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INFLUENCE OF D120-SOLUTION ON LIFE SPAN AND FERTILITY IN MICE

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ABSTRACT Prolonged administration to mice of the medicinal product with the working title "D120" appreciably increase the average and maximum lifespan of the mice either with different health status or having severe pathologies. Under some experimental conditions, fertility of the animals dramatically increased, which was manifested in a litter born by the animals in the 3rd and 4th year of their life.

KEYWORDS : life span, fertility, cumulative effect.

INTRODUCTION

The number of studies devoted to the fight against aging has markedly increased in recent years. Various research teams make their contribution to an increase in the life span of animals using different approaches to animal experiments. For example, by restricting calorie intake (Heilbornn & Ravussin, 2003), inhibiting cell signaling pathways with rapamycin (Wilkinson et al., 2012), using administration of nicotinamide (Cambronne et al., 2016) and other antioxidants (Shabalina et al., 2017), transplanting sex glands of young individuals into old ones (Goodman et al., 2016), with the help of pharmaceutical medications to "cleanse" the body from old cells (Baker et al., 2016), by reprogramming mature cells into stem cells (Ocampo et al., 2016), suppressing the work of the hypothalamus (Zhang et al., 2017), by transplanting the bone marrow of young laboratory mice into old mice (Das et al., 2019), or by using other techniques.

This report presents the findings of the research into the effects of the medicinal product "D120" on the life span of mammals. For this purpose, the authors used outwardly healthy mice of the same strain differing in the life span, as well as the animals suffering from severe lethal pathologies.

MATERIALS AND METHODS

Animals.

Three groups of mice were used to perform the experiments: the first group contained outwardly healthy 6-month-old C57Black/6 mice with a life span of about 2 years, the second group contained outwardly healthy 3-month-old C57Black/6 mice ("weak"), with a life span of about 1 year and the third group consisted of short-lived mutant B/KS-db +/ + m mice, representing the genetic model of type 2 diabetes. The animals of the latter group are considered an appropriate model of type 2 diabetes, suitable for researching and evaluating new effective treatments for the disease in the experiment. This model reproduces characteristic features of the stages in the development of type 2 diabetes:insulin resistance of tissues develops at the early stages (1-2 months), which is compensated by islet cell hypertrophy; the stage of absolute insulin deficiency, as in type 1 diabetes, develops within the period of 3-4 months; the late stage of diabetes mellitus develops in the 5th-6th months, which leads to a profound disruption of metabolism, cachexia and death of animals.

Observation of mice throughout the entire experiment was carried out in the premises of the Department of Experimental Animals (License № 00075-LS, distributed 15.12.2014). It is a 1-storey brick building with the area of 538 m^2. The mice were kept in a room of 16.5 m^2 with a large window, equipped with a supply and exhaust ventilation system, electric illumination, central heating, hot and cold water. The mice were placed in plastic cages (5-10 individuals in each) of 25x40 cm (1000 cm^2) and 20 cm high. The cages had a solid floor and were covered with a metal grid, on which food and water in special drinking containers were provided. Daily cage cleaning included removing the bedding (sawdust) and replacing food and water. The diet of the animals consisted of standard full-value feed in pellets and ordinary tap water.

Produkt D120 (Patent. Postnov, 2013). Only the mice from the experimental groups additionally received *per os* 0,25 µl of D120 in the mornings according to different schemes. Committee on Biomedical Ethics of the National Research Center for Epidemiology and Microbiology named after Prof. N.F. Gamaleya confirms (Protocol No. 12, 2017) that research studies aimed to expand life span of mice were performed in compliance with the moral and ethical norms and recommendations for humane treatment of laboratory animals reflected in the European Convention for the Protection of Vertebrates used for Experimental and other Scientific Purposes adopted on March 18, 1986 in Strasbourg.

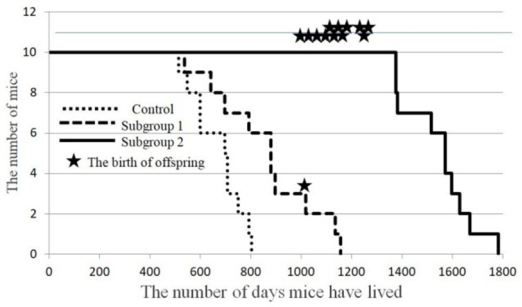
The statistical analysis was performed using the Kaplan-Meier multiplicative estimators (Kaplan & Meier, 1958), Gehan's generalized Wilcoxon test (Gehan, 1965) and the Cox-Mantel test F-criterion (Mantel, 1966) (*see Supplement:* "Cumulative proportion of mice surviving in the subgroups of the 1st group", "Cumulative proportion of "weak" mice surviving", "Cumulative proportion of diabetic mice surviving").

RESULTS

The animals from the first group - outwardly healthy 6-month-old C57Black / 6 mice - were divided into 3 subgroups of 10 animals in each. The animals of the 1st subgroup received *per os* 0,25 µl of D120 daily for 2,5 months. The animals of the 2nd subgroup received D120 in the same way daily over the entire observation period. Ten mice served as the control. Each subgroup of animals included both males and females: there were 8 females and 2 males in the first subgroup, 8 females and 2 males in the second subgroup and the control group contained 7 females and 3 males.

Starting from the 513-th day of life of the animals, the onset of their death was first recorded in the control subgroup, and soon in the 1st experimental subgroup (Fig. 1).

Figure 1. Dynamics in the death rate of the mice from different subgroups and the birth of offspring at the advanced periods of life



Subsequently, the mortality rate of the mice from the 1st experimental subgroup somewhat slowed down, as compared to the mortality rate of the animals from the control subgroup. This ultimately affected the indicators for the average and maximum life span of the animals in this subgroup.

A specific nature of the mortality rate was observed in the animals from the 2nd subgroup, where the onset of death was recorded when the animals reached the age of over 3,7 years. The mortality curve for the mice from this subgroup was less "stretched" in comparison with that for the animals in the 1st experimental subgroup. The data on the survival rate of each individual in the control and both experimental subgroups made it possible to determine the average and maximum life span of the animals in these subgroups (Table 1).

As shown in Table. 1, the average and maximum life span of the animals receiving D120 just for 2.5 months turned out to be 28% and 44%, respectively, longer as compared to the control.

Table 1. Average and maximum life span of mice in each group

Group	Number of days each of 10 mice have lived	Total number of days	Life span (days)	
			Average	Maximum
Control	513, 549, 598,598, 698, 708,708, 751, 793, 803	6719	671.9 **100%**	803 **100%**
1st subgroup	537, 642, 698, 793, 880, 880, 897, 1018, 1135, 1157	8637	863.7 **+28%**	1157 **+44%**
2nd subgroup	1376, 1376, 1382, 1517,1573, 1573, 1598, 1630, 1670, 1762	15457	1545.7 **+130%**	1762 **+119%**

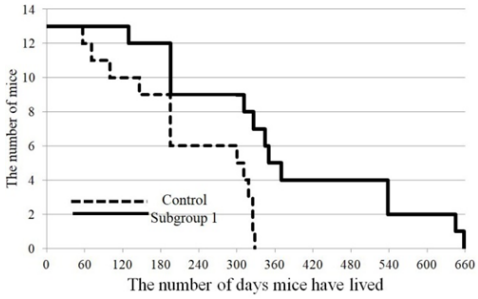
At the same time, prolonged administration of D120 into the animals of the 2nd subgroup allowed the authors to increase their average life span more than twice (by 130%) and maximum life span by 119%. (See *Supplement* "Cumulative proportion of mice surviving in the subgroups of the 1st group").

At the beginning of the study, we did not aim to determine fertility of the animals. However, the animals from the 1st and 2nd subgroups gave birth to offspring at the advanced periods of life, when they reached almost 3 years of age, which made us focus particular attention on this fact. By the end of the observation period, when all the mice in the control group had already died, we were able to record that the animals from the 1st subgroup gave birth to a single litter, whereas the animals from the 2nd experimental subgroup produced the offspring 12 times (Fig. 1). The total number of mouse pups born by the animals from the 1st and 2nd experimental subgroups was equal to 7 and 96 individuals, correspondingly.

The group of so-called "weak" C57Black/6 mice with a shorter life span consisted of two subgroups - the control and experimental ones of 13 animals in each. Control animals received ordinary tap water and standard feed in pellets. Mice from the experimental group additionally received *per os* 0,25 µl of the medicinal product D120 daily.

As anticipated, the onset of death in the control mice from the weakened group of animals was recorded already in the second or third months of their first year of life. Unlike previous observations, the control mice did not survive until the end of their first year of life (Fig. 2).

Figure 2. Dynamics in the death rate of the "weak" mice from different subgroups



In the experimental subgroup, the mice started dying in the fifth month of life. It took place at prolonged time intervals. The obtained data allowed the authors to determine the average and maximum life span of the animals (Table 2)

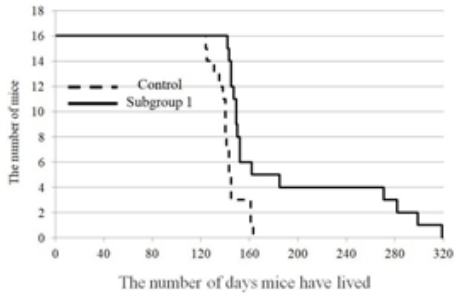
Table 2. Average and maximum life span of "weak" mice in each subgroup

Group	Number of days each of 13 mice have lived	Total number of days	Life span (days)	
			Average	Maximum
Control	57, 71, 100, 146, 195, 195, 195, 300, 311, 318, 325, 325, 328	2866	220,5 100%	328 100%
1st subgroup	129, 195, 195, 195, 311, 326, 344, 350, 370, 538, 538, 645, 658	4794	368,8 +67,3%	658 +100,6%

D120, was placed in a cage with experimental animals at the end of the working day.

As anticipated, the onset of mortality in the mice from the control group (Fig. 3) started from the 4th month of their life and was rather sudden. As expected, all control mice died before they had reached 6 months of age.

Figure 3. Dynamics in the death rate of the "diabetic" mice from different subgroups



At the same time, mortality of the mice that were receiving D120 was also characterized by a rather "steep" dynamics. However, the onset of their death started somewhat later. The process of a gradual death lasted much longer, as compared with the control. These differences are also expressed in the values of the average and maximum life span of the animals (Table 3).

Table 3. Average and maximum life span of "diabetic" mice in each subgroup

Group	Number of days each of 16 mice have lived	Total number of days	Life span (days)	
			Average	Maximum
Control	124, 125, 131, 135, 138, 140, 140, 140, 141, 143, 143, 145, 145, 161, 161, 163	2275	142,2 100%	163 100%
1st subgroup	142, 143, 145, 145, 147, 149, 149, 150, 152, 152, 162, 185, 271, 282, 299, 319	2992	187,0 **+31,5%**	319 **+95,7%**

So, owing to the administration of D120, the average and maximum life span of diabetic mice have increased by 31,5% and 95,7%, respectively, as compared to the control. (See *Supplement* "Cumulative proportion of diabetic mice surviving").

DISCUSSION

The obtained data make it possible to speculate about an obviously positive effect of D120 on the indicators for life span of the animals. This is clearly demonstrated by a significant increase in the average

and maximum life span in the animals of all three experimental groups. A comparative analysis of the survival curves of the healthy animals from the 1st group (Fig. 1) marks a certain similarity in the nature of the curves for the animals from the first and second experimental subgroups. However, analysis of the survival curves of the "weak" and diabetic mice treated with D120 exhibits some particular characteristic features: a distinctive "roughness", with the curve reaching the "plateau" of different lengths, is observed in both cases. It is likely to be caused by a positive cumulative effect of D120, which is realized as a result of its accumulation in the body over time. Also this effect could possibly be more pronounced in more severe pathologies. On the other hand, the reason for such cumulative effect may be associated with the varying sensitivity of animals to the effects of D120, as well.

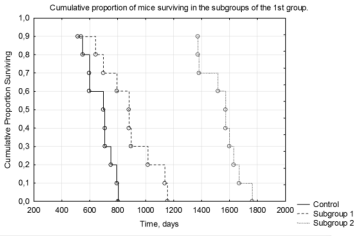
And finally, completely unexpected fertility at the advanced periods of life in the mice from the first group treated with D120 should be recognized as a direct argument in favor of the positive effect of D120 on the changes in general life indicators of the mice. This result allows the authors to speculate about the ability of D120 to preserve, to a certain extent, the quality of life typical of the young organism even at the mature age.

The noteworthy feature is the qualitative similarity in the positive effects of D120 on the general life indicators of the mice from the first group despite different schemes of D120 administration. Although the quantitative indicators of these processes were different, the similarity mentioned above may be attributed to the fact that D120 was administered to already mature individuals both in the 1st and 2nd experimental subgroups. More pronounced changes in the 2nd experimental subgroup may be associated with a more prolonged administration of D120 itself. Accordingly, an increase in the average and maximum life span, as well as fertility of the animals, indicates a significant increase in the duration of the active phase of animal life under the influence of the medicinal product D120.

Taking into account a recently appeared statement that «lifespan and sexual maturity depends on your brain more than your body» (Vandwerbilt University, 2018; Herculano-Houzel, 2018), we allow ourselves to suggest that in each of the three groups of the animals, D120 should have a positive impact on the mammalian brain; and humans are unlikely to be the exception (Herculano-Houzel, 2018).

Supplement

Comparison of control and subgroup 1



Survival Time	Variables: Time by Group(3 groups) (Survival Analysis) Censoring var.: Censor (Censored cases are marked with +) Chi? = 21,4114 df = 2 p = ,00002 Group	Score
513,00	Contro	-29,000(
537,00	Subgroup '	-27,000(
549,00	Contro	-25,000(
598,00	Contro	-22,000(
598,00	Contro	-22,000(
642,00	Subgroup '	-19,000(
698,00	Subgroup '	-16,000(
698,00	Contro	-16,000(
708,00	Contro	-12,000(
708,00	Contro	-12,000(
751,00	Contro	-9,000(
793,00	Contro	-6,000(
793,00	Subgroup '	-6,000(
803,00	Contro	-3,000(
880,00	Subgroup '	0,000(
880,00	Subgroup '	0,000(
897,00	Subgroup '	3,000(
1018,0	Subgroup '	5,000(
1135,0	Subgroup '	7,000(
1157,0	Subgroup '	9,000(
1376,0	Subgroup 2	12,000(
1376,0	Subgroup 2	12,000(
1382,0	Subgroup 2	15,000(
1517,0	Subgroup 2	17,000(
1573,0	Subgroup 2	20,000(
1573,0	Subgroup 2	20,000(
1598,0	Subgroup 2	23,000(
1630,0	Subgroup 2	25,000(
1670,0	Subgroup 2	27,000(
1762,0	Subgroup 2	29,000(

Group	Descriptive statistics for each group (Survival Analysis) Median	Mean	Std.Dv.	No.uncsd	N.censrd	Total N
Control	703,000	671,900	101,4741	10	0	10
Subgroup 1	880,000	863,700	203,6926	10	0	10
Subgroup 2	1573,000	1545,700	132,8265	10	0	10
Total	880,000	1027,100	408,6563	30	0	30

Comparison of control and subgroup 1

Survival Time	Gehan's Wilcoxon Test (Survival Analysis) WW = -56,00 Sum = 2650,0 Var = 697,37 Test statistic = -2,08272 p = ,03728 Group	R1	R2
513,00	Control	1,00000	20,00000
537,00	Subgroup 1	2,00000	19,00000
549,00	Control	3,00000	18,00000
598,00	Control	4,00000	16,00000
598,00	Control	4,00000	16,00000
642,00	Subgroup 1	6,00000	15,00000
698,00	Subgroup 1	7,00000	13,00000
698,00	Control	7,00000	13,00000
708,00	Control	9,00000	11,00000
708,00	Control	9,00000	11,00000
751,00	Control	11,00000	10,00000
793,00	Subgroup 1	12,00000	8,00000
793,00	Control	12,00000	8,00000
803,00	Control	14,00000	7,00000
880,00	Subgroup 1	15,00000	5,00000
880,00	Subgroup 1	15,00000	5,00000
897,00	Subgroup 1	17,00000	4,00000
1018,0	Subgroup 1	18,00000	3,00000
1135,0	Subgroup 1	19,00000	2,00000
1157,0	Subgroup 1	20,00000	1,00000

Risk	Cox-Mantel Test (Survival Analysis) I = 2,985392 U = -4,60736 Test statistic = -2,66656 p = ,00766 A(I)
10,00000	0,500000
10,00000	0,526316
9,000000	0,500000
9,000000	0,529412
9,000000	0,600000
8,000000	0,571429
7,000000	0,583333
7,000000	0,700000
7,000000	0,777778
6,000000	0,857143
6,000000	1,000000
4,000000	1,000000
3,000000	1,000000
2,000000	1,000000
1,000000	1,000000

Comparison of control and subgroup 2

Comparison of control and subgroup 2

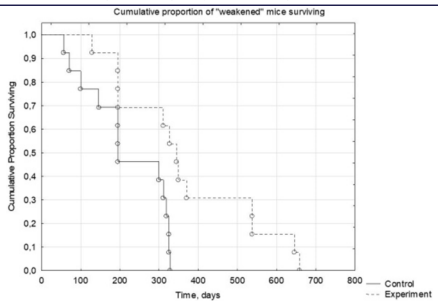
Survival Time	Gehan's Wilcoxon Test (Survival Analysis) WW = -100,0 Sum = 2652,0 Var = 697,89 Test statistic = -3,74749 p = ,00018 Group	R1	R2
513,00	Control	1,00000	20,00000
549,00	Control	2,00000	19,00000
598,00	Control	3,00000	17,00000
598,00	Control	3,00000	17,00000
698,00	Control	5,00000	16,00000
708,00	Control	6,00000	14,00000
708,00	Control	6,00000	14,00000
751,00	Control	8,00000	13,00000
793,00	Control	9,00000	12,00000
803,00	Control	10,00000	11,00000
1376,0	Subgroup 2	11,00000	9,00000
1376,0	Subgroup 2	11,00000	9,00000
1382,0	Subgroup 2	13,00000	8,00000
1517,0	Subgroup 2	14,00000	7,00000
1573,0	Subgroup 2	15,00000	5,00000
1573,0	Subgroup 2	15,00000	5,00000
1598,0	Subgroup 2	17,00000	4,00000
1630,0	Subgroup 2	18,00000	3,00000
1670,0	Subgroup 2	19,00000	2,00000
1762,0	Subgroup 2	20,00000	1,00000

	Cox-Mantel Test (Survival Analysis) I = 2,010208 U = -6,60742 Test statistic = -4,66027 p = ,00000
Risk	A(I)
10,00000	0,500000
10,00000	0,526316
10,00000	0,555556
10,00000	0,625000
10,00000	0,666667
10,00000	0,769231
10,00000	0,833333
10,00000	0,909091
10,00000	1,000000
8,000000	1,000000
7,000000	1,000000
6,000000	1,000000
4,000000	1,000000
3,000000	1,000000
2,000000	1,000000
1,000000	1,000000

Comparison of subgroups 1 and 2

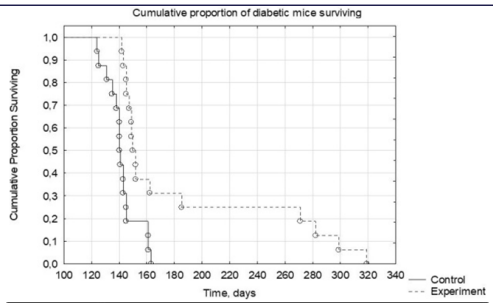
Survival Time	Gehan's Wilcoxon Test (Survival Analysis) WW = -100,0 Sum = 2654,0 Var = 698,42 Test statistic = -3,74608 p = ,00018		
	Group	R1	R2
537,00	Subgroup 1	1,00000	20,00000
642,00	Subgroup 1	2,00000	19,00000
698,00	Subgroup 1	3,00000	18,00000
793,00	Subgroup 1	4,00000	17,00000
880,00	Subgroup 1	5,00000	15,00000
880,00	Subgroup 1	5,00000	15,00000
897,00	Subgroup 1	7,00000	14,00000
1018,0	Subgroup 1	8,00000	13,00000
1135,0	Subgroup 1	9,00000	12,00000
1157,0	Subgroup 1	10,00000	11,00000
1376,0	Subgroup 2	11,00000	9,00000
1376,0	Subgroup 2	11,00000	9,00000
1382,0	Subgroup 2	13,00000	8,00000
1517,0	Subgroup 2	14,00000	7,00000
1573,0	Subgroup 2	15,00000	5,00000
1573,0	Subgroup 2	15,00000	5,00000
1598,0	Subgroup 2	17,00000	4,00000
1630,0	Subgroup 2	18,00000	3,00000
1670,0	Subgroup 2	19,00000	2,00000
1762,0	Subgroup 2	20,00000	1,00000

	Cox-Mantel Test (Survival Analysis) I = 2,029066 U = -6,64605 Test statistic = -4,66568 p = ,00000
Risk	A(I)
10,00000	0,500000
10,00000	0,526316
10,00000	0,555556
10,00000	0,588235
10,00000	0,625000
10,00000	0,714286
10,00000	0,769231
10,00000	0,833333
10,00000	0,909091
10,00000	1,000000
8,000000	1,000000
7,000000	1,000000
6,000000	1,000000
4,000000	1,000000
3,000000	1,000000
2,000000	1,000000
1,000000	1,000000



Survival Time	Gehan's Wilcoxon Test (Survival Analysis) WW = -93,00 Sum = 5774,0 Var = 1501,2 Test statistic = -2,37445 p = ,01758		
	Group	R1	R2
57,000	Control	1,00000	26,00000
71,000	Control	2,00000	25,00000
100,00	Control	3,00000	24,00000
129,00	Experiment	4,00000	23,00000
146,00	Control	5,00000	22,00000
195,00	Control	6,00000	16,00000
195,00	Control	6,00000	16,00000
195,00	Experiment	6,00000	16,00000
195,00	Control	6,00000	16,00000
195,00	Experiment	6,00000	16,00000
195,00	Experiment	6,00000	16,00000
300,00	Control	12,00000	15,00000
311,00	Control	13,00000	13,00000
311,00	Experiment	13,00000	13,00000
318,00	Control	15,00000	12,00000
325,00	Control	16,00000	10,00000
325,00	Control	16,00000	10,00000
326,00	Experiment	18,00000	9,00000
328,00	Control	19,00000	8,00000
344,00	Experiment	20,00000	7,00000
350,00	Experiment	21,00000	6,00000
370,00	Experiment	22,00000	5,00000
538,00	Experiment	23,00000	3,00000
538,00	Experiment	23,00000	3,00000
645,00	Experiment	25,00000	2,00000
658,00	Experiment	26,00000	1,00000

	Cox-Mantel Test (Survival Analysis) I = 3,794835 U = -5,87173 Test statistic = -3,01418 p = ,00258
Risk	A(I)
13,00000	0,500000
13,00000	0,520000
13,00000	0,541667
13,00000	0,565217
12,00000	0,545455
12,00000	0,571429
9,000000	0,600000
9,000000	0,642857
8,000000	0,666667
8,000000	0,727273
8,000000	0,888889
7,000000	0,875000
7,000000	1,000000
6,000000	1,000000
5,000000	1,000000
4,000000	1,000000
2,000000	1,000000
1,000000	1,000000



Survival Time	Gehan's Wilcoxon Test (Survival Analysis) WW = -176,0 Sum = 10870, Var = 2805,2 Test statistic = -3,30415 p = ,00095 Group	R1	R2
124,00	Contro	1,0000(	32,0000(
125,00	Contro	2,0000(	31,0000(
131,00	Contro	3,0000(	30,0000(
135,00	Contro	4,0000(	29,0000(
138,00	Contro	5,0000(	28,0000(
140,00	Contro	6,0000(	25,0000(
140,00	Contro	6,0000(	25,0000(
140,00	Contro	6,0000(	25,0000(
141,00	Contro	9,0000(	24,0000(
142,00	Experiment	10,0000(	23,0000(
143,00	Contro	11,0000(	20,0000(
143,00	Contro	11,0000(	20,0000(
143,00	Experiment	11,0000(	20,0000(
145,00	Contro	14,0000(	16,0000(
145,00	Contro	14,0000(	16,0000(
145,00	Experiment	14,0000(	16,0000(
145,00	Experiment	14,0000(	16,0000(
147,00	Experiment	18,0000(	15,0000(
149,00	Experiment	19,0000(	13,0000(
149,00	Experiment	19,0000(	13,0000(
150,00	Experiment	21,0000(	12,0000(
152,00	Experiment	22,0000(	10,0000(
152,00	Experiment	22,0000(	10,0000(
161,00	Contro	24,0000(	8,0000(
161,00	Contro	24,0000(	8,0000(
162,00	Experiment	26,0000(	7,0000(
163,00	Contro	27,0000(	6,0000(
185,00	Experiment	28,0000(	5,0000(
271,00	Experiment	29,0000(	4,0000(
282,00	Experiment	30,0000(	3,0000(
299,00	Experiment	31,0000(	2,0000(
319,00	Experiment	32,0000(	1,0000(

Risk	Cox-Mantel Test (Survival Analysis) I = 5,244139 U = -7,40532 Test statistic = -3,23375 p = ,00122 A(I)
16,00000	0,500000
16,00000	0,516129
16,00000	0,533333
16,00000	0,551724
16,00000	0,571429
16,00000	0,592593
16,00000	0,666667
16,00000	0,695652
15,00000	0,681818
14,00000	0,736842
12,00000	0,800000
11,00000	0,785714
9,000000	0,750000
8,000000	0,727273
6,000000	0,666667
6,000000	0,857143
5,000000	0,833333
5,000000	1,000000
4,000000	1,000000
3,000000	1,000000
2,000000	1,000000
1,000000	1,000000

Conflict of interests:

the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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