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Teratogenic assessment of 'Winniecure' in pregnant Wistar rats

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Abstract
The study to determine the possible teratogenicity of Winniecure (a multicomponent herbal remedy) was carried out on pregnant female Wistar rats. A single dose of Winniecure suspension (8.4mg/kg) was administered intraperitoneally to 3 groups of pregnant Wistar rats on the 1st, 2nd and 3rd weeks of gestation, respectively. The control group received corresponding dose of normal saline during gestation. On day 20 of gestation, the litter of each rat was delivered by hysterectomy and examined for gross anomalies. The colour, activity, weight and length of the litters were also assessed and recorded. The results showed no teratogenic effect of the single dose of Winniecure, during any of the trimesters of pregnancy. However, it is possible that multiple and/or higher doses of Winniecure, administered in the 1st week of gestation may produce teratogenicity as anomalies or abortions.

Keywords: Winniecure; Teratogenicity, Wistar rats; Litter; Gestation

INTRODUCTION

Several herbs have been found useful for the treatment of ailments and have been regarded as medicinal plants. But like most drugs certain medicinal plants present contraindications; their use produce some undesired effects that make them not good enough or not to be recommended in some situations (Farnsworth and Bingel,1977). Plants that present contraindications do so because of the presence of specific bioactive agents which are responsible for the production of undesirable effects. As a result of this, the screening of plants for bioactive principles is important if the plants must be used for drug development. Drug regulatory agencies like the National Agency for Food and Drug Administration and Control (NAFDAC), Food and Drug Administration (FDA) of the United States and the Dunlop Committee of the United Kingdom require some information about the nature of bioactive agents present in a "vegetable drug" or medicinal plant before the drug can be approved for general use (Finnel, 1999). This regulation is very important for the assessment of lethal toxicity or side effects produced by the drug. Among the undesirable effects produced by the bioactive agents in medicinal plants are cognitive malformations seen in neonates. The study of undesirable effects on intrauterine development following exposure to certain drugs is called development toxicology. Since toxicology is

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the study of poisonous materials and their effect on living organisms (Martin, 1999); developmental toxicology therefore can be described as the study of poisonous materials and their effects on the development of living organisms.

Many medicinal plants have been discovered in recent times and they have been used successfully for the treatment of different types of ailments, but some of them as well as other drugs have been shown to have adverse effects on intrauterine development using animals. The result of these medicinal testing on animals have been extrapolated to humans based on comparative developmental studies made by Carnegie Research Institute and based on these findings, the use of such medicinal plants or drugs have been avoided or modified in pregnant women.

The safety profile in pregnant rats of an anti-diabetic agent was extracted from *Momordica charantia* in 1999. In this study, Fernandopullae and Ratnasooriya (1999) discovered that administration of the water extract of *Momordica charantia* L. to pregnant rats resulted in malformations, so they concluded that it induced teratogenesis in rodents. The *M. charantia* extract is also used in Sri Lanka as a herbal therapy for diabetes mellitus. Kim *et al.* (1993) carried out a teratogenicity study in pregnant Sprague Dawley rats. The animals were treated with the water extract concentrates of the medicinal herbs, *Scutellariae radix* by daily oral administration. Three different doses were administered to different groups of animals and the foetuses obtained from the treated pregnant rats were observed. The study revealed an increase in the incidence of skeletal variations; presence of lumbar ribs and increase in the incidence of abnormal urinary system, especially dilatation of the ureter. The incidence of the variation was dose-dependent.

Mackler *et al.*, (1975) also studied the effects of the inhibition of mitochondrial energy systems on the embryonic development of rats. Pregnant rats treated with inhibitors of mitochondrial oxidative energy metabolism which lowered energy tension and the foetuses were examined for the occurrence of congenital malformations and for changes in enzymatic activities. All the agents used resulted in skeletal anomalies. This showed that the process of production of congenital malformation by such inhibitory drugs may either be by interruption of the supply of energy source to the embryo, interruption or disruption of the enzymatic function, or both. Among the drugs used, the most teratogenic inhibitor tested is phenobarbital. This produced a high incidence of malformation including cleft palate, tail anomalies, spinal retroflexion and facial hypoplasia. Diphenylhydantoin was also tested but it produced a low incidence of malformations like syndactyly and oligodactyly. Chloramphenicol was shown to inhibit mitochondrial enzymes, which interferes with implantation on testing.

Dejan *et al.*, (2004) reported that prenatal exposure of foetal rats to adriamycin during gestational days 6-9 induced congenital disorders such as oesophageal atresia, tracheo-oesophageal fistula, with all the consistent features of the VATER (Vertebral anomalies, Anorectal abnormalities, Congenital heart disease, Esophagial and/or Renal abnormalities) syndrome, reduced reproductive ability, deformed notochord and undivided foregut. Nishimura *et al.*, (1979) also demonstrated some cardiovascular abnormalities in the rat foetuses induced by drug phenobarbital (PB) administered on days 7-17 of gestation.

Other research findings on teratology study in rats with the aim of examining the sensitive period of cardiovascular abnormalities induced by PB as day 8-11 of gestation have been documented (Okuda *et*

al., 1992). The main cardiovascular defects detected were Ventricular Septal Defects (VSD), overriding aorta and transposition of the great vessels. (Aorta and pulmonary artery). Some congenital malformations have also been demonstrated in the rat forelimb. In the study, teratogenesis was induced in the forelimb of rat foetuses by retinoic-acid and the main defect observed after the complete gestation of 21 days was adactyly (Newall and Ward, 1990).

In a separate study, Yoshida *et al.*, (1994) demonstrated the developmental feature of hyperphalangism induced by nifedipine in rat foetuses. He suggested that the development of the extraphalangeal bone might be related to perturbation and differentiation of limb bud cells around the region between the middle and distal phalanges, where the extraphalangeal bone was formed. This is just to note that hyperphalangism can be induced in the rat foetus as a congenital defect induced by drugs. Some other external abnormalities like cleft mandible, cleft lower lip, ankyloglossia, schistogiossia and exencephaly have also been demonstrated in the rat foetuses. The above features were demonstrated in a teratogenic study by Noda, (1992) and were induced by the drug, di-*n*- butylin diacetate. In the study, he tried to find out the critical gestation day of teratogenesis as well as the greatest number of malformations which occur following administration of the drug on the critical day(s). Following oral administration of the drug, the Investigator concluded that day 8 of gestation is the critical gestational day of teratogenesis.

Other researchers like Tachikura, (1988) while studying the teratogenic effects of E- 64 on rat embryogenesis demonstrated some nervous system defects which include hydrocephaly, exencephaly, exophthalmia, microphthalmia, as well as hydronephrosis and renal hypoplasia. Using the Sprague-Dawley rats as a model, Khera, (1993) demonstrated teratogenicity by the administration of different doses of valproic acid. Each administered dose caused teratogenic effects such as: tail defects, rib defects, phalangeal defects, degenerative changes in the labyrinth, reduced mean foetal weight and absorptions. This data of comparison can be very useful when extrapolating experimental results of teratology testing, especially when the critical days of teratogenesis are the concern to humans.

Drugs made from medicinal plants also go through teratology testing in order to prevent congenital anomalies in the neonates following the exposure of the pregnant mother to those drugs. Teratology testing is the process by which a chemical is evaluated for its ability to cause developmental toxicity in humans and/or animals (Francis, 2003). Animals are used in teratology testing for obvious ethical considerations instead of humans (Wilson, 1973). After the thalidomide episode of the 1960s, drug regulatory agencies in the U.S. and in the U.K. put together requirements for the testing of drugs in animals, before they can be approved for the market (Finnel, 1999).

The past decade has witnessed a significant increase in the use of herbal medicines. As a result of World Health Organization's (WHO) promotion of traditional medicine, countries have been seeking the assistance of WHO in identifying safe and effective herbal medicines for use in National health care systems. In 1989, one of the many resolutions adopted by the World Health Assembly in support of National traditional medicine programmes drew attention to herbal medicines as being of great importance to the health of individuals and communities (WHO, 1991).

'Winniecure' is a hot aqueous extract of four medicinal plants, sterilized, lyophilized and formulated into capsule. Small scale clinical trial on the drug showed

that it brings about a marked reduction of HIV-RNA copies up to detectable levels in asymptomatic HIV-Positive cohorts (Agbonlahor *et al.*, 2003). This marked reduction is brought about by the effect of the drug on the CD4+ lymphocyte count. A controlled clinical evaluation of the effect of the herbal extract on CD4+ T lymphocyte count in HIV- infection was carried out and it was shown that CD4+ T lymphocyte count increased following the administration of the drug. The four medicinal plants combined in this herbal remedy are *Ficus asperifolia*, *Ficus exasperate*, *Ficus sur* and *Sida acuta*. *Ficus asperifolia* is also known as sandpaper tree. Ficus species belong to the family Moraceae. Shellac, a resinous excretion of the insect laccifer lacca is produced on several species of the Moraceae family including Ficus (Jordaan, 2000). This excretory product is known to produce allergic contact dermatitis (Finnel, 1999). The presence of shellac on any of the herbs used for making the drug Winniecure may pose danger to development if present in the extract, as resins are generally known to be poisonous. The phytochemical analysis of Winniecure revealed the presence of alkaloids and tannins and the absence of cyanogenic glycosides and saponins (Agbonlahor *et al.*, 2003). Many alkaloids have been implicated in the development of congenital malformations and even abortion (Farnsworth and Bingel, 1977).

There are two areas of great concern in teratology which are abortion and congenital anomalies. Some plants are known to have abortifacient effects while others have been shown to produce congenital abnormalities in developing foetus. With the use of plants with abortifacient properties in lower doses, congenital anomalies could result. This was seen in neonates that escaped death as intended by abortifacient plants (Farnsworth and Bingel, 1977). The other concern is congenital malformations induced by drugs. In this case, the drugs do not have any visible/noticeable side effect on the pregnant mother but the neonate presents with some structural or functional abnormalities. During development, if the sequential events are not disrupted, the result is the birth of a normal child. However certain substances and some drugs may disrupt the synchrony in the events of development, resulting in congenital anomalies in the newborn. In the 1950s, it was observed that administration of aminopterin, a drug for abortion, to pregnant women failed to terminate the pregnancies, but produced several malformations in the children born to such mothers (Finnel, 1999). The thalidomide disaster between 1959 and 1962 heightened the awareness of the vulnerability of the human embryo to environmental insult and led to the consequent emphasis on reproductive testing of drugs and other chemicals to which pregnant women might be exposed (Francis, 2003).

It is important to note that there are four manifestations of developmental toxicity (Francis, 2003). These manifestations include death, malformation, growth retardation and functional deficit. This is why the development toxicology of bioactive principles present in the herbal extract, Winniecure cannot be exhausted as this study only focuses on the gross structural malformation and others like decreased birth weight, decreased size (crown-rump length) and decreased activity. Although species-dependent, congenital anomalies will result with varying severity depending on the developmental age at which the mother is exposed to the teratogen. Generally the first trimester is believed to be the most sensitive period because of the process of organogenesis. However, the second trimester is the most sensitive period in rats (Francis, 2003; Finnel, 1999). The dose of teratogens is equally important because the deviant development may finally manifest in death, malformation, growth retardation or

functional deficit (Francis, 2003; Finnel, 1999; Berlinska and Sitarek, 1997).

Winniecure is an antiretroviral drug taken regularly by HIV/AIDS patients which may be contraindicated in pregnancy. It is important to verify this so as to allow for continued use or discontinuation of use, by infected pregnant mothers. This study is aimed at evaluating the effect of the bioactive agents in Winniecure and to establish its possible teratogenic effect on the on the developing foetuses on the 1st, 2nd or /and 3rd trimesters of pregnancy of female Wistar rats.

The reproductive cycle of female rats, called the oestrous cycle, has four phases: Proestrous; Oestrous; Metoestrous (or Dioestrous I); and Dioestrous (or dioestrous II). Rats have a mean cycle length of 4 days from the onset of sexual maturity up to the age of 12 months (Long and Evans, 1922; Mandl, 1951). During the oestrous cycle, prolactin, Leutinising hormone (LH) and Follicle stimulating hormone (FSH) are low but increase in the proestrous phase. Estradiol levels reaches the peak during proestrous following increase at metoestrous estradiol returns to its baseline levels at oestrous Progesterone secretion also increases at metoestrous and reaches its peak towards proestrous. Ovulation occurs between the beginning of proestrous and the end of oestrous. The cytology of vaginal smear is used for the determination of the phase of the oestrous cycle (Mandl, 1951). The relative proportions of the three types of cells observed in the vaginal smear (epithelial cells, cornified cells and leucocytes) are used to determine the phases.

During the proestrous phase, the smear contains both epithelial cells and leucocytes. The epithelial cells appear as small rounded nucleated cells with a granular cytoplasm. Towards the end of proestrous, the number of epithelial cells increases while the number of leucocytes decreases. Cornfield cells are present in the oestrous phase. They appear like scale and leaves No leucocyte is seen at this stage, but cornified cells dominate the smear. Behavioural oestrous is reflected in increased running activity of the female rats, quivering of their ears and receptivity of the male counterpart.

During the metoestrous phase, the vaginal smear contains cornfield cells mainly. However, leucocytes reappear towards the end of the phase. Epithelial cells are small, round, nucleated and have some mucus shred. The dioestrous phase is the longest and most variable phase of the oestrous cycle. Leucocytes and mucus strands are seen in the vaginal smear. Occasionally, small epithelial cells may be seen.

EXPERIMENTAL

Twenty cyclic Wistar rats were obtained from the Experimental Animal House of the University of Jos and acclimatized under favourable laboratory conditions with photoperiodicity of 12 hour light and l2 hour darkness. They were fed with Growers mash Vital Feed from Grand Cereals and Oil mills Ltd, Jos and water *ad libitum* until they attained a weight range of 150-180g. The rats were divided into four groups, n=5. Their oestrous pattern was established by daily vaginal smear examination for two weeks. Only four-day cyclic rats were used for this study. The rats were mated with proven males at the proestrous phase in a 3:1 (F/M ratio). The vaginal smear analyses of the female rats were carried out and the presence of sperms confirmed that mating had taken place according to the method of Long and Evans, (1922) and Mandl (1951). The sperm-positive day was considered as day 0 of pregnancy. The drug was administered to the three groups intraperitoneally on the 1st, 2nd, and 3weeks of pregnancy at a dose of 8.4mg/kg. On the 20th day of gestation, the animals were delivered by caesarean section and the foetuses were removed, blotted dry and the

following morphological examinations were carried out.

(i) Litter number
(ii) Litter weight
(iii) Litter crown-rump length (CRL)
(iv) Gross morphology of limbs, face, and vertebrae
(v) Litter colour
(vi) Activity

RESULTS AND DISCUSSION

The weight and the crown-rump length of each foetus were measured using a Mettler balance and a divider together with a meter rule. The colour of each foetus was determined. This indicates the degree of blood supplied to the foetus and thus the amount of metabolites the foetus was exposed to during the period of gestation. The activity of each foetus was determined by how long they lived after they were brought out. There were no dead foetuses; else their activity would have been graded. The animals that died almost immediately they were brought out were noted. Those that remained alive and active for more than 6 hours were equally noted.

Spinal malformations such as spinal bifida and abnormal ribs (such as cervical ribs and lumbar ribs) were checked for, in each foetus. Limb abnormalities such as polydactyly, syndactyly, adactyly and phocomelia were checked for and noted. Abnonnalities of the face such as cleft lip/palate and facial cleft were checked for and noted as well. Each foetus was thoroughly observed using a hand lens and no abnormalities were observed and thus none was recorded in all the groups. Comparing the results obtained from both the treated and control groups; there were no marked variation in the parameters of the foetuses of the rats. The average litter number of all the groups was normal at 7-12 litters, which agrees with the findings of Carlos, (2002). This shows that the drug, Winniecure, has no effect on litter number when administered to pregnant Wistar rats in the 1^{st}, 2^{nd} or 3^{rd} trimesters of pregnancy. During intrauterine development, there has been record of spontaneous death. This is one of the manifestations of teratology (Francis, 2003). The cause of this death may be due to intrinsic factors such as the genotype of the developing foetus itself or due to extrinsic factors such as maternal exposure to teratogens. The dependence of teratogens on the genotype of conceptus is based on variable sensitivity. For example, less than 10% of foetuses exposed to hydantoins had congenital defects. (Finnel, 1999).

The average litter weight range in the groups obtained was 1.0 -4.5g. This is at variance with the findings of Noda, (1992) who observed a range of 5-6 grams at birth. The reduction in weight of litters is usually due to intrauterine growth retardation and this occurs in the last trimester of gestation. Any drug that would cause intrauterine growth retardation is believed to exert its effect on pregnancy only during the third trimester. This is because, organogenesis occurs during the second trimester and all the organs are formed in the third trimester. Growth is the major event that takes place in the third trimester. However, CNS defects may be produced as a result of teratogenic insult during the third trimester because of the myelination of neurons (Finnel, 1999).

Comparing the weight of litters in the treated and control groups, it was observed that there were no variations, the litters in all the groups had the same range of litter weight. The reduction in the litter weight cannot therefore be attributed to the administration of the single dose (8.4mg/kg) of the drug, Winniecure during gestation.

Based on the observations made on the litters of the treated and control groups, the herbal extract, Winniecure produced no toxic or teratogenic effect on the foetuses of Wistar rats. It can therefore be concluded from this study that Winniecure, an

antiretroviral herbal preparation, is not teratogenic in Wistar rats when administered in the 1st, 2nd, or 3rd trimester of pregnancy at a dose of 8.4mg/kg. However, further work needs to be carried out to determine its safety in pregnant women at the same or higher doses of administration.

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