Teratogenic activity of methadone hydrochloride in mouse and chick embryos

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SUMMARY

Teratogenic activity of methadone HCl (Physeptone, Burroughs Wellcome and Co.) was tested on inbred JBT/Jd and outbred Q strain mouse embryos and on chick embryos. 22–24 mg/kg injected subcutaneously on the 9th day of pregnancy caused by the 13th day exencephaly in 56 out of 479 JBT/Jd embryos but after 32 mg/kg only in 1 out of 220 of the Q strain. Some affected JBT/Jd embryos showed also rachischisis in the cervical area.

The second abnormality shown by the embryos of both strains is Z-shaped kinkage of the spinal cord.

In explanted chick embryos cultured in vitro as well as in embryos treated in ovo methadone causes non-closure of the neural tube with extensive necrosis of the neural plate cells in the cephalic region.

The results of this study indicate that methadone, which is a neutropic drug, has an embryo-toxic activity directed against the developing central nervous system.

INTRODUCTION

Methadone hydrochloride (2-dimethylamino-4,4-diphenyl-heptanone(5) hydrochloride) is a synthetic drug originally used as a potent analgesic alternative to morphine or heroin. Its chemical constitution is different from that of the opiate alkaloids although in general it may be regarded as a synthetic alkaloid with a simplified structure in comparison with natural products. Pharmacologically it is a typical narcotic and its repeated use may lead to addiction. However, the withdrawal symptoms in methadone addicts are much milder than those during morphine or heroin abstinence and this is why methadone is widely used, particularly in the U.S.A., in the treatment of heroin addiction, as a transitory substitute for heroin preceding the complete withdrawal of narcotics.

Methadone was subjected previously to some experimental teratological investigation although not on a very wide scale. In hamsters Geber & Schramm (1969) reported in a short abstract which does not give any statistical data that methadone caused gross anomalies of which the most commonly observed were exencephaly and cranioccephaly.

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In rabbits and rats, Markham, Emmerson & Owen (1971) did not observe any drug related defects after oral administration of 20 or 40 mg of methadone per kg body weight.

The experiments reported here were undertaken to investigate whether methadone as a neurotropic drug has an adverse effect on the development of the central nervous system, particularly during the process of neural tube closure. Secondly, it was also intended to compare methadone in respect of its potential teratogenic activity with that of morphine as they are pharmacologically to some extent similar.

MATERIAL AND METHODS

In these investigations the JBT/Jd strain of the laboratory mouse inbred for 45 generations (Jurand, 1968) and an outbred strain Q were used. Females 2–5 months old were kept with males overnight and on the next morning were examined for the presence of copulation plugs. The convention adopted in this report is that the day on which plugs were found was considered to be the first day of pregnancy.

Methadone hydrochloride used in these experiments was in the form of 1% solution in ampoules manufactured by Burroughs Wellcome and Co. and marketed in the United Kingdom under the name of Physeptone. For experimental purposes it was diluted to contain the required dose for 1 kg in 40 ml of physiological saline solution. In this way 1 ml of this solution was the exact volume to contain the dose for a 25 g mouse. Before injections pregnant females were weighed and the required volume of the methadone solution was injected.

In the first series of experiments the litter LD_{50} of methadone for the JBT/Jd strain embryos was estimated and it was found to be 25 mg/kg when administered on the 9th day of pregnancy. For the Q strain embryos the litter LD_{50} was higher, namely 35 mg/kg. In the main series of experiments, JBT/Jd pregnant females were treated on the 9th day of pregnancy with doses ranging from 22 to 24 mg per kg. Pregnant Q strain females were treated in the same manner on the 9th day of pregnancy with 32 mg per kg of methadone. In addition, some of the JBT/Jd pregnant females were injected with 23 mg per kg in the same way on the 8th or on the 10th day of pregnancy.

In all experiments methadone was administered subcutaneously under light ether anaesthesia and the females were sacrificed on the 13th day of pregnancy by cervical dislocation. After dissection the embryos were fixed while still in utero by immersion in 5% trichloroacetic acid solution with 1.37% lanthanum acetate at room temperature. After 1–2 h the embryos were dissected out from the uteri, examined for gross malformations and postfixed in the same fixative for 24 h. Selected embryos were examined histologically by dehydrating and

1 Litter LD_{50} for methadone is defined as the dose which if administered subcutaneously on the 9th day of pregnancy kills about 50% of implanted embryos by the 14th day of pregnancy.
embedding in 56 °C paraffin in the conventional way. The methyl green-pyronine stain was used for staining paraffin sections.

For control purposes, both JBT/Jd and Q strains were collected on the 13th day of pregnancy from 29 and 14 pregnant females respectively. The control females had been lightly etherized on the 9th day.

Further experiments were conducted to investigate the influence of methadone on the morphogenesis of the central nervous system of Brown Leghorn chick embryos explanted or in ovo. The embryos were explanted at the primitive streak stage (stage 4 according to Hamburger & Hamilton, 1951) after pre-incubation for 28 h at the temperature 38.5 °C and cultured in vitro according to the method of New (1955). Methadone was administered in 0.5 ml of solution containing 1, 2.5 or 4 mg of the drug per ml. The solutions were prepared so as to contain 50% of egg albumen and were applied on the egg albumen side of the blastoderm. After incubation for another 30 h embryos were fixed with 5% trichloroacetic acid with 1.37% lanthanum acetate and embedded for histological examination or mounted as whole mounts. For histological purposes the embryos were sectioned longitudinally or transversely and stained with methyl green-pyronine. For experiments on chick embryos in ovo the eggs were preincubated for 28 h and then injected with 0.75, 1, 1.5, 2, 3 or 5 mg of methadone in 1% solution into egg white and further incubated for 36 h at 38.5 °C. For control purposes, explanted chick embryos were incubated in the presence of saline solution containing 50% of egg white and the embryos in ovo were treated with 0.5 ml saline solution.

RESULTS

In the JBT/Jd strain mouse embryos methadone at a dose of 22-24 mg/kg given on the 9th day of pregnancy as a single subcutaneous injection caused two main abnormalities, namely exencephaly in 11.6% of the embryos (Figs. 1-3) accompanied occasionally by rachischisis at the level of the fourth brain ventricle or the cervical region and by more or less extensive spina bifida with myeloschisis (Fig. 4). The second abnormality found was kinking of the spinal cord (Fig. 5).

In the Q strain embryos treated with methadone at 32 mg/kg showed a remarkably low incidence of exencephaly (3.4%) and a lower incidence of kinkage in the spinal cord.

In control experiments none of these abnormalities were present. The numerical data of these results are presented in Table 1.

Histological examination of embryos with exencephaly shows eversion of the prosencephalon and mesencephalon so that the ventricular surfaces are exposed due to the failure of the corresponding parts of the neural tube to close. The skull vault is absent, the optic vesicles develop normally but the other parts of the
Fig. 1. An example of a litter of 13-day-old JBT/Jd embryos after treatment of the mother with a subcutaneous injection of 24 mg methadone per kg body weight on the 9th day of pregnancy. x 3.5.

Fig. 2. An exencephalic embryo after treatment as those in Fig. 1 (side view). x 6.

Fig. 3. An exencephalic embryo after the same treatment as in Fig. 1 (rear view). The arrow points to the anterior neuropore. x 6.

Fig. 4. A case of exencephaly, rachischisis and spina bifida after the same treatment as in Fig. 1. x 6.
Table 1. The effect of methadone in producing abnormalities

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>Treatment</th>
<th>No. of litters</th>
<th>No. of embryos</th>
<th>Normal</th>
<th>With exencephaly</th>
<th>With kinkage of the spinal cord</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>JBT/Jd</td>
<td>22-24 mg/kg</td>
<td>56</td>
<td>268 (55.9 %)</td>
<td>56 (11.6 %)</td>
<td>163 (34.0 %)</td>
<td>—</td>
<td>479</td>
</tr>
<tr>
<td>JBT/Jd</td>
<td>Control</td>
<td>29</td>
<td>235</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>235</td>
</tr>
<tr>
<td>Q</td>
<td>32 mg/kg</td>
<td>25</td>
<td>188 (81.8 %)</td>
<td>1 (3.4 %)</td>
<td>31 (14.0 %)</td>
<td>—</td>
<td>220</td>
</tr>
<tr>
<td>Q</td>
<td>Control</td>
<td>14</td>
<td>123</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>123</td>
</tr>
</tbody>
</table>

Diencephalon such as thalamus and hypothalamus, although recognizable, are exposed and the anterior neuropore is opened to the exterior (Fig. 6).

The protruding part of the brain which is exposed is most probably formed by an extensive proliferation of the cranial flexure together with the cerebral peduncle. Ventral to both lateral parts of the protruding hemispheres, there is a dense network of dilated blood vessels which are probably branches and anastomoses of the basilar and internal carotid arteries (Fig. 7).

The second abnormality found after treatment with the teratological dose of methadone was a characteristic Z-shaped kinkage of the spinal cord. It occurred with a frequency of 34 % in the JBT/Jd embryos and in 14.0 % of the Q strain embryos.

In additional experiments, JBT/Jd pregnant females were injected subcutaneously with 23 mg/kg methadone HCl on the 8th or 10th day of pregnancy. All the 11 females injected on the 8th day showed, on the 13th day, an advanced reabsorption of embryos with no survivors present. In the second series of 15 females injected with the same dose on the 10th day of pregnancy, 115 embryos showed no abnormality.

In experiments with chick embryos explanted and cultured in vitro or injected in ovo the most consistent results were observed after methadone treatment of 2.5 mg/ml to explanted embryos and after injections of 3 mg into the eggs. The results for chick embryos indicated that methadone has an affinity for the developing central nervous system, particularly the brain. The explanted embryos, as well as those treated in ovo, exhibited extensive disturbance to the closure of the neural tube and a selective necrotic damage of the neural tube in the area of the developing brain (Figs. 8-10). In addition, an exencephalic condition could be recognized in chick embryos at a slightly later stage of development (Figs. 11, 12).

**DISCUSSION**

According to Geber & Schramm (1969) methadone was found to cause exencephaly and cranioschisis in hamster embryos. In rabbits, however, it did
Fig. 5. Z-shaped kinkage of the spinal cord after treatment with 23 mg of methadone injected subcutaneously on the 9th day of pregnancy. ×6.

Fig. 6. Serial sagittal section through the exencephalic brain showing communication of the spinal canal with the exterior through the anterior neuropore (arrow). ×29.

Fig. 7. Parasagittal section through the left exencephalic brain hemisphere showing the network of dilated branches of the basilar and internal carotid arteries. ×21.

Fig. 8. Longitudinal section through the head region of a control chick embryo at stage 12 (Hamburger & Hamilton, 1951) cultured in vitro. ×37.5.

Fig. 9. Longitudinal section through the head region of a chick embryo cultured in vitro and treated with 2.5 mg/ml methadone. ×37.5.

Fig. 10. Similar section to Fig. 9 at higher magnification showing extensive necrosis of the nervous tissue in the brain area and unaffected other tissues. ×95.

Fig. 11. Control chick embryo at stage 13 (whole mount). ×29.

Fig. 12. Chick embryo, stage 13, after treatment in ovo with 3 mg/ml methadone (whole mount). Note that the closure of the neural tube is affected and diencephalon and telencephalon are not covered by the head ectoderm. ×29.
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not appear to be teratogenic (Markham, Emmerson & Owen, 1971). From the results reported here, it can be concluded that methadone hydrochloride is teratogenic for the JBT/Jd strain mouse embryos if administered subcutaneously at a dose between 22 and 24 mg per kg body weight on the 9th day of pregnancy. On the other hand, embryos of the Q strain of mice in similar conditions are much more resistant to methadone.

The proportion of the exencephalic embryos of the JBT/Jd strain is 56 out of the total of 479 embryos, i.e. about 11.6%, which is fairly high in comparison with other teratogens which can cause exencephaly. For instance, morphine sulphate at comparatively higher doses (100–500 mg/kg) administered subcutaneously on the 8th or 9th day was reported to cause exencephaly only in 25 out of 730 (3.4%) mouse embryos (Harpel & Gautieri, 1968). Other teratogenic factors such as hypervitaminosis A induce exencephaly in mice in about 5% of embryos (Kalter & Warkany, 1961), X-irradiation in about 16.7% (Kaven, 1938), urethane in up to 30% in the C3HeB/Fe strain of mice and none in the CBA strain (Tsuchikawa & Akabori, 1964, 1965) and hypoglycemia induced by insulin in 30% of 129 strain mouse embryos and only in 3% of BALB/c strain embryos (Smithberg & Runner, 1963). A similar difference in the sensitivity of different strains is reported here to exist between the JBT/Jd and the Q strains.

Exencephaly induced by methadone hydrochloride is here classified as such because its general appearance corresponds to the descriptions and definitions of exencephaly in the literature (Kalter, 1968). This malformation, characterized by a complete extroversion of the cerebral hemispheres, is called also pseudencephaly (Willis, 1962). Extensive dilatation of blood vessels in the everted brain hemispheres is most probably due to an abnormal angiogenesis which was observed also in human anencephaly. Abnormal angiogenesis is thought to play an important causative role in the pathogenesis of anencephaly due to the failure of integration of primordial cerebral vessels into the systemic circulation (Vogel, 1958, 1961).

It has to be emphasized that, for exencephaly to be induced, treatment with teratogens has to take place at a critical specific stage of the neural tube closure. Out of 25 cases of exencephaly reported for mice after administration of morphine sulphate, 22 cases were found after treatment on the 8th day of pregnancy (i.e. on the 9th day according to the convention of staging mouse embryos adopted in this paper). The remaining three cases were found after administration on the 9th (i.e. 10th) day (Harpel & Gautieri, 1968). These findings, as well as the results reported here, indicate that there is a stage in the development of mouse embryos, during the 9th day of pregnancy, when a specific disturbance of the normal morphogenesis of the brain and skull leads to exencephaly. Moreover, this sensitive stage is evidently of such a short duration that, due to the intralitter variability of developmental age, only up to about 50% and never all of the embryos are simultaneously affected in one litter. The unaffected
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embryos presumably have not yet reached the sensitive stage at the time of treatment or have already passed this stage.

There is a marked difference between the results of treatment with the same dose of methadone administered to pregnant mice on the 8th or 10th day of pregnancy in comparison with the critical 9th day. In general during these 3 days methadone becomes less and less embryotoxic. It is probable that during this period of time a placental barrier for methadone develops concurrently with the development of the placenta.

Z-shaped kinkage of the spinal cord, which occurs at a fairly high frequency after methadone treatment, is probably caused by disturbance of the neural tube closure in non-cephalic regions. A similar effect was observed in mouse embryos after triethanomelamine (TEM) treatment (Jurand, 1959).

Experiments with chick embryos have confirmed that methadone hydrochloride affects the development of the central nervous system in this experimental system also.

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REFERENCES


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