THE CASE OF DIETHYLSILBESTROL TREATED VEAL CONTAINED IN HOMOGENIZED BABY-FOODS IN ITALY.

METHODOLOGICAL AND TOXICOLOGICAL ASPECTS

A. LOIZZO (a), G.L. GATTI (b), A. MACRI’ (c), G. MORETTI (d), F. ORTOLANI (a) and S. PALAZZESI (a)

(a) Laboratorio di Farmacologia; (b) Laboratorio di Tossicologia Applicata; (c) Laboratorio di Tossicologia Comparata ed Ecotoxicologia; (d) Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, Roma

Summary. – From previous studies carried out in the Istituto Superiore di Sanità, Rome, over the years 1979-1980, it was hypothesized that calf meat-derived, homogenized and lyophilized baby food might contain high doses of compounds endowed with oestrogenic activity. Thus, analytical investigations performed on 450 samples of commercial baby-food products showed that 150 of them contained a powerful oestrogenic substance, later on identified as diethylstilbestrol (DES), in high amounts (20 to 140 μg/kg food). Since meat from calves illegally treated with xenobiotic drugs having hormonal activity generally contains modest yet potentially dangerous levels of such compounds (in the range 0.1 to 2 μg/kg meat), it was derived that the elevated DES levels in homogenized products could be originated from working plout or injectable preparation DES residues, given to animals as auxin. Similar considerations are advanced to explain the etiopathogenesis of gynaecomastia, early pseudopuberty, or troubles in the sex organs of school-age children described in Italy as well as in other countries during these latter years.

Riassunto. – Da studi precedenti condotti presso l’Istituto Superiore di Sanità, Roma nel periodo 1979-80, si era ipotesi che il cibo per neonati contenente carni di vitelli trattati con dietilstilbestrol potesse contenere sostanze ormonalmente attive. L’esame di 450 campioni di alimenti per neonati commerciali dimostrò che 150 di essi contenevano un ammontare notevole di DES, un farmaco ormonale. Data la presenza di DES in carni di vitelli trattati illegalmente con farmaci xenobiotici aventi attività ormonale, si ipotizza che possano contaminare i prodotti alimentari per neonati. Considerazioni analoghe sono state avanzate per spiegare la causa dei disturbi sessuali precoci in bambini descritti in Italia e in altri paesi nel corso degli ultimi anni.

Introduction

On April 1980 the Veterinary Services of Italian Ministry of Health became aware of increasing trade abuse of synthetic anabolic hormones as auxinic agents in animal farms [1]. Moreover, a report from paediatric hospitals on cryptogenic breast enlargement cases in prepuberal children [2], induced Italian Health Authorities to consider the possibility that the use of hormones as auxinic agents in animal breeding was much larger than official information could suggest. Following indications contained in these studies [1], a nationwide program on hormones in calf meat was devised in May 1980, and widespread controls were therefore carried out by regional laboratories on calf meat-containing children food. Over the period May–July 1980, 450 samples of baby food manufactured by 5 major producers in Italy (daily production of about 300,000 jars) randomly selected from 26 towns scattered all over Italy, were examined. The meat was 37% of Italian origin, 25% imported from other EEC countries, 2% from European non EEC countries, 29% from South American countries, and 7% from others. These 450 samples underwent biological assay for estrogenic activity in regional control laboratories (see "Biological method").
dicas, 150 were found to be positive for estrogenic activity, therefore 82 out of these 150 were forwarded to our laboratory for a second instance control, while the remaining 68 were sent elsewhere.

This report deals with the results obtained from analyses performed on these 82 samples. Potential effects induced by anabolic treated meat on public health are also discussed.

**Methods**

a) **Biological method.** - The method was based on the weight increase of uteri in immature female mice, originally set forth by Umberger et al. [3] and later modified by Tiecco [4], as adopted by the italian health authorities [5]. Groups of six 21 ± 1 days old female mice were fed for 3 consecutive days with the sample to be analyzed, mingled with an equal quantity (w/w) of ground pellet food. On the 4th day the mice were sacrificed, and uterus weight (expressed as percent of body weight) was compared with uterus weight of groups of control mice, fed with the usual food, and of groups of mice fed estrogen free calf meat mingled with an equal quantity (w/w) of ground pellet food. Where estrogenic activity was found, an estimate of its amount in terms of diethylstilbestrol (DES) equivalents was made by serial dilution of the sample with the control meat, and by comparing the response thereby obtained with a standard dose/response curve simultaneously obtained by adding known amounts of DES to the control meat diet. Moreover, since total amount of each sample (60 to 120 g per jar) did not always allow complete analysis (serial dilution sample plus chemical analysis) semiquantitative measurement of uterotrophic activity was carried out also by dilution on 4 samples randomly chosen among those with marked uterotrophic activity (range: 344 up to 503 percent of control).

b) **Chemical method and mass-spectrometry.** - The chemical method described by Verbeke [6] for the detection of anabolic substances in animal tissues was employed on a total of 10 samples selected at random from the samples which resulted positive by the biological method described above.

Baby food samples, 40-50 g, were extracted with methanol after enzymic hydrolysis with glucuronidase-sulphatase mixture in buffer solution. The aqueous methanol phase was washed with n-hexane, and extracted with dichloromethane. The extract was evaporated to dryness. The residue was dissolved in water and allowed to percolate through an Amberlite XAD-2 column; the anabolic substances were eluted with methanol. The eluate was concentrated and then passed through a basic Extrelut column; estrogenic substances were then eluted with diethyl ether from the column. After concentration the extract was analysed by twodimensional thin layer chromatography using precoated silica gel 60 HPTLC plates. Estrogenic substances were detected by spraying the plates with sulphuric acid in acetic anhydride, heating and observing typical fluorescence at 366 nm. As shown with added DES, the detection limit of this method was 2-3 μg/kg food. A direct inlet mass spectrometry procedure was also used [7] in two out of the 10 samples. A part of the extract prepared for TLC analysis (see above) was treated with pentafluorobenzoyl chloride and triethylamine in benzene solution. Then, the reaction mixture was purified by silica gel column chromatography.

The part of eluate containing the pentafluorobenzoyl derivative of DES was concentrated and transferred into the probe of the mass spectrometer (LKB 2091 instrument). The probe temperature was programmed up to 250 °C and the ionization energy was 70 eV. Mass spectra were elaborated by a computer system by repetitive scanning.

**Results**

Seventy-eight of the eighty-two baby food samples showed marked uterotrophic activity. In Table 1 is depicted the distribution in classes of mean uterus weight of mice treated with baby food samples, expressed as percent of the control, and the corresponding dose of DES, estimated on a previously established titration curve [8]. Only four out of 82 samples did not confirm regional laboratories analysis at the 1% significance level (biological method). The measurement of uterotrophic activity obtained by dilution of 4 samples, moreover, confirmed the afore mentioned estimation (Fig. 1).

**Table 1.** - Distribution in classes of mean uterus weight in immature mice fed with baby food mingled 50% with usual food, for 3 days

<table>
<thead>
<tr>
<th>Uterus weight increase (in percent of control)</th>
<th>Number of samples</th>
<th>Estimated DES content (in μg/kg food)</th>
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<td>from &lt; 90</td>
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<td>540</td>
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<tr>
<td>&gt; 690</td>
<td>5</td>
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<td>82</td>
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Results are expressed as percent of control. Four out of 82 samples did not confirm peripheral laboratories analysis at the 1% significance level. A normal distribution of values is evident. In the right part of the table an estimated DES content was drawn, comparing the uterine weight with a titration curve obtained with several doses of DES (for details see ref. n. 8)
The chemical method used for 10 out of the 78 biologically positive samples revealed the presence of DES: no other estrogenic substances were detected. A semiquantitative estimation of DES content in the samples was possible by visual comparison on silica gel plates between sample and reference DES spots. These estimations were in good agreement with the evaluations performed with the biological assay. Mass spectrometry further confirmed the identity of DES in the two already biologically and chemically tested baby food samples. Figure 2A shows the mass spectrum obtained for the reference DES derivative, and Fig. 2B shows the mass spectrum obtained from a sample positive by biological and chemical analyses; in this latter spectrum DES is evidenced as pentafluorobenzoate derivative (molecular ion $[M]^{+}$ m/z 656).

**Discussion**

The presence of a potent estrogenic substance was demonstrated in 78 out of 82 samples of commercial, homogenized baby food. Analytical investigations performed in 10 out of 78 samples demonstrated that the estrogenic substance responsible for the uterus enlargement was DES and in two cases the presence of DES was confirmed by mass spectrometry. It is reasonable, therefore, to hypothesize that all other samples with marked uterotrophic activity contained the same or equivalent compound. Overall, a figure of 33% of estrogen-positive jars among those analyzed all over the national territory was found. If we postulate the presence in baby food of 20 to 140 μg/kg of DES in 1/3 of baby food jars on sale in that period (see "Results"), we can estimate that children regularly fed with those baby food jars may have ingested 0.10 to 0.70 μg DES per kg body weight per day, perhaps for several months after weaning.

In the last few years sporadic reports have been published on cryptogenic epidemics of gynaecomastia, or on cases of pseudopubertas praecox in children, even aging 1 year [9,10]. In these reports, hypothesis of estrogen contaminated food had been forwarded, but no definite cause-effect relationship was demonstrated under controlled experimental conditions.

According to the data shown by Weber et al. [10] doses of DES similar to the ones found in baby food, are capable, upon chronic administration, to induce in children various disturbances, chiefly consisting of breast enlargement, vaginal bleeding and pseudoprecocious puberty [11]. However, symptoms described in older children by Pasquino et al. [11], as well as in children described by Fara et al. [9], could not be induced by estrogens contained in baby food, which is seldom administered to children after 18 months of age,
Therefore a different mechanism of ingestion of the drug should be hypothesized. The illegal use of anabolic agents in cattle generally occurs through injection or subcutaneous pellet implantation, of 30-72 mg DES [12, 13]. When implant site is discarded just before or after slaughtering, DES levels in meat or liver are usually low, up to a few μg DES per kg edible tissue [13]. However, subcutaneously, or intramuscularly, implanted DES is often not withdrawn, therefore massive amounts of DES (10 to 20% of the original amount of 30-72 mg) remain in the tissue, even for several months, and are processed when the cattle is being prepared for baby food, meat-balls, hamburgers, or as food stuff in general (i.e. 3 to 14 mg DES per carcass, which is 150 to 200 kg).

These amounts are potentially high enough to induce scattered episodes of hormonal hyperdosage effects, as probably happened in some of the cases described by Fara et al. [9] and by Pasquino et al. [11].

If the evaluation of risk inherent to the presence of DES in foodstuffs is considered, various aspects of DES toxicity in animals may be quoted versus the quantities of DES found in the course of food controls or studies; minimal dose ranges (i.e. DES levels between 0.1 and 2 μg/kg food) are usually found in meat of implanted veals up to 30-40 days after treatment, and, conversely, in rats fed with this meat for 1 to 2 years histological and functional alteration of reproductive organs and fertility are induced [12-15]. DES levels between 1 and 10 μg/kg food are often found in liver or kidney of implanted veals, sometimes in plasma or meat [13, 16, 17]; a diet containing such amount of DES induces weight increment of uterus and cornification of vaginal epithelium in prepuberal or castrated mice or rats, after 3 to 7 days [3, 12, 18]. Higher DES levels induced linear log dose–response curves with significant slopes for neoplasm formation in female mice or castrate male mice (25-500 μg DES/kg food) with a latency of 31 to 69 weeks [19], i.e. about 40 to 80% of expected duration of life in a mouse.

Data collected from animal species have but indicative values, and cannot be extrapolated on the human species.

Our data suggest that an unexpectedly great number of children were probably exposed to high doses of DES. The possible damage induced by DES on such children, cannot be easily established, but epidemics of pseudoprecocious puberty have been described both in Italy and abroad. Anabolic agents such as DES or zeranol, present through the production of meat could be responsible for such episodes [2, 8, 20-22].

We would therefore underline that our data, as well as conclusions given in recent International Symposium on anabolic hormones [23], strongly support conclusions [24] given by EEC Scientific Committee: i.e. studies on acceptable daily intake values of xenobiotic hormones should consider more carefully bioavailability, pharmacology and toxicology of minute amounts of these substances before accepting them as auxinic agents in animal farms [25].

Acknowledgements

This work is dedicated to the memory of G.L. Gatti.

The authors wish to thank L. Boniforti for mass spectrometer analyses. Thanks are also expressed to R. Boscherini, G. De Felip, F. Di Ruzza, E. Palliola, R. Sahai, V. Silano and G.A. Zapponi for discussion and suggestions, and to P. Campagna for accurate editing of the manuscript.

Ricevuto il 2 luglio 1984
Accettato il 22 dicembre 1984
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