PRESERVATION OF BONE MARROW CELLS OF DOG WITH HEPARIN AND EDTA *

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The extension of the experimental works on blood and bone marrow cell cultures in genetics, as well as in transplantation of the bone marrow has pointed out the problem of a better study about an eventual activity of anticoagulants on the viability of cells. Goerner (3) had reported that in the presence of 1% heparin, cells of carcinoma of Flexner-Jobling became not transplantable. Fisher (2) observed that even 0.05% heparin prevented the cellular division of fibroblasts and condrioblasts of chicken embryo, and according to Heilbrunn and Wilson (4) this same concentration inhibited the mitosis of eggs of Arbacis and Chaetopterus. In vivo, heparin was found by Lippman (7) to produce mitotic inhibition of cells of Ehrlich's ascitic tumor. In experiments with human bone marrow, Kurnick, Montano, Gerdes and Feder (6) verified that 5 unit concentrations, corresponding to 0.5 mg per ml of human bone marrow did not damage the cells, but this effect was produced by increased concentrations. Kreisler (5) reported that in transplantation of lymphosarcoma to mice, intravenous injections of heparin up to 15 units in intervals of 12 hours did not show any disadvantage, but this criterion can not be applied in confront to the others above mentioned.

On the other hand, some experiments with EDTA were also published, as those of McDonald and Kaufmann (9) in which ethylene-diamine tetracetate (EDTA) at the concentration level of 0.0004 to 0.002 M were found to cause mitotic changes in cells of the onion root. This fact was also observed by Davidson (1).

Soon after the experiments reported in this paper had been finished, Lochte, Ferrebee and Thomas (8) published results obtained through a comparative study between the action of heparin and EDTA on the DNA synthesis "in vitro" by bone marrow cells. They observed that concentrations of 0.025 to 1.0 mg of heparin/ml did not have an inhibiting activity, while 0.5 mg of EDTA/ml depressed

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the DNA synthesis after 4 hours of incubation, but this did not occur before 3 hours. They also observed that the phenol sometimes added to heparin had an inhibitory effect when in quantities above 0.01 g%.

The purpose of this work was to compare heparin and EDTA as to their ability to preserve bone marrow cells when used as anticoagulants. This was accomplished through the number of cells found on several days to verify their resistance of survival.

Material and methods

Normal dogs were anesthetized with intra-peritoneal injections of 15 mg nembutal and 10 mg morphine chlorhydrate per kg of body weight. Aspiration of 0.5 ml bone marrow was made from different ribs using needles with a lumen of 10/10 mm and syringes already containing the anticoagulant. Glassware was coated with silicone.

Commercial heparin for therapeutical use was employed in 1% solutions containing 0.18% of methyl *p*-hydroxybenzoate and 0.02% of propyl *p*-hydroxybenzoate. It was observed that 0.1 ml was the minimum volume required to moisten the foremost part of a 20 ml syringe, this procedure was carried out because it is the most frequently used. As a consequence, the heparin quantity used was 2 mg per ml of marrow. As for EDTA, 0.05 ml of a 10% solution of the disodic salt was used in the syringe, in the same way, corresponding to 10 mg EDTA concentration per ml of marrow.

The resulting suspension of aspirated marrow containing either heparin or EDTA was distributed in two flasks, one being stored at +5°C and the other at −15°C. The cells were counted in a counting chamber and before drawing the marrow from the flasks, they were throughly shaken for 1 minute. The cell countings were carried out immediately after obtaining the marrow suspensions and at intervals of 24 hours for three days. Thereafter, flasks kept at −15°C were defrosted at room temperature every day for the three days.

For observations of the red blood cells, six dogs were used and the same number for the nucleated cells maintained at +5°C. Four dogs were utilized for counting of these cells in the bone marrow preserved at −15°C.

Results

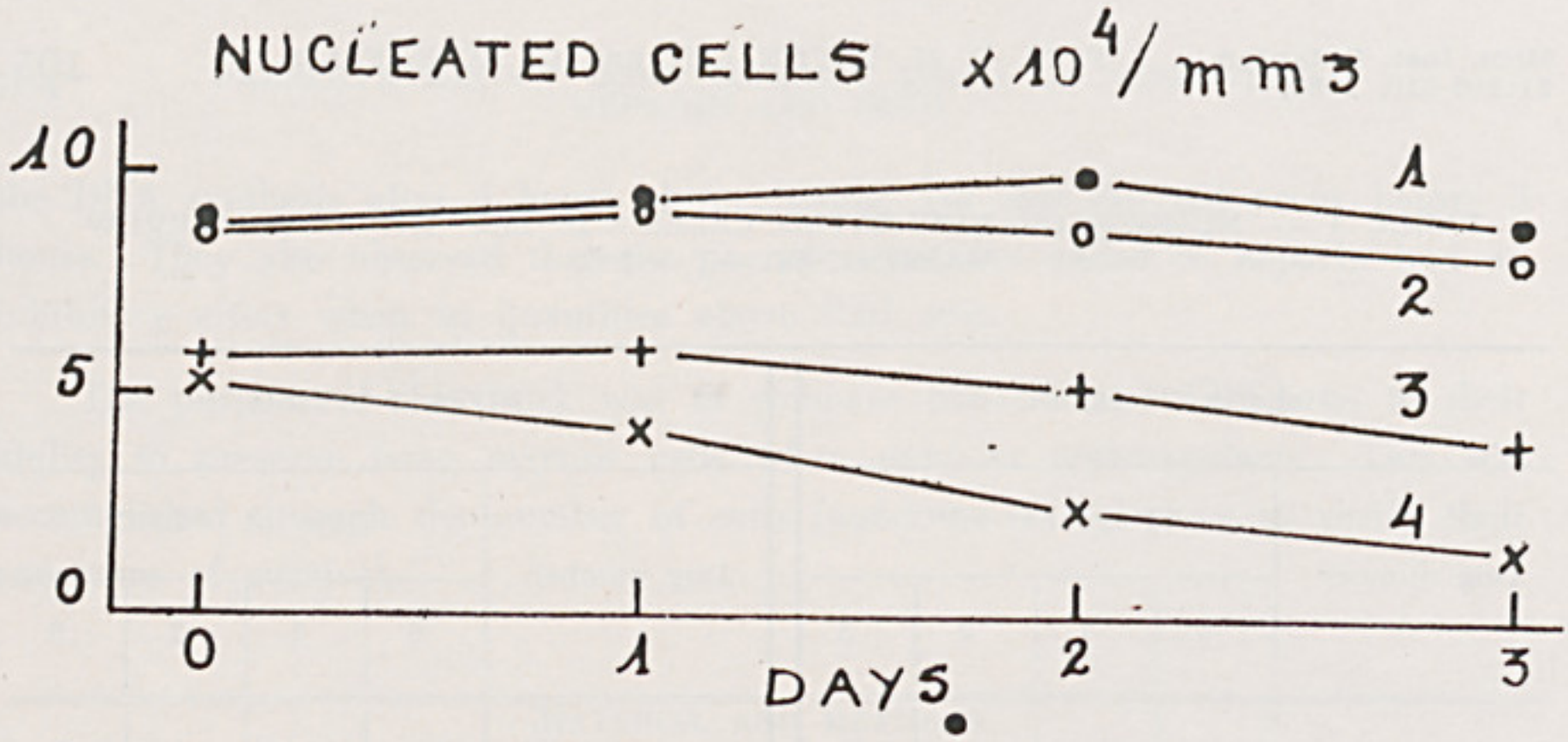
Bone marrow with heparin maintained at +5°C had a slightly increased number of nucleated cells after 24 hours, afterwards it decreased and finally, on the third day, it was very little below the initial number, but these modifications

TABLE 1 — NUMBER OF NUCLEATED CELLS × 10^3/mm³ IN BONE MARROW MAINTAINED AT +5ºC

Heparin 2 mg/ml					EDTA 5 mg/ml				
Dog number	Days at +5ºC				Dog number	Days at +15ºC			
	0	1	2	3		0	1	2	3
12	1044	1262	958	1024	12	672	641	804	741
13	319	301	284	309	13	572	545	658	532
14	928	1034	956	889	14	854	1029	1074	939
15	1029	968	1088	984	15	827	878	1087	936
17	1247	1312	1320	1169	17	1600	1909	1797	1626
18	628	638	638	534	18	722	633	552	633
Mean	866	919	874	818	Mean	874	939	995	901

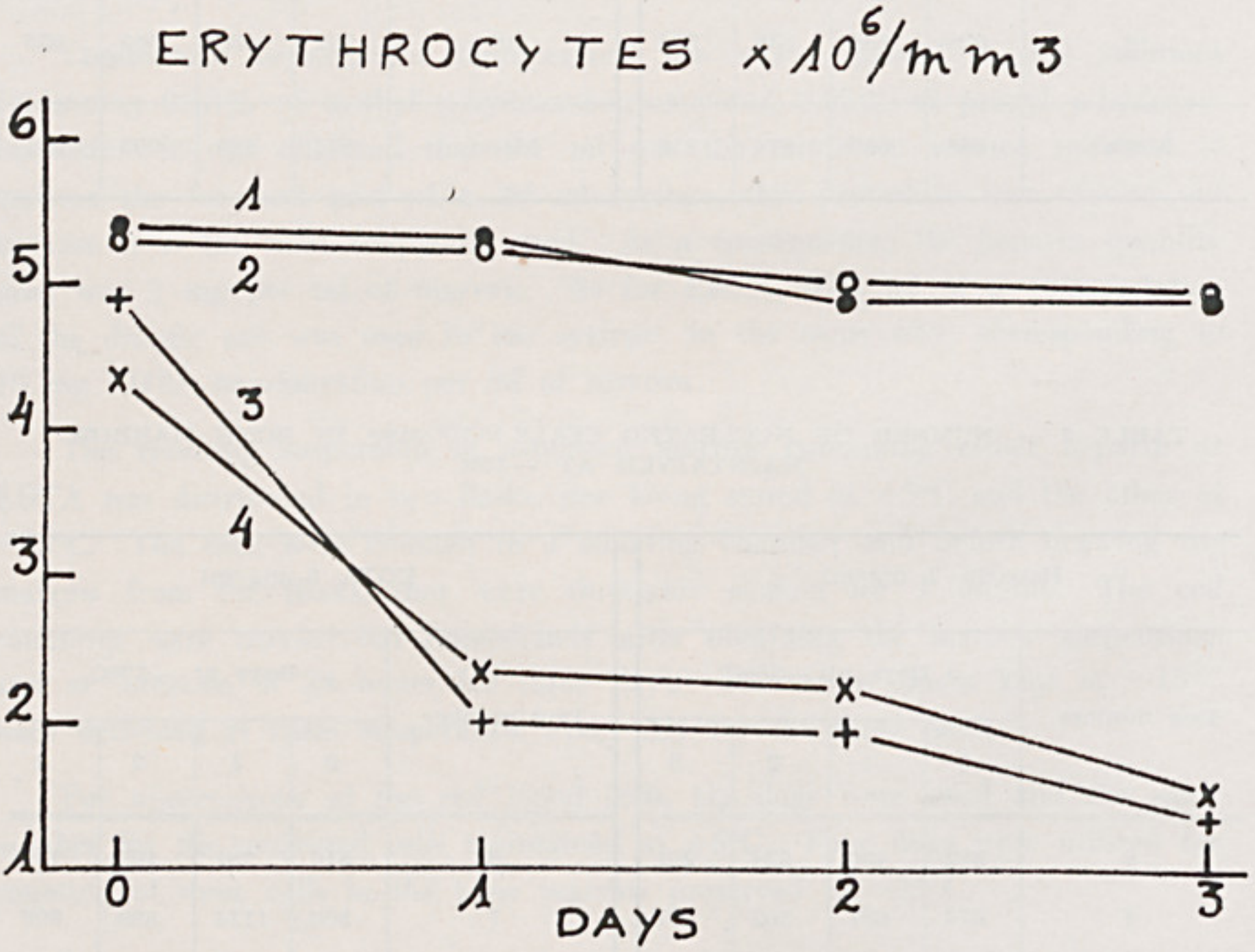
TABLE 2 — NUMBER OF NUCLEATED CELLS × 10^3/mm³ IN BONE MARROW MAINTAINED AT —15ºC

Heparin 2 mg/ml					EDTA 5 mg/ml				
Dog number	Days at —15ºC				Dog number	Days at —15ºC			
	0	1	2	3		0	1	2	3
6	382	490	224	201	6	310	231	195	159
7	674	687	502	287	7	800	1114	889	506
10	567	114	48	44	10	519	490	555	401
11	465	353	165	123	11	680	534	461	520
Mean	522	411	235	164	Mean	577	592	525	396



Graph 1 — Number of nucleated cells on bone marrow maintained at +5°C (mean numbers of 6 dogs) and −15°C (mean numbers of 4 dogs).

Curve
1 — EDTA +5°C
2 — Heparin +5°C
3 — EDTA −15°C
4 — Heparin −15°C



Graph 2 — Number of red blood cells in bone marrow maintained at +5°C and −15°C. Mean numbers of 6 dogs each.

Curve
1 — EDTA +5°C
2 — Heparin +5°C
3 — EDTA −15°C
4 — Heparin −15°C

were found not to be significant (Table 1, graph 1, curve 2). With EDTA, the increase of nucleated cells went forward until 48 hours and then decrease took place on the third day, when the same quantity of cells was recorded as in the beginning (Table 1, graph 1, curve 1), but the changes observed were found to be statistically non-significant.

Bone marrow kept at −15°C had a statistically significant decrease of nucleated cells, when mixed with heparin (Table 2, graph 1, curve 1), while the changes observed with EDTA were found to be statistically non-significant until the third day (Table 2, graph 1, curve 3).

As to the erythrocytes, their behavior was identical in regard to both anticoagulants. In the sample kept at +5°C, the number of cells diminished very little (Graph 2, curves 1 and 2), while in the frozen marrow there was a very marked decrease (Graph 2, curves 3 and 4).

Discussion

According to Rosenfeld (10) 1 mg/ml is the best concentration for EDTA to be used as anticoagulant when it is also able to preserve cells, as well as or better than heparin, and if heparin is used, the best concentration is 0.1 mg per ml of blood. In the experiments reported in this paper, greater quantities were used in order to maintain the commonly used conditions for preparing bone-marrow suspensions, such as syringe moistening with sterile solutions of anticoagulants, and also to give a better evidence on the unfavourable activity of these substances on cells.

As to the erythrocytes preservation, no difference between the two anticoagulants was observed. The rapid decrease of cell number in the frozen sample was obviously due to the successive thawing and freezing of samples. But in what nucleated cells are concerned, EDTA showed advantages in relation to heparin, not only because the increase of these cells went on for more time, indicating a smaller inhibition on their multiplication, but also because of a better protection in a period of three days.

It seems that EDTA can be used with advantages for replacing heparin as an anticoagulant, when bone-marrow may not be immediately used for purpose of cells culture or transplantation. The one thing necessary is to use measured quantities of any anticoagulant and not the syringe moistening, as it is usual, in order to avoid variations or excess of substances that could disturb preservation and viability of cells.

Summary

Dog bone marrow obtained by rib puncture was added to heparin in proportion of 2 mg/ml or to EDTA, 10 mg/ml. The samples were kept at +5°C or −15°C and the cell countings were made in 24 hours intervals up to the third day.

In marrow kept at +5°C, the number of nucleated cells seemed to increase slightly during the first day, decreasing afterwards in the course, on the last day, to values not far from the initial ones when heparin was used. With EDTA, the increase of cells continued up to 48 hours, descending on the third day to almost the same quantity, as that found in the beginning. In the frozen samples, there was a gradual decrease of the number of cells with heparin, while with EDTA there was still a slight addition in 24 hours and smaller decay on the third day, than with heparin. For erythrocyte preservation, there was no difference between heparin and EDTA.

The usual method of moistening the syringe with the anticoagulant result in the use of excess of substance that can be prejudicial, it is necessary to measure the volume of the anticoagulant solution.

Resumo

Medula óssea de cão obtida pela punção de costelas foi adicionada de heparina na proporção de 2 mg/ml ou de EDTA na proporção de 10 mg/ml. O material foi conservado em temperatura de +5°C ou −15°C e contagens de glóbulos foram feitas com intervalos de 24 horas até o terceiro dia.

Na medula conservada a +5°C, o número de células nucleadas aumentou ligeiramente no primeiro dia, descendo depois no último dia para valor próximo do inicial quando se usou heparina. Com o EDTA, o aumento das células continuou até 48 horas depois, descendo no terceiro dia para uma quantidade quase igual à inicial. No material congelado houve queda gradual do número de células com heparina, enquanto que, com o EDTA ainda houve ligeiro acréscimo nas 24 horas e queda menor do que com a heparina no terceiro dia. Para preservação de hemácias não houve diferença entre a heparina e o EDTA.

O EDTA apresentou algumas vantagens sôbre a heparina, permitindo uma melhor multiplicação das células e, preservando-as melhor quando congeladas.

O método usual de umedecer a seringa com o anticoagulante resulta do uso de quantidades excessivas que podem prejudicar, é necessário medir o volume da solução anticoagulante.

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