Control of variance in experimental studies of hyperlipidemia using the WHHL rabbit

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Abstract Between-animal variability has frustrated many experimental studies in outbred animal models of human disease. Variability that arises from genetic heterozygosity can be minimized by use of experimental designs that match littermates (polyzygotic twins) across control and treatment groups. Poor breeding vigor has prevented use of this experimental design in the WHHL rabbit model of hyperlipidemia and atherosclerosis. A comparison of reproduction in WHHL and normal rabbits demonstrated that litter size is limited by functional deficits at ovulation, implantation, and gestation in WHHL females. Superovulation of females reliably produced expanded litters of WHHL rabbits. Plasma lipids were measured in expanded litters of Japanese White WHHL (JW-WW) and English Half-lop WHHL (EHL-WW) rabbits. The variance of plasma cholesterol within sibships was two- to three-fold less than that between-litters. Intraclass correlation of total cholesterol within litters of EHL-WW was 0.72 and within litters of JW-WW was 0.67. These data provide evidence of genetic modulation of hypercholesterolemia in WHHL rabbits and demonstrate that experimental designs in which littermates are paired across groups can decrease the number of animals needed or increase the sensitivity of hypothesis tests by two- to threefold. — **Donnelly, T. M., S. F. Kelsey, D. M. Levine, and T. S. Parker.** Control of variance in experimental studies of hyperlipidemia using the WHHL rabbit. *J. Lipid Res.* 1991. **32:** 1089–1098.

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The introduction of the Watanabe Heritable Hyperlipidemic (WHHL) rabbit as a model of familial hypercholesterolemia (FH) has renewed interest in the rabbit as a small animal model for studies of atherosclerosis. WHHL rabbits offer important advantages over the classical, cholesterol-fed rabbit model of atherosclerosis. Plasma cholesterol concentrations, which range from 350 to 1200 mg/dl, are similar to those seen in human FH. The WHHL rabbit does not develop hepatic cholesterol toxicity (or other organ damage) as does the cholesterol-fed rabbit (1–3). Cholesterol-fed rabbits develop aortic lesions that contain foam cells predominantly (4, 5), unless the diet is carefully adjusted to match plasma lipid concentrations in WHHL rabbits (6–8). Homozygous WHHL Japanese White rabbits (JW-WW) and English Half-lop rabbits[2] (EHL-WW) develop fibrous lesions and complicated and calcified atherosclerotic plaques (9–11). Finally, type 1 JW-WW rabbits (see Discussion) develop coronary artery disease (CAD) (12), lose coronary vascular reserve (13), suffer ischemic myocardial injury, and live shorter lives than other rabbits (14). Atherogenesis in WHHL rabbits is accelerated by hypertension (15) and slowed by cholesterol-lowering drugs (16) and antioxidant drugs (17, 18).

The WHHL trait is maintained in outbred rabbit lines. Experimental studies that use the WHHL rabbit must be designed with sufficient numbers of animals to cope with the problem of between-animal variability. It is a fundamental principle of statistics that the number of samples (animals) needed to test an hypothesis is directly proportional to the variance of the measure of outcome (19). Variance can be minimized by carefully matching

Abbreviations: JW, Japanese White rabbit; NZW, New Zealand White rabbit; EHL English Half-lop rabbit; WHHL is used to indicate collectively the homozygous Watanabe Heritable Hyperlipidemic phenotype for rabbits of all strains; ww, wW, and WW are, respectively, normal, heterozygous, and homozygous Watanabe Heritable Hyperlipidemic phenotypes, i.e., EHL-ww, EHL-wW, and EHL-WW refer to normal, heterozygous, and homozygous WHHL English Half-lop rabbits; FH, familial hypercholesterolemia; FSH, follicle-stimulating hormone; HCG, human chorionic gonadotropin.

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[2]The name, English Half-lop, is the name of the "DxL" rabbits, which Richards (24) used to derive the modified WHHL rabbit. We and others have previously referred to these animals as British Halflop rabbits (25, 48).

animals in control and experimental groups for age, sex, weight, and genetic background, ideally by matching littermates (polyzygotic twins). This approach has had limited application (17) in the WHHL rabbit model because of the poor reproductive success with which these animals breed. Outbred, wild-type rabbits give birth to litters of 8–10 young (20). By contrast, litter sizes reported for WHHL rabbits are 2–4 in inbred lines (21), 3–6 in closed colonies (22, 23), and 4–8 in an outbred line (24).

We compared the outcomes of key stages of reproduction in WHHL and normal rabbits and found abnormalities in both sexes. WHHL males frequently produce sperm of poor morphology and motility. WHHL females have defective function during ovulation, implantation, gestation, and nursing (25). On the basis of these findings, we developed a method for expanding WHHL litters which uses superovulation and artificial insemination with pre-screened semen, either alone or together with embryo transfer. Plasma lipids were measured in these expanded litters and the within-litter and between-litter variances in plasma cholesterol were compared. The data were then used to determine the degree to which the sensitivity (or power) of experimental studies of hyperlipidemia and atherosclerosis could be increased by matching litter-mates (polyzygotic twins) across control and test groups.

METHODS

Materials

FSH and HCG were obtained from Sigma. Enzymatic triglyceride and cholesterol kits were purchased from Boehringer Mannheim.

Animals

The JW-WW rabbits have been maintained as a closed colony bred from three fertile males and two fertile females generously provided by Dr. Y. Watanabe in 1981. The JW-WHHL colony was monitored for specific pathogens and found to carry *Pasteurella multocida*, *Treponema cuniculi*, and enteric coccidia. In order to minimize chronic differences between healthy and sick animals and transient variations in plasma lipids, due to intermittent infection, an effort was made to free the colony of these pathogens. *Treponema cuniculi* and enteric coccidia were eliminated by 1983 by treatment with penicillin and amprolium. All data reported here are from animals born after 1983 spanning generations 2 through 6. The term "partially-inbred" is applied to these JW-WW rabbits to indicate that this degree of closed-colony breeding has increased the coefficient of inbreeding to some moderate, but unknown extent. Litter size was not significantly correlated with generation over this period of time (data from the 24 successful natural matings presented in Table 1; generations 2–6; $P > 0.05$).

Outbred, EHL-WW rabbits, originally described by Richards, Horlock, and Gallagher (24) were purchased from Froxfield Farms, Unit 3, King Lane, Froxfield, Hampshire, UK GU321DR. EHL-WW rabbits were maintained in specific pathogen-free (SPF) rooms at the Laboratory Animal Research Facility (LARC) at The Rockefeller University. Continuous monitoring for *Pasteurella multocida*, *Treponema cuniculi*, and enteric coccidia show the colony to be free of these pathogens. This colony is maintained by mating homozygous males with heterozygous females. New outbred breeders are obtained from Froxfield Farms each year. Offspring with plasma cholesterol concentrations of 400 mg/dl or higher between 6 and 12 weeks of age are considered homozygotes. Animals with plasma cholesterol concentrations between 250 and 400 mg/dl are resampled and regarded as homozygotes when the plasma cholesterol is consistently above 300 mg/dl.

Pregnant or nursing females and weanling animals have unrestricted access to Purina Lab Rabbit Chow low-fiber chow (#5321) up to the time blood is drawn to screen for lipid phenotype. All other adult rabbits above 16 weeks of age that are not in studies and breeding females between matings are fed Purina Lab Rabbit Chow high-fiber (#5326) in 125 g/day rations to prevent obesity.

Experimental Studies

All female WHHL rabbits used in the comparisons of experimental methods of reproduction were healthy, nonobese adults between 6 months and 14 months of age and had undergone induced ovulation or pregnancy at least once before the study. Breeding animals were kept separately in a quiet room with limited human access. Mated females were moved to double-sized cages and provided with cotton for nesting material. The maternity cages of expectant and new mothers were inspected at 8 AM and 5 PM each day. Newborn WHHL pups were given to NZW foster nurses when the natural mother was not adequately nursing her young (see Induction of parturition and wet nurses below).

Natural mating. The data in Table 1 are from natural 1-day matings. Gestation time was calculated from the 1-day natural matings of NZW-ww × NZW-ww rabbits and JW-WW × JW-WW rabbits. The data presented in Table 2 are from natural 3-day matings of homozygous with heterozygous or homozygous females.

Exploratory laparotomy. Laparotomy was performed after mating healthy adult NZW and WHHL females (26). The number of ova released was estimated by counting ovulation points. Exploratory laparotomy was repeated 2 weeks after mating to count the number of fetuses that had implanted.

Superovulation. Post-pubescent WHHL females (>6 months of age) were superovulated by subcutaneous injection of 0.5 units of porcine follicle-stimulating hormone (FSH) twice per day for 3 days. On the fourth day, females were inseminated and immediately given 100 IU of human chorionic gonadotrophin (HCG) intravenously.

Artificial insemination. Semen was collected from homozygous WHHL males, 6–12 months of age, using an artificial vagina. The semen was then grossly examined immediately for consistency (e.g., absence of urine), volume, and microscopically for sperm concentration, motility and morphology. Those samples qualitatively estimated to be fertile were used immediately to inseminate the doe. Each doe was inseminated with at least 100 million motile spermatozoa into the cranial aspect of the vagina. Semen samples from multiple donors were not pooled.

Superovulation without embryo transfer. Superovulated females were fertilized by artificial insemination. Mothers were allowed to carry the embryos to term. The litters produced by this method are larger (4–13 pups) than those born by females mated naturally. The pups, particularly those from the largest litters, are small in size and weight. When mothers did not nurse, the pups were given to foster nurses.

Embryo transfer. Embryos (one-cell and two-cell zygotes) were collected 18–24 h post HCG injection. Superovulation and artificial insemination result in a range of 28–68 (mean of 46) embryos recovered per female, with a failure of embryo pick-up in about 15% of the animals. After midline laparotomy, a slim glass cannula was inserted into the fimbriated ends of the oviducts and a blunt 20-gauge needle was introduced via the uterus and the utero-tubal junction into the isthmus of the oviduct. The oviducts were flushed with approximately 5 ml each of the optimal medium of Kane and Foote, plus 10% heat-inactivated, sterile rabbit serum, and the zygotes were collected in a sterile petri dish (27). This particular approach allows later reutilization of the donor mothers for further collection of zygotes from identical parents. Zygotes were examined and counted under a dissecting microscope and, after exteriorization of the oviducts through a flank laparotomy, up to 7 embryos were transferred into each ampulla in proven breeder NZW rabbits. Except where specifically noted, embryos from multiple donors were not pooled. The number of foster mothers receiving embryos ranged from 2 to 5 depending on the number of embryos that were collected. Surrogate mothers were injected 18–24 h previously with 100 IU HCG to induce ovulation and thus a synchronized pseudopregnancy.

Induction of parturition and wet nurses. To provide wet nurses, normal NZW females were bred to normal males when WHHL rabbits were bred. NZW females were given 10 units of oxytocin subcutaneously to induce delivery when needed. The fraction of pups raised by wet nurses ranged from none to 30% and averages approximately 20%. Most pups that survive the first 48 h after transfer to wet nurses survive to weaning (>90% and develop into normal weight adults.

Blood and plasma lipid determinations

Weanling animals were separated from their mothers between 6 and 10 weeks of age. Blood was taken from the marginal ear vein of nonfasted animals 1 or more weeks after they had been weaned to the rabbit chow diet. Blood was drawn with a 24-gauge intravenous Teflon catheter (Angiocath, Becton Dickinson and Co.) to avoid hemolysis which interferes with the enzymatic method used for determination of cholesterol. Blood was transferred to a tube containing disodium EDTA to a final concentration of 1 mg/ml, immediately refrigerated, and separated within 3 h. Total cholesterol and triglycerides were measured by enzymatic assay (28–30) using a Roche COBAS Fara analyzer. Long-term standardization of both assays was maintained by use of three-level, serum-based, control reference pools (kept at −70°C) that recovered the CDC cholesterol and triglyceride standards to within ± 5% of the targeted values (31). Cholesterol and triglycerides were measured once for each animal. The total imprecision (expressed as coefficient of variation %) of the cholesterol method was 2% (0.9% within run, 1.1% between run) and 1.4% between day. The total imprecision of the triglyceride method was 5% (2% within run, 3% between run) and 3.7% between day (32). The enzymatic triglyceride measurements were not corrected for free glycerol; a preliminary survey of 33 plasma samples from JW, NZW, and EHL normal and WHHL rabbits showed that free glycerol concentrations were low (equivalent to 13.5 mg/dl triglyceride-equivalents; 95% confidence limits −9.3 to 36.3 mg/dl).

Plasma progesterone concentrations were measured by extraction and radioimmunoassay as previously described (33).

A two-tailed Student's *t*-test was used to test differences in mean values of total cholesterol and triglycerides between sexes and breeds of rabbit. The same test was applied to breeding data. For all animals within a breed and separately for males and females, total variances for cholesterol and triglycerides were calculated. This total variance does not take into account litter and therefore represents the magnitude of variance that must be considered when litter is ignored in an experimental design.

A one-way analysis of variance assuming a random effect model (34) was used to estimate the between- and within-litter variability of total cholesterol for each breed. Intraclass correlations were estimated and an approximate 95% lower confidence limit was calculated. The in-

TABLE 1. Yields from 24 pregnancies each in normal NZW and homozygous WHHL rabbits

Stage[a]	NZW	JW-WW
Abortions[b]	0	4
Stillborn pups	11	18
Live-born pups	228	97
Weanlings	208	43

[a]The results of 24 successful matings of NZW (wild type) rabbits and 24 successful matings of male and female JW-WW rabbits. All data are from natural 1-day matings. Males were not screened for quality and quantity of semen. The percentages of successful matings for NZW and JW-WW were: 24/34 = 71% and 24/56 = 43%, respectively.
[b]All abortions occurred after the 20th day of gestation.

traclass correlation, which has a maximum of 1, is large when the variation between litters is large in relation to the total variation. For this analysis, a large intraclass correlation indicates that animals within a litter have similar cholesterol levels. This was done for all animals and repeated for males and females. Similar analyses were performed for triglycerides.

RESULTS

Reproductive yields from natural matings

The data shown in **Table 1** are the outcomes of 24 successful pregnancies from natural 1-day matings of JW-WW × JW-WW rabbits and NZW-ww × NZW-ww rabbits. JW-WW females gave birth at 29–31 days compared to 30–32 days for NZW-ww females (30.6 ± 1.2 days, n = 13 vs. 31.8 ± 0.4 days, n = 13; $P < 0.005$). Pregnancy in WHHL rabbits ended more often with spontaneous abortion or delivery of stillborn pups than in NZW rabbits. WHHL mothers bore fewer live-born pups and nurtured fewer to weaning. Mismothering, mostly failure to nurse, claimed 56% and 5% of live-born pups in WHHL and normal litters, respectively. The smaller number of live-born pups in the WHHL litters together with the large number of spontaneous abortions and stillborn young suggested that the WHHL females were losing embryos early in pregnancy.

Reproductive function in WHHL rabbits

To test this hypothesis, we studied ovulation and implantation by exploratory laparotomy in another eight WHHL and four NZW rabbits. By counting ovulation points, we infer that WHHL and NZW females released respectively: 9.4 ± 2.1 and 12.5 ± 1.9 ova ($P < 0.05$). A second laparotomy 2 weeks later indicated that significantly fewer ova had been fertilized and become implanted in the JW-WW mothers: 3.6 ± 3.2 vs. 8.5 ± 2.4 ($P < 0.05$). Implantation rates (number implanted/number of ova) were not significantly lower in JW-WW mothers compared to NZW mothers ($P > 0.05$). Of these implanted embryos, 0.75 ± 1.4 vs. 6.5 ± 2.4 (per-litter mean) survived to parturition ($P < 0.001$). The rate of parturition (number of live-born/number implanted) was significantly lower in JW-WW mothers ($P < 0.005$).

Gestation is dependent on progesterone. In the rabbit, plasma progesterone concentrations peak between the 10th and 25th day of gestation (35). Progesterone concentrations measured in two each of the JW-WW and NZW mothers described above were in the low-normal range in JW-WW mothers (2–7 ng/ml) until the 15th day of gestation. At day 20, concentrations remained below 10 ng/ml (range 7–8 ng/ml) in the JW-WW mothers and rose above 10 ng/ml (range 11–25) in the NZW mothers. This evidence of suboptimal progesterone production during pregnancy led us to test several reproductive techniques that would help maintain pregnancy by supplementing progesterone production in WHHL females or would transfer the burden of gestation to normal surrogate mothers.

Expansion of litters

Natural matings. The data shown in **Table 2** are the outcomes of natural 3-day matings and three experimental breeding techniques. Pregnancy rates and the number of rabbits weaned per litter are presented because they determine the success of a breeding program, but these measures are affected by many factors (age, health, obesity, prior breeding, and nursing experience and animal care) that are not related directly to reproductive function. Only litter size directly reflects reproductive losses during ovulation, implantation, and gestation. Homozygous WHHL females, either partially inbred JW-WW or outbred EHL-WW rabbits, produced small litters: 4.0 ± 2.1 and 5.5 ± 2.9, respectively, in natural matings with homozygous WHHL males. This is approximately half the 8–10 pups produced per litter by normal rabbits (see Table 1 or ref. 20).

Artificial insemination without superovulation was tried in 13 JW-WW females and 10 EHL-WW females. This is not strictly a "natural" method of mating, but neither is it an experimental method in the sense that it supplements progesterone production or transfers the burden of gestation to heterozygous or normal females. Artificial insemination without superovulation gave litter sizes of 4.5 ± 2.5 and 5.6 ± 2.5 live-born pups in JW-WW and EHL-WW rabbits, respectively, compared to a mean litter size of 6 ± 2 reported by Phelan et al. (23). In our hands, artificial insemination without superovulation did not significantly increase litter size compared to natural mating.

TABLE 2. Breeding methods and yields in JW-WW and EHL-W rabbits

Method[a]	Cross[b] (M × F)	Pregnancy Rate[c]	All Live Born[d]	Homozygous Weanlings[d]
			mean/litter ± STD	
JW rabbits				
Natural	WW × WW	23/42 (55%)	4.0 ± 2.1	2.1 ± 2.6
EHL rabbits				
Natural	WW × WW	10/14 (71%)	5.5 ± 2.9[e]	1.9 ± 2.6[e]
Natural	WW × wW	49/108 (45%)	6.5 ± 2.7[f]	2.0 ± 2.6[g]
S.O.	WW × WW	10/14 (71%)	9.6 ± 3.3[h]	5.1 ± 3.3[i]
S.O. with E.T.	WW × WW	3/5 (60%)	6.2 ± 1.9[j]	4.0 ± 1.6[k]

[a]S.O. is superovulation; E.T. is embryo transfer.
[b]WW × WW: homozygous male mated to homozygous female; WW × wW: homozygous male mated to heterozygous female.
[c]Pregnancy rate is given as litters/matings with percent yield in parentheses. The higher efficiency of natural matings compared to Table 1 is explained by the use of males that were screened for normal sperm production. The pregnancy rate data for embryo transfer experiments are: number of donors that produced any live born pups/total number of donors.
[d]The numbers of live born and weanling pups are given as mean per litter ± standard deviation. The total number of pups can be calculated by multiplying by the number of pregnancies for each category. All live-born/litter refers to homozygous WHHL rabbits, except for male EHL-WW × female EHL-wW matings where heterozygous and homozygous newborns could not be distinguished. Heterozygous EHL-wW rabbits were not included in the count of homozygous weanlings.
[e]Naturally bred JW-WW × WW vs. EHL-WW × WW: NS ($P > 0.05$).
[f]Live-born JW-WW × WW vs. EHL- wW × WW: $P < 0.001$.
[g]Homozygous weanlings JW-WW × WW vs. EHL WW × wW: NS ($P > 0.05$).
[h]Live-born EHL-WW × WW natural mating vs. superovulation: $P < 0.01$.
[i]Weanling EHL-WW × WW natural mating vs. superovulation: $P < 0.05$.
[j]Live-born EHL-WW × WW natural mating vs. superovulation with embryo transfer: NS ($P > 0.05$).
[k]Weanling EHL WW × WW natural mating vs. superovulation with embryo transfer: $P < 0.05$.

Experimental reproductive methods. Outbred EHL rabbits were used to compare experimental methods of reproduction to avoid the question of inbreeding depression which would have been an issue had we used partially inbred females from the closed colony of JW-WW rabbits. In the first experimental method shown in the lower portion of Table 2, heterozygous females were mated to homozygous males to transfer the burden of pregnancy away from homozygous females. Heterozygous WHHL females gave birth to larger (but not significantly larger, $P > 0.05$) litters than were those produced by homozygous mothers, but only about half of these were homozygous WHHL rabbits. The over-all production of weanling homozygous WHHL rabbits by heterozygous females was less than that from matings between homozygous animals.

In the second method, homozygous WHHL females were superovulated, artificially inseminated, and then left to carry their embryos to term. We confirmed by laparotomy that this procedure induced ovulation from 40–60 corpora lutea. The data in Table 2 show that the litters produced by superovulated mothers were approximately twice as large as those produced by naturally mated homozygous or heterozygous mothers. However, the pups from the largest litters with 10–13 new-borns were small in size and weight and the mothers occasionally needed help from foster nurses to feed part of the litter. This was the simplest and most productive method of those tested, but success required that foster mothers were prepared and available at the time of delivery.

The third method was superovulation, artificial insemination, and transfer of embryos from superovulated homozygous females to normal NZW surrogate mothers. The data reported in Table 2 are the results of embryo transfer experiments carried out with five superovulated EHL-WW donors. The average yield of embryos was good: mean, 46; range, 20–67. In pilot studies it was observed that surrogate mothers that received fewer than 5 embryos per ampulla (total < 10) did not produce live-born pups. For this reason, each NZW surrogate mother received from 10–15 embryos, so that the from two to four surrogate mothers were used for each donor mother. Included in Table 2 are data from two unsuccessful superovulation and embryo transfer experiments that gave no live-born pups and three successful experiments that gave an average of 6.2 ± 1.9 live-born pups. Of these pups, 4.0 ± 1.6 were weaned per superovulated donor.

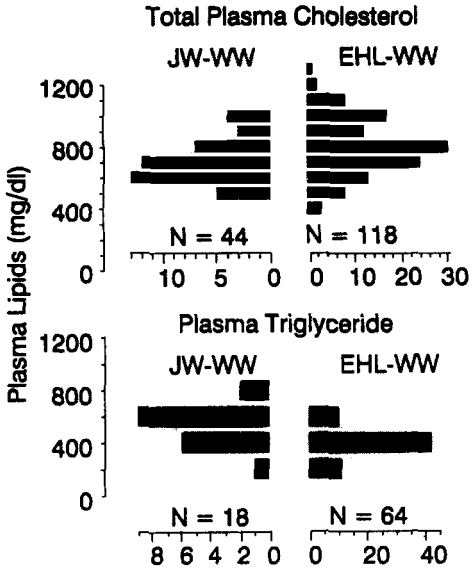

Fig. 1. Distribution of plasma cholesterol and triglycerides in nonfasting JW-WW and EHL-WW rabbits of 6 to 15 weeks of age. Range of cholesterol is divided into 100 mg/dl intervals; range of triglyceride is divided into 200 mg/dl intervals. All animals reared under the same conditions. See Table 3 for means by breed and sex.

Superovulation and embryo transfer gave larger litters (but not significantly so) and more weanlings ($P < 0.05$) than natural EHL-WW × EHL-WW matings. The better weaning rate may be partly explained by the fact that the nursing mothers are wild-type NZW rabbits. We believe that success with this method was due more to the large number of embryos that were transferred than to any beneficial effect on survivial. For example, in an experiment that is not included in Table 2, 80 embryos were collected from two donors (45 from one and 35 from the other) and pooled for transfer; 60 embryos were transferred to 4 surrogate mothers (15 embryos each); 16 pups were born and 14 survived to weaning.

Plasma lipids

Plasma lipid concentrations in WHHL rabbits plateau during the period of rapid growth from 6 to ~15 weeks of age and then begin to fall to levels 50% to 60% of peak between 6 and 12 months of age (36). By limiting the plasma lipid data to the relatively stable period between 6 and 15 weeks of age, the contribution of age to between-litter variability was minimized: 10 JW-WW litters containing 44 rabbits and 35 EHL-WW litters containing 118 animals met this criterion. Plasma triglyceride data were available from only 64 of the 118 EHL-WW rabbits and from 18 of the 44 JW-WW rabbits. The frequency distributions of nonfasting plasma total cholesterol and triglycerides are shown for all JW-WW and EHL-WW rabbits in **Fig. 1**. Mean plasma lipid concentrations of males and females of both breeds are given in **Table 3**. Plasma cholesterol tended to be higher in male and female EHL-WW rabbits compared to their JW-WW counterparts, but the difference was statistically significant in females only. Nonfasting plasma triglyceride concentrations were significantly lower in EHL-WW males compared to JW-WW males. These measurements of plasma cholesterol and triglyceride concentrations are in agreement with those reported by Watanabe, Takashi, and Shiomi (36) and by Gallagher et al. (10) for the respective founder colonies. Thus our data, from animals reared under identical conditions, confirm the differences in plasma cholesterol and triglyceride concentration between these two strains of WHHL rabbit. Preliminary data from our animals suggest that the between-strain difference in hypertriglyceridemia is even more pronounced in male rabbits after a 12-h overnight fast: 180 mg/dl versus 430 mg/dl in EHL-WW and JW-WW, respectively.

Between- and within-litter associations of plasma lipid concentration

The variance of plasma cholesterol among littermates was compared to that between litters. The results in **Table**

TABLE 3. Plasma lipids in JW-WW and EHL-WW rabbits

	Total Cholesterol			Triglyceride		
	Males	Females	All	Males	Females	All
		mg/dl			*mg/dl*	
JW-WW[a]	664 ± 162 (22)	633 ± 133 (22)	648 ± 147 (44)	498 ± 144 (11)	330 ± 116 (7)	433 ± 155 (18)
EHL-WW	721 ± 175 (59)	774 ± 194 (59)	748 ± 186 (118)	267 ± 86 (35)	318 ± 102 (29)	290 ± 96 (64)
JW vs. EHL[b]	NS	$P = 0.0005$	$P = 0.0006$	$P < 0.0001$	NS	$P < 0.005$

Data are given as means ± standard deviation with the number of animals in parentheses.
[a]Within JW-WW rabbits: males vs. females: TC not significant; TG, $P < 0.02$.
[b]Between JW and EHL: P values are two-sided t tests; NS, not significant.

TABLE 4. Within and between litter variance of plasma cholesterol[a]

	JW-WW	EHL-WW
Males	10 litters	28 litters
Variance	and 22 pups	and 59 pups
Between litter	18,018	21,411
Within litter	9,676[d]	9,891[d]
R[b]	0.65	0.68
CL[c]	0.26	0.48
Females	10 litters	30 litters
Variance	and 22 pups	and 59 pups
Between litter	14,092	30,300
Within litter	4,758[d]	8,151[e]
R	0.75	0.79
CL	0.43	0.64
Males and females	10 litters	35 litters
Variance	and 44 pups	and 118 pups
Between litter	15,409	25,405
Within litter	7,710[d]	9,944[d]
R	0.67	0.72
CL	0.45	0.60

[a]Variance is expressed in units of mg/dl squared with the number of litters and pups given for each group.
[b]Intraclass correlation.
[c]Ninety-five percent lower confidence limit of intraclass correlation.
[d]Between litters vs. within litter variance difference: $P < 0.001$.
[e]Between litters vs. within litter variance difference: $P < 0.01$.

4 show that the between-litter variance in plasma cholesterol is significantly larger than the within-litter variance when males or females are considered separately or together. This was true in both strains. Intraclass correlations were high: 0.72 for EHL-WW rabbits and 0.67 for JW rabbits. Male and female animals had similar intraclass correlations of plasma cholesterol. The analysis was repeated for triglyceride concentration, but there was no evidence of intraclass correlation for this measure within the smaller sample size that was available.

DISCUSSION

Expanded litters

The two- to threefold lower variance of plasma cholesterol found within litters as compared to between litters has important implications for the design of experiments with WHHL rabbits. The number of animals needed to detect a difference between a control and an experimental group at a given level of statistical significance is directly proportional to the variance of the experimental measurement. Pairing littermates can increase sensitivity by making a smaller difference detectable, increase the statistical power of a given experimental design, reduce the number of animals used in each experimental group and thereby lower cost, or enable more effective use of experimental agents that are often in short supply.

To realize the benefit from pairing WHHL littermates, it was necessary to develop breeding methods that produced expanded litters in large numbers. This is because plasma cholesterol, triglycerides, and incidence of coronary artery disease vary with age and sex in WHHL rabbits. Not only must littermates be paired, but the paired animals should also be sex-matched and the litters should be age-matched (ideally within 7 to 10 days of age). Superovulation of WHHL rabbits produces litters 70% of the time and gives the investigator control over the time of birth. On average, 5 of the 9 animals in each litter survive to weaning and most litters produce two pairs of same-sex littermates. Superovulation is a relatively simple nonsurgical procedure that can be applied with equal ease to the smaller JW-WW rabbits. It requires no more expertise than the method of induction of ovulation recommended by Phelan et al. (23), but larger litters are produced. Superovulation with embryo transfer offers the promise of further expansion of litters, but this approach remains technically complex and less reliable than superovulation alone.

Reproduction in WHHL rabbits

Our data clearly demonstrate reproductive dysfuction at specific stages in pregnancy in WHHL females. Losses at ovulation, implantation, and during gestation accounted for the poor reproductive vigor of WHHL rabbits.

Chai (37) demonstrated by laparotomy that implantation was impaired in inbred females that presumably had normal LDL-receptor function. He interpreted this increased mortality of zygotes as evidence of an unfavorable uterine environment due perhaps to lack of hormonal stimulation. This same mechanism has been advanced to explain the rapid decrease in litter size observed with serial inbreeding of JW-WW rabbits (21).[3] However, this explanation is ruled out in the case of the outbred EHL-WW rabbits and seems unlikely in the case of our partially inbred JW-WW rabbits. Watanabe and his coworkers (21) have shown that closed-colony breeding of JW-WW rabbits does not cause the same rapid fall in reproductive vigor that occurs with each generation of father-daughter inbreeding.

Our finding that plasma progesterone concentrations did not rise in pregnant WHHL females supports the hypothesis that reproductive dysfunction at these steroid hormone-dependent stages in pregnancy may be linked in some undefined way to the loss of LDL-receptor function.

[3]Shiomi, Ito, and Watanabe (21) quote C. K. Chai (37) as having shown that reproductive hormone function is decreased with inbreeding. Chai only proposed this as one of several hypothetical mechanisms that could explain the reproductive depression of inbred rabbits.

Basal cortisol production is normal in WHHL rabbits, but adrenal reserve is limited (38). Similarly, plasma progesterone concentrations were in the low range of normal until the 20th day of gestation in pregnant WHHL rabbits. The drop-off in plasma progesterone occurred at the time of gestation when plasma cholesterol concentrations fall to very low levels in the rabbit: to below 50 mg/dl in normal rabbits (39), below 100 mg/dl in cholesterol-fed rabbits (40–42), and below 200 mg/dl in WHHL rabbits (43). The combination of low plasma LDL concentration with genetically defective LDL receptor function may limit delivery of cholesteryl ester to critical steroidogenic tissues, such as the ovary and placenta (44) in the rabbit. Sub-optimal production of progesterone may then result in smaller litter size and shorter gestation time.

Popják (40) has described a severe lipid storage disorder in the fetal placenta of cholesterol-fed rabbits that is associated with shortened gestation time, miscarriage, low birth weight, and small litter size. Popják suggested that fetal nutrition might be compromised, but Zilversmit, Remington, and Hughes (45) found no evidence to support this hypothesis. We have observed a lipid storage disorder in 28-day fetal placentas taken from EHL-WW rabbits compared to EHL-ww rabbits. However, the accumulation of lipid was moderate compared to that observed in placentas taken from cholesterol-fed EHL-ww rabbits. Nevertheless, these preliminary findings confirm the earlier work of Popják (40), Ross and Zilversmit (42), and Zilversmit et al. (45) and extend their observations to genetically hypercholesterolemic WHHL rabbits. Although many questions remain unanswered, two mechanisms can be proposed: placental lipid storage (presumably in non-steriodogenic cells of cholesterol-fed and WHHL rabbits) and LDL-receptor-limited steriodogenesis (WHHL rabbits) that could account for reproductive deficits in early pregnancy.

Genetic backgrounds of WHHL rabbits

Our data show that genetic background strongly affects the expression of hyperlipidemia in LDL-receptor-deficient WHHL rabbits. WHHL rabbits of different breeds cannot be regarded as equivalent. Even rabbits of the same breed from different closed colonies are best regarded as distinct sublines. This is well illustrated by the experience of the colony at Kobe University Institute for Experimental Animals. The hyperlipidemic rabbit (HLR) trait was discovered in a Japanese White rabbit in 1973 (46). Two partially inbred lines of WHHL rabbit were developed from the original HLR stock: WHHL-02 (brother-sister mating of the original HLR rabbits) and WHHL-04 (parent offspring mating and back-crossing) (47). Between the first and fourth filial generations, the frequency of hyperlipidemia increased from 15% to 56%, the coefficient of inbreeding rose to 50%, and the litter size decreased from 6.6 to 3.6. Three sublines were developed to maintain reproductive vigor: WHHL-M (crossing -02 and -04), WHHL-B (crossing -04 and JW-NIBS strain) and WHHL-06 (crossing -04 and JW rabbits). Then in 1985 Watanabe divided his colony into two major sublines. The original WHHL line was designated type 2 and a new line was designated type 1. The new type 1 WHHL rabbits were derived by selective breeding of three coronary artery disease-positive males from the B, M, and -04 sublines. After selective breeding, WHHL rabbits from matings between two type-1 parents had higher plasma cholesterol, 713 ± 181 mg/dl versus 530 ± 144 mg/dl, and higher triglyceride, 643 ± 204 mg/dl versus 478 ± 160 mg/dl, concentrations compared to type 2 WHHL rabbits. Aortic atherosclerosis is reported to progress at the same rate in both strains, but the ages at which 50% of offspring develop coronary lesions are 6–8 months and 16–18 months for types 1 and 2, respectively. The degree to which genetic background and breeding history can affect hyperlipidemia and atherosclerosis in WHHL rabbits is not widely appreciated and breeding history is often inadequately described in reports of experimental studies of WHHL rabbits.

Summary

We have shown that specific progesterone-dependent stages of reproduction are defective in female WHHL rabbits. This deficit can be partially overcome by superovulation of WHHL females prior to mating them with males. Superovulation is a relatively simple method that has the further benefit of producing larger (expanded) litters. We have reported between-breed and between-litter differences in plasma cholesterol and triglyceride concentrations that demonstrate the contribution of genetic heterozygosity to the total variance of plasma lipid levels in WHHL rabbits, by matching same-sex littermates across experimental groups, the power of a given experimental design can be increased substantially. This advantage is gained through the control over between-litter variation which is achieved by sib-matching. The result is either a significant gain in sensitivity or a proportional reduction of cost.

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REFERENCES

1. Clarkson, T. B. 1963. Atherosclerosis—spontaneous and induced. *Adv. Lipid Res.* **1:** 211–252.
2. Anderson, J. M., S. D. Turley, and J. M. Dietschy. 1982. Relative rates of sterol synthesis in the liver and various extrahepatic tissues of normal and cholesterol-fed rabbits. Relationship to plasma lipoprotein and tissue cholesterol levels. *Biochim. Biophys. Acta.* **711:** 421–430.
3. Dietschy, J. M., T. Kita, K. E. Suckling, J. L. Goldstein, and M. S. Brown. 1983. Cholesterol synthesis in vivo and in vitro in the WHHL rabbit, an animal with defective low density lipoprotein receptors. *J. Lipid Res.* **24:** 469–480.
4. Imai, H., K. T. Lee, S. Pastori, E. Panlilio, R. A. Florentin, and W. A. Thomas. 1966. Atherosclerosis in rabbits: architectural and subcellular alterations of smooth muscle cells of aortas in response to hyperlipidemia. *Exp. Mol. Pathol.* **5:** 273–310.
5. Scott, R. F., A. S. Daoud, and R. A. Florentin. 1971. Animal models in atherosclerosis. *In* The Pathogenesis of Atherosclerosis. R. W. Wissler and J. C. Greer, editors. Williams and Wilkins, Baltimore. 120–146.
6. Rosenfeld, W. E., T. Tsukada, A. M. Gown, and R. Ross. 1987. Fatty streak initiation in Watanabe Heritable Hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis.* **7:** 9–23.
7. Rosenfeld, W. E., T. Tsukada, A. M. Gown, and R. Ross. 1987. Fatty streak expansion in Watanabe Heritable Hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis.* **7:** 24–34.
8. Rosenfeld, W. E., A. Chait, E. Bierman, W. King, P. Goodwin, C. E. Walden, and R. Ross. 1988. Lipid composition of aorta of Watanabe Heritable Hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. Plasma lipid composition determines aortic lipid composition of hypercholesterolemic rabbits. *Arteriosclerosis.* **8:** 338–347.
9. Buja, L. M., T. Kita, J. L. Goldstein, Y. Watanabe, and M. S. Brown. 1983. Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia. *Arteriosclerosis.* **3:** 87–101.
10. Gallagher, P. J., N. Nanjee, T. Richards, W. R. Roche, and N. E. Miller. 1988. Biochemical and pathological features of a modified strain of WHHL rabbits. *Atherosclerosis.* **71:** 173–183.
11. Nolte, C. J., A. M. Tercyak, H. M. Wu, and D. M. Small. 1990. Chemical and physiochemical comparison of advanced atherosclerotic lesions of similar size and cholesterol content in cholesterol-fed New Zealand White and Watanabe Heritable Hyperlipidemic rabbits. *Lab. Invest.* **62:** 213–222.
12. Kaoru, H., T. Ito, M. Shiomi, A. Yamamoto, and Y. Watanabe. 1987. Ischemic heart disease in the WHHL rabbit: a model for myocardial injury in genetically hyperlipidemic animals. *Am. Heart J.* **113:** 280–288.
13. Clozel, J. P., H. Lengsfeld, H. Kuhn, and H. R. Baumgartner. 1988. Decreased coronary vascular reserve in Watanabe Heritable Hyperlipidemic rabbits. *Arteriosclerosis.* **8:** 310–320.
14. Ito, T., Y. Watanabe, and M. Shiomi. 1982. Spontaneous atherosclerosis in immature of WHHL rabbits [in Japanese]. *J. Jap. Coll. Angiol.* **22:** 229.
15. Chobanian, A. V., A. H. Lichtenstein, V. Nilakhe, C. C. Haudenschild, R. Drago, and C. Nickerson. 1989. Influence of hypertension on aortic atherosclerosis in the Watanabe rabbit. *Hypertension.* **14:** 203–209.
16. Shiomi, M., T. Ito, Y. Watanabe, Y. Tsujita, M. Kuroda, M. Arai, M. Fukami, J. Fukushige, and A. Tamura. 1990. Suppression of established atherosclerosis and xanthomas in mature WHHL rabbits by keeping their serum cholesterol levels extremely low. *Atherosclerosis.* **83:** 69–80.
17. Carew, T. E., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe Heritable Hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
18. Mao, S. J. T., M. T. Yates, A. E. Rechtin, R. L. Jackson, and W. A. Van Sickle. 1991. Antioxidant activity of probucol and its analogues in hypercholesterolemic Watanabe rabbits. *J. Med. Chem.* **34:** 298–302.
19. Snedecor, G. W., and W. G. Cochran. 1980. Statistical Methods. 7th ed. Iowa State University Press, Ames, IA. 64.
20. Hagen, K. W. 1974. Colony Husbandry. *In* The Biology of the Laboratory Rabbit. S. H. Weisbroth, R. E. Flatt, and A. L. Kraus, editors. Academic Press, New York.
21. Shiomi, M., T. Ito, and Y. Watanabe. 1987. Effects of hyperlipidemia on nursing ability of WHHL rabbits. *Lab Anim. Sci.* **37:** 84–88.
22. Watanabe, Y., I. Takashi, and T. Kondo. 1977. Breeding of a rabbit strain of hyperlipidemia and characteristics of this strain. *Exp. Anim.* **26:** 35–42.
23. Phelan, J. P., B. J. Van Lenten, A. M. Fogelman, C. Kean, M. E. Haberland, and P. A. Edwards. 1985. Notes on the breeding of the WHHL rabbit: an animal model of familial hypercholesterolemia. *J. Lipid Res.* **26:** 776–778.
24. Richards, T., H. Horlock, and P. Gallagher. 1986. Atherosclerosis in modified WHHL rabbits. *Animal Tech.* **37:** 1–6.
25. Donnelly, T. M., and T. S. Parker. 1986. Localization of the reproductive deficit of the WHHL rabbit. *Arteriosclerosis.* **6:** 539a.
26. Overstreet, J. W., and J. M. Bedford. 1976. Embryonic mortality in the rabbit is not increased after fertilization by young epididymal spermatozoa. *Biol. Reprod.* **15:** 54–57.
27. Kane, M. T., and R. H. Foote. 1971. Factors affecting blastocyst expansion of rabbit zygotes and young embryos in defined media. *Biol. Reprod.* **4:** 41–47.
28. Siedel, J., E. O. Hagele, J. Siegenhorn, and A. W. Wahlefeld. 1983. Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. *Clin. Chem.* **29:** 1075–1080.
29. Wiebe, D. A., and J. T. Bernert, Jr. 1984. Influence of incomplete cholesteryl ester hydrolysis on enzymic measurements of cholesterol. *Clin. Chem.* **30:** 352–356.
30. Nagele, U., E. O. Hagele, G. Sauer, E. Wiedemann, P. Lehmann, A. W. Wahlefeld, and W. Gruber. 1984. Reagent for the enzymatic determination of total triglycerides with improved lipolytic efficiency. *Clin. Chem.* **29:** 1075–1080.
31. Hainline, A., J. Karon, and K. Lippell, editors. 1982. Manual of Laboratory Operations. Lipid Research Clinics Program: Lipid and Lipoprotein Analysis. 2nd edition. DHEW-NIH, Washington, DC.

32. Imprecision was estimated according to the National Committee for Clinical Laboratory Standards (NCCLS) protocol EP5-T. 1984. Volume 4, No. 8. 185–218.
33. Aufrere, M. E., and H. Benson. 1976. Progesterone, an overview and recent advances. *J. Pharm. Sci.* **65:** 783–800.
34. Fleiss, J. L. 1986. The Design and Analysis of Clinical Experiments. John Wiley and Sons, NY. 8–14.
35. Challis, J. R. G., I. J. Daves, and K. J. Ryan. 1973. The concentrations of progesterone, estrone and estradiol-17B in the plasma of pregnant rabbits. *Endocrinology.* **93:** 971–978.
36. Watanabe, Y., I. Takashi, and M. Shiomi. 1985. The effect of selective breeding on the development of coronary atherosclerosis in WHHL rabbits. *Atherosclerosis.* **56:** 71–79.
37. Chai, C. K. 1968. Effects of inbreeding in rabbits. Inbred lines, discrete characters, breeding performance and mortality. *J. Hered.* **60:** 64–70.
38. Hoeg, J. M., L. Loriaux, R. E. Gregg, W. R. Green, and H. B. Brewer, Jr. 1985. Impaired adrenal reserve in the Watanabe Heritable Hyperlipidemic rabbit: implications for LDL-receptor function in steriodogenesis. *Metabolism.* **34:** 194–197.
39. Baumann, E. J., and O. M. Holly. 1925. Cholesterol and phosphatide metabolism in pregnancy. *Am. J. Physiol.* **75:** 618–632.
40. Popják, G. 1950. Maternal and foetal tissue- and plasma-lipids in normal and cholesterol-fed rabbits. *Biochem. J.* **46:** 547–561.
41. Zilversmit, D. B., L. B. Hughes, and M. Remington. 1972. Hypolipidemic effect of pregnancy in the rabbit. *J. Lipid Res.* **13:** 750–756.
42. Ross, A. C., and D. B. Zilversmit. 1976. Disposition of plasma cholesterol in the pregnant rabbit. *Am. J. Physiol.* **230:** 754–761.
43. Shiomi, M., T. Ito, and Y. Watanabe. 1987. Increase in hepatic low-density lipoprotein receptor activity during pregnancy in Watanabe heritable hyperlipidemic rabbits: an animal model for familial hypercholesterolemia. *Biochim. Biophys. Acta.* **917:** 92–100.
44. Thau, R., and J. T. Lanman. 1974. Evaluation of progesterone synthesis in rabbit placentas. *Endocrinology.* **94:** 925–926.
45. Zilversmit, D. B., M. Remington, and B. Hughes. 1972. Fetal growth and placental permeability in rabbits fed cholesterol. *J. Nutr.* **102:** 1681–1688.
46. Kondo, T., and Y. Watanabe. 1975. A genetic hyperlipidemic rabbit. *Exp. Anim.* **24:** 89–94.
47. Watanabe, Y., 1980. Serial inbreeding of rabbits with hereditary hyperlipidemia (WHHL-rabbit). *Atherosclerosis.* **36:** 261–268.
48. Olsson, G., A. M. Ostlund-Lindquist, G. Bondjers, O. Wiklund, and S. O. Olofsson. 1988. Quantification of plasma lipid and apolipoproteins in British Halflop rabbits: a comparison between normocholesterolemic rabbits, hypercholesterolemic rabbits (modified WHHL rabbits) and rabbits fed an atherosclerotic diet. *Atherosclerosis.* **70:** 81–94.