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CALCIUM CHANNEL BLOCKER VERAPAMIL STIMULATES OVULATION AND INDUCES FETAL REABSORPTION IN RATS

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ABSTRACT - Calcium channel blockers are widely used in cardiovascular diseases, usually in long-term treatments, occasionally in pregnant women. The effects of verapamil were studied on uterine implantation, fetal reabsorption and mother's and offspring's plasmatic levels of calcium, phosphate and total protein. Seventy-five quality-controlled female Wistar rats were used. The drug-treated animals were divided in two groups: the first received verapamil in doses of 2.4 mg/rat/day and the second, doses of 24 mg/rat/day. The drug was administered by means of the drinking water for 10 weeks: 7 weeks before mating and 3 weeks during pregnancy; cesarean surgery was performed on the 21st. day. On the occasion of the cesarean, mother's and newborn's blood samples were taken. Verapamil, in the higher dose, caused a lowering of plasmatic calcium and phosphate in mothers and reduced body weight in offspring. In the young, plasmatic calcium, phosphate and total proteins were comparable in verapamil treated and control. However, these values were significantly lower in newborn of treated and control groups, as compared to their respective mothers. Results show that verapamil stimulated ovulation, increasing the uterine implantation but induced early fetal death and reabsorption.

RESUMO - Os bloqueadores dos canais de cálcio são largamente utilizados em distúrbios cardiovasculares, usualmente em tratamentos prolongados, ocasionalmente em mulheres grávidas. Foram estudados os efeitos do verapamil sobre a implantação uterina, a reabsorção fetal e os níveis de cálcio, fosfato e proteínas totais plasmáticos de mães e filhotes. Foram utilizadas 75 ratas fêmeas Wistar, com qualidade controlada. Os animais tratados foram divididos em dois grupos: o primeiro recebeu verapamil na dose 2,4 mg/rato/dia de e o segundo, 24 mg/rato/dia. A droga foi administrado na água de beber durante 10 semanas: 7 semanas antes do acasalamento e 3 semanas durante a gestação, sendo a operação cesariana realizada ao 21º dia. Por ocasião da cesariana, foram tomadas amostras de sangue das mães e filhotes. O verapamil, na dose mais elevada, causou diminuição dos níveis plasmáticos de cálcio e fosfato nas mães a par de diminuir o peso corpóreo dos filhotes. Os níveis séricos de cálcio, fosfato e proteínas foram comparáveis nos filhotes dos grupos controle e tratado. Contudo, foram significativamente mais baixos em todos os filhotes, em comparação com as respectivas mães. Os resultados mostraram que o verapamil estimulou a ovulação, aumentando o número de implantações uterinas, enquanto induziu morte fetal precoce e reabsorção fetal.

Introduction

Calcium channel blockers are important therapeutic agents, used as vasodilator and antiarrhythmic drugs, often in long-term treatments. They prevent ionized calcium from entering the cell and thus influencing calcium-dependent processes (FLECKESTEIN *et al*., 1967, 1969). Verapamil, chemically a phenylalkylamine, was the first drug used and is nowadays extensively used in the treatment of essential hypertension, cardiac arrhythmias and *angina pectoris*. It is proposed to act on slow voltage dependent calcium channels of the L type (NOWICKY *et al*., 1985) of excitable cells. An extensive revision on the pharmacology and clinical research of calcium antagonists was published by VANHOUTTE *et al*., (1988). The calcium channel blocking agents are often administered to women in reproductive age and during pregnancy. It is clinically considered to be safe and no teratogenic effect of verapamil has been referred. During our experiments, intending to observe the calcification of dental germ in rats born from verapamil-treated mothers, a very significant reduction in the number of offspring was observed. Such result leaded the present research.

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Material and Methods

For the experiments, 57 Wistar rats in controlled good health conditions were used. Thirty-eight were virgin females, aged 90-100 days at the onset of the experiments and 19 were reproductive male rats. The animals were divided in two groups according to the dose of verapamil: the first one received the dose of 2.4 mg/rat/day (D1) named "lower" dose and the second received 24 mg/rat/day (D2) named "higher" dose. Verapamil

was added to the drinking water. Sucrose (0.5 g/rat/day) was mixed to the water of control and treated groups. A third group of untreated rats under the same experimental conditions was run in parallel as control. All the animals were weighed weekly. After 7 weeks of treatment with verapamil the estrous phase was determined, and the female and male rats were put together overnight for mating. Diagnosis of pregnancy was determined by presence of spermatozoa in the vaginal secretion (see NICHOLAS, 1949).

Cesarean operated rats. On the 21st. day of pregnancy, the uterus of mothers was exposed by cesarean surgery under anesthesia and the number of fetuses, the embryonic reabsorption and uterine embryonic implantation (= number of embryos + number of embryonic reabsorption) were observed. The ovary was removed and the number of corresponding ovules released during fertilization was counted.

Offspring external somatic examination. The newborn rats were cleaned, weighed and somatic external characteristics examined under lens, 3 woth times magnification. Ears and eyes implantation, palate, labial cleft, anterior and posterior limbs and tail were examined.

Determination of plasmatic calcium, phosphate and total protein. On the occasion of the cesarean surgery, blood samples from mothers and offspring were collected in heparin and obtained plasma. Biochemical determination of calcium, inorganic phosphate and total protein were carried out using a Beckman-Du-600 Spectrophotometer. Plasmatic calcium was determined by complexiметric method of o-cresolphtalein-complexone using Merck Reagents. For plasmatic inorganic phosphate determination FISKE and SUBAROW (1925) method was used. Total proteins were determined by Biuret method, using a "Bioclin" kit reagents.

Mathematical-statistical processing: significance of the difference between groups was assessed by non-parametric variance analysis of Kruskal Wallis. A multiple comparison among groups was carried out by using the GMC computer program version 7.0, according to CONOVER (1980).

Results

1 - Fetal uterine implantation. The mean number of fetal uterine implantation in control mothers was of 8.1(±2.4). In treated mothers this mean was significantly increased, that is: 13.0(±2.7) with the lower dose (D1) and 13.3(±4.2) with the higher dose (D2) of verapamil. The number of uterine implantation corresponded to the number of ovarian luteinizing bodies.

Table 1. Uterine implantation, fetal reabsorption and alive offspring (mean values) from control and verapamil treated (10 weeks)*** group with doses of 2.4 mg/rat/day (D1) and 24.0 mg/rat/day (D2)

	Control	Verapamil D1(n=18)	Verapamil D2 (n=10)
Uterine implant.	8.1(±2.4)	13.0 (2.7%)	13.3 (±4.2)
Fetal reabsorption	0	5.8 (45%)*	10.0 (75%)*
Alive offspring	8.1	7.2 (55%)**	3.3 (25%)**

* Percentage of fetal reabsorption; ** Percentage of alive young at cesarean surgery. *** Seven weeks before mating and 3 weeks during pregnancy.

2 - Fetal uterine reabsorption. No fetal uterine reabsorption was observed in control mothers. However, in the verapamil treated animals, a fetal uterine reabsorption of 45% occurred with doses of 2.4 mg/rat/day (D1) and 75% with doses of 24 mg/rat/day (D2).

3 - Alive offspring and somatic characteristics. In control rats, alive newborn rats corresponded to 100% of uterine implantation. In verapamil treated group, the uterine implantation/alive offspring ratio was significantly reduced: with the lower dose, it corresponded to 25% and with the higher dose, to 55%. The weight of newborn rats from treated mothers was reduced in 10% (D1) and 18% (D2) as compared to control. No macroscopic malformations in control and treated groups were observed. Results of 1, 2 and 3 are summarized in Table 1.

4 - Plasmatic calcium, phosphate and total protein. Plasmatic calcium and phosphate were significantly reduced in mothers of the groups treated with the higher dose (24mg/rat/day) but did not change when lower doses (2.4 mg/rat/day) were used. Total protein was not changed by the treatment with verapamil. In offspring, plasmatic calcium, phosphate and total protein values were not changed by the verapamil treatment. However, a significant decrease in the values of calcium, phosphate and protein was observed as compared to their respective mothers, either in treated or control. Results are summarized in Table 2.

Table 2. Rats plasmatic calcium (mg/100 ml), phosphate (mg/100 ml) and total protein (g/100 ml) from control and verapamil treated groups (10 weeks)*** with doses of 2.4mg/rat/day (D1) and 24.0mg/rat/day (D2). A. – Mothers; B – Offspring.

A- Mothers	Control	Verapamil D1	Verapamil D2
Calcium	6.13 (±0.28)	6.0 (±0.27)	5.41 (±0.68)*
Phosphate	3.97 (±0.6)	3.90 (±0.12)	2.79 (±0.74)*
Total protein	7.66 (±0.8)	7.99 (±0.52)	7.07 (±0.80)
B- Offspring	Control	Verapamil D1	Verapamil D2
Calcium	5.27 (±0.55)**	5.20 (±0.19)**	5.08 (±0.15)**
Phosphate	2.48 (±0.42)**	2.42 (±0.42)**	2.47 (±0.99)**
Total protein	3.39 (±0.33)**	3.36 (± 0.33)**	2.86 (±0.75)**

Significant as compared to control. ** Significant as compared to their respective mothers. *** Seven weeks before mating and 3 weeks during pregnancy.

Discussion

There is a great medical, scientific and social interest and care about the teratogenicity of drugs when given to mothers, specially if administered early in the course of pregnancy. Since the dramatic episode of embryonic malformation – phocomelia – induced by thalidomide, detected in the 1960s, scientists and pharmaceutical industry have exhaustively studied the teratogenic potential of drugs, within a pharmacological and toxicological screening. In the United States, the Harris-Kefauver Amendments (NIES and SPIELBERG, 1996) are sound legislation and the total time of a drug development and final approval averages 8 to 9 years (DiMASI *et al*., 1994). Clinical aspects of teratogenicity of drugs were revised by NISHMURA and TANIMURA (1976). In reference to verapamil, its toxicity is very well known and no data associates its use to congenital anomalies, and the drug is considered appropriated to administration during pregnancy. However, in the course of our studies on the effect verapamil on dental germ of offspring born from treated mothers, we observed a significant reduction of birth or no birth at all. This observation led to the present research. The doses of verapamil used in our experiments can be considered to be adequate, because during the long-term treatment the mother rats seemed to thrive, increased their body weight, no disturbing behavior was observed and no death occurred. In the offspring. Verapamil caused a dose-related reduction of body weight, but no malformation was observed. To assure a regular chronic administration and to avoid daily stressful injections, the drug was added to the drinking water. To a better acceptance of the verapamil solution, sucrose was added, in both control and treated groups. Both doses of verapamil were chosen on the basis of the approximate maximal doses to human, that is about 7.0 mg/kg/day. To the rat the "small" dose (D1) corresponded to 8.0mg/kg/day and the "high" one, was ten times higher - 80 mg/kg/day (D2). Our doses are somewhat similar to those used by SAMNEGARD and SJÖDÉN (1992) who studied the influence of verapamil on mineralization of male and female bones. The dietary calcium-phosphate was adequate as shown by normal growth of control and treated rats. It is well known that the ratio of calcium to phosphorous in the diet exerts a marked effect upon growth and calcium and inorganic phosphorous content in the blood serum. No sign of rickets were observed. The daily dietary calcium and phosphate intake was within the range proposed by McCOY (1949) to normal development of the rat. Verapamil diminishes intestinal absorption of calcium in isolated and *in vivo* intestinal wall (WROBEL and MICHALSKA, 1977; SJÖDÉN *et al*., 1983; PENTO and JOHNSON, 1983) but such effect does not occur in pregnant rats, in which calcium absorption is increased (BROMMAGE *et al*., 1990). In the present research, plasmatic calcium, phosphate and total protein were determined in both mother and offspring to control how could they be influenced by the doses of verapamil and eventually explain results or health condition of the animals. Regarding calcium and phosphate concentrations, they were diminished by verapamil in mothers treated with the higher dose. This event could be related with ovulation and fetal reabsorption, but this could not be demonstrated, because ovulation was enhanced also with lower doses, which did not alter calcemia. As calcium and phosphate levels were not affected in young of any group, whether treated or not, a protective balance mechanism between mother and fetuses is indicated. An interesting finding was that plasmatic calcium, phosphate and total protein were significantly lower in offspring of treated and control groups (see Table 2). Total proteins depression was over 50%. The lower values can be considered "normal" to offspring at birth, because such a lower values were observed in control young, born from untreated mothers. The increased ovulation and higher embryonic uterine implantation in verapamil treated rats were a consistent finding. However, such implantation did not correspond to the fetuses at term, since a high degree of uterine reabsorption occurred. Results are suggestive in indicating that verapamil stimulated ovarian follicles, liberating

immature ova, which were fertilized but did not succeed in giving rise to functional ovum and fetus. One can speculate whether verapamil enhanced ovulation by itself, influencing calcium-dependent mechanisms or acted indirectly by stimulating luteinizing hormone (LH) or follicle-stimulating hormone (FSH), which respond to ovulation and corpora lutea formation. On the other hand the drug could act by inhibiting atresia of ovarian follicles not destined to produce functional ovum. Could verapamil be tried in experimental polycystic ovarian syndrome (see TOLIS *et al*., 1993) a condition in which ovulation is inhibited? As a matter of fact, ovulation was significantly stimulated even with the lower dose, which is similar to that therapeutically indicated in human. So, such effect cannot be considered a toxic but rather a normal one. However, no data from the literature refers to an enhanced ovulation by verapamil in women. One must consider that the present results, although consistent and significant, are preliminary, not allowing a further discussion, but are suggestive to inspire further research. In a high percentage of the fertilized rats, verapamil induced partial or total uterine reabsorption. The drug is likely to have acted during blastogenesis, that in rats occurs from the 1st to the 6th day, a period when toxic action of drugs leads to embryo death and fetal reabsorption. The drug did not certainly acted during organogenesis (after the 6th day) because in doing so, it could lead to fetal malformations (teratogenesis), not observed.

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