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**Original article**

# Sperm quality and reproductive traits in male offspring of female rabbits exposed to Lindane (γ-HCH) during pregnancy and lactation

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**Abstract** — Fifteen Grimaud female hybrid rabbits, 135 days old and weighting an average of 3.74 ± 0.01 kg each, were administered an oral dose of 1 mg·kg$^{-1}$ body weight of Lindane during gestation and lactation period. Fertility rate, libido, volume of ejaculate, concentration and morphology of spermatozoa were investigated to test the effects of the treatment on reproductive traits of first generation male rabbits. Ultrastructure of abnormal spermatozoa was described by Transmission Electron Microscopy and the different abnormalities were quantified. The results obtained indicate that low dose exposure of Lindane has effects on spermatozoa ultrastructure that proved to be susceptible to the treatment with the pesticide (cytoplasmic droplets: 5.3% in control group and 10.3% in Lindane group, $P \leq 0.05$; coiled tails: 1.3% in control group and 4.3% in Lindane group, $P \leq 0.05$) and could be utilised as a good marker of toxicity.

**Lindane / rabbit reproduction / spermatozoa / transmission electron microscopy**

**Résumé — Qualité du sperme et caractères reproductifs de la première génération de lapins mâles nés de femelles exposées au Lindane (γ-HCH) pendant la gestation et l'allaitement.** Quinze lapines hybrides Grimaud, âgées de 135 jours et d'un poids moyen de 3,74 ± 0,01 kg ont reçu pendant la gestation et la période d'allaitement du Lindane à raison de 1 mg·kg$^{-1}$ poids corporel. La fertilité, la libido, le volume du sperme éjaculé, la concentration et la morphologie des spermatozoïdes ont été suivis chez les mâles de la première génération. L'ultrastructure des spermatozoïdes anormaux a été étudiée au microscope électronique à transmission et les différentes anomalies ont été quantifiées. Les résultats obtenus indiquent qu'une faible dose de ce pesticide a affecté l'ultrastructure des spermatozoïdes (gouttelettes cytoplasmiques : 5,3 % dans le groupe des témoins et 10,3 %

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dans le groupe traité avec le Lindane, $P \leq 0,05$ ; queue en épingle: 1,3 % dans le groupe des témoins et 4,3 % dans le groupe traité avec le Lindane, $P \leq 0,05$), ce qui pourrait être utilisé comme indicateur de toxicité.

**Lindane / reproduction du lapin / spermatozoïdes / microscopie électronique à transmission**

## 1. INTRODUCTION

The γ-isomer of 1,2,3,4,5,6- hexachlorocyclohexane (γ-HCH, Lindane) is present in a wide variety of agricultural, medical and veterinary products [32]. Although its registration and production have been withdrawn in many countries, Lindane is still present, in small quantities, as a pesticide [5, 6, 22, 23].

The lipophilic nature of Lindane, like other chlorinated hydrocarbons, and its resistance to chemical degradation led to the ubiquitous bioaccumulation within the food web [1]. Consequently, humans and animals could be constantly exposed to these substances. It has been reported, that the daily intake an adult person can receive from food is on an average of 0.14 μg of Lindane, while its concentration in drinking water is of 0.05–0.1 ppb [13].

Lindane is one of the environmental chemicals known to have an endocrine effect and has been associated with male reproductive alterations [6, 9, 10, 11, 25, 27, 30, 31]. Exposure to Lindane in utero is particularly harmful for the organogenesis [15]. In fact Lindane crosses the placenta and is detected in the fetus [17, 21]. Moreover, the substance is known to accumulate in fat tissues, to produce a store of metabolites, like pentachlorobenzene, and to be slowly eliminated in milk during lactation [10, 24]. As a result, the neonate could be exposed to lipophilic persistent substances during a very critical and vulnerable period in its development. For these reasons, recently, the possibility of disturbances induced during the pre/perinatal period had received special attention [6, 9, 10, 11].

The aim of this paper is to verify whether a low dose of Lindane, mimicking closely the dosage present in the environment, administered to female rabbits during pregnancy and lactation, produces effects on reproductive functions in first generation male rabbits. To reach this objective, fertility rate, libido and ejaculate characteristics were analysed. Particular attention has been given to the effects of pesticides on ultrastructural features of sperm considered to be sensitive to the environmental conditions [2, 12, 14, 28].

## 2. MATERIALS AND METHODS

### 2.1. Animals and experimental conditions

Two groups of fifteen Grimaud hybrid female rabbits, from an industrial farm, 135 days old and weighting an average of $3.74 \pm 0.01$ kg each, were artificially inseminated. To make receptive all does at the same time, they were synchronised by 20 IU of PMSG (Ciclogonine, Prochena), given 72 h earlier. Each female received a dose of 0.5 mL of semen diluted in Tris-buffer and containing about $10 \times 10^6$ of spermatozoa. 20 μg of GnRH per dose and per doe (Gonadoreline, Fertagyl, Intervet Lab.) were injected in the thigh muscle to induce ovulation.

One group of female rabbits were administered, by a precision syringe, an oral dose of 1 $mg \cdot kg^{-1}$ of body weight per day of Lindane diluted in 0.5 mL of corn oil and the control group received the same volume of solvent. Treatment began eight days after insemination not to interfere with the first

delicate period of gestation. The pesticide was administered daily for two weeks, then every two days until the end of lactation period. The selected dose was a little higher than the amount present in polluted soil and water to show any potential toxic effects, but low enough to avoid any overt toxicity [5, 6, 22]. The offspring of females exposed to Lindane or not were weaned at 35 days postpartum and housed in single metal cages ($33 \times 42$ cm$^2$, height 40 cm). At puberty, twenty males, 173 days old, were randomly chosen to form two experimental groups ($n$ = 10 males per group): Lindane group, LG (receiving Lindane during prenatal period through maternal blood and after birth till weaning trough maternal milk), and control group, CG. All animals used in this study were fed commercial pellets (Val Serra, Terni, Italy; 16% crude protein, 16.5% crude fibre, 2.9% crude fat). Drinking water was also provided ad libitum. Care and treatment of animals were given according to the Farm Animal Welfare Council.

### 2.2. Parameters examined

*Body weight* of ten litters per group at 7, 15, 22 and 35 days was recorded. *Fertility rate* (parturitions/mating) was controlled on 22 mating with untreated females for each group. *Litter size* and percentage of *live born/total born* were also considered. *Libido* was measured weekly, during ten weeks, as collection time of ejaculate from the moment of the introduction of the doe in the buck cage [19].

*Sperm analysis* were made on semen collected by an artificial vagina from each male once a week to evaluate: *volume of ejaculate*, determined by a graduated tube directly connected to the artificial vagina, *concentration of spermatozoa*, determined on fresh sperm diluted 1:100 in a 3% NaCl solution to kill the cells and count them by a Bürker chamber. For each sample 400 spermatozoa were observed by a phase contrast Light Microscope (LM; Nikon SE) at $\times$ 400.

*Spermatozoa morphology* was examined by the Transmission Electron Microscope (TEM; 1200 JEOL E XII) to carry out qualitative and quantitative analyses.

Spermatozoa from each collection were fixed soon after ejaculation for 2 h at 4 °C in 4% paraformaldehyde and 5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 [16]. They were rinsed overnight in cacodylate buffer, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series and embedded in Epon-Araldite mixture. Thin sections were cut with Reichert Ultracut and LKB Nova ultramicrotomes, stained with uranyl acetate and lead citrate and examined with a 1200 JEOL EX II electron microscope.

To characterise the occurrence of ultrastructural sperm defects in CG and LG the following parameters were used:

– number of head abnormalities / 600 sections of the sperm head;

– number of abnormalities of the tail midpiece / 600 sections of the intermediate region;

– number of abnormalities of the tail principal piece / 600 sections of the principal piece.

Different sections of each sample were randomly examined, counted and classified in terms of visible alterations. The indices were transformed to percentages.

### 2.3. Statistical analyses

Weight of kits, volume of ejaculates and concentration of spermatozoa were statistically analysed using a two-way analysis of variance with repeated measures by the general linear models procedure of SAS [29]. The effects of treatment on litter size were assayed by the independent Student's t test. Fertility rate, percentages of live born/total born and percentages of spermatozoa with abnormalities were compared in the two groups by chi-squared analysis.

$P$ values $\leq$ 0.05 were considered significant.

## 3. RESULTS

### 3.1. Growth performance

There was no significant difference between CG and LG in postnatal growth performance of young rabbits from birth to weaning.

### 3.2. Reproductive parameters

Percentages of *fertility rate* were: 86.4% and 95.4% in CG and LG respectively; the average of birth litter size was 10.3 ± 0.8 and 9.4 ± 1.2 in CG and LG respectively. These values represent the normal means in rabbit breeding [7, 26]. The pesticide treatment did not affect significantly these parameters. Percentages of *live born/total born* observed in both groups (CG, 88.8%; LG, 89.0%) did not differ significantly.

*Mating time* decreased in both groups according to training from the beginning of male sexual activity [19]. In total, libido manifested itself at the maximum level after nine weeks from the beginning of the collection (Fig. 1). Regression curves showed no difference between the two groups.

*Volume of ejaculates* at the beginning of reproductive activity is illustrated in Figure 2. It was in the normal range of values [3]. Although from the 4th semen collection the mean values of sperm volume of bucks born from treated mothers were higher than the CG, no significant differences were observed. Variability was very high.

*Concentration of spermatozoa* in ejaculates was similar in the two groups. It showed a significant increase by 2.5 times, within a four weeks period, from the beginning of the semen collection (Fig. 3). Later on, both groups showed lower mean values. This trend was more accentuated in LG, but also in this case, differences were not statistically significant.

*Quantitative analysis on sperm* collected at three different times, 194, 215 and 236 days of age, was made. The morphological abnormalities present in both CG and LG were grouped into three different categories:

– head abnormalities: acrosome abnormalities (swollen or vesiculated) and nuclear abnormalities;

– abnormalities of midpieces: bent tails, presence of cytoplasmic droplets at proximal

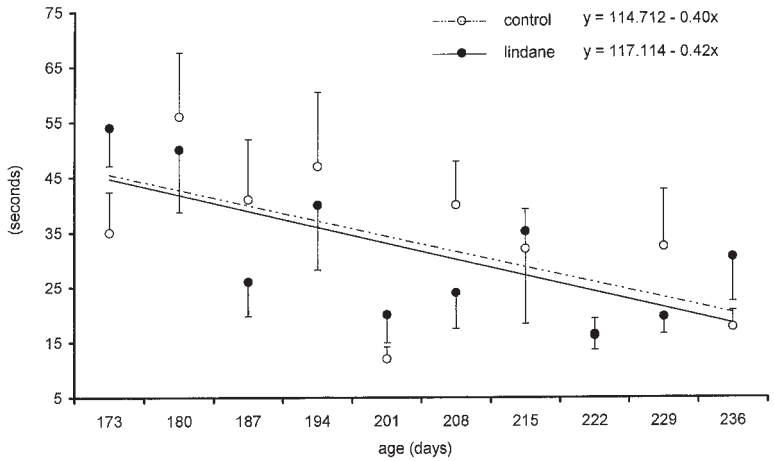


**Figure 1.** Mating times and their regression lines in male rabbits (10 animals/point, mean ± SEM) born from mothers receiving Lindane (—●—) or not (– – ○ – –).

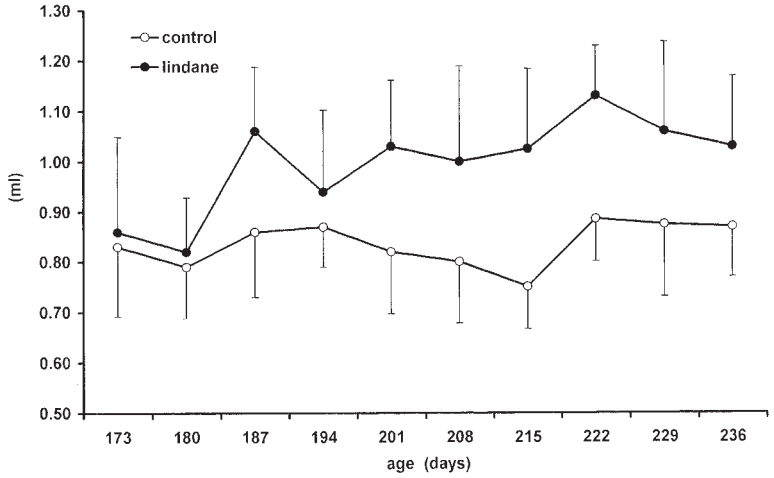


**Figure 2.** Volume of ejaculate in male rabbits at the beginning of reproductive activity (mean and SEM).

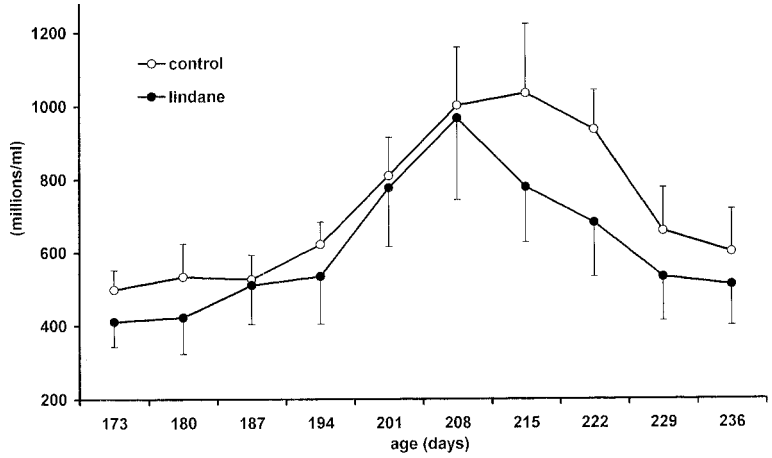


**Figure 3.** Concentration of spermatozoa in ejaculates at the beginning of reproductive activity (mean and SEM).

(Fig. 4a) or distal (Figs. 4b, 4c and 4d) level and mitochondria abnormalities;

– abnormalities of tail principal piece: presence of cytoplasmic droplets, coiled tails folded once (Fig. 4e) or more times and often entrapped by a cytoplasmic droplets (Fig. 4f), and axonemal abnormalities, such as ruptures (Fig. 4c) or lack (Fig. 4f) of some doublets.

The mean of frequencies of each category was calculated in CG and LG. The results obtained arises that Lindane treatment did not affect significantly the ultrastructure of three different sperm head abnormalities between CG and LG respectively: swollen acrosome 3.0% and 2.0%; vesiculated acrosome 5.0% and 6.0%; nuclear abnormalities 3.7% and 3.0%.

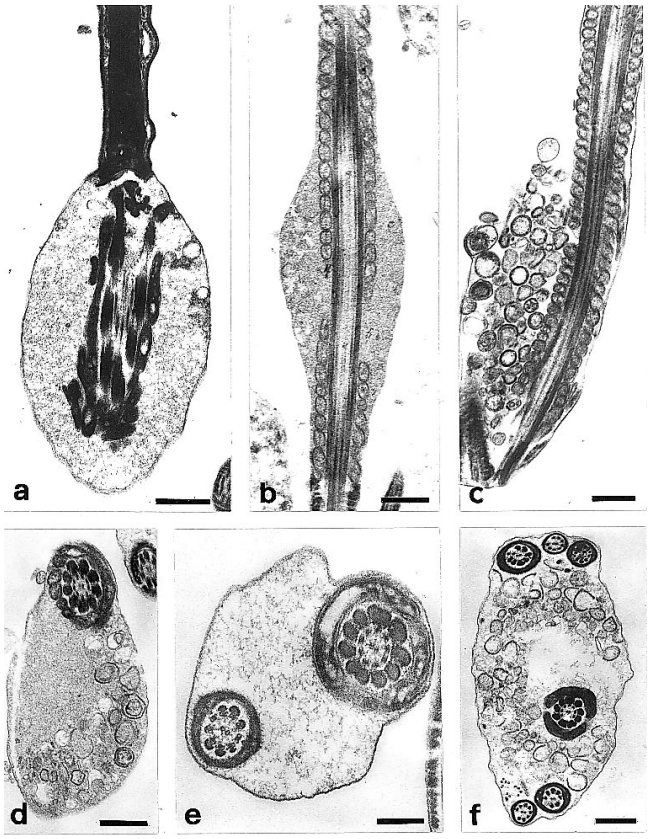


**Figure 4.** TEM images of sperm tail abnormalities from LG. (**a**), (**b**) cytoplasmic droplets in proximal (**a**) and distal (**b**) portions of midpiece. Note missing mitochondria associated with cytoplasmic droplets. Bar = 500 μm. (**c**) cytoplasmic droplet in the distal region of the midpiece with a marked angular deviation of the broken axoneme. Bar = 500 μm. (**d**) cross section through a midpiece with a cytoplasmic droplet showing a system of vesicles inside. Bar = 500 μm. (**e**) cross sections through coiled tail. Note the fusion of sperm membrane between the two adjacent portions of the spermatozoon. Bar = 200 μm. (**f**) cross section through a coiled tail folded more times. Note the lack of doublets in axonemal arrangement. Bar = 200 μm.

On the contrary, tail morphology appeared sensitive to the treatment. In fact (Fig. 5) the only differences were due in particular to a significant increase of the percentage of cytoplasmic droplets (5.3% in CG and 10.3% in LG; $P \leq 0.05$) and coiled tails (1.3% in CG and 4.3% in LG; $P \leq 0.05$). No significant differences have been found for the others tail abnormalities: bent tails (2.3% in CG and 3.0% in LG), mitochondria abnormalities (6.3% in CG and 7.0% in LG) and axonemal abnormalities (4.3% in CG and 6.3% in LG).

## 4. DISCUSSION

The current study shows that the spermatozoa ultrastructure is the only reproductive parameter significantly affected by a treatment with a low dose of Lindane, carried out during prenatal and lactation period, although the sperm ultrastructural analysis is not commonly utilised to evaluate the males reproductive efficiency. A significant increase in the number of sperm tails with cytoplasmic droplets and coiled tails were observed in response to Lindane treatment. These defects can be related to an incomplete maturation of spermatozoa [2, 8]. In fact, cytoplasmic droplets are present around midpiece during spermiation. They slip backwards from a proximal to a distal position during epididymal transit and are expelled during the resting in cauda epididymis. However, a low percentage of spermatozoa with cytoplasmic droplets has been found in normal rabbit ejaculates [14, 20].

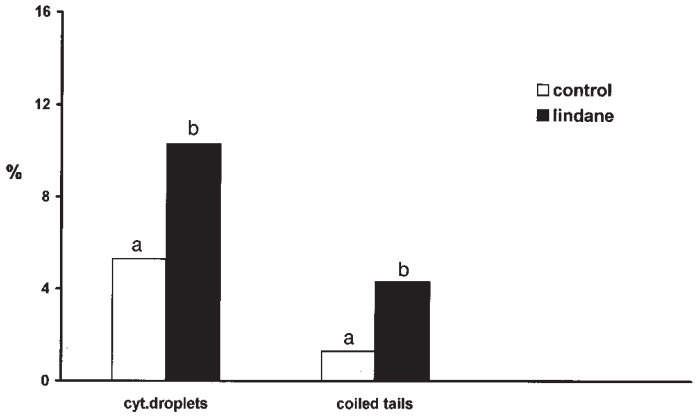


**Figure 5.** Percentages of cytoplasmic droplets and coiled tails of spermatozoa observed by TEM in the control (□) and Lindane (■) group (different letters within categories represent $P < 0.05$).

We can hypothesise that the increase of sperm tail with cytoplasmic droplets in LG is due to an alteration of epididymis function that can cause maturation defects of spermatozoa, as Kumar and Susheela [18] demonstrated.

The remained cytoplasmic droplets could be also associated with coiled tail defect. In fact, an hypothesis regarding the formation mechanism of this defect suggests a premature release of hydrolytic enzymes from the droplet, resulting in decomposition of the structural components of the tail [2]. According to literature, the coiled tail defect can be also due to other factors, such as cell death, heat stress or osmotic shock [4, 14]. Regarding the last point, the increase of coiled tails in LG could be related to quantitative and/or qualitative differences in seminal plasma. Our results show that the quantitative production of seminal plasma is not significantly influenced by Lindane treatment, that could instead influence the seminal plasma composition, although direct effects of Lindane on glandular secretion are not reported in literature.

The presence of an increased number of abnormal spermatozoa is not inconsistent with the obtained normal fertility since the sperm output per ejaculate exceeds far more the amount required for a normal fertility. On the contrary, experiments performed in minks and rams have demonstrated that exposure to a low dose of Lindane, from conception to sexual maturity, produces a decrease in reproductive efficiency [5]. This could suggest that there is a species specific response to Lindane treatment.

The other reproductive parameters analysed in the present work are not significantly affected by Lindane. Nevertheless, the trend of mean values of sperm volume and concentration of spermatozoa of bucks born from treated mothers appeared equally interesting. In fact, in these animals, the mean values of volume and concentration, from the 4th and 6th semen collection, respectively, were constantly and consistently different from the controls, but because of very high variability the observed differences were not statistically significant. In particular, it is not easy to explain the observed trend of concentration in both groups. In fact, the increase of spermatozoa output, 2.5 times within a 1 month period in comparison to the beginning values, can be correlated to the age of the animals, more sexually mature. On the contrary, it is not so understandable the reason of the decrease in the following month. Concentration of spermatozoa in ejaculates usually decreases because of high ambient temperature or pathologic events, but the present study was carried out in the spring season and no diseases were observed in the bucks.

In conclusion, the obtained results indicate that exposure to a low dosage of Lindane, carried out during prenatal and lactation period, induces a certain toxicity on spermatozoa ultrastructure of male rabbits, although general toxic symptoms were not observed in the animals. Sperm could represent a store of low level damages, but does not produce macroscopic effects. Sperm cell is more susceptible to the Lindane treatment than the other investigated end points, thus revealing itself as a sensitive marker of toxicity.

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