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# Extension of the lifespan of cultured normal human diploid cells by vitamin E: A reevaluation

**(cell aging/WI-38 cells/human diploid fibroblasts/cell pathology)**

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**ABSTRACT** **Previously we reported [Packer, L. & Smith, J. R. (1974) *Proc. Natl. Acad. Sci. USA* 71, 4763–4767] that the lifespan of WI-38 human diploid fibroblasts *in vitro* was significantly increased by continuously growing the cell cultures in the presence of vitamin E (*dl*-α-tocopherol), but in 19 subsequent subcultivation series we were unable to reproduce these findings. While vitamin E is incorporated into the cells and is able to act effectively as an antioxidant, apparently its intracellular antioxidant properties alone do not routinely result in an increase of cell lifespan. A synergism between vitamin E and some component(s) in the first of two lots of serum used in the original experiments seems the most likely explanation for our earlier findings.**

Normal human diploid fibroblasts manifest a finite *in vitro* lifespan, but it is not known to what extent this is a genetically determined and/or environmentally limited phenomenon. We previously reported a significant increase in the lifespan of WI-38 fetal human lung diploid fibroblasts cultured *in vitro* when the cultures were grown continuously in the presence of vitamin E (*dl*-α-tocopherol) (1), suggesting that environmental oxidative damage may be a significant factor in *in vitro* aging. In subsequent experiments we have been unable to repeat our previous results. The results from those experiments are reported here.

## MATERIALS AND METHODS

WI-38 cells were obtained from L. Hayflick of Stanford University, and used throughout these experiments. The details of culture procedures have been reported (1). Vitamin E (Sigma Chemical Co., St. Louis) was added to the culture medium from concentrated preparations made by blending vitamin E in complete medium (1) or by dissolving vitamin E in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the cell culture medium was 0.01% (vol/vol). Fetal bovine serum was obtained from Pacific Biologicals Co., Berkeley, CA and Reheis Chemical Co., Kankakee, IL.

## RESULTS

In our original experiments, vitamin E (10 μg/ml and 100 μg/ml) with and without hydrocortisone (5 μg/ml) was added to parallel cultures at the 45th population doubling level (PDL). The vitamin E was removed from some cultures at the 75th PDL, while some cultures were continuously exposed to vitamin E. Control cultures maintained in the absence of vitamin E ceased proliferation after 65 population doublings. All cultures were grown in Eagle's basal medium supplemented with 10% fetal bovine serum. For the early experiments, a single lot of fetal bovine serum was exhausted by the time the vitamin-E-treated cultures had reached about the 100th PDL. In Fig. 1, the cumulative number of population doublings is plotted as a function of time for cultures containing 10 and 100 μg/ml of vitamin E. The control cultures ceased proliferation after the 65th PDL. There was no sudden decrease in growth rate when the serum was changed (to Lot no. 4041c, Pacific Biological Co.); however, the use of the new lot of serum for the serial subcultivation of these cultures led to a loss of proliferative capacity within an additional 10 to 15 PDL.

In subsequent experiments, more than 19 subcultivation series were grown in medium that contained vitamin E. In these experiments serum from Pacific Biological Co. different from the lots mentioned above and serum from Reheis Chemical Co. were used. Vitamin E was added to the medium from blended concentrates and from dimethyl sulfoxide solutions. The mean lifespans of all these vitamin-E-treated cultures compared with the appropriate controls showed no significant increase (Table 1). Other investigators have indicated similar results (ref. 2; C. Ceccarini and L. Hayflick, personal communications), but have not published details. We have found that cells are able to incorporate vitamin E (3) and that the vitamin E is able to act effectively as an antioxidant and to protect the cells from some

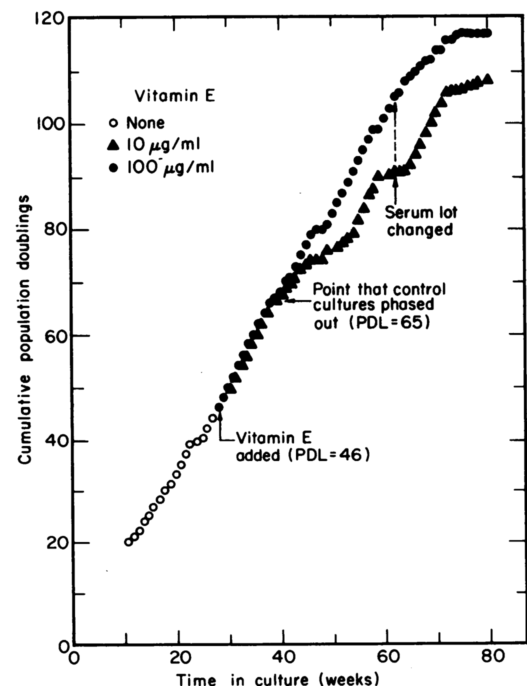


FIG. 1. Cumulative population doublings as a function of time in culture. Control cultures (O) were compared with cultures grown in medium containing vitamin E at 10 μg/ml (▲) or 100 μg/ml (●). Vitamin E treatment was begun at the 46th PDL. The serum lot was changed 33 weeks after initiation of vitamin E treatment. Control cultures stopped proliferation at the 65th PDL.

Abbreviation: PDL, population doubling level.

Table 1. *In vitro* lifespan of control and vitamin-E-treated cultures

| Vitamin E concentration, μg/ml | Culture series* | Lifespan, population doublings | |
|---|---|---|---|
| | | Control† | Vitamin-E-treated |
| 100‡ | A | 72 | 82 |
| | B | 76 | 71 |
| | B | 74 | 72 |
| | B | 72 | 74 |
| | C | 42 | 50 |
| | D | 58 | 58 |
| | Mean ± SD | 65.7 ± 13.2 | 67.8 ± 11.7 |
| 50‡ | C | 42 | 50 |
| | E | 63 | 50 |
| | F | 48 | 47 |
| | G | 49 | 50 |
| | H | 48 | 47 |
| | I | 51 | 51 |
| | I | 56 | 56 |
| | J | 52 | 49 |
| | K | 50 | 51 |
| | Mean ± SD | 51 ± 5.8 | 50.1 ± 2.7 |
| 10‡ | G | 49 | 51 |
| 10§ | L | 62 (60) | 51 |
| | M | 54 (56) | 45 |
| | N | 57 (59) | 57 |
| | Mean ± SD | 57.7 ± 4.0 (58 ± 2.9) | 51.0 ± 6.0 |

Vitamin E treatment was started by the 20th population doubling level in all cases and was continued throughout the *in vitro* lifespan.

* Each different culture series originated from a different starter culture obtained from L. Hayflick.

† Numbers in parentheses are for controls grown in the presence of 0.01% dimethyl sulfoxide.

‡ Vitamin E was added from a blended concentrate.

§ Vitamin E was added from a dimethyl sulfoxide concentrate.

forms of oxidative environmental stress (3, 4). Because vitamin E is able to act as an intracellular antioxidant when used in conjunction with several lots of fetal bovine sera but was not able to cause any increase in the lifespan, we have concluded that intracellular antioxidant properties alone were not responsible for the increase in lifespan we previously observed. However, it may be significant that we have found that WI-38 or IMR 90 human lung cells grown *in vitro* in 10% $O_2$ show a 20–40% increased lifespan (5). Because acute $O_2$ toxicity is partially reversed by vitamin E and/or selenium (4), it would appear that oxidative damage is an important factor in both short- and long-term cell viability in culture.

Other investigators have found that different serum batches result in different *in vitro* lifespans of diploid fibroblasts (6; E. L. Schneider, Y. Mitsui, and K. Braunschweiger, personal communication). However, because the lifespan of control cultures in the original lot of serum was not unusually long, the serum alone could not be responsible for the increased lifespan we observed previously. We feel that a likely explanation of our results is a synergism between some component(s) in the original lot of serum and vitamin E. However, we cannot as yet identify this presumptive component(s). Two of the batches of serum employed in this experiment were analyzed for their fatty acid composition profile. Marked differences were not observed except that the first serum (no. 1023) contained less arachidonic acid (20:4) and about half the total free fatty acids compared to the second batch of serum (no. 4041c), which was the one in use when culture senescence occurred. Total levels of unsaturated fatty acids were similar. Vitamin E, at the levels employed, might be anticipated to inhibit oxidative damage promoted by the presence of higher levels of free fatty acids in the "toxic" serum, but this cannot be unambiguously proven. Further studies on the requirements for hydrophobic molecules by cells in culture under conditions of oxidative stress seem warranted.

Our present knowledge of the nutrient requirements of normal cells in culture is too limited for us to be able to explain why the inclusion of vitamin E in medium during one set of experiments resulted in an extension of the *in vitro* lifespan of WI-38 cells, yet when vitamin E was used in other experiments no effect was observed.

These studies point out the need for a systematic approach to the study of the effect of cell nutrition on the *in vitro* lifespan of normal human cells in culture.

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