Steroid-induced intrahepatic cholestasis in mice

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It has been reported that administration of orally-active steroids such as norethandrolone (1, 2), norethisterone (3), methyltestosterone (4) and methandienone (5) can cause in some patients a type of cholestatic jaundice characterized by accumulation of bile in the liver without extrahepatic biliary obstruction (intrahepatic cholestasis), occurrence of canalicular bile-plugs, absence of either parenchymal necrosis or portal inflammation, and an elevation of conjugated bilirubin in the serum. In addition, Schaffner and Popper (6) have shown that ultrastructural alterations of the liver comprising a wide-spread dilatation of the biliary canaliculi and distortion of the canalicular microvilli are invariably associated with the intrahepatic cholestasis.

Several authors have reported experimental studies in relation to the cholestatic effect of steroids. Schaffner and Popper (6) have demonstrated the occurrence of canalicular dilatation in the liver of rats following administration of norethandrolone. Retardation of bromosulphalein excretion is known to occur in animals treated with methyltestosterone and norethandrolone (7-9). However, the experimental model with definite manifestations of intrahepatic bile-stasis such as conjugated hyperbilirubinemia or canalicular bile-plugs has not been established.

In the course of studying the acute toxicity of norethisterone, 17α-ethinyl-19-nortestosterone, we found a consistent appearance of jaundice in DS mice following administration of a large amount of norethisterone as well as other C17 α-alkyl or alkynyl substituted steroids. This experiment attempted to evaluate the pathological entities of the norethisterone-induced jaundice in mice in comparison with the intrahepatic cholestasis in men caused by various steroid drugs.

Materials and Methods

Norethisterone-induced hepatic alterations in mice

Male DS mice, weighing around 25 g were used. Norethisterone was suspended in 10% aqueous solution of gum acacia and orally administered to mice using a gastric tube. Experiments were conducted in the following two series;

In the first series, five groups of 20 mice each received oral administration of norethisterone in doses of 25 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg or 500 mg/kg for 5 consecutive days. Additional 20 mice served as the control group and were given 0.1 ml of the vehicle for the same period. At 24 hours after the last administration, all mice
were sacrificed under nembutal anesthesia. Blood samples were collected from the inferior caval vein using a heparinized syringe, and centrifuged at 3,000 rpm for 10 minutes. The blood-plasma was assayed as to alkaline phosphatase activity (10), glutamic-oxalo-acetic transaminase activity (11), glutamic-pyruvic transaminase activity and the total bilirubin concentration (12). A differential analysis of direct-reacting bilirubin and indirect-reacting bilirubin (13) was made on the blood-plasma from mice treated with 500 mg/kg of norethisterone. The liver was excised and fixed in 10% formalin for histological examination. For electron microscopy, representative hepatic samples were fixed in cacodylate-buffered 3% glutaraldehyde for 40 minutes, post-fixed in phosphate-buffered 2% osmium tetroxide for 30 minutes, and embedded in Epon 812. Sections were cut in a LKB Ultrotome, stained with lead (13) and uranium (14), and observed with a JEM-6C electron microscope.

In the second experiment, four groups of 20 mice each were given daily oral administration of norethisterone in a dose of 100 mg/kg for 1, 3 or 5 days, and sacrificed 24 hours after the last administration. Total bilirubin concentration, glutamic-oxalo-acetic transaminase activity, glutamic-pyruvic transaminase activity and alkaline phosphatase activity of the blood-plasma was estimated. The liver was removed, weighed and fixed in 10% formalin for histology.

Strain and species difference
 Male mice of ICR, C3H, CBA, NA2, C57BL and DS strain, weighing around 25 g, and male rats of Sprague-Dawley strain, weighing around 100 g were used. About twenty mice of each strain were divided into 2 groups, and given daily oral administration of norethisterone in dose levels of either 100 mg/kg or 50 mg/kg for 5 days. Twenty Sprague-Dawley rats received oral administration of 1,000 mg norethisterone/kg for 5 days. All animals were sacrificed 24 hours after the last administration, and the liver was weighed and fixed in 10% formalin for histology.

Chemical structure-activity relationship
 Eight steroids including norethisterone, norethandrolone, methyltestosterone, oxymetholone, mestranol, testosterone propionate, estradiol-17β and progesterone were tested. These compounds were suspended in 10% gum acacia, and administered to male DS mice either orally or intraperitoneally for 5 days in dose levels as shown in Table 6. The animals were sacrificed 24 hours after the last administration. The liver was excised and studied histologically.

RESULTS

Norethisterone-induced hepatic alterations in mice
 Gross and histological findings: The livers of norethisterone-treated mice were enlarged and colored dark brown. An icteric coloration of the skin was noted in the high dosage groups. Histological examination of the liver invariably revealed occurrence of bile-plugs in the biliary canaliculi as well as in the interlobular biliary ducts. The hepatic cells appeared to be swollen, exhibited a decrease of cytoplasmic basophilia and
TALE 1. Occurrence of intrahepatic cholestasis in mice following administration of various amounts of norethisterone.

| Administration | Liver |
|----------------|-------|
| Daily dose mg/kg | Period days | Weight g/10 g b.w. | Bile-stasis |
| --- | --- | --- | --- |
| 0 | 0/10 | 0/10 | (−,−,−,−,−,−,−,−,−,−,−,−) ** |
| 25 | 5 | 0.57 ± 0.02 | 0/10 | (−,−,−,−,−,−,−,−,−,−,−,−) |
| 50 | 5 | 0.63 ± 0.01 | 6/10 | (+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+,+ | Occasionally contained bile pigment granules. Single cell necrosis of the parenchymal cells was occasionally noted in the groups treated with more than 200 mg/kg of norethisterone. Round cell infiltration at the peripoportal area was also noted in these high dosage groups. As shown in Table 1, the severity of the hepatic lesions as indicated by the liver weight and incidence of the bile-plug formation appeared to be dose-dependent.

**Electron microscopic findings:** In norethisterone-treated mice, dilatation of the biliary canaliculi together with distortion or reduction of the canalicular microvilli was frequently noted. Amorphous, fine granular or fibrillar materials were occasionally seen to fill the diluted bile canaliculi. These substances are assumed to correspond to the bile-plugs noted in histological sections. In the parenchymal cells, parallel arrays of granular endoplasmic reticulum were reduced; while vesicles of agranular endoplasmic reticulum were markedly increased. Occasionally, these vesicles contained amor-

TABLE 2. Biochemical changes of blood-plasma in mice following administration of various amounts of norethisterone.

| Administration | Total bilirubin concentration mg/dl | Alkaline phosphatase activity units* | Glutamic-oxaloacetic transaminase activity units** | Glutamic-pyruvic transaminase activity units** |
|----------------|-------------------------------------|-------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Daily dose mg/kg | Period days | 0.37 ± 0.05 | 2.58 ± 0.14 | 7.7 ± 1.2 | 1.9 ± 0.3 |
| 25 | 5 | 0.39 ± 0.03 | 1.71 ± 0.14 | 7.5 ± 0.7 | 2.4 ± 0.7 |
| 50 | 5 | 0.64 ± 0.07 | 3.19 ± 0.33 | 10.9 ± 0.5 | 3.2 ± 0.5 |
| 100 | 5 | 0.77 ± 0.07 | 3.57 ± 0.30 | 41.6 ± 6.4 | 13.8 ± 2.7 |
| 200 | 5 | 0.99 ± 0.25 | 7.24 ± 0.91 | 46.4 ± 3.9 | 19.5 ± 2.2 |
| 500 | 5 | 2.57 ± 0.56 | 5.21 ± 0.83 | 59.3 ± 2.9 | 31.4 ± 1.4 |

* Bessey-Lowry unit (10).

** The activity of GOT and GPT was estimated according to a modification of Karmen's method (11).
phous or fine granular materials. The ectoplasma surrounding the dilated bile canaliculi was shown to be thickend.

**Biochemical findings:** As shown in Table 2, the plasma bilirubin level was significantly elevated in norethisterone-treated mice. In the 500 mg/kg group, the total bilirubin concentration reached 8 times of the control value, and 87% of the total amount was estimated as a direct-reacting type (Table 3). The time course of hyperbilirubinemia during daily administration of 100 mg norethisterone/kg is shown in Table 4; an elevation of the total bilirubin concentration is apparent after 3 day administration.

The activity of plasma alkaline phosphatase, glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase was also shown to be increased in norethisterone-treated mice. As shown in Table 4, significant elevation of these enzyme activities appeared in mice which recieved 50 mg/kg of norethisterone for 5 days; while administration of 100 mg/kg for 3 days did not result in elevation of these enzyme activities.

| TABLE 3. Differential analysis of plasma bilirubin in mice recieved either administration of norethisterone or ligation of the common bile-duct. |
|---------------------------------------------------------------|
| **Treatment** | **Total mg/dl** | **Direct mg/dl** | **Direct/Total %** |
| Administration of norethisterone for 5 consecutive days | 2.57±0.56 | 2.24±0.47 | 87.2 |
| Bile-duct ligation* | 5.34±0.34 | 5.05±0.31 | 94.6 |

* Blood samples were collected from the inferior caval vein of mice 18 hours after ligation of the common bile-duct.

| TABLE 4. Liver weight and biochemical changes of blood-plasma in mice by daily oral administration of 100 mg norethisterone/kg. |
|---------------------------------------------------------------|
| **Dosage period days** | **Liver weight g/10 g b.w.** | **Bilirubin mg/dl** | **Alkaline phosphatase units** | **Glutamic-oxaloacetic transaminase units** | **Glutamic-pyruvic transaminase units** |
|----|----------------|----------------|-----------------|----------------|----------------|
| 0.48±0.01 | 0.37±0.05 | 2.85±0.14 | 8.8±0.6 | 2.6±0.2 |
| 0.54±0.01 | 0.36±0.07 | 2.10±0.15 | 8.1±0.8 | 2.3±0.5 |
| 0.67±0.02 | 0.59±0.09 | 2.42±0.13 | 6.4±0.3 | 2.3±0.6 |
| 0.86±0.02 | 0.77±0.07 | 3.57±0.30 | 21.1±1.9 | 12.0±2.2 |

* Bessey-Lowry unit (10).
** The activity of GOT and GPT was estimated according to a modification of Karmen's method (11).

**Strain and species difference**

Following administration of norethisterone, the bile-stasis as indicated by dark brownish discoloration of the liver and canalicular bile-plug formation occurred not only in DS mice but also in the other 5 strains of mice tested. Of these strains, DS mice and C57BL mice appeared to be most sensitive; ICR mice were least sensitive (Table 5). In contrast to mice, Sprague-Dawley rats did not exhibit such morphological alternations even after administration of 1,000 mg norethisterone/kg for 5 days.
### TABLE 5. Cholestatic effects of norethisterone on various strains of mice.

| Strain | Administration | Liver |
|--------|----------------|-------|
|        | Daily dose mg/kg | Period days | Weight g/10 g b.w. | Bile-stasis |
| DS     | 100            | 5      | 0.86±0.02       | 10/10* (++)(++,+++,+++,+++,+++,++,++)** |
|        | 50             | 5      | 0.62±0.01       | 6/10 (++,++,++,++,++,++,++,++,++,++,++,++,++) |
| C5BL   | 100            | 5      | 0.81±0.01       | 10/10 (++,++,++,++,++,++,++,++,++,++,++,++) |
|        | 50             | 5      | 0.70±0.02       | 6/10 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |
| CBA    | 100            | 5      | 0.81±0.03       | 9/9 (++,++,++,++,++,++,++,++,++,++,++,++,++) |
|        | 50             | 5      | 0.68±0.02       | 5/8 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |
| Na2    | 100            | 5      | 0.77±0.02       | 9/9 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |
|        | 50             | 5      | 0.66±0.03       | 2/10 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |
| C3H    | 100            | 5      | 0.72±0.03       | 9/10 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |
|        | 50             | 5      | 0.67±0.03       | 2/9 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |
| ICR    | 100            | 5      | 0.84±0.01       | 3/10 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |
|        | 50             | 5      | 0.73±0.03       | 0/10 (++,++,++,++,++,++,++,++,++,++,++,++,++,++) |

* No. of mice with bile-stasis/No. of mice examined.

** Degrees of bile-stasis were estimated according to the criteria shown in Table 1.

### TABLE 6. Occurrence of canalicular bile-plug in DS mice following administration of various steroids.

| Test compounds | Administration | Route | Daily dose mg/kg | Period days | Bile-plug |
|----------------|----------------|-------|------------------|-------------|-----------|
| Norethisterone |                | Oral  | 50               | 5           | +         |
| Methyltestosterone |        | »      | 200              | 5           | +         |
| Oxynetholone   |                | »      | 500              | 5           | +         |
| Mestranol      |                | »      | 500              | 5           | +         |
| Norethandrolone|                | »      | 250              | 5           | +         |
| Testosterone propionate | Oral | 1000 | 5 | - |
| Progesterone   |                | »      | 1000             | 5           | -         |
| Estradiol-17β |                | »      | 1000             | 5           | -         |
| Testosterone propionate | i.p. | 250 | 5 | - |
| Progesterone   |                | »      | 250              | 5           | -         |
| Estradiol-17β |                | »      | 250              | 5           | -         |

**Chemical structure-activity relationship**

Besides norethisterone, the other 5 steroidal compounds with C17 α-alkyl or alkynyl-substitution were shown to elicit cholestatic effects (Table 6). Administration of testosterone propionate, progesterone or estradiol-17β did not cause bile-stasis in mice.

**DISCUSSION**

This experiment indicates that oral administration of norethisterone can cause bile-stasis in mice as indicated by canalicular bile-plug formation in the liver and elevation of plasma bilirubin concentration. Ultrastructurally, the biliary canaliculi were dilated,
and the canalicular microvilli were decreased or distorted. These findings correspond to the pathological entities of intrahepatic cholestasis in men caused by various steroid compounds having an alkyl or alkynyl-substitution at C17 (15, 16). A similar situation was also shown in this experiment, where the bile-stasis was produced in mice following administration of C17α-substituted steroids including norethandrolone, methyltestosterone and mestranol; while the steroidal compounds without such chemical configuration did not elicit the cholestatic effect.

Previous reports suggest that experimental animals including rats (9), rabbits (7, 8) and dogs (17) are very little susceptible to the cholestatic effect of the steroids. It was reported that hepatic dysfunction as measured by bromosulphalein retention appeared in patients receiving C17α-substituted steroids in therapeutic range (18, 3), whereas a large amount of the steroids, more than 10 folds of the therapeutic dose was required to cause significant retardation of bromosulphalein excretion in animals (7-9). Schaffner and Popper (6) demonstrated invariable occurrence of ultrastructural alterations of biliary canaliculi in rats following administration of 60 mg norethandrolone/kg for 8 days, but the morphological lesions indicating an accumulation of bile in the liver were not seen in these animals. Therefore, the present study which disclosed a consistent production of bile-stasis in mice following administration of norethisterone as well as other C17α-substituted steroids may provide with a more straight-forward experimental model of the steroid-induced intrahepatic cholestasis.

At present, the mechanism of cholestatic effect of the steroids is not fully elucidated. The maximum rate of hepatic bilirubin clearance in rats is known to be reduced by administration of norethandrolone, methyltestosterone or norethynodrel (19). Arias reported that norethandrolone blocked the excretion of conjugated bilirubin in rats (20). In an in vitro experiment using rat-liver slices, Hargreaves (19) disclosed an interesting fact that the steroids caused an accumulation of conjugated bilirubin in the slice; while phenothiazines, another type of cholestatic agent increased the unconjugated bilirubin in the slice. These data together with the occurrence of conjugated hyperbilirubinemia and canalicular bile-plug noted in this experiment may support the view that the steroids prevent the transportation of conjugated bilirubin into the biliary canaliculi by blocking a stage after conjugation.

**SUMMARY**

Oral administration of norethisterone in does levels of more than 50 mg/kg for 5 consecutive days produced in mice a hepatic alteration characterized by bile plug formation in the intrahepatic biliary tracts together with an elevation of plasma bilirubin concentration. The activity of plasma alkaline phosphatase, glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase were also shown to be elevated. Electron microscopic examination invariably revealed dilatation of the bile canaliculi in association with a decrease of microvilli and occasional appearance of bile plugs. Various sized vesicles of agranular endoplasmic reticulum, occasionally containing amorphous...
or fine granular materials appeared in the cytoplasm of the hepatic cells. Besides nor-ethisterone, various other C17 α-substituted steroids, including methyltestosterone, oxymetholone and norethandrolone, caused intrahepatic bile-plug formation in mice; while neither testosterone propionate, progesterone or estradiol-17β elicited such effects. In contrast to mice, rats did not exhibit bile-plug formation even after high dosage of norethisterone.

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FIG. 1. The liver on the right is from a mouse treated with oral administration of 100 mg norethisterone/kg for 5 consecutive days showing enlargement and dark-brown discoloration. The liver on the left is from a control mouse.

FIG. 2. An area of the liver from a mouse treated with oral administration of 200 mg norethisterone/kg for 5 consecutive days, showing bile-plug formation in the interlobular bile duct and slight periporal cell infiltration. Hematoxylin-eosin. 75x.

FIG. 3. An area of the liver from a mouse treated with oral administration of 100 mg norethisterone/kg for 5 consecutive days, showing bile-plug formation in the interlobular bile duct and bile-pigment granules in the hepatic cells (arrow). Hematoxylin-eosin. 250x.
Fig. 4. An area of the liver from a mouse treated with oral administration of 100 mg norethisterone/kg for 5 consecutive days, showing canalicular bile-plug formation and bile-pigment granules in the hepatic cells. Hematoxylin-eosin. 250 x.

Fig. 5. An electron micrograph of the liver from a mouse treated with oral administration of 200 mg norethisterone/kg for 5 consecutive days, showing a dilatation of the bile canaliculus together with reduction of the canalicular microvilli. Fine granular materials are seen to fill the lumen. The pericanalicular cytoplasm is shown to be thickened and numerous vesicles of agranular endoplasmic reticulum are distributed in the cytoplasm. 25,000 x.