Demographic Approaches to the Study of Aging on Cell Cultures

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Abstract—Aging organisms die out in accordance with the “Gompertz law,” i.e., the probability of their death increases with age. Survival curve construction is the main tool for gerontologists to study aging and test anti-aging drugs. The analysis of survival curves includes obtaining some indices characterizing aging of the population, for example, the average and maximum lifespan, the mortality rate, and the aging rate. Testing geroprotectors can be correctly performed only by obtaining such curves. The dying out of stationary cell populations—bacteria, yeast, and mammalian cell cultures—also occurs in accordance with the Gompertz equation. In this regard, it is reasonable to use the construction of survival curves and their analysis to study the “aging” of non-subcultured cell cultures and testing anti-aging drugs on them. We used this approach in our experiments, due to which we were able to detect the positive anti-aging effect of the Quinton Marine Plasma on stationary phase aging culture of Chinese hamster cells.

Keywords: survival curves, Gompertz law, lifespan, cell aging, stationary phase aging, geroprotectors.

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Currently, the construction of survival curves for cohorts of animals/humans is the most reliable method for assessing the efficiency of influence of physical factors or biologically active compounds on the aging process. It is this approach but not the manifestation of age biomarkers (about which many researchers were so enthusiastic) that actually makes it possible to see how aging proceeds in groups of test animals as well as to test geroprotectors. Unfortunately, the survival curve construction is a labor-, time-, and cost-intensive process, whereas the technique of using biomarkers is much easier. Manifestation of biomarkers may correlate well with the chronological age of test organisms but not with aging (i.e., the time-dependent increase in the probability of death); however, many researchers ignore this fact.

Aging organisms die out in accordance with the Gompertz law. There are also non-aging organisms, the probability of death of which does not increase with age and sometimes even decreases [1]. To distinguish the former from the latter, it is necessary to analyze the shape of the survival curves of respective cohorts of organisms [2–5]. In very rare cases of complete lack of death, for example, in the case of freshwater hydra (Hydra magnipapillata or Hydra vulgaris) under certain conditions [1, 6], the survival curve is a horizontal line. The conclusion of whether or not one or another factor affects the aging process is made on the basis of changes in the shape of survival curves of aging organisms under its influence. It can be assumed that a true geroprotector (any agent that slows down the aging process) should cause a rightward shift in the survival curve without changing its shape (that is, it should increase both the average and maximum lifespan).

Survival curves are constructed not only for humans and animals but also for cell populations. For example, the dying out of a cell population is studied in radiobiology (dying out under exposure to increasing doses of radiation) [7–9]. In addition, survival curves are constructed in experimental studies of aging of yeast and bacteria in the chronological/“stationary phase” aging model [10–14]. We have previously shown that a mammalian cell culture in the “stationary phase aging” model dies out in accordance with the Gompertz law [15]. The aim of the present study was to attempt to use the procedures that are used for the analysis of the survival curves of experimental animals in experiments with non-subcultured mammalian cell cultures dying out in the course of stationary phase aging.

MATERIALS AND METHODS

Experiments were performed on transformed Chinese hamster cells of the established line B11-dii-FAF28 (clone 237), which was obtained from the Medical Genetics Research Center (Moscow). The cells were cultured at 37°C in Carrel glass flasks using
Dulbecco’s Modified Eagle’s Medium (HyClone, United States) supplemented with 5–10% bovine serum (PAA, Austria), penicillin (100 U/mL), and streptomycin (100 μg/mL). To maintain the mass cell culture, cells were subcultured at a ratio of 1 : 10–1 : 3 every 3–4 days. The cells were removed from the growth surface using a mixture (1 : 1) of 0.02% versene and 0.25% trypsin (Ivanovsky Institute of Virology, Ministry of Health of the Russian Federation, Moscow).

To assess the effect of various agents on the cell culture growth kinetics and subsequent dying out of cells in the stationary phase, 3-day-old cells were seeded into hermetically sealed glass flasks at a density of 20000–40000 cells/cm². The next day, the adherent cells were counted, and a medium containing the test compound was added to the flasks. The control flasks were supplemented with the medium containing a respective volume of solvent. At certain time intervals, the cells were removed from the growth surface with a mixture of trypsin and Versene solutions, and their number was evaluated in the standard hemocytometer (three or four flasks per point, four chambers per flask).

The obtained data were used to construct the growth, stationary phase, and death curves of cells in the control and experimental groups. Using the data on the kinetics of the cell culture dying out, the survival curves were constructed, the average lifespan (ALS) of the cell population was calculated, and the time at which 50 and 90% of the population in each group will die (the median lifespan (LS50) and 90% mortality (LS90), respectively) was determined (Fig. 1). In some cases, we used the Gompertz equation parameters that characterize survival, such as the modal lifespan (MLS, the time at which the population mortality rate is maximum, which corresponds to the inflection point on the Gompertz curve), the force of mortality at time zero (R₀), and the cell culture aging rate (α). To obtain these indices, the day on the time axis on which the “plateau” phase on the growth curve started was taken as the starting point (t₀) (Fig. 1).

ALS was calculated using the formula

\[
ALS = \frac{1}{N_0} \sum_{i=1}^{n} \left( t_{i+1} - t_{i} \right) \left( N_{i-1} - N_i \right).
\]

where \(N_0\) is the maximum cell culture density and \(t_i\) is the time at which the cell culture reached density \(N_i\).

The resulting cell survival curves were approximated using the Gompertz equation. The survival rates in the control and experimental groups were compared using the Kolmogorov–Smirnov test. Differences were considered statistically significant at \(p < 0.05\). Mathematical calculations and statistical data were processed using the SigmaPlot software ver. 12.0 (Systat Software Inc., United States). All results are represented as the mean value and the standard error of the mean.

**RESULTS AND DISCUSSION**

In the study, we obtained several survival curves for the non-subcultured cultures. In our experiments, cells, indeed, die out in accordance with the
Gompertz equation, i.e., undergo aging (Fig. 2). This fact suggests that their death probability increases exponentially with time, similarly to that of aging animals or humans [15, 16].

As mentioned earlier, the best way to assess the effect of various geroprotectors on aging is to construct survival/death curves for the studied model objects. The construction and analysis of survival curves for non-subcultured cell cultures allow us to test geroprotectors. In addition, we obtain a set of extra parameters—the average and maximum lifespan, the mortality rate, and the force of mortality. Since the variance in the “tail” of the curve rises due to increased heterogeneity of the population and reduced absolute number of cells, we have replaced the concept of the maximum lifespan with LS90. In most experiments, we assessed the full life cycle of the culture—growth, staying in the stationary phase, and dying out [17–19]. This approach has allowed us to conditionally divide cultures by “age;” a culture in the logarithmic growth phase is “young,” a culture in the stationary phase for 2–4 days is “mature,” and a culture in the dying out phase is “old.”

It is easier to obtain curves for cell populations than for some model organisms that may exist for a long time. However, the researcher faces another problem: it is unclear at which time a cohort can be regarded as formed. At the beginning of an experiment, cells first actively proliferate; later, their proliferation slows down and eventually stops, which coincides with the beginning of the “plateau” phase. We decided to take the moment of transition of a culture from the logarithmic growth phase to the stationary phase as time zero (cohort “birth” time) \( t_0 \). From this time moment, we perform approximation using the Gompertz equation and calculate all indices.

It is important to note that, in experiments in which the stationary phase aging model and analysis of cell culture survival curves are used, it is inadvisable to use the normal (diploid) cells, which have the “telomeric counter,” because this may additionally bias the resulting curves. In particular, growth will unequivocally differ for two cell cultures at different passages, and the dying out of a culture directly depends on the nature of its growth. For this reason, it is better to use transformed or immortalized cells, which do not have proliferative constraints [20].

It should be noted that, in studies with the use of the chronological yeast aging models, the chronological lifespan of a culture (its average and maximum lifespan) is also assessed [21, 22]. In such studies, the survival curves are constructed. However, we found no studies in which the approximation of results was performed. Moreover, it can often be seen from the shape of yeast survival curves that they cannot be approximated using the Gompertz equation because they do not have the so-called “arm.” This “arm” appeared in some studies where culturing conditions are changed, e.g., a buffer is added [23] or the composition of the culture media is changed [11]. This means that we can talk about “aging” of yeast only under certain conditions [24]. In many cases, researchers study dying out of cell cultures for other causes (depletion of nutrients or poisoning with decay products) but not aging.

In our experiment, which was performed in 2015, we found no anti-aging effect of the isotonic Quinton Marine Plasma (QMP) solution after our usual data processing [17]. We assessed differences only in several
points and found that, when 44.4% of Dulbecco’s Modified Eagle Medium was replaced with saline or QMP, the density of Chinese hamster cell culture on day 5 in the “saline” group was significantly higher than in the other two groups (Fig. 3a). It was also higher in the same group on days 31 and 36, though only when compared to the control indices (Fig. 3a). At first sight, these findings suggest that the addition of saline enhances the proliferative activity of cells and their survival in the late stationary phase, whereas QMP has no effect on the culture. However, a more detailed analysis of the data on the dying out of the cell culture using our cell kinetics approach showed that the ALS of the culture grown in saline was 17% lower than in the control, whereas the ALS of the culture grown in QMP was 7% higher than in the control (ALS\textsubscript{control} = 20.2 days, ALS\textsubscript{saline} = 16.8 days, and ALS\textsubscript{QMP} = 21.56 days; \( t_0 \) was 5 days). The cell culture in the group “saline” underwent dying out in accordance with a straight line rather than in accordance with the Gompertz equation. Data are represented as the mean value ± standard error of the mean. * Significant difference from the control group, # significant difference from both the control group and group QMP; \( p < 0.05 \).

Fig. 3. Effect of dilution of Dulbecco’s Modified Eagle Medium by 44.4% with saline or the isotonic Quinton Marine Plasma (QMP) solution on the kinetics of both growth and stationary phase aging of Chinese hamster cell culture. (a) Experimental data with superimposed approximation (dashed lines). (b) Approximation only; data were approximated in accordance with the Gompertz equation. Data are represented as the mean value ± standard error of the mean. * Significant difference from the control group, # significant difference from both the control group and group QMP; \( p < 0.05 \).
with the Gompertz equation (Fig. 3b), which indicates that the state of this culture was worse than in the other two groups. It can be assumed that a small part of the cell population in this group survives for a long time (even longer than in the other two groups: LS90_{control} = 27 days, and LS90_{QMP} = 30 days; however, even a smaller number of cells of this group than in the other two groups survives to the middle of the experiment (approximately day 20) (LS50_{saline} = 16 days, LS50_{control} = 22 days, and LS50_{QMP} = 23 days). In group “QMP,” index MLS, similarly to ALS and LS90, was higher than in the control group (MLS_{control} = 22.8 days, MLS_{QMP} = 23.7 days, and MLS_{saline} = 11 days). In all three groups, the distribution patterns of lifespan values significantly differed. The approximated survival curve in group “QMP” lies to the right of the control throughout the period of the culture dying out (Fig. 3b), suggesting the presence of anti-aging properties in isotonic QMP solution.

Thus, our new technique with the use of analysis of the survival curves makes it possible to more comprehensively analyze the nature of “aging” of the cell culture. This allows us to test potential anti-aging drugs, select conditions that are optimal for long-term culturing, and study aging of diverse cell lines and strains. It remains unclear whether stationary phase cell populations of the organism (e.g., neuronal populations) die out in accordance with the Gompertz law. Since it is known that the dying out of system’s components does not necessarily proceed in accordance with the same laws as the dying out of systems themselves, cell populations constituting the organism do not necessarily die out in accordance with the Gompertz law. Nevertheless, it can be assumed that this law applies to them as well.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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