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Cardioprotective Effect of Total Saponins from Three Medicinal Species of *Dioscorea*
Against Isoprenaline-Induced Myocardial Ischemia

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1 Introduction

Ischemic heart disease (IHD) is the leading cause of morbidity and mortality in the western world, even in China. It is estimated by the World Health Organization that IHD will be the leading cause of death in the world in the coming decades (Yang et al., 2013).

To combat this serious disease, people have been trying to investigate and develop herbal medicines from traditional herbs based on experiences from antecessors. In particular, of the 49 species of the genus Dioscorea distributed in China (Editorial Board of Flora of China, 1985), three species, namely, Dioscorea nipponica Makino (DN), D. panthaica Prain et Burkill (DP), and D. zingiberensis C. H. Wright (DZ), have been used as folk medicine since 1950s, and are regarded as having more or less the same traditional therapeutic actions, such as activating blood, relieving pain, and dispersing swelling (Chinese Pharmacopeia Committee, 2010; Commission of Chinese Materia Medica, 1999); finally the bioactive steroidal saponins from these three medicinal species were successfully developed as several effective single-herb medicines by the pharmaceutical industry for treating IHD, and have been in use since the 1970s in China even in the former Soviet Union (Research coordination group of Dunye Guanxinnng Tablet, 1985; Sichuan Biology Research Institute, 1977, Leskov et al., 1976; Dutch Medicines Evaluation Board, 2012).

In order to discover and develop more new drugs from these Dioscorea herbs, understanding the mechanism of Dioscorea saponins for treating IHD could be important. In our previous study, it was found that the chemical compositions of DN and DP were similar, and both were remarkably
different from DZ (Tang et al., 2013 and 2014; Zhu et al., 2010; Yi et al., 2014). Hence, whether their
different chemical profiles support their anti-IHD activity in common still needs to be answered.

Further, although there are a few of studies on these three herbs attenuating experimental
hyperlipidemia and ischemia-perfusion injury or clinical angina pectoris, in forms of a single
compound of saponin, total saponins or a patent Chinese Medicine (Lu et al., 2008; Wang et al., 2009;
Wang et al., 2012), so far the efficacies of these herbs have not been compared in the same animal
model or a unified clinical trial. Thus, it is still unknown that whether these three herbs act via
similar mechanism and whether they possess comparable therapeutic efficacy for experimental MI.

As it is widely accepted that isoprenaline (ISO) injection can readily induce acute MI in rats and
as antioxidant activity is one of the key mechanisms of anti-MI efficacy (Long et al., 2012; Song et
al., 2013; Cokkinos et al., 2006), it is reasonable to use this model to compare the therapeutic effect
of these three herbs with respect to antioxidant activity. So far, the published studies concerning
anti-MI activity of DN, DP and DZ monitored only four indices related to the antioxidant activity in
MI model. These were: creatine kinase (CK), lactate dehydrogenase (LDH); malondialdehyde
(MDA); and total superoxide dismutases (SOD) (Ning et al., 2008; Wang et al., 2009; Wang et al.,
2012). But nonenzymatic antioxidants, which are also part of the antioxidant defense system, have
not been reported for the bioactivity of these Dioscorea species in MI animals.

To comprehensively compare the anti-MI effect of DN, DP and DZ, in the present study, an
additional myocardial injury marker enzyme, aspartate aminotransferase (AST), two enzymatic
antioxidants namely, catalase (CAT) and glutathione peroxidase (GPx), as well as an indicator of nonenzymatic antioxidants, namely, total antioxidant capacity (T-AOC) were assayed for different rat groups administered with total saponins from these three Dioscorea herbs. In addition, histological changes of experimental rat groups were examined by hematoxylin and eosin (H&E) staining and light microscopy observation.

2 Material and methods

2.1 Materials, chemicals and reagents

The rhizomes of DN, DP, and DZ were collected from Lingbao in Henan Province, Xichang in Sichuan Province and Enshi in Hubei Province, China. All the crude drugs were of high quality and authenticated by Dr. Hubiao Chen, School of Chinese Medicine, Hong Kong Baptist University (HKBU). Corresponding voucher specimens were deposited in the Phytochemistry and Quality Research Lab of HKBU. Eight reference standards were used for qualitative and quantitative analysis, namely seven glycosides (protodioscin, protogracillin, pseudoprotodioscin, dioscin, gracillin, polyphyllin V and trillin) and one aglycone (diosgenin) (Fig. 1); their purity and the commercial sources were the same as that in our previous study (Tang et al., 2015). Isoprenaline hydrochloride (purity > 98.5%) was purchased from Sigma (St. Louis, US). Propranolol was purchased from Kangpu Pharmaceutical Co., Ltd. (Changzhou, China). Test kits for CK, LDH, AST, SOD, CAT, GPx, T-AOC and MDA were all purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China).
2.2 Preparation and quantification of Dioscorea total saponins

The total saponins (TS) from DN, DP and DZ (abbreviated as DNTS, DPTS and DZTS, respectively) were prepared separately. The detail procedures (Section 2.2 in supplementary material) were the same as that in our previous study (Tang et al., 2015). These obtained extracts were individually suspended in 0.5% (w/v) aqueous sodium carboxymethyl cellulose (CMC-Na) for animal administration.

The quantification of DNTS, DPTS and DZTS was performed using ultra-high performance liquid chromatography with ultra-high definition accurate mass quadrupole time-of-flight mass spectrometry (Agilent Technologies, G6540A). The chromatographic conditions were the same as for our previous study (Tang et al., 2013). For details, see Section 2.2 in supplement material. The base peak chromatograms of DNTS, DPTS and DZTS are shown in Fig. 1.

Seven glycosides (as indicated in Fig. 1) and one aglycone (diosgenin, the main constituent of acid hydrolyzed total saponins), were identified by reference standards, and quantified in DNTS, DPTS and/or DZTS. For tentatively identifying other compounds in DZTS, see Table S1 in supplementary material.
<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Molecular formula</th>
<th>R</th>
<th>Content of quantified analytes (mg/g)</th>
<th>DNTS</th>
<th>DPTS</th>
<th>DZTS</th>
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<tr>
<td>Diosgenin</td>
<td>C_{27}H_{42}O_{3}</td>
<td>R_{1}</td>
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<td>248.56 ± 7.95</td>
<td>169.10 ± 4.78</td>
<td>194.44 ± 4.62</td>
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<td>Dioscin</td>
<td>C_{45}H_{70}O_{16}</td>
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<td>156.12 ± 4.06</td>
<td>144.62 ± 4.69</td>
<td>1.85 ± 0.07</td>
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<tr>
<td>Gracillin</td>
<td>C_{43}H_{72}O_{17}</td>
<td></td>
<td></td>
<td>27.73 ± 0.99</td>
<td>38.62 ± 1.02</td>
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<tr>
<td>Polyphyllin V</td>
<td>C_{39}H_{62}O_{12}</td>
<td></td>
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<td>5.96 ± 0.26</td>
<td>19.79 ± 0.62</td>
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<tr>
<td>Trillin</td>
<td>C_{33}H_{52}O_{8}</td>
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<td></td>
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<td>N.D.</td>
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<tr>
<td>Protodioscin</td>
<td>C_{51}H_{84}O_{22}</td>
<td>R_{2}</td>
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<td>235.29 ± 4.61</td>
<td>140.05 ± 2.35</td>
<td>7.72 ± 0.16</td>
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<tr>
<td>Protogracillin</td>
<td>C_{51}H_{84}O_{23}</td>
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<td></td>
<td>39.16 ± 0.98</td>
<td>157.62 ± 4.59</td>
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<tr>
<td>Pseudoprotodioscin</td>
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<td>19.05 ± 0.63</td>
<td>10.77 ± 0.41</td>
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</tr>
</tbody>
</table>

Note: Glc = β-D-glucopyranosyl, Rha = α-L-rhamnopyranosyl; N.D. = not detected.

**Fig. 1 Chemical characterization of DNNTS, DPTS and DZTS.**

A: Chemical structures and content of quantified analytes; B: Base peak chromatograms.


For identifying Diosgenin (peak 17), see **Fig. S1** in supplement material.
2.3 Animals and acute myocardial ischemia induced by isoprenalineline

Male Sprague-Dawley rats (200 ± 20g) were purchased from Laboratory Animal Services Center, the Chinese University of Hong Kong, Hong Kong. The rats were bred in a breeding room with temperature of 23 ± 1 °C, humidity of 60 ± 5%, and 12 h dark – light cycle. They were given tap water and fed normal food ad libitum. All the experimental animals were housed under the above conditions for 3 days’ acclimation. All experimental protocols were approved by the Committee on the Use of Human & Animal Subjects in Teaching and Research of Hong Kong Baptist University (No. of License: 12-17 in DH/HA&P/8/2/6 Pt.2), in accordance with the Animals Ordinance (Department of Health, Hong Kong).

A total of 72 rats were randomly divided into 9 groups: (1) normal control (0.5% w/v aqueous CMC-Na, i.g.); (2) model group (ISO injection only); (3) positive group (propranolol, 15 mg/kg i.g. for 3 days after ISO injection); (4)–(6) post-ISO groups: each group administered with DNTS, DPTS or DZTS; 300 mg/kg for 3 days after ISO injection ; (7)–(9) pre- and post- ISO groups: each group administered with DNTS, DPTS or DZTS; 150 and 300 mg/kg, respectively for 3 days both before and after ISO injection). All TS and propranolol were administered once daily except the day on which ISO was injected. For illustrated administration protocol, see Fig. S2 in supplementary material.

Animals were treated with isoprenalineline (1 mg/kg, s.c.) to induce experimental MI twice at an interval of 8 hours on the 4th day. On the last day of experiment (8th day), the animals were sacrificed.
The blood samples were collected from the femoral arteries of rats anesthetized with diethyl ether.

Serum was saved at -80 °C following centrifugation at 4 °C at 4000 rpm for 20 min.

2.3.1 Histological examination of myocardium

Immediately after the sacrifice of the rats, the hearts were removed, washed with iced normal saline and fixed in 10% neutral formalin solution. The heart tissue was processed for sectioning and staining by standard histological methods. Sections (5 μm, Leica RM 2125, Germany) from the left ventricle were stained with hematoxylin and eosin (H&E) and examined by light microscopy (Leica DMR, Germany) at 200x magnification.

2.3.2 Assays for biological activities

Activities of CK, LDH, AST, SOD, GPx, CAT, T-AOC and MDA were measured using kits according to the manufacturer’s instructions.

2.3.3 Data analyses

Data were expressed as means ± SD. A one-way analysis of variance (ANOVA) was used to compare the means among different groups. Post hoc multiple comparisons were done with Bonferroni and Turkey tests to further compare group data.

Analyses were conducted with SPSS 18.0 software. P value < 0.05 was considered to be statistically significant.
3 Results and discussion

3.1 Effects of Dioscorea TS on CK, LDH, and AST serum levels

The relatively high content of CK, LDH and AST in cardiac tissue accounts for the clinical usefulness of measuring the activity of these enzymes in serum to diagnose and monitor MI. This is because leakage of these enzymes acted as a marker of cell membrane damage (Cokkinos et al., 2006). Thus, CK, LDH, and AST were measured in serum from experimental group animals (Fig. 2 A-C). Compared with levels in the normal control group, ISO injection resulted in significantly increased activities of CK, LDH and AST in ISO model group (##P < 0.01), clearly suggesting that ISO produced myocardial damage.

Although there are some minor differences in values of enzyme activities among groups treated with DNTS, DPTS or DZTS, in general, no remarkable differences were observed. In other words, all three species restore the activities of myocardial injury marker enzymes more or less to the same extent. For the change percentage of CK, LDH and AST (defined in Fig. 2 ii), three pre- and post-ISO groups given different total saponins show greater changes than the positive group did except the LDH% of DP-PP group, and even DN-P and DP-P group exert greater changes than the positive group did in AST%. Thus it appears that these three TS could attenuate the cell membrane injury almost as potent as the positive drug.
Fig. 2 Effects of *Dioscorea* saponins on acute experimental myocardial ischemia. i) A-H: CK, LDH, AST, SOD, CAT, GPx, T-AOC and MDA serum levels.

ii) Change percentage of assay markers for each group administered with different total saponins or positive drug. Compared to the model group, the change percentage of each marker was calculated as indicated in Fig. 2 ii. Values indicate greater changes than in positive group. Normal: normal control; ISO: model group only injected with isoprenaline; Pos: positive control (propranolol, 15 mg/kg); DN-P, DP-P and DZ-P: orally given DNTS, DPTS and DZTS, respectively, after ISO injection (300 mg/kg); DN-PP, DP-PP and DZ-PP: orally given DNTS, DPTS and DZTS, respectively, both before (150 mg/kg) and after ISO injection (300 mg/kg). Data are expressed as mean ± SD (n = 8 for each group). ** P < 0.01 vs. normal control; * P < 0.05, ** P < 0.01 vs. ISO group.

iii) I-Q: Histological observations of the hearts from each experimental group. I: Normal, showing normal myocardial fibres with normal cell structure; J: ISO, showing sever inflammatory infiltration and hemorrhage; K: Pos; L: DN-P; M: DN-PP; N: DP-P; O: DP-PP; P: DZ-P; Q: DZ-PP. L-Q: DNTS, DPTS or DZTS treated groups showing almost normal cardiac fibers with mild hemorrhage and inflammatory infiltration when compared to the heart of model group, indicating a protective effect. Magnification: 200x. Arrow indicates inflammatory infiltration; circle indicates hemorrhage.
3.2 Effects of Dioscorea TS on SOD, CAT, GPx, T-AOC and MDA serum levels

Oxidative stress plays an essential role in the pathogenesis of MI injury. The major reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$), superoxide and hydroxyl radicals, are generated during ischemia and particularly during reperfusion (Cokkinos et al., 2006). However, these potentially deleterious ROS are controlled by a system of enzymatic and nonenzymatic antioxidants which eliminate prooxidants and scavenge free radicals. As for enzymatic antioxidants, superoxide, is converted to H$_2$O$_2$ by superoxide dismutase; then, excess H$_2$O$_2$ can be reduced to H$_2$O via the catalase or the glutathione peroxidase system. Thus, SOD, CAT and GPx were chosen to assay. As for nonenzymatic antioxidants, these involve myocardial hydrophilic antioxidants such as ascorbate and glutathione, and lipophilic antioxidants, such as ubiquinol 9, vitamin E (Haramaki et al., 1998; Noori, 2012). Total antioxidant capacity is used to characterize the total level of antioxidants, particularly the nonenzymatic in biosamples by accessing ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996). MDA, as a lipid peroxidation product, can be regarded as an index for the severity of cellular damage caused by ROS.

Compared with the normal control group, SOD, CAT, GPx and T-AOC levels in the ISO group decreased significantly (##$P < 0.01$), while MDA levels increased significantly (##$P < 0.01$) (Fig. 2 D-H). Although groups administered with different TS, either post-ISO or pre- and post- ISO groups, did not show greater change percentage in all these five markers than the positive group did (Fig. 2 ii), pathological levels of SOD, CAT, GPx and T-AOC and MDA in experimental MI rats
were almost normalized by TS treatment compared with ISO group (**P < 0.01 or *P < 0.05).

Further, comparing the serum antioxidant activities of the post-ISO groups and both pre- and post-ISO groups administered with the same Dioscorea TS, revealed that the latter — i.e., all groups that received Dioscorea TS, both before and after ISO injection, showed greater improvement. These results represent evidence that Dioscorea saponins have measurable, distinct and specific ability to prevent MI.

Compared with the relevant reports published, namely, methyl protodioscin, a single compound isolated from Dioscorea spp. (Ning et al., 2008), and total saponins extracted from DP increased the antioxidative ability of myocardium in rats with ischemia-reperfusion model induced by coronary artery ligation (Wang et al., 2009), the decreased activities of CK and MDA and enhanced activity of SOD were in accordance with our results; thus, the effect of Dioscorea saponins on CK, MDA and SOD were verified in our experiment. Furthermore, our findings revealed that the anti-MI mechanism of Dioscorea saponins is related to not only more varieties of enzymatic antioxidant, such as GPx and CAT, but also to nonenzymatic antioxidants. Our recent study shows that diosgenin, which is the main metabolite found in plasma and feces samples from all rat groups individually administered with DNTS, DPTS or DZTS (Tang et al., 2015); thus, we hypothesize that diosgenin is one of the bioactive compounds responsible for the amelioration effect of these three TS on MI, and the related research is ongoing.

3.3 Effects of Dioscorea TS on myocardial histology
Histopathology of rat heart from normal control showed a normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils (Fig. 2 I-Q). Tissue from the ISO group revealed marked infiltrating inflammatory cells and loss of striations with nuclear changes. Tissue sections from the positive group showed approximately normal myofibrillar structure with clear striations and slight inflammatory cell infiltration. Post-ISO groups showed diminished myocardial cell swelling, unclear transverse striations, and reduced inflammatory cell infiltration. Compared with these groups, tissues from both pre- and post-ISO groups revealed less severe histological damage, namely, normal myocardial arrangement, clear transverse striations, and few invasive inflammatory cells. Groups treated with different TS, namely DNTS, DPTS or DZTS, did not show significant differences in improvement of pathological changes.

4 Conclusion

The findings of the present study provide evidence that DNTS, DPTS and DZTS can prevent MI and that they can protect the myocardium against ischemic insult. Furthermore, the protective effect can be attributed to the increase of enzymatic and nonenzymatic antioxidant levels in vivo, and decrease of lipid peroxidation formed. Although the saponin profiles of DNTS and DPTS were similar, and distinct from DZTS, in general, the cardioprotective efficacy of these three TS for rat myocardial ischemia were closely comparable based on LDH, CK, AST, SOD, GPx, CAT, T-AOC and MDA levels, as well as on myocardial histology, thereby explaining the similarity in their clinical efficacy as anti-MI drugs.
Conflict of interest

All authors have no conflict of interest to disclose.

Acknowledgements

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Wang, T.J., Choi, R.C., Li, J., Bi, C.W., Ran, W., Chen, X.H., Dong, T.T., Bi, K.S., and Tsim, K.W.,


Supplementary material

2.2 Preparation and quantification of *Dioscorea* total saponins

Table S1 Tentatively identified constituents from DZTS by UPLC-QTOF-MS

Figure S1 Chromatograms of acid hydrolyzed DNTS, DPTS and DZTS.

Figure S2 Illustrated administration protocol
2.2 Preparation and quantification of Dioscorea total saponins

The total saponins from DN, DP and DZ were prepared separately. The dried rhizomes of these herbs were crushed and 1 kg portions were refluxed thrice with 8 L of 70% ethanol for 2 hours each time at 80 °C. The mixture was filtered and the combined ethanolic extracts were rotary evaporated under reduced pressure at 60 °C to concentrated aqueous extract (equivalent to 1.5 g of crude drug/mL). A portion of 200 mL was successively extracted with 200 mL n-butanol pre-saturated with water three times. The upper layer (n-butanol layer) was combined and evaporated to dryness. The residue was dissolved in methanol, and centrifuged. The supernatant was dried under vacuum and re-dissolved in a small amount of methanol. The methanolic solution was added, drop by drop, into approximately 20-fold volume of ethyl acetate while stirring. The precipitate was filtered under vacuum and washed with fresh ethyl acetate. The total saponins were obtained after drying the precipitate at 50 °C. These extracts were individually suspended in 0.5% (w/v) aqueous sodium carboxymethyl cellulose (CMC-Na) for animal administration.

The chromatographic conditions were the same as for our previous study (Tang et al., 2013). For the determination of glycosides, the mobile phase consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). Gradient elution program was used: 0-2 min, 20-20% B; 2-12min, 20-28% B; 12-20 min, 28-45% B; 20-35min, 45-48% B. The flow rate was 0.4 mL/min. The column temperature was set at 25 °C. The MS spectra were acquired in negative ion mode for detecting glycosides and positive ion mode for detecting aglycones. High-purity nitrogen was used as nebulizer and auxiliary gas. The mass scan was over the range of m/z
50-1700 for negative ion mode with electrospray ionization (ESI) interface. The ESI capillary voltage was set at -3.5kV for negative mode.

For the determination of aglycones, the mobile phase consisted of water (A) and methanol (B). Elution program was set as follows: 0-10 min, 55-95% B; 10-18min, 95-95% B. The flow rate was 0.45 mL/min. The column temperature was set at 45 °C. The mass scan was over the range of m/z 100-1000 for positive ion mode with an atmospheric-pressure chemical ionization (APCI) source. The capillary voltage was set at +4.5kV. All the results were analyzed by Aglient MassHunter Workstation Qualitative Analysis B.04.00 and Quantitative Analysis (Q-TOF) B.04.00.
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<td></td>
<td>397.3059</td>
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<td></td>
<td>[M+H-Glc-Rha-H₂O]⁺</td>
</tr>
</tbody>
</table>

*indicates precursor ion for MS²; N.D. indicates “related MS fragments not detected”.

Note: The determination of diosgenin underwent acid hydrolysis. The detailed procedure was described in our previous study (Tang et al., 2013). The chromatogram of hydrolyzed DZTS, as well as DNNTS and DZTS, was shown in Fig. S1.
Figure S1 Chromatograms of acid hydrolyzed DNTS, DPTS and DZTS. 17. Diosgenin
Figure S2 illustrated administration protocol

<table>
<thead>
<tr>
<th>Pre-ISO</th>
<th>ISO Injection</th>
<th>Post-ISO</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Saponins: 150mg/kg</td>
<td>1 mg/kg, s.c., twice</td>
<td>Total Saponins: 300 mg/kg</td>
<td>All groups</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Normal control: given vehicle, without ISO injection
Both pre- and post-ISO group: DN-PP; DP-PP; DZ-PP

Model group (ISO)
Post-ISO group: DN-P; DP-P; DZ-P;
Positive group

Pre-ISO Total Saponins: 150mg/kg
ISO Injection. 1 mg/kg, s.c., twice
Post-ISO Total Saponins: 300 mg/kg
Positive (propranolol): 15 mg/kg
Sacrifice All groups

1 2 3 4 5 6 7 8 day

DN-P, DP-P and DZ-P: orally given DNTS, DPTS and DZTS, respectively, after ISO injection;
DN-PP, DP-PP and DZ-PP: orally given DNTS, DPTS and DZTS, respectively, both before and after ISO injection;