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The Influence of the Xymedon Conjugate with L-Methionine on the Regeneration of *Schmidtea mediterranea* Planarians

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Abstract
The effect of the Xymedon conjugate with methionine on blastema regeneration in *Schmidtea mediterranea* planarians was investigated. It was established that the preparation has low toxicity towards planarians, which is evidenced by its CL50 values (lethal concentration). The results of the vital computer morphometry and flow cytofluorometry confirm that Xymedon with methionine induces regeneration at high concentrations only: 0.01 g/100 mL, 0.05 g/100 mL, and 0.1 mg/100 mL. Low concentrations of the preparation have no influence on blastema regeneration in planarians.

Keywords Regeneration · Planarians · Pyrimidine derivatives · Xymedon conjugates · Hepatoprotectors

1 Introduction

Xymedon (1,2-dihydro-4,6-dimethyl-1-*N*-(2-hydroxyethyl)-pyrimidine-2-one) is a pyrimidine derivative with proved regenerative, anti-inflammatory, wound healing, and immunomodulatory properties [1] and marked hepatoprotective effect [2].

Here, with the help of vital computer morphometry and flow cytometry of the blastemic cells, we studied how the proliferative activity of a conjugate of Xymedon with L-methionine (1,2-dihydro-4,6-dimethyl-1-(2-hydroxyethyl)-pyrimidine-2-one L-methionate) (Fig. 1) [3] affects the regeneration ability of *Schmidtea mediterranea* (Benazzi, Baguñà, Ballester, Puccinelli & Del Papa, 1975) planarians. Our previous studies on Xymedon show that this compound is effective as a regeneration-promoting agent in *Dugesia tigrina* planarians [4]. Xymedon with methionine was also found to have a neuroprotective potential [5]. Interestingly, it has been recently reported [6] that the Xymedon conjugate with methionine has less prominent hepatoprotective properties than Xymedon itself.

Invertebrate hydrobionts fit well in many cases as alternative model objects for explaining the action of medications: flatworms have been successfully used to investigate stem cell biology and regeneration mechanisms [7]. In this study, we used cultured *S. mediterranea* planarians, a popular experimental model for getting a better insight into the regenerative abilities [7], as a test object to assess the effect of Xymedon on regeneration.

2 Material and Methods

The study was performed on the laboratory culture of *Schmidtea mediterranea* (Platyhelminthes, Tricladida) asexual planarians (800 specimens in total). The planarians were precultured under laboratory conditions at 26 °C and fed on larval dipterans. Experiments were performed with specimens 7–8 mm long. They were kept in starvation for 7 days and then divided into the experimental groups ($n = 30$ per group) and the control group ($n = 30$). Regeneration was initiated by amputation of the head end in the area of eyes (Fig. 2). First, the lethal (CL50) and maximum no-observed-effect (NOEC) concentrations of Xymedon with methionine were determined. Based on the CL50 and NOEC values, the optimal working concentrations were selected. The kinetics of head end growth in the decapitated planarians was measured by vital computer morphometry [8, 9]: a photocontrast between old pigmented

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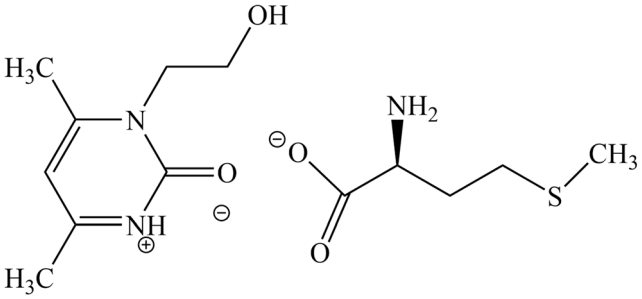


Fig. 1 Structure of conjugate of Xymedon with L-methionine

and new (regenerating) unpigmented body parts was recorded. Blastema growth was registered 72 h after the decapitation. All experiments were carried out in triplicate. The regeneration index was calculated by the following formula as a ratio of the regenerating blastema area to the total body area of a decapitated planarian: $R\,(\%) = (s/S) \times 100$, where s is the blastema area, S is the area of an entire regenerating planarian [9]. The blastema images were taken under a HIROX KH-7700 microscope.

The quantitative characteristics of the groups were compared with the non-normal distribution values using the Kruskal–Wallis H test. Post hoc multiple comparisons were performed with the help of the Dunn test. The differences were considered statistically significant at $P \leq 0.05$ and insignificant at $P > 0.10$. Borderline cases ($0.05 < P \leq 0.10$) were accepted as tendencies to differences. All calculations and diagrams were made using the Past V. 3.26 (Paleontological Statistics) software package.

The effect of the Xymedon conjugate with methionine on the proliferation intensity of planarian neoblasts was studied by flow cytofluorometry [8]. Quantitative estimation of the cell cycle-related changes in the system of planarian stem cells was performed in various physiological conditions (intact and regenerating specimens) and under the effect of the preparation. For this purpose, a cell suspension was made from the tissue of the postblastemic region incubated for 3 days at the investigated concentrations of the preparation. Blastemic pieces were placed at room temperature for 10 min into an Eppendorf tube containing 300 μL of 0.1 M citric acid and 1.5 μL Tween 20. Then, the tissue was suspended by shaking it for 10 s in a Vortex. The obtained cell suspension was mixed with 1 mL of 0.4 M NaH2PO4 of the EtBr-containing Hoechst 33342 fluorescent dye (10 μg/mL; Sigma). The cells were incubated at room temperature for 30 min and analyzed with a Millipore Guava EasyCyte 12HT cytometer at a rate of 100–200 cells/s. Cytometric profiles of the cell suspensions showing the total number of cells and cell cycle phases (G0/G1, S, G2/M) of the regenerating planarians were obtained.

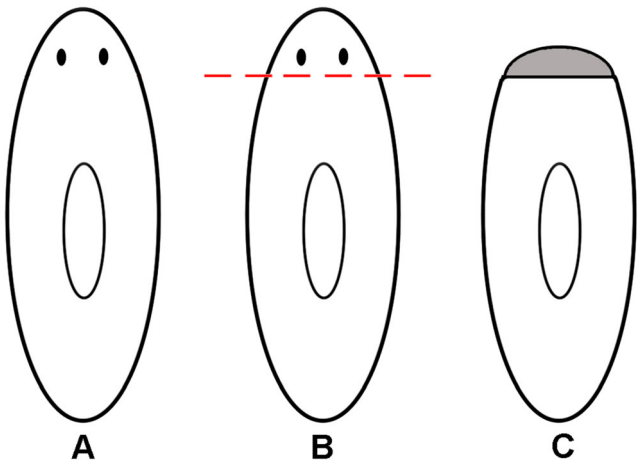


Fig. 2 Scheme of the operation (**a**). Animals were operated behind the eyes (**b**). Planarians after decapitation with blastema area (**c**)

3 Results and Discussion

The following concentrations were selected to determine CL50 values: 1 mg/100 mL, 0.5 mg/100 mL, 0.1 mg/100 mL, and 0.05 mg/100 mL of water. The analysis shows that these concentrations have no toxic effect (such as motion suppression or tissue lysis) on planarians, thereby confirming that the Xymedon conjugate with methionine is practically non-toxic. These results agree with our previous studies on Xymedon [4]. According to [10, 11], the concentration of any substance higher than 2 g/L is critical for hydrobionts.

The working concentrations were as follows: 0.001 mg/100 mL, 0.01 mg/100 mL, 0.03 mg/100 mL, 0.01 g/100 mL, 0.05 g/100 mL, and 0.1 g/100 mL.

Highly significant differences were found between the mean R values at different concentrations of the preparation: $H_{(6)} = 65.75$; $P < 0.001$. The post hoc multiple comparisons of the mean R values with the control values indicate that they occur due to the differences at the concentration of 0.1 g/100 mL ($P = 0.005$) of the Xymedon conjugate with methionine (Table 1). The differences from the control group were also statistically significant at the low concentrations of the preparation (0.001 mg/100 mL ($P = 0.001$); 0.01 mg/100 mL ($P = 0.003$); 0.03 mg/100 mL (P = 0.001). The R value decreased at the low concentrations of the preparation and, on the contrary, increased at the high ones (Fig. 3).

For studying the cell cycle in the postblastemic tissue of the regenerating *S. mediterranea* by the method of flow cytometry, only the highest concentrations of the preparation were considered, i.e., those that were effective during the vital computer morphometry.

Table 1 The comparison of the regeneration index for planarians at different concentration levels of the Xymedon conjugate with L-methionine and in the control group, based on Dunn's post hoc (*P* values, the level of significance)

	Control	0.001 mg/100 mL	0.01 mg/100 mL	0.03 mg/100 mL	0.01 g/100 mL	0.05 g/100 mL	0.1 g/100 mL
Control		0.001093	0.003144	0.001219	0.1388	0.09169	0.004812
0.001 mg/100 mL	0.001093		0.7117	0.9321	1718E-05	8505E-06	3745E-07
0.01 mg/100 mL	0.003144	0.7117		0.7735	5495E-05	2751E-05	3745E-07
0.03 mg/100 mL	0.001219	0.9321	0.7735		1795E-05	8768E-06	1051E-07
0.01 g/100 mL	0.1388	1718E-05	5495E-05	1795E-05		0.8421	0.2034
0.05 g/100 mL	0.09169	8505E-06	2751E-05	8768E-06	0.8421		0.2845
0.1 g /100 mL	0.004812	3745E-07	3745E-07	1051E-07	0.2034	0.2845	

The number of cells in the S and G2/M phases was higher in the experimental groups than in the control group. In the G2/M phase, Xymedon with methionine at the concentrations of 0.01 and 0.05 g/100 mL of water enhanced cell proliferation by 11% and 13%, respectively, compared with the control group. Upon the treatment with the preparation at 0.1 g/100 mL of water, this parameter in the same phase increased insignificantly (by 4%). In the control group, the percentage of cells in the G2/M phase exhibited only a minor increase (0.9%) (Fig. 4). In the S phase, the number of cells grew from 1.62% (control group) to 1.87% (at the concentration of 0.1 g/100 mL). The proportion of cells in the S phase multiplied significantly at 0.01 g/100 mL of the preparation (3.88%), reaching its maximum value at the concentration of 0.05 g/100 mL (6.48%) (Fig. 4).

The scatter diagrams showing a relationship between the cell size and cell content heterogeneity (Fig. 5) were built. The Y-axis represents side scattering (cell content heterogeneity); the X-axis represents forward scattering (cell size). Diagram A is characterized by low dispersion, skewed linearity. It suggests that there are two different neoblast populations. Diagrams B and C are clearly non-linear as well. On diagrams B, C, and D, the scattered points cover a wider area. Thus, the cells can be divided into two groups: (1) small cells with a more homogeneous content, such as somatic cells in the G0/G1 phase (in the left bottom part of the diagrams) and (2) large, possibly proliferating, cells with heterogeneous content in the S and G2/M phases (in the right upper part of the diagram). The heterogeneity of the cell content may be attributed to DNA duplication, protein synthesis, and early spindle assembly and nuclear membrane degradation during the S and G2/M phases.

The results of the flow cytometry of the blastomic cells add to the data obtained by other methods and confirm that the Xymedon conjugate with methionine induces cell proliferation at extremely high concentrations only.

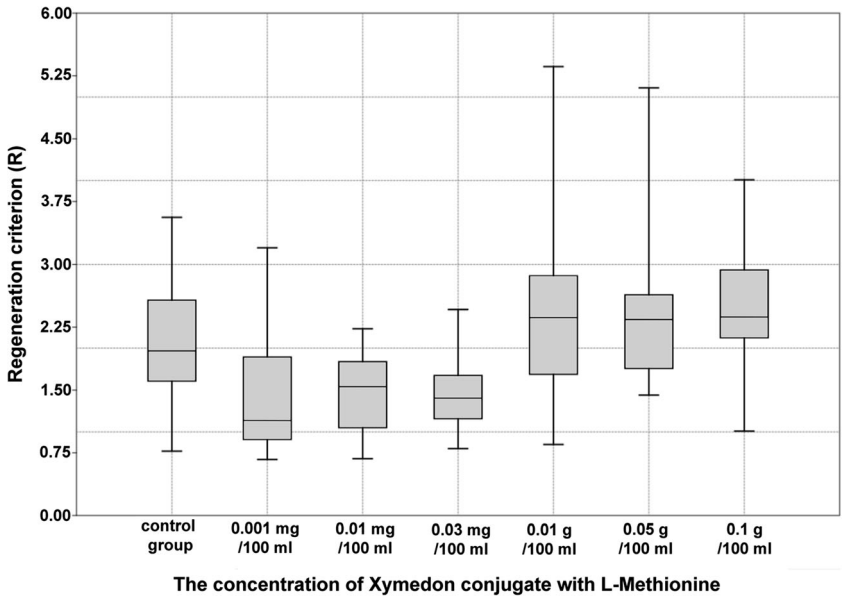
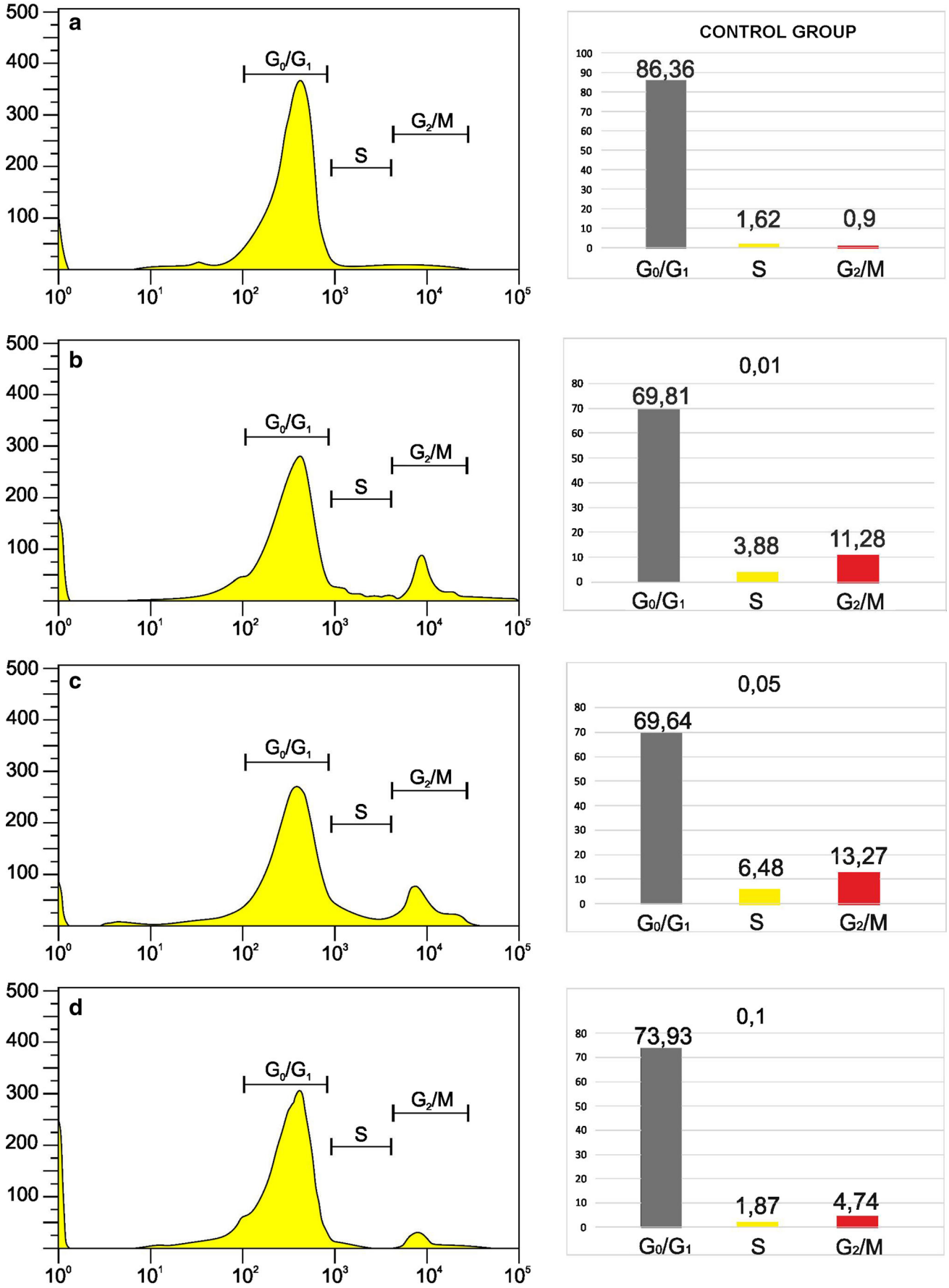


Fig. 3 Regeneration index for various concentrations of the Xymedon conjugate with L-methionine (median (Q1–Q3))


a
G0/G1
S
G2/M
CONTROL GROUP
86,36
1,62
0,9
G0/G1
S
G2/M
b
0,01
69,81
3,88
11,28
c
0,05
69,64
6,48
13,27
d
0,1
73,93
1,87
4,74

◀ **Fig. 4** Comparison of the flow cytometric profiles of the cell suspensions obtained from the blastema of the regenerating *S. mediterranea* specimens (**a** control group) and from the regenerants incubated for 72 h at different concentrations of the preparation dissolved in water: **b** 0.01 g/100 mL; **c** 0.05 g/100 mL; **d** 0.1 g/100 mL. On the right side of the cytograms, their histograms of cell distribution in each phase are provided for all groups

4 Conclusions

We established that the Xymedon conjugate with methionine has low toxicity towards planarians. The obtained CL50 values testify to the extremely low toxicity of the preparation. The results of the vital computer morphometry suggest that the high concentrations (0.01 g/100 mL; 0.05 g/100 mL, and 0.1 mg/100 mL) of Xymedon with methionine induce blastema proliferation in *S. mediterranea* planarians. The flow cytometry showed that the effect of the preparation is strongest at the concentration of 0.05 g/100 mL of water. Even the lowest concentrations of the preparation increased the number of cells in the S and G2/M phases. Thus, the Xymedon conjugate with methionine is a potent proliferation inducer.

Author's Contribution Equal contribution.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval *Schmidtea mediterranea* planarians are well-known objects for the study of regeneration in the world. The formation of the blastema is always reached by cutting off the edges of the planarian body. Blastema formation occurs due to migration and division of neoblasts—pluripotent cells. Planarians have a very simple nervous system and do not have the sensitivity like mammals. All planarians used in the experiment survived and restored their front ends with eyes.

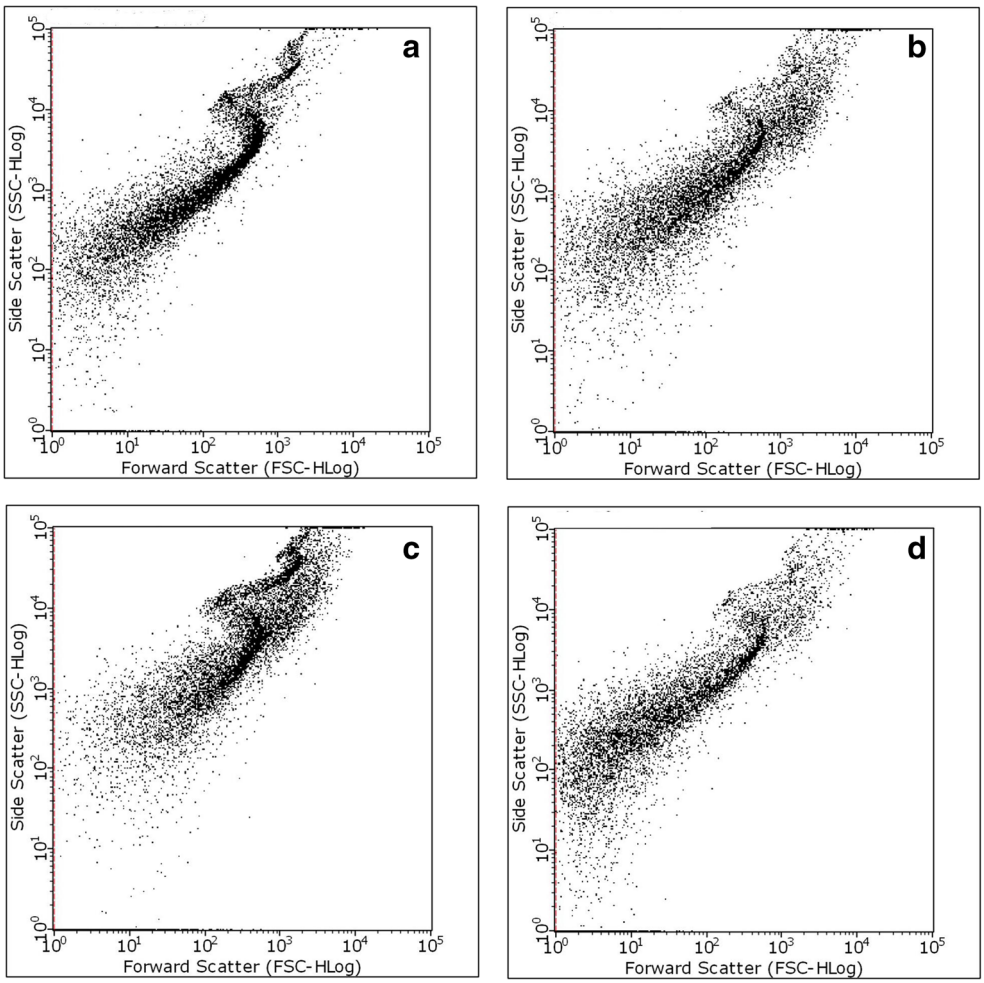

Fig. 5 Scatter diagrams showing the relationship between the cell size and cell content heterogeneity. **a** Control group; **b** 0.01 g/100 mL; **c** 0.05 g/100 mL; **d** 0.1 g/100 mL

Consent to Participate All authors agree to participate in this investigation.

Consent for Publication All authors agree to participate in this article.

Code Availability Not applicable.

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