Validity for Epidemiological Studies of Long-Term Cryoconservation of Steroid and Protein Hormones in Serum and Plasma[1]

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Abstract

Prospective studies based on the storage of biological samples at low temperature have opened new perspectives in etiological research on cancer. In planning these studies a crucial question is to evaluate whether the long-term preservation of the samples is able to affect the categorization of the subjects involved. In the frame of the ORDET project, a prospective study of hormones and diet in the etiology of breast cancer provided with a −80°C biological bank, we have evaluated the stability of estradiol, free and total testosterone, progesterone, and prolactin in serum and plasma samples over 3 years of cryoconservation.

Study results showed that the subjects maintained almost the same rank by hormonal concentration throughout the 3-year period for all hormones. Looking at the stability over time, estradiol, prolactin, and total testosterone had fairly good performance for both serum and plasma. Serum-free testosterone increased in time up to 30%, whereas progesterone decreased by about 40% of the initial concentration. However, the reliability of the individual categorization by hormonal level suggests the validity of low temperature storage for epidemiological purposes, at least for hormonal parameters.

Introduction

In the last 2 decades several prospective studies have considered blood and urinary specimens stored at low temperature in biological banks (1) as a source of information. Biological markers from samples collected before the onset of disease and stored until clinical expression may provide essential information on exposure to endogenous and exogenous factors not biased by the metabolic effects of the illness.

A few epidemiological studies on cardiovascular disease (2–3) and cancer (4) observed changes, usually a decline, in the concentration of a number of hormonal and nutritional parameters after 10–12 years of storage at low temperature. However, most of these observations were not planned, temperature was not monitored, and, in many cases, the effect of low temperature storage could not be differentiated from that of repeated thawing of material. Therefore, systematic and quantitative information regarding the effect of cryoconservation on relevant endocrine parameters is still lacking.

The ORDET study (5) is a prospective cohort of 10,788 healthy women volunteers with the aim of investigating hormones and diet in the etiology of breast cancer; the biological bank includes aliquots of serum, plasma, 12-h urine, RBC membranes, and leukocytes, all preserved at −80°C. A corollary study is being carried out to monitor the validity of biological banking and to evaluate the long-term storage effect on the concentration of relevant hormones.

This report shows the results of 3 years of storage on estradiol, total testosterone, progesterone and prolactin in serum and plasma, and free testosterone in serum. The reason for choosing these five hormones is derived from their possible role in breast cancer etiology (6) and from the belief that they could be representative of the behavior of other steroid and protein hormones.

Materials and Methods

Subjects. Sixteen women from an Italian blood donor's association (Associazione Volontazi Italiani Sangue) were recruited for this study; 8 were in premenopause (luteal phase) and 8 were in postmenopause (not menstruating at least within the last 12 months before recruitment).

Specimen Collection and Handling. A total blood volume of 300 ml was collected from each subject by conventional venepuncture and was divided in equal volume in glass centrifuge bottles to obtain serum and plasma with sodium heparinate as anticoagulant (1 ml in 150 ml of whole blood). After clotting of the serum sample at room temperature, the bottles were centrifuged at 2000 × *g* for 15 min at room temperature.

Plasma and serum were divided into 1.5-ml aliquots in polypropylene cryotubes (Sarstedt, West Germany) without any preservative and stored at −80°C until analysis. On the day of assay, the aliquots were thawed at room temperature and thoroughly mixed before determination.

Assay Time Intervals. Blood samples were taken in June to July, 1988; baseline hormonal determinations were carried out in January 1989, and from that moment (hereafter referred to as B) the assay timing was the following: 1 year from B, all hormones; 1.5 years from B, free testosterone and progesterone; 2 years from B, plasma steroids only; and 3 years from B, all hormones.

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Table 1 Mean concentration levels of control sera at different assay times and the intra- and interassay CVs[a, b]

Hormone	Baseline	1 yr	1.5 yr	2 yr	3 yr	Cumulative intraassay CV %[b]	Interassay CV %
Estradiol (pmol/liter)	324 (15.2)	409 (13.5)		305 (14.6)	394 (10.6)	13.6	14.6
Total testosterone (nmol/liter)	2.39 (9.1)	1.70 (10.2)		2.53 (9.5)	2.19 (8.6)	9.2	16.9
Free testosterone (pmol/liter)	13.7 (8.2)	14.7 (7.9)	13.8 (8.9)		14.8 (8.6)	8.3	3.7
Progesterone (nmol/liter)	22.1 (10.2)	30.9 (11.4)	21.2 (12.5)	29.5 (9.9)	21.1 (10.5)	10.7	19.6
Prolactin (ng/ml)	10.4 (4.3)	10.1 (3.7)			10.7 (4.6)	4.2	2.6

[a] CV, coefficient of variation.
[b] Each mean value is calculated as the mean of three duplicates. Numbers in parentheses, intraassay CV percentage at each assay time.

Baseline values were determined in triplicate by assaying the samples in three different runs. Single assays were carried out on subsequent time stations. All determinations have been performed blind; however, the laboratory needed to know the menopausal status.

To save aliquots for future monitoring, the more stable hormones were assayed less frequently. Prolactin, for instance, proved to be very stable at 1 year, so that 1.5- and 2-year stations were dropped. Progesterone, however, was determined more frequently because of its notable decrease after 1 year.

Assay Techniques. Estradiol, total testosterone, and progesterone (the latter evaluated in premenopausal samples only) were measured with the use of in-house RIA, involving extraction from the sample with diethyl ether for estradiol and total testosterone and with petroleum ether for progesterone. The immunological step was performed directly on the ether extract with the use of tritium tracer and dextran-coated charcoal for the bound/free separation. Highly specific antisera were prepared by our own method (7).

The anti-estradiol-6-carboxymethyloxime-BSA showed a cross-reactivity of 1.6 and 0.6% for estrone and estriol, respectively; the anti-progesterone-11-succinyl-BSA showed a cross-reactivity of 0.2% and <0.01% for 17-hydroxyprogesterone and cortisol, respectively; and the anti-testosterone-3-carboxymethyloxime-BSA showed a cross-reactivity of 4.0 and 1.6% for androstenedione and dihydrotestosterone, respectively.

Extraction recoveries were 84 ± 7, 76 ± 15, and 88 ± 7% for estradiol, progesterone, and total testosterone, respectively. The experimental values were corrected for procedural losses.

Free testosterone was measured by coated-tube RIA with a "coat-a-counts" kit (Diagnostic Products Corp., Medical Systems, Genoa, Italy); prolactin with a "Maiaclone" kit (Ares-Serono Diagnostics, Milan, Italy) based on immunoradiometric assay technique with mAbs and a second antibody supported on magnetizable particles was used as a separating agent.

The intra- and interassay coefficients of variation percentage, measured during the study period with the use of a commercial lyophilized control sera (Lyphochek; Bio-Rad, Milan, Italy), were 13.6 and 14.6 for estradiol, 9.2 and 16.9 for total testosterone, 8.3 and 3.7 for free testosterone, 10.7 and 19.6 for progesterone, and 4.2 and 2.6 for prolactin, respectively. No drift of the control values was observed over the study period, indicating the absence of a systematic error during the experience, as shown in Table 1.

Statistical Methods. The level of agreement between the distribution at baseline *versus* each subsequent time station was evaluated by Pearson's r and Kendall's τ, respectively, for numerical and categorical distributions. The rank distributions at the first and third stations were compared with the baseline rank distribution adopted as reference. At each time station, mean hormonal level between subjects was computed. To account for nonnormality, Wilcoxon's nonparametric test for paired data was chosen to test the within-subject difference from baseline.

Results

Table 2 presents the mean concentrations of the considered hormones in serum and plasma at various times after cryoconservation for premenopausal and postmenopausal women. Indexes of agreement (r and τ) with the baseline distribution are also shown.

Table 3 shows the ranking of subjects at 1 and 3 years, respectively, with reference to the hormonal rank distribution at baseline.

Estradiol in serum showed quite similar mean values at baseline and after 1 and 3 years, both in high and low concentration samples (*i.e.*, in premenopausal and postmenopausal women, respectively). The correlations (r) were high, and the within-subject differences were not significant. On the contrary, the mean values of plasma estradiol displayed a large variability over time, and the within-subject differences from the baseline were large and significantly different. In spite of this variability, the correlation with the baseline distribution was very strong in all comparisons. However, the rank distribution (see τ and Table 3) showed a fairly good agreement with the baseline values in both serum and plasma. The swinging mean between subjects in plasma, together with the large within-subject differences and the high level of agreement of the distributions, suggest a between-assay laboratory effect.

Concentrations of total testosterone in premenopausal samples were similar or slightly lower (the differences were not significant) than in postmenopausal samples; therefore, comparisons can be made on the total number of subjects. Serum total testosterone levels showed only minor differences over 3-year periods, whereas in plasma total testosterone presented a larger variability. The individual categorization was quite good in both plasma and serum, particularly in postmenopausal women whose distributions by rank at 1 and 3 years were the same as at baseline (Table 3).

Serum-free testosterone levels were similar in pre- and postmenopausal samples. Looking at the whole set of samples, the trend of the between-subject mean suggests a systematic increase after the baseline. The differences within subjects were often important, but the correlation indexes were systematically high. The rank of the subjects did not substantially differ over the considered period.

Serum and plasma progesterone showed a progressive concentration decrease from the baseline to the third year of the study; despite this trend, the indexes of agreement among distributions remained very high, and the baseline rank was maintained almost perfectly at 1 and 3 years.

Table 2 Steroid and protein hormones in plasma and serum at different times of storage for 8 premenopausal (Pre) and 8 postmenopausal (Post) women. Mean values ($\bar{X}$) and Pearson's r and Kendall's τ indexes *versus* baseline

	Baseline	1 Yr			1.5 Yr[h]			3 Yr		
	$\bar{X}$	$\bar{X}$[a]	r	τ	$\bar{X}$[a]	r	τ	$\bar{X}$[a, b]	r	τ
Serum										
Estradiol (pmol/liter)										
Pre	344.1	350.9[c]	0.96	0.91				342.7[c]	0.93	0.64
Post	157.3	133.0[c]	0.99	0.69				158.4[c]	0.99	0.60
Total testosterone (nmol/liter)										
Pre	0.98	1.02[c]	0.73	0.40				1.27[d]	0.57	0.43
Post	1.32	1.23[c]	0.99	1				1.26[c]	0.94	1
All	1.15	1.12[c]	0.93	0.76				1.27[c]	0.80	0.73
Free testosterone (pmol/liter)										
Pre	4.47	5.09[c]	0.97	0.79	4.90[c]	0.93	0.79	5.51[d]	0.95	0.79
Post	4.02	5.28[d]	0.97	0.86	5.70[d]	0.89	0.71	5.59[d]	0.75	0.60
All	4.24	5.18[e]	0.96	0.87	5.30[e]	0.87	0.70	5.54[f]	0.89	0.67
Progesterone (nmol/liter)										
Pre	21.5	19.2[d]	0.99	1	16.2[d]	0.99	0.93	12.2[d]	0.98	1
Prolactin (ng/ml)										
Pre	10.38	10.63[c]	0.98	0.86				10.81[c]	0.97	0.86
Post	8.94	8.72[c]	0.95	0.79				8.56[c]	0.98	0.87
Plasma										
Estradiol (pmol/liter)										
Pre	426.6	358.7[d]	0.96	0.86	578.0[d]	0.95	0.71	355.1[d]	0.95	0.79
Post	170.0	119.7[d]	0.99	0.76	134.0[d]	0.99	0.67	135.3[d]	0.99	0.79
Total testosterone (nmol/liter)										
Pre	1.41	1.42[c]	0.98	0.93	1.46[c]	0.86	0.84	1.57[d]	0.91	0.74
Post	1.52	1.44[d]	0.99	1	2.16[d]	0.52	0.69	1.68[d]	0.96	1
All	1.47	1.43[c]	0.98	0.92	1.81[d]	0.55	0.78	1.62[f]	0.95	0.86
Progesterone (nmol/liter)[g]										
Pre	20.5	18.9[d]	0.99	0.93	13.0[d]	0.97	0.79	12.4[d]	0.99	1
Prolactin (ng/ml)										
Pre	11.03	10.92[c]	0.99	0.86				11.48[c]	0.98	0.86
Post	8.75	8.74[c]	0.92	0.79				9.35[c]	0.88	0.79

[a] P for Wilcoxon's test for matched-pair ranks *versus* baseline.
[b] At 3 years analyses were based on 6 subjects for estradiol in post, the corresponding baseline value is 186.1; 7 subjects for total testosterone in pre, the corresponding baseline value is 0.96; 6 subjects for total testosterone in post, the corresponding baseline value is 1.13; 13 subjects for total testosterone in all, the corresponding baseline value is 1.04; 6 subjects for free testosterone in post, the corresponding baseline value is 3.97; 14 subjects for free testosterone in all, the corresponding baseline value is 4.26; 6 subjects for prolactine in post, the corresponding baseline value is 8.91.
[c] Not significant.
[d] $P = 0.05$.
[e] $P = 0.005$.
[f] $P = 0.001$.
[g] Progesterone was also analyzed at 1.5 years from baseline with the following values: $\bar{X} = 14.8$; $r = 0.98$; $\tau = 0.86$.
[h] The intermediate evaluation in plasma samples was performed at 2 years instead of at 1.5 years from baseline.

Prolactin proved to be quite stable throughout the whole study period in serum and plasma for premenopausal and postmenopausal women. Correlation indexes were always very strong, and the individual ranks were almost the same over the 3-year period.

Discussion

We studied the long-term stability of estradiol, total testosterone, free testosterone, progesterone, and prolactin in frozen samples of plasma and serum stored at −80°C. Prolactin in both serum and plasma and estradiol in serum are stable, and total testosterone is also rather stable; conversely, progesterone and free testosterone concentrations seem to undergo progressive modification, with a 40% decrease and a 30% increase, respectively, over 3 years. However, this study shows that the subjects stayed in almost the same rank during the study period, even for progesterone and free testosterone, which were affected by stability problems over time.

To limit any source of variability that might affect the evaluation of cryoconservation, the following procedures have been strictly controlled: (*a*) blood was collected between 8 and 9 a.m. from healthy women in fasting conditions; (*b*) plasma and serum were obtained with a standardized procedure and all the aliquots were prepared in the same way; (*c*) freezer temperature was registered continuously (no accident occurred during the study period); (*d*) time spent for transportation of samples from the blood bank to the laboratory for hormone assays was the same for all the time stations, with aliquots packed in dry ice; and (*e*) to prevent any influence on behalf of laboratory technicians, all hormone assays were performed blind.

The major problem in comparing laboratory results in long-term studies is between-assay variability, which may be

Table 3 Hormonal rank distribution after 1 year and 3 years compared with the baseline rank distribution for serum and plasma for premenopausal (Pre) and postmenopausal (Post) women[a]

Baseline ranks by increasing hormonal values	1	2	3	4	5	6	7	8
Estradiol								
Serum								
Pre (yr)								
1	1	2	3	5–6	4	5–6	7	8
3	1	2	3	6	7	5	4	8
Post (yr)								
1	4–5	1	3	2	4–5	6	7	8
3	3	2	1			6	7	8
Plasma								
Pre (yr)								
1	1	2	3	5	4	6	8	7
3	1	2	3	4	7	6	5	8
Post (yr)								
1	3	2	1	4–5	4–5	6	7	8
3	3	1	2	4–6	4–6	4–6	7	8
Total testosterone								
Serum								
Pre (yr)								
1	2	7	1	3	5–6	5–6	4	8
3	1	7	2	4		6	3	8
Post (yr)								
1	1	2	3	4	5	6	7	8
3	1	2	3	4	5			8
Plasma								
Pre (yr)								
1	1	3	2	4	5	6	7–8	7–8
3	1	2	3	5–6	4	8	7	5–6
Post (yr)								
1	1	2	3	4	5	6	7	8
3	1	2	3	4	5	6	7	8
Free testosterone								
Serum								
Pre (yr)								
1	1	2	3	5	7	4	6	8
3	1	2	4	3	6	7	5	8
Post (yr)								
1	2	1	3	4	5	6	8	7
3		2	5	3	6	4		8
Progesterone								
Serum								
Pre (yr)								
1	1	2	3	4	5	6	7	8
3	1	2	3	4	5	6	7	8
Plasma								
Pre (yr)								
1	1	2	3	4	5	7	6	8
3	1	2	3	4	5	6	7	8
Prolactin								
Serum								
Pre (yr)								
1	1	2	4	3	5	6	8	7
3	1	2	3	4	6	5	8	7
Post (yr)								
1	1	3	2	4	5	8	6	7
3	1	2		4	5		8	7
Plasma								
Pre (yr)								
1	1	2	4	3	6	5	7	8
3	1	2	4	3	5	6	8	7
Post (yr)								
1	1	2	3	4	5	8	7	6
3	1	2	3	4	5	8	7	6

[a] Note: subjects presenting tie ranks were compared with all their ranks at baseline, *e.g.*, subjects with ranks 4 and 6 at baseline were ties at 1 year for estradiol in premenopausal serum.

affected by several factors, such as change of commercial kits, instrumental calibration, and environmental conditions. When the study was being planned, it was a period of major changes for assay technologies. Therefore, to avoid depending on the market, whenever possible, extraction methods for hormone assays were used instead of direct methods, which may not be available with the same performance for a period of many years. Actually, for estradiol, total testosterone, and progesterone, most of the kits that were available at baseline were subsequently withdrawn from commerce, and those still in commerce changed, both for the quality of antisera and for methodological conditions. Prolactin and free testosterone, however, were determined by direct method, which did not change over time.

The coefficients of variation were controlled constantly with the use of the same batch of commercial lyophilized sera with 3-year nominal stability, and no time drift was observed (Table 1).

In the statistical analysis of the experiment, it is not possible to separate cryoconservation and between-assay laboratory effects. It is reasonable to infer, however, that systematic laboratory errors are the main cause of variability when the mean values are swinging over time but the individual data tend to keep the same ranking, as in the case of estradiol in plasma. On the other hand, when the analysis discloses a monotonic trend, as in the case of progesterone and free testosterone, we cannot rule out a laboratory effect, but a cryoconservation effect is far more likely, especially if one takes into account the absence of drift with lyophilized control sera. For epidemiological purposes, however, what is most important in these cases is that the indexes of correlation with the baseline remain very high, suggesting a systematic effect of cryoconservation rather than a random effect. Actually, the rank of subjects according to the progesterone concentration after 3 years is exactly the same as at baseline, and the rank of free testosterone does not differ substantially (Table 3).

The 30% increase of free testosterone levels could be due to the progressive dissociation of the steroid-protein complex in frozen sera; the relative stability of total testosterone confirms this hypothesis, excluding structural modifications of the molecule. Some hints pointing to the occurrence of a dissociation of the steroid-protein complexes in stored sera were already available from epidemiological studies on free estradiol and breast cancer. Langley *et al.* (8) reported that the dissociation of dihydrotestosterone and estradiol from sex hormone-binding globulin increases with time in frozen serum samples. In a few studies a strong association of free estradiol with breast cancer is likely to have been caused by the fact that the samples of cases had been stored for a longer period than were those of controls (6).

The progressive decrease of about 40% on the initial concentration of progesterone over the 3-year period could be due to either a modification of the molecule or an interaction between the steroid and the material of the cryotube used in low temperature storage, despite declarations to the contrary by the manufacturer. In fact, the lipophil characteristics of this hormone might lead to absorption into the plastic walls of the cryotube. The plausibility of this interpretation is supported by experiments showing that several plastic materials, in particular polyvinyl chloride and siliconated rubber, may absorb steroids hormones and especially progesterone (9). On the other hand, Key *et al.* (10) reported apparently normal progesterone concentrations in serum stored at −20°C for about 7 years. We are not aware, however, of other formal studies of validity of sera cryoconservation for progesterone assay.

Several authors reported either stability over time (11–12) or some degradation (2–3, 13) of estradiol and/or testosterone in frozen serum, without providing full details of their experiments.

The interest in hormonal cryoconservation concerns both clinical and epidemiological studies. Although the actual hormonal concentration is the issue of major interest for clinicians, epidemiologists are more interested in understanding whether storage can modify the individual ranking. From the results of our study, we can draw the conclusion that serum stored at −80°C provides reliable quantitative information for the concentration of estradiol and prolactin and fairly reliable information on total testosterone; that plasma is reliable for prolactin and total testosterone; and that epidemiologists can also rely on the rank distribution of values for progesterone, free testosterone, and plasma estradiol, at least within 3 years from the time of storage.

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