALKP),

153 gamma glutamyl transpeptidase (GGT), total bilirubin (TBIL), cholesterol (CHOL),
154 amylase (AMYL), lipase (LIPA). Creatinine (CREA), blood urea nitrogen (BUN),
155 BUN/CREA ratio, total protein (TP), albumin globulin (ALB), globulin (GLOB),
156 ALB/GLOB ratio, alanine aminotransferase (ALT), gamma glutamyl transpeptidase
157 (GGT), amylase (AMYL), and lipase (LIPA) showed significant differences from those
158 of the control ($p < 0.05$). In particular, CREA decreased significantly in the T4 and T5
159 treatment groups compared with control ($p < 0.05$). BUN, ALT, and LIPA showed a
160 significant increase in all treatment groups than that of control ($p < 0.05$). No significant
161 differences were detected in the other biochemical parameters (i.e., GLU, PHOS, CA,
162 ALKP, TBIL, and CHOL). The principal component analysis did not show a difference
163 between control and treatment groups (data not shown).

164 Blood biochemistry values provides important biological information to humans and
165 animals. The results of our study showed that pesticide exposure affects pigs, resulting
166 in significant differences in parameters such as CREA, BUN, ALT, and LIPA. These
167 biochemical changes can lead to destructive and degenerative changes in the renal
168 corpuscles based on pesticides (Khan et al., 2013; Mossalam et al., 2011). Farmers
169 exposed to pesticides had significantly higher serum levels of urea and CREA
170 (Haghighizadeh et al., 2015; Ritu et al., 2013). The urea and CREA levels showed
171 significant differences between control and treatment groups. However, CREA levels in
172 the T5 group were lower than those in control. Urea is formed by ammonia produced in
173 the liver and is excreted through the kidney. Urea and CREA excreted by the kidneys
174 are used as biomarkers to determine kidney damage (Singh et al. 2011).
175 Organophosphates are widely used pesticides. The organophosphate pesticides increase
176 CREA levels because of impaired glomerular function and damage to the renal tubules

177 (Mohssen, 2001). ALT, also known as transaminases, provide important information
178 about damaged hepatocytes (Hernandez et al. 2013). The ALT levels caused by
179 pesticide-induced stress are associated with the production of reactive oxygen species
180 and oxidative tissue damage (Patil et al. 2009, Singh et al. 2011). In particular, increase
181 in blood glucose, insulin, triglycerides, and lipases exposed to organophosphorus
182 pesticides have been reported in several studies (Romero-Navarro et al., 2006; Gangemi
183 et al., 2016; Kamath and Rajini, 2007; Rodrigues et al., 1986). Immobilized lipase is
184 used as a biosensor to determine TG due to its accuracy and efficiency (Chandra et al.,
185 2020; Escamilla-Mejía et al., 2014). Significant elevations were observed in urea and
186 CREA concentrations of serum samples exposed to pesticides. These elevations
187 correspond to renal impairment and renal dysfunction (Pandya et al., 2016). Serum
188 CREA and urea, known as renal biochemical markers, were significantly different
189 between control and treatment groups. The elevated serum urea observed in response to
190 pesticide exposure in this study could be explained by impaired synthesis and protein
191 metabolism due to hepatic dysfunction. Together, CREA, BUN, ALT, and LIPA can act
192 as potential biomarkers to detect exposure to flutriafol.

193

194 **Flutriafol residue analysis**

195 The linear and quadratic equations of flutriafol exposure concentrations were used for
196 determining maximum residue limits in liver (Fig. 1A), kidney (Fig. 1B), fat (Fig. 1C),
197 muscle (Fig. 1D), and blood (Fig. 1E). The residual levels of all tissues increased with
198 an increase in flutriafol concentration. The quadratic equations for liver ($R^2 = 0.9982$, p
199 < 0.001), kidney ($R^2 = 0.9960$, $p < 0.01$), muscle ($R^2 = 0.9928$, $p < 0.05$), and blood (R^2
200 $= 0.9856$, $p < 0.01$) showed significant differences between control and treatment

201 groups (Fig. 1). The linear equations of liver ($R^2 = 0.9951$), kidney ($R^2 = 0.9599$),
202 muscle ($R^2 = 0.9092$), and blood ($R^2 = 0.9847$) were concentration dependent (Fig. 1B-
203 D). However, although the residual levels increased according to treated flutriafol
204 concentrations in fat, there was no significant differences between the treatment groups
205 (Fig. 1C).

206 Flutriafol, known as conazole fungicide, is used to control leaf and ear diseases in
207 cereals (FAO, 2011) and to prevent fungal diseases (Bhuiyan et al., 2015; Lass-Flörl,
208 2011). Exposure to pesticides through oral, dermal, or inhalation routes is associated
209 with low or moderate toxicity (Kwon et al., 2021; Shahinasi et al., 2017; Toumi et al.,
210 2017;). The high performance liquid chromatography method was used to establish the
211 maximum residue limits of flutriafol exposure in wheat and soil (Pingzhong et al., 2012,
212 Zhang et al., 2014). Therefore, our results suggest that the residual values for different
213 tissues showed variations according to pesticide concentrations. Taken together, the
214 equations for graded levels of flutriafol will help predict the risk assessment and
215 maximum residue limits in pig production and meat safety.

216

217 **Histological analysis**

218 In this study, the histological changes in liver, kidney, muscle, fat, and ileum tissues
219 of pig due to exposure to flutriafol were assessed using MT staining (Fig. 2). Fibrosis
220 deposition and tissue destruction at different flutriafol concentrations were observed for
221 all treatment groups. Fibrosis in treatment groups was measured by MT staining on the
222 portal areas and lobular boundary of the liver. The glomeruli, tubulus, and vasculature in
223 kidney tissues were stained blue. Kidneys showed interstitial fibrosis and
224 glomerulosclerosis at different flutriafol concentrations. Villus form and lamina propria

225 in ileum were deteriorated and exhibited prominent blue staining after flutriafol
226 exposure as compared to that in control. Muscle and fat tissues were stained with blue
227 after being exposed to flutriafol. Collagen fibrosis also showed concentration-dependent
228 effects in the treatment groups than in control. The ethylated dialkylphosphates, known
229 as metabolites of organophosphorus pesticides, are known to aggravate heart fibrosis
230 and inflammation (Medina-Buelvas et al., 2019). In our study, the flutriafol also showed
231 changes in vacuoles of the proximal tubules causing necrosis and hepatocyte damage in
232 the liver and kidney. Fibrosis lead to ectopic fat accumulation, resulting in non-alcoholic
233 fatty liver disease (Akbel et al., 2018; Khan et al., 2016; Kwon et al., 2021; Ojha et al.,
234 2011). These histological changes were also observed in our study. Fibrosis due to
235 exposure to flutriafol affected the morphological characteristics in liver, kidney, muscle,
236 fat, and ileum tissue of pigs.

237

238 **Conclusion**

239 The results of the present study suggest that flutriafol exposure affects pig tissues
240 (e.g., muscle, fat, blood, liver, kidney, and ileum), causing significant alterations in
241 some biochemical parameters including BUN, CREA, ALT, and LIPA. In particular, the
242 linear and quadratic equations for liver and blood showed a significant increase ($p <$
243 0.05) after exposure to different flutriafol concentrations. Flutriafol also can lead to
244 morphological changes related to fibrosis in several tissues. Therefore, these results
245 indicate that pesticides such as flutriafol can provide the basis for risk assessment and
246 safety based on maximum residue limits in pig meat and related products.

247

248

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253

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ACCEPTED

401 **Figure legends**

402 Fig. 1. Residue levels in different tissues of pigs fed on a flutriafol-exposed diet. Liver
403 (A), kidney (B), fat (C), muscle (D), and blood (E).

404 Fig. 2. Histology of flutriafol exposure in pig liver, kidney, muscle, fat, and ileum
405 tissues as represented by Masson's trichrome staining. Original magnification at 100x
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423 **Table 1.** Changes of blood biochemical characteristics by exposed to flutriafol in finishing pigs

Biochemical parameter	Control	T1(x1)	T2(x5)	T3(x10)	T4(x50)	T5(x100)	<i>p</i> value
GLU, mg/dL	87.40 ± 2.89	89.89 ± 3.19	85.07 ± 3.30	83.08 ± 2.30	82.19 ± 2.86	85.81 ± 3.75	0.5524
CREA, mg/dL	0.95 ± 0.03 ^a	1.05 ± 0.02 ^a	0.97 ± 0.05 ^a	1.03 ± 0.02 ^a	0.91 ± 0.03 ^b	0.89 ± 0.03 ^b	0.0047
BUN, mg/dL	9.67 ± 0.44 ^b	11.07 ± 0.57 ^a	8.13 ± 0.70 ^b	11.17 ± 0.81 ^a	12.38 ± 0.58 ^a	11.56 ± 0.52 ^a	<0.0001
BUN/CREA	10.40 ± 0.68 ^b	10.60 ± 0.64 ^b	8.33 ± 0.54 ^b	11.00 ± 0.94 ^b	13.94 ± 0.85 ^a	13.06 ± 0.47 ^a	<0.0001
PHOS, mg/dL	7.31 ± 0.23	7.23 ± 0.27	7.18 ± 0.23	7.15 ± 0.23	7.36 ± 0.20	7.56 ± 0.20	0.8184
CA, mg/dL	11.01 ± 0.14	11.15 ± 0.12	10.96 ± 0.14	10.93 ± 0.33	11.19 ± 0.14	11.09 ± 0.16	0.7952
TP, g/dL	8.05 ± 0.11 ^a	7.95 ± 0.06 ^b	7.45 ± 0.11 ^b	7.61 ± 0.25 ^b	7.91 ± 0.14 ^b	8.48 ± 0.15 ^a	<0.0001
ALB, g/dL	3.87 ± 0.11 ^a	4.00 ± 0.07 ^a	3.57 ± 0.08 ^b	3.47 ± 0.20 ^b	3.73 ± 0.10 ^a	3.75 ± 0.10 ^a	0.0138
GLOB, g/dL	4.19 ± 0.11 ^b	3.94 ± 0.11 ^b	3.88 ± 0.09 ^b	4.14 ± 0.13 ^b	4.18 ± 0.09 ^b	4.73 ± 0.17 ^a	<0.0001
ALB/GLOB	0.93 ± 0.05 ^b	1.05 ± 0.04 ^a	0.93 ± 0.03 ^b	0.84 ± 0.05 ^b	0.91 ± 0.03 ^b	0.82 ± 0.05 ^b	0.0068
ALT, U/L	45.93 ± 2.18 ^b	54.40 ± 1.87 ^b	49.13 ± 1.65 ^b	51.25 ± 3.88 ^b	44.88 ± 2.75 ^b	94.44 ± 10.66 ^a	<0.0001
ALKP, U/L	129.9 ± 7.46	164.7 ± 4.93	142.4 ± 5.19	130.8 ± 11.75	154.2 ± 12.37	145.0 ± 11.60	0.0898
GGT, U/L	25.13 ± 3.74 ^b	14.93 ± 1.32 ^b	25.53 ± 2.64 ^b	22.58 ± 1.58 ^b	23.94 ± 1.78 ^b	29.13 ± 4.36 ^a	0.0242
TBIL, mg/dL	0.32 ± 0.13	0.21 ± 0.02	0.19 ± 0.02	0.26 ± 0.02	0.35 ± 0.11	0.39 ± 0.07	0.4072

CHOL, mg/dL	73.87 ± 4.49	74.07 ± 4.32	64.80 ± 3.61	73.50 ± 5.30	76.94 ± 3.55	65.25 ± 6.52	0.3195
AMYL, U/L	539.7 ± 30.28 ^a	542.3 ± 48.86 ^a	523.9 ± 26.11 ^a	344.3 ± 17.08 ^b	521.8 ± 62.43 ^a	603.6 ± 37.66 ^a	0.0011
LIPA, U/L	13.27 ± 1.73 ^b	16.07 ± 2.11 ^b	15.40 ± 1.41 ^b	12.00 ± 0.91 ^b	28.75 ± 6.05 ^a	20.88 ± 2.76 ^a	0.0038

424 Values are mean ± SEM. n = 20. Reference ranges : GLU (85-160), CREA (0.5-2.1), BUN (6-30), PHOS (3.6-9.2), CA (6.5-11.4), TP (6.0-8.0),
425 ALB (1.8-3.3),), GLOB (2.5-4.5), ALT (9-43), ALKP (92-294), GGT (16-30), TBIL (0.1-0.3), CHOL (18-79), AMYL (271-1198), LIPA (10-44).
426 GLU, glucose; CREA, creatinine; BUN, blood urea nitrogen; PHOS, phosphate; CA, calcium; TP, total protein (TP=ALB+GLOB); ALB,
427 albumin globulin; ALT, alanine aminotransferase; ALKP, alkalinephosphatase; GGT, gamma glutamyl transpeptidase; TBIL, total bilirubin,
428 CHOL, cholesterol; AMYL, amylase; LIPA, lipase. All traits in the table were analyzed by one-way ANOVA with Tukey's multiple comparison
429 test.

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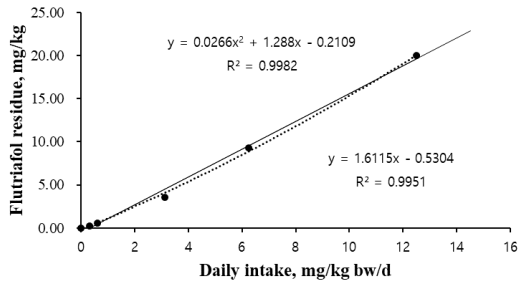
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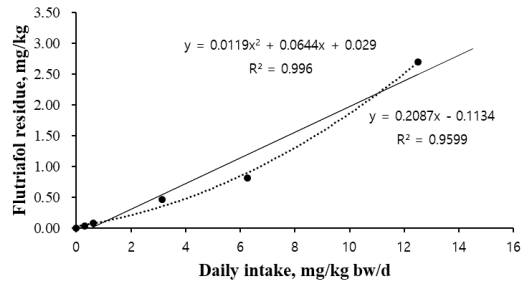
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436 **Fig. 1.**

437 **A. liver**

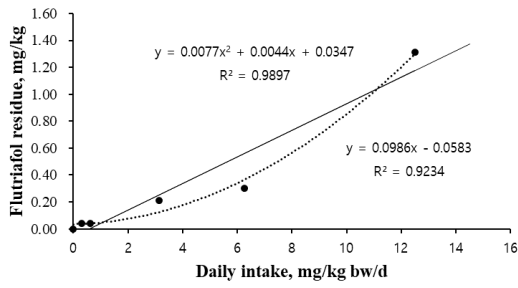


B. kidney

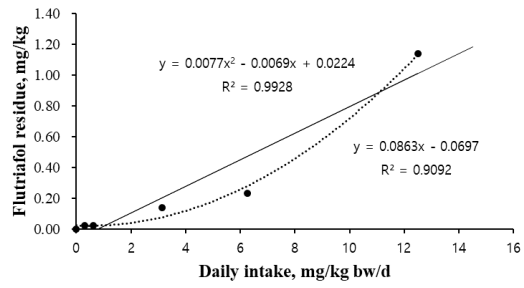


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439 **C. fat**

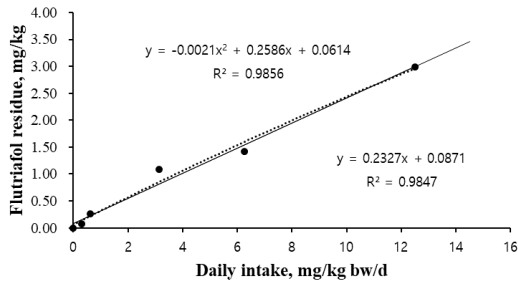


D. muscle



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441 **E. blood**



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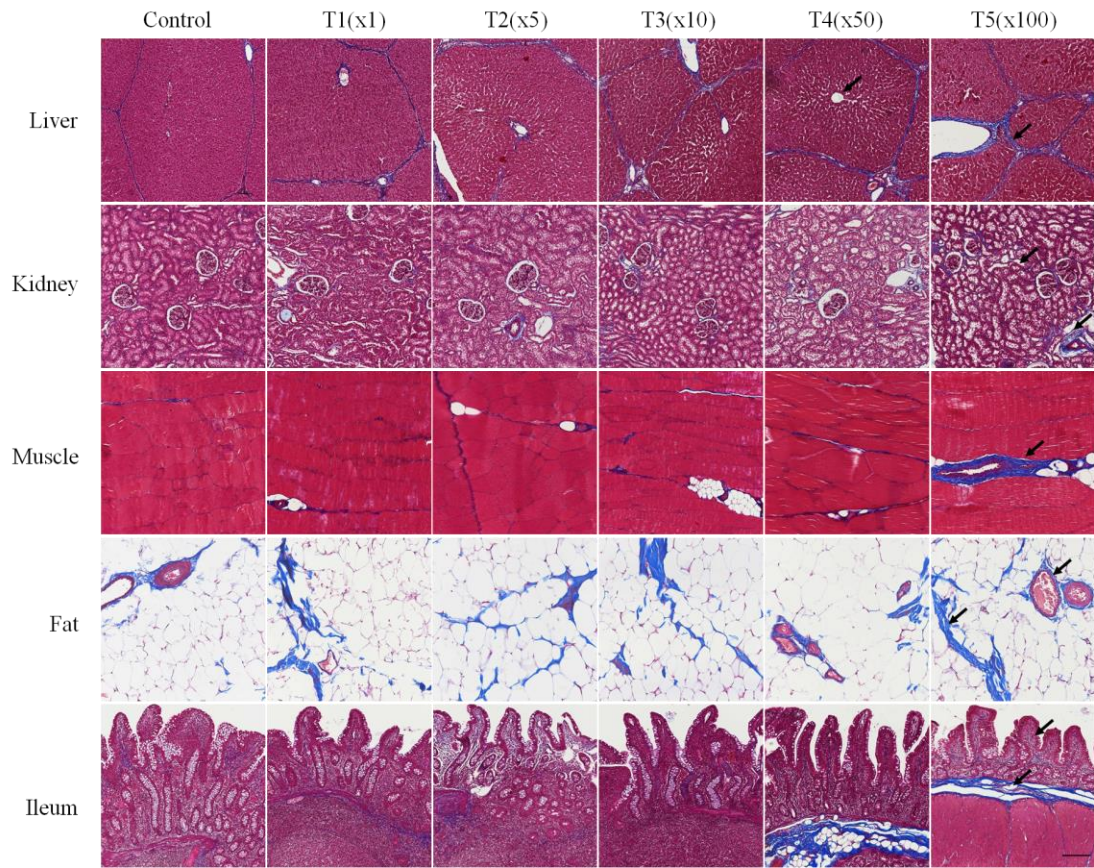
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Fig. 2.



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