Letter to the Editor

S Phase Cell Percentage Normalized BrdU Incorporation Rate, a New Parameter for Determining S Arrest*

ZHAO Peng[1,2], FU Juan Ling[1], YAO Bi Yun[1,2], JIA Yong Rui[3], and ZHOU Zong Can[1,#]

In this study, a new parameter, S phase cell percentage (S fraction) normalized BrdU (SFN-BrdU) incorporation rate, was introduced to detect S arrest. The results showed a positive linear correlation between the BrdU incorporation rate and the S fraction in unperturbed 16HBE cells. Theoretical analysis indicated that only S arrest could result in a decrease in the SFN-BrdU incorporation rate. Additionally, the decrease in SFN-BrdU incorporation rate and the activation of DNA damage checkpoints further demonstrated that S arrest was induced by diethyl sulfate treatment of 16HBE cells. In conclusion, SFN-BrdU incorporation rate can be used to detecting S arrest.

Cell proliferation is an important feature of cell viability. It is not only essential for the growth and development of an organism, but also plays a crucial role in maintaining the normal physiological function and the maintenance of the body's cell number. In addition, cell proliferation is a key event involved in tissue repair following injury. The cell cycle is the foundation of cell proliferation. Various toxicants can influence cell proliferation by disrupting its progression. Exploring the effects of chemicals on cell cycle progression, particularly the S phase, has been the focus of extensive toxicological research. DNA replication is the most important biological event in the S phase. The mechanisms involved in the regulation of S phase progression are essential for maintaining genome integrity and fidelity. Cells can incorporate the thymidine analog 5-bromo-2' -deoxyuridine (BrdU) into their DNA during DNA synthesis, producing a nucleoside analog that serves as a marker of the S phase and cell proliferation[1]. The detection of BrdU incorporation offers all the benefits of [^{3}H]TdR autoradiography; however, compared to the latter, is less cumbersome and thus is of wider application[2]. At present, the percentage of S phase cells (S fraction) and the BrdU incorporation rate are widely used to assess S phase progression in relevant toxicological studies. However, their actual significance has never been considered in full detail. Are the two parameters, the S fraction and the BrdU incorporation rate, effective in assessing S phase progression? Since BrdU incorporation mainly occurs in the S phase[3], the BrdU incorporation rate should be equal to the S fraction as measured by flow cytometry, and there should be an interdependent relationship between the two parameters. Based on this hypothesis, we investigated the relationship between BrdU incorporation rate and the S fraction in human bronchial epithelial 16HBE cells, and reanalyzed the significance of the BrdU incorporation rate and the S fraction in assessing S phase progression. Moreover, we introduce a new parameter, which we call the S fraction normalized BrdU incorporation rate or SFN-BrdU incorporation rate (BrdU incorporation rate/S fraction, or the number of BrdU-positive cells/the number of S phase cells), to determine S arrest. The terms BrdU incorporation rate and SFN-BrdU incorporation rate mentioned in this paper are restricted to pulse-labeling BrdU incorporations. BrdU incorporation can be detected by microscopy and flow cytometry. Both methods have their advantages. However, if conditions permit, it is better to use flow cytometry in determining BrdU incorporation because it has a higher sensitivity. Detailed experimental methods are described in Supplementary Material 1.

To obtain cell samples of different S fractions, we released the serum-starved 16HBE cells to a DMEM complete medium containing 10% (v/v) fetal bovine serum, and then collected cells at 12, 16, 20, 24, and 28 h after release for cell cycle distribution and BrdU incorporation measurement. As shown in Figure 1A, the cell cycle distributions at the indicated

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1. Department of Toxicology, Peking University Health Science Center, Beijing 100191, China; 2. Beijing Key Laboratory of Toxicological Research and Risk Assessment for Food Safety, Peking University Health Science Center, Beijing 100191, China; 3. Medical and Healthy Analytical Center, Peking University Health Science Center, Beijing 100191, China

time points were different (typical histograms are shown in Supplementary Material 2). During the observation period, a decrease in the G1 fraction and an increase in the S fraction were observed. The apoptotic fraction visible as a sub-G1 peak was not detected. Significant differences among the BrdU incorporation rates at the indicated time points were also observed (Figure 1B), and interestingly, the changing trend in the BrdU incorporation rate (Figure 1B) was similar to that of the S fraction (Figure 1A).

Since the changing trends in the BrdU incorporation rate and the S fraction were similar, we investigated their relationship by using regression and correlation analyses. As exhibited in Figure 1C, there was a positive linear correlation between the BrdU incorporation rate and the S fraction (R^2>0.9; P<0.001). We then normalized the BrdU incorporation rate by using the S fraction at the same time point. The SFN-BrdU incorporation rates at the indicated time points were consistent, and slightly higher than 100% (Figure 1D).

Several biological events could influence the total cell number, S phase cell number, and DNA synthesis, thereby inducing alteration in the S fraction, BrdU incorporation rate, and SFN-BrdU incorporation rate. Table 1 lists the biological event-induced corresponding theoretical changes of the indicated parameters in perturbed cells compared to that in unperturbed cells. For example, when mitosis arrest was sustained for an appropriate period, the total number of cells decreased relative to that of unperturbed cells, although the numbers of S phase cells and BrdU-positive cells remained the same. Therefore, the S fraction (the number of S phase cells/the total

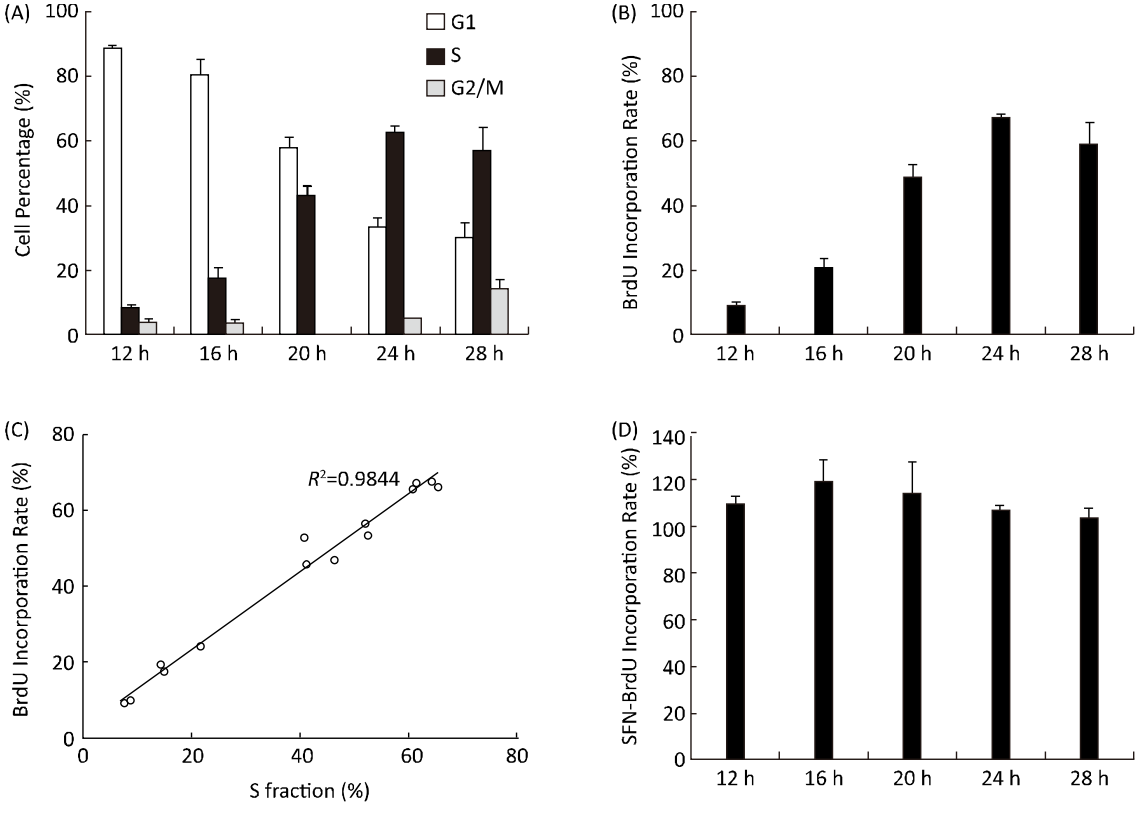

Figure 1. The relationship between BrdU incorporation rate and S fraction. The serum-starved G1 cells were seeded in DMEM complete medium in 25-cm^2 flasks, and then cell cycle distribution and BrdU incorporation rates were determined at the indicated time points after seeding. (A) Shows the G1, S, and G2/M fractions. G2/M fraction at 20 h was 0.013%±0.023%. (B) Shows the BrdU incorporation rate. (C) Shows the positive linear correlation between the BrdU incorporation rate and the S fraction (R^2>0.9; P<0.001). (D) Shows the SFN-BrdU incorporation rate, which denotes the S fraction normalized BrdU incorporation rate (BrdU incorporation rate/S fraction). Each value represents the mean±standard deviation of three experiments.

number of cells) and the BrdU incorporation rate (the number of BrdU-positive cells/the total number of cells) increased, whereas the SFN-BrdU incorporation rate (the number of BrdU-positive cells/the number of S phase cells) did not significantly change. The detailed analysis is presented in Supplementary Material 3. Only an S arrest can induce a decrease in the SFN-BrdU incorporation rate, which indicates that the parameter, SFN-BrdU incorporation rate, is a more reasonable approach in detecting S arrest than either the non-normalized BrdU incorporation rate or S fraction.

We previously demonstrated that diethyl sulfate (DES) induces S arrest in 16HBE cells 20 h after treatment[4]. In this study, we found that the S fractions and the BrdU incorporation rates of DES-treated cells 20 h after treatment were significantly higher than those of DMSO control cells (P<0.01) (Figures 2A-D). When the BrdU incorporation rate is exclusively used to assess S phase progression as previously described, it is expected that DES treatment will accelerate the S phase progression 20 h after treatment. In fact, the increase in the DNA damage checkpoint proteins p-Chk1 (Ser345), Chk1, p-Chk2 (Thr68), Chk2, and p53 in the S phase cells was induced by DES treatment at the 20-h time point (Figures 2G and H), which indicates the activation of DNA damage checkpoints, and supports the conclusion that DES induces an S arrest. The new parameter, SFN-BrdU incorporation rate, might further enhance this response. Therefore, we further analyzed the data on the effects of DES on cell cycle progression in 16HBE cells by using the SFN-BrdU incorporation rate. As shown in Figure 2F, compared to DMSO control cells, the SFN-BrdU incorporation rates of DES-treated cells significantly decreased. Additionally, by overlapping the BrdU incorporation double-parameter scatters of the DES-treated and DMSO control cells, we observed that the S phase cells in the purple rectangle marquee area indicated by an arrow (Figures 2D and E) did not undergo DNA synthesis 20 h after DES treatment. These results further demonstrate that DES induced the S arrest of 16HBE cells.

The results presented in this paper demonstrate that there is indeed a positive linear correlation between the BrdU incorporation rate and the S fraction in unperturbed cells (Figure 1C), which to some degree indicates that their significance in the assessment of S phase progression are equivalent. As mentioned in our previous paper, the increase in the S fraction may be due to any of the following reasons: (a) S arrest; (b) G1/S transition acceleration; (c) both (a) and (b)[5]. Likewise, the alteration in the BrdU incorporation rate may not be attributable to a single mechanism. As shown in Table 1, G1/S transition acceleration, mitosis arrest, and DNA damage-induced unscheduled DNA synthesis (UDS) can result in an increase in the BrdU incorporation rate, whereas S arrest, G1/S transition inhibition (G1 arrest), and mitosis acceleration can lead to a decrease in the BrdU incorporation rate. Since the reasons that induce the alteration of neither BrdU incorporation rate nor the S fraction are unique, it is not sufficient to judge S phase progression only by using the BrdU incorporation rate or the S fraction. The SFN-BrdU incorporation rate can be easily calculated by using the classical BrdU incorporation rate and S fraction. S arrest is the only plausible reason that could induce a decrease in the SFN-BrdU incorporation rate, and therefore it can be used to determine S arrest. In this study, both S fraction and BrdU incorporation rate significantly increased in DES-treated 16HBE cells at the 20-h time point, but

Table 1. Biological Event-induced Corresponding Theoretical Changes in the Parameters S Fraction, BrdU Incorporation Rate, and SFN-BrdU Incorporation Rate in Perturbed Cells Compared to Unperturbed Cells

Biological Event	Corresponding Theoretical Changes		
	S fraction	BrdU incorporation rate	SFN-BrdU incorporation rate
G1 arrest	Decrease	Decrease	No significant change
G1/S transition acceleration	Increase	Increase	No significant change
S arrest	Increase	Decrease	Decrease
S/G2 transition acceleration	Decrease	No significant change	Increase
Mitosis arrest	Increase	Increase	No significant change
Mitosis acceleration	Decrease	Decrease	No significant change
Unscheduled DNA synthesis	No significant change	Increase	Increase

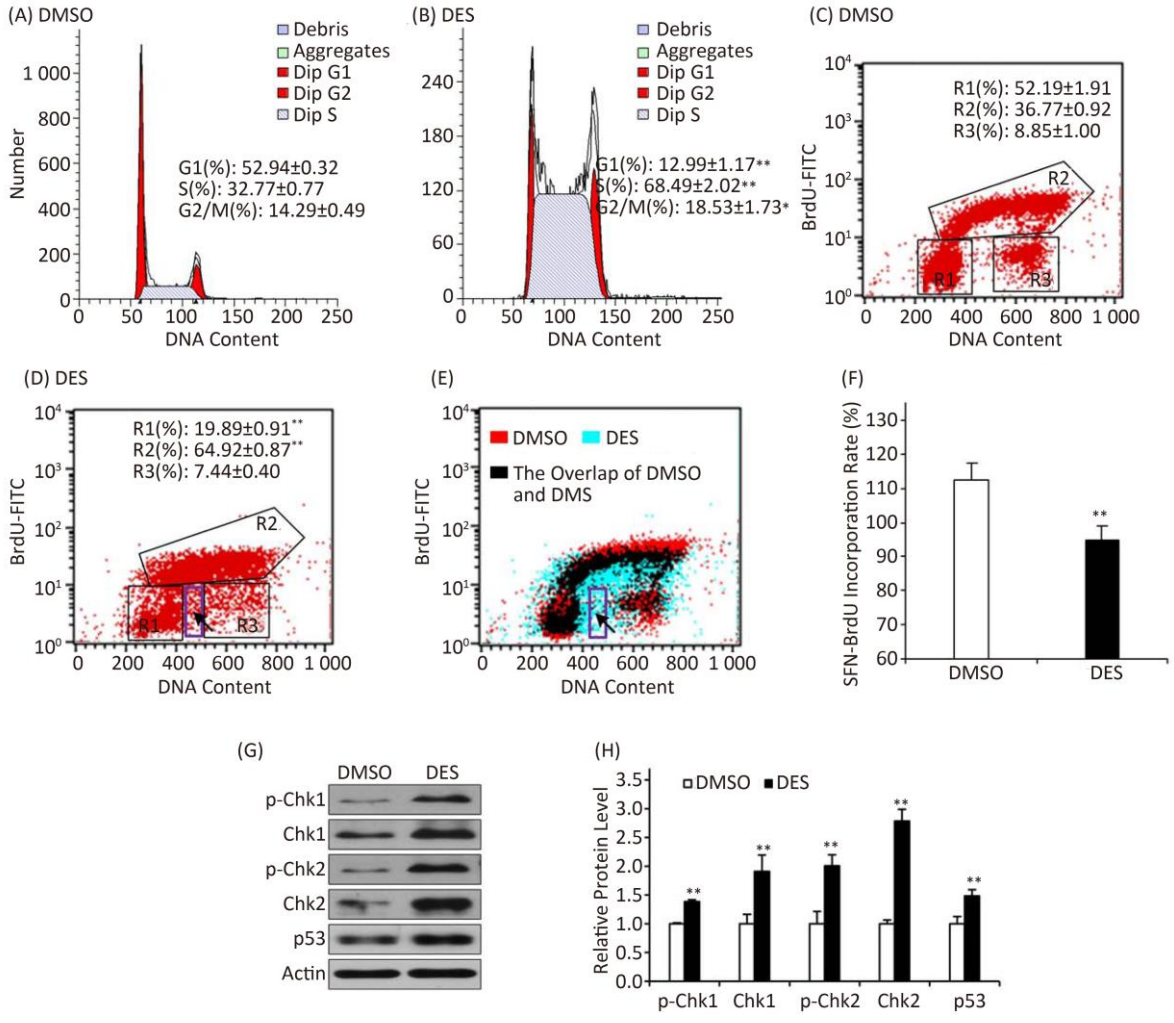

Figure 2. The effects of DES on cell cycle distribution, BrdU incorporation, and cellular levels of DNA damage checkpoint proteins in 16HBE cells. After treatment with 2.0 mmol/L of DES for 20 h, cell cycle distribution, BrdU incorporation rate, and cellular levels of DNA damage checkpoint proteins p-Chk1 (Ser345), Chk1, p-Chk2 (Thr68), Chk2, and p53 were measured. (A) and (B) Show the results of the cell cycle distribution analysis. (C) and (D) Show the results of BrdU incorporation rates (R2). (E) The overlap of (C) and (D), the cells in the purple rectangle marquee area indicated by an arrow are S phase cells that did not incorporate BrdU. (F) Shows the SFN-BrdU incorporation rate. (G) and (H) Show the cellular levels of p-Chk1 (Ser345), Chk1, p-Chk2 (Thr68), Chk2, and p53 in S phase cells treated with 2.0 mmol/L of DES for 20 h. (G) Shows the representative immunoblots obtained with the indicated antibodies. (H) Densitometric analysis of (G). The DMSO control value was set to 1. Each value represents the mean± standard deviation of three experiments. The asterisk indicates a significant difference between DES-treated and DMSO control cells as analyzed by the student's *t*-test ($^{*}P<0.05$; $^{**}P<0.01$).

the increase in the amount of BrdU incorporation rate was lower than the S fraction, which indicates that a fraction of the S phase cells did not incorporate BrdU and that S arrest occurred. The SFN-BrdU incorporation rate of DES-treated cells decreased 20 h after treatment, which directly indicates that DES induced the S arrest in 16HBE cells. Certainly, the activation of DNA damage checkpoints in DES-treated S phase cells supports the conclusion based on the SFN-BrdU incorporation rate. The new parameter, the SFN-BrdU incorporation rate, introduced in this paper is more reasonable and suitable for determining S arrest than either non-normalized BrdU incorporation rate or S fraction.

Why were the SFN-BrdU incorporation rates of

unperturbed 16HBE cell samples slightly higher than 100%? During the BrdU labeling period, three populations of cells, including the cells that entered S phase, the cells that were still in the S phase, and the cells that entered G2 phase can be labeled and then detected as BrdU-positive cells. Only two cell populations, which include the cells that entered S phase and the cells that were still in S phase during the labeling period, could be measured as S phase cells. Thus, the SFN-BrdU incorporation rates (the number of BrdU-positive cells/the number of S phase cells) of unperturbed 16HBE cell samples were consistent and slightly higher than 100%.

This study has a number of limitations. Only one cell line and one chemical have been analyzed in our experiments. SFN-BrdU incorporation rate is obtained by using cell cycle distribution measurements (DNA content measurement) and BrdU incorporation assays, which are regarded as two classical methods for studying the cell cycle. Since the two classical methods are neither cell type-specific nor chemical-specific, SFN-BrdU incorporation rate offer the advantage of being universally applicable. In addition, a decrease in the SFN-BrdU incorporation rate was also observed in benzo(a)pyrene-treated 16HBE cells 12 h after treatment[6]. Nevertheless, this study may contribute to further understanding and interpreting the results of cell cycle distribution and BrdU incorporation.

In summary, BrdU incorporation rate is positively correlated with the S fraction in unperturbed 16HBE cells. The new parameter, SFN-BrdU incorporation rate, is more reasonable to use in assessing S arrest than either the non-normalized BrdU incorporation rate or S fraction and can be used to determine S arrest.

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#Correspondence should be addressed to Professor ZHOU Zong Can. Tel: 86-10-82801531, E-mail: zhouzc@bjmu.edu.cn

Biographical note of the first author: ZHAO Peng, PhD, Tel: 86-10-82801531, E-mail: zhaopeng@bjmu.edu.cn, majoring in cellular and molecular toxicology.

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