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Study on Impact Resistance of PC12 Cells*

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Abstract

In this study, the effects of a dynamic strain in the cytotoxicity and mortality of the PC12 cell line were evaluated by using impact experiment with huge acceleration. In order to consider the influence of axonal damage on nerve cells, 2 types of nerve cells were used for the impact experiments, i.e. with and without axons. The cytotoxicity and mortality of cells were evaluated by the input acceleration, strain and strain rate and the strain rate seemed to be the most appropriate to evaluate the cytotoxicity and mortality of cells. Cells with axons showed higher cytotoxicity and mortality than cells without axons, when the strain rate was larger than 13.11 (1/s). Damage to axons was confirmed by terminal swellings and beadings of the axons. These data indicated that the presence of axons increased the cytotoxicity and mortality of cells.

Key words: Diffuse Axonal Injury, Strain Rate, Neuronal Cell, Axon, PC12 Cell

1. Introduction

When a human receives a heavy impact on the head, a skull fracture, intracranial hemorrhage, or cerebral injury can be caused. A cerebral contusion, which is local damage to the brain, and diffuse axonal injury (DAI), which is diffuse damage to the brain, are practically important in medico-legal cases of the closed head injuries. In various head injuries caused by outside impact loading, cerebral contusion and DAI are the result of a direct failure of the cerebral parenchyma. DAI is considered to be caused by strain to the brainstem due to the rotational movement of the head. When a human head receives a dynamic impact, the cranium moves first and then the brain follows its movement; this delay becomes more remarkable inside the brain, and a large strain is generated in the deep brain. This large strain causes damage to the axons of nerve cells and results in DAI [(1)(2)].

Gennarelli *et al.* characterized the strain caused by a rotational acceleration load to the head, and proposed the strain threshold of DAI by using finite element models for the crania of human and baboon [(3)(4)]. They observed the shear deformation generated in each part of the brain with a high speed camera when the head was rotated, and assumed that the strain that caused DAI was larger than 0.094. Pfister *et al.* developed a device which could generate shear deformation of cells cultured on a plane by pulling the ground substance and made it possible to produce a strain up to 0.7 and a strain rate up to 90 (1/s) [(5)]. Laplaca *et al.* cultured cells in a gel and generated 3D deformation of the cells by producing a shear deformation of the gel (strain < 0.5, strain rate < 30 (1/s)). Neuronal cells showed a lower tolerance to this strain than the glial cells [(6)(7)].

Tamura *et al.* analyzed the difference in strain caused by a tensile test between porcine

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brain tissue and nerve fiber in the white matter, and reported that the maximum neural fiber strain was ~25% of the level in the surrounding tissue [8]. Nakayama *et al.* showed morphological changes of axons and the progress of this damage over time caused by one-dimensional, horizontal oscillations of nerve cells [9]. These data suggested that an axon would receive damage with a strain of larger than 0.1 and a strain rate of larger than 10(1/s).

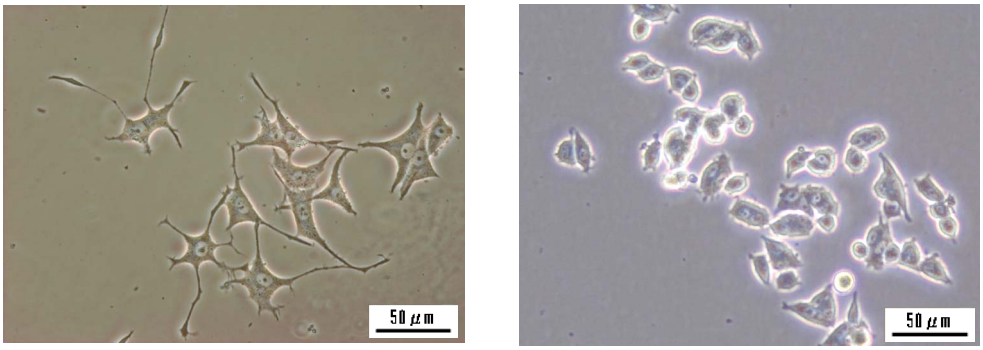
In this study, in order to study the influence of the axonal damage on the damage of cells, the cytotoxicity and mortality of PC12 cell (rat adrenal pheochromocytoma cell) line were evaluated by applying huge acceleration to cells. Huge acceleration was generated by an impact machine and was given to 2 kinds of cells, i.e. with and without axons.

2. Materials and Methods

2.1 Cell culture

In this study, PC12 cell line (obtained from Riken Cell Bank, Tsukuba, Japan) was used. Cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium; Gibco, Gland Island, NY, USA) supplemented with 10% FBS (fetal bovine serum), 10% HS (horse serum), and Pennicillin-Streptmycin (10U/ml, 100ng/ml, Sigma-Aldrich, St. Louis, MO, USA) in 95%Air, 5% CO_2 at 37℃.

In the impact experiment, the PC12 cells with and without axons were used. Axons were developed by adding 50ng/ml NGF (nerve growth factor, 2.5S; Invitrogen, Carlsbad, CA, USA). The cells were seeded in PLL (poly-L-lysine)-coated dishes (Φ35mm) at a density of $1\times10^4/cm^2$, and incubated for 5 days. Phase-contrast images of cells are shown in Fig. 1.



(a) PC12 cells with axons (b) PC12 cells without axons

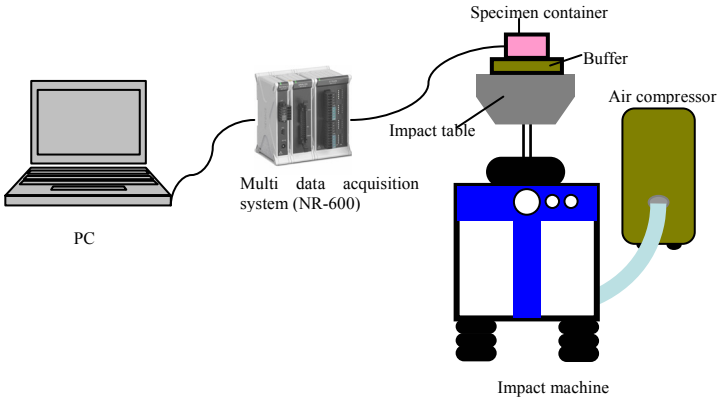
Fig. 1 Phase-contrast images of PC12 cells cultured for 5days

2.2 Impact experiment with huge acceleration

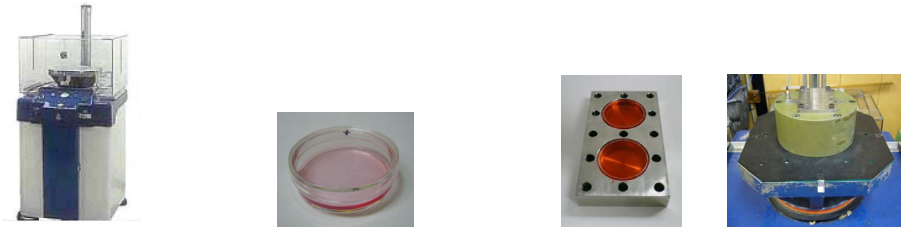
The outline of the impact experiment is shown in Fig. 2a. The impact experiments were carried out with an SM-100-3P impact machine (Fig. 2b, AVEX Electronics Inc., PA, USA). The impact machine can accelerate the specimen, with a range of acceleration of 3~20000 G and for duration of 0.1~60 ms.

A dish seeded with PC12 cells was filled with culture solution (Fig. 2c), and fixed in position in a stainless plate (Fig. 2d). The plate was then fixed on the impact table (Fig. 2e).The impact table was elevated and dropped by the compressor. The impact table collided against the buffer generating an impact with huge velocity to the dish.

The strain applied to the cultured dish was measured with a strain gauge (KFG-2N-120-C1; Kyowa Electronic Instruments Co.), which was attached to the bottom of the dish and connected to a multi-data acquisition system (NR-600; Keyence Co.). A total of 72 impact experiments were carried out, i.e. 6 experiments per condition. The input acceleration ranged from 3000 G to 10000 G, and the duration of all accelerations was 0.1ms.



(a) Devices used for the impact experiments



(b) Impact machine (c) Cultured dish and culture solution (d) Specimen container (e) Impact table

Fig. 2 Major components of the impact experiment

2.3 Evaluation of injury

2.3.1 Cytotoxicity of PC12 cells

Cytotoxicity of cells was measured by the LDH (lactate dehydrogenase) assay. LDH is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis.

After the impact, the culture solution in the dish was centrifuged for 10 minutes at 250 G, 100 µl of the reaction solution (cytotoxicity detection kit (LDH), Roche Diagnostics) was added to 100 µl of the supernatant. The reactions were incubated at room temperature for 30 min using 96-well plates (96-well Micro Test III assay Plate, BD Falcon), and 1N-HCl was added as a stop solution. The absorbance of each specimen was measured at 490 nm by a Microplate Reader (Model 680; Bio-Rad Laboratories). Similarly, the absorbance of the low control specimens (no load cells) and high control specimens (cells dissolved by 1% TritonX-100 in PBS) were measured. The cytotoxicity of PC12 cells was calculated by the following equation.

$$\text{Cytotoxicity}\,(\%) = (C - LC)/HC \times 100\% \qquad (1)$$

where C is the LDH quantity (IU/l) obtained from the impact experiment specimen, LC is the LDH quantity (IU/l) obtained from the low control specimen, and HC is the LDH quantity (IU/l) obtained from the high control specimen.

2.3.2 Mortality of PC12 cells

Mortality of cells was measured by the dye exclusion method with trypan blue dye. This method determines cell viability by mixing a suspension of live cells with a dilute solution of trypan blue; cells that exclude dye are considered to be alive, while stained cells are considered to be dead.

After the impact, cells were separated from the dish with 0.25% Trypsin-EDTA (Gibco), collected in a microcentrifuge tube (1.5 ml), and centrifuged for 30 seconds at 2000 G.

$$\text{Mortality}\,(\%) = N/M \times 100\% \quad (2)$$

where N is the number of the dead cells and M is the total number of cells.

2.3.3 Morphological change of axon

When an axon is damaged, terminal swellings coincide with the detachment of the growth cones from the substrate. The detachment of the growth cones from the substrate destroys the cytoskeletal network, which determines and maintains cell shape, resulting in a spherical deformation of the axon. Terminal swellings form in the early stages of the injury. When the cytoskeletal destruction occurs at non-terminal sites along the axon, spherical deformations develop slowly, and these appear as beads. Beadings grow in the later stages of injury [9] [10].

After the impact, the morphological changes of the axons were observed with a phase-contrast microscope.

2.3.4 Statistical analysis

Statistical analysis of the cytotoxicity and mortality of cells with axons and without axons for each experimental condition were assessed with the *t*-test; $p < 0.05$ was considered to be statistically significant. Data were expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1 Strain and strain rate obtained from the impact experiments

The average strain at the bottom of dish and the average strain rate are shown in Table2. As an example, the strain fluctuation when the peak of acceleration was 7000G is shown in Fig.3.

A strain from 0.035% to 0.201% and a strain rate from 6.67 (1/s) to19.02 (1/s) were measurable on the bottom of dish with the input accelerations from 3000 G to 10000 G. The strain measured at the bottom of dish had increased linearly as the input acceleration increased.

The strain rate tended to increase linearly as the input acceleration increased. However, when the input acceleration was 5000, 6000 and 8000 G, the strain rate was 13.02 (1/s), 13.11 (1/s), and 13.36 (1/s), respectively. There was no significant difference between these strain rates. The strain rate obtained at 7000 G was 14.62 (1/s), which was larger than the strain rate obtained at 8000G. It was difficult to control the duration of the acceleration accurately in the impact experiment when the input acceleration was very powerful. Although the strain obtained at 8000 G was larger than the strain obtained at 7000G, the duration of 8000 G became longer than the duration of 7000 G, resulting in the smaller strain rate at 8000 G.

Table 1 Strain and strain rates obtained from the impact experiments

Condition number	Peak acceleration [G]	Duration [ms]	Average strain [%]	Average strain rate (1/s)
1	3000	0.1	0.035	6.67
2	5000	0.1	0.065	13.02
3	6000	0.1	0.148	13.11
4	7000	0.1	0.164	14.62
5	8000	0.1	0.187	13.36
6	10000	0.1	0.201	19.02

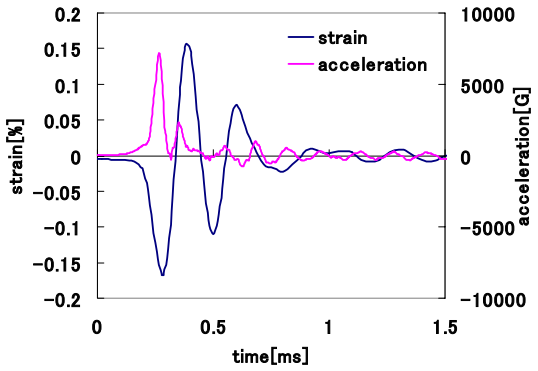
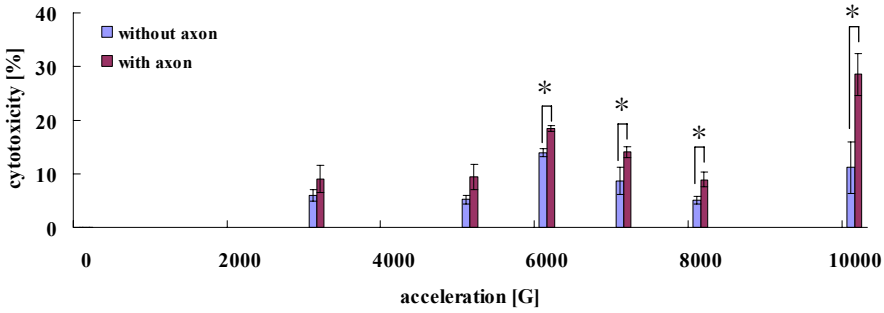


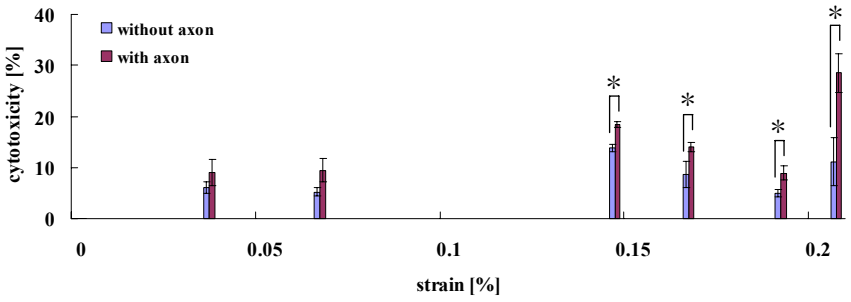
Fig. 3 Strain fluctuation when the peak of the acceleration was 7000 G.

3.2 Cytotoxicity of PC12 cells

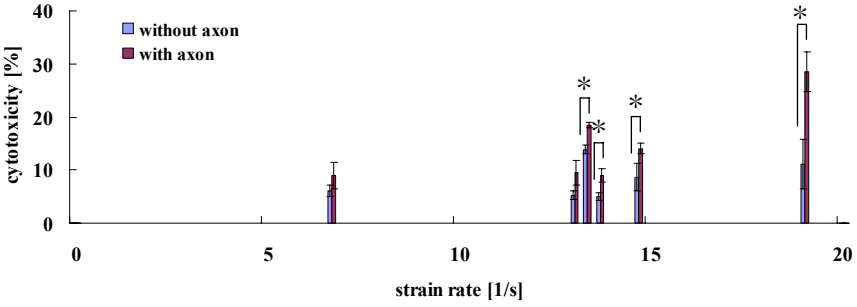
Cytotoxicity of cells with and without axons immediately after the impact experiment is shown in Fig.4. The relationship between the input acceleration and cytotoxicity of cells is shown in Fig.4a, the relationship between the strain and cytotoxicity of cells is shown in Fig.4b, and the relationship between the strain rate and cytotoxicity of cells is shown in Fig.4c. Since the input of the impact experiment was acceleration, the strain rate could not be controlled in detail; the data obtained from 5000-8000G were concentrated on around 14 (1/s) as shown in Table 1.



(a) Relationship between the input acceleration and cytotoxicity of PC12 cells



(b) Relationship between the strain and cytotoxicity of PC12 cells



(c) Relationship between the strain rate and cytotoxicity of PC12 cells

Fig. 4 Experimental results of cytotoxicity of PC12 cells. Error bars represent SEM. (* $p < 0.05$)

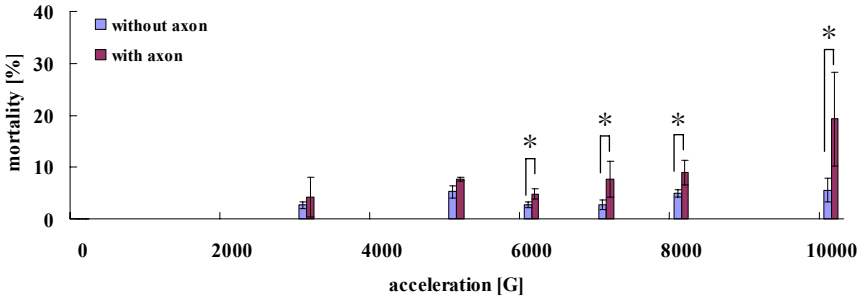
Cytotoxicity of cells seemed to increase as the input acceleration and strain increased, but these relationships did not show strong correlations (Figs. 4a, and 4b). Although the tendency that cytotoxicity of cells increased as the strain rate increased was shown, the correlation could not be quantitatively evaluated, because in the small strain rate range around 14(1/s) cytotoxicity of cells did not increase monotonically.

For the two results obtained from 3000 and 5000 G (correspond to 0.035% and 0.065% in the strain, and to 6.67 and 13.02(1/s) in the strain rate), cytotoxicity of cells with axons was not significantly higher than in cells without axons. When the input acceleration was larger than 6000 G, the strain was larger than 0.15%, and the strain rate was larger than 13.11 (1/s), cytotoxicity of cells with axons was significantly higher than in cells without axons (Figs. 4a, 4b, and 4c; $*p<0.05$).

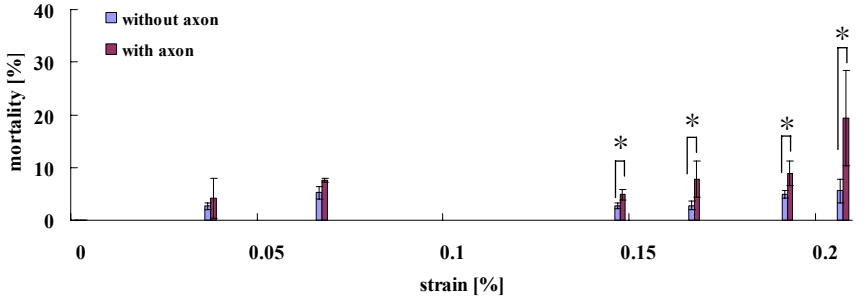
Therefore, it appeared that cytotoxicity of cells increased as the strain rate increased, and cells with axons were more easily damaged than cells without axons when the strain rate was larger than 13.11 (1/s).

3.3 Mortality of PC12 cells

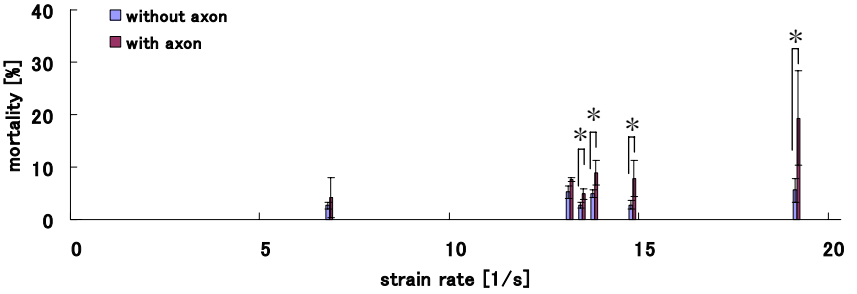
Mortality of cells with and without axons immediately after the impact experiment is shown in Fig. 5. The relationship between the input acceleration and mortality of cells is shown in Fig. 5a, the relationship between the strain and mortality of cells is shown in Fig. 5b, and the relationship between the strain rate and mortality of cells is shown in Fig. 5c.



(a) Relationship between the input acceleration and mortality of PC12 cells



(b) Relationship between the strain and mortality of PC12 cells



(c) Relationship between the strain rate and mortality of PC12 cells

Fig. 5 Experimental result of mortality of PC12 cells. Error bars represent SEM. ($* \ p < 0.05$)

Mortality of cells seemed to increase as the input acceleration and strain increased, but these relationships did not show strong correlations (Fig. 5a, and 5b). Although The tendency that mortality of cells increased as the strain rate increased was shown, the correlation could not be quantitatively evaluated, because in the small strain rate range around 14(1/s) mortality of cells did not increase monotonically.

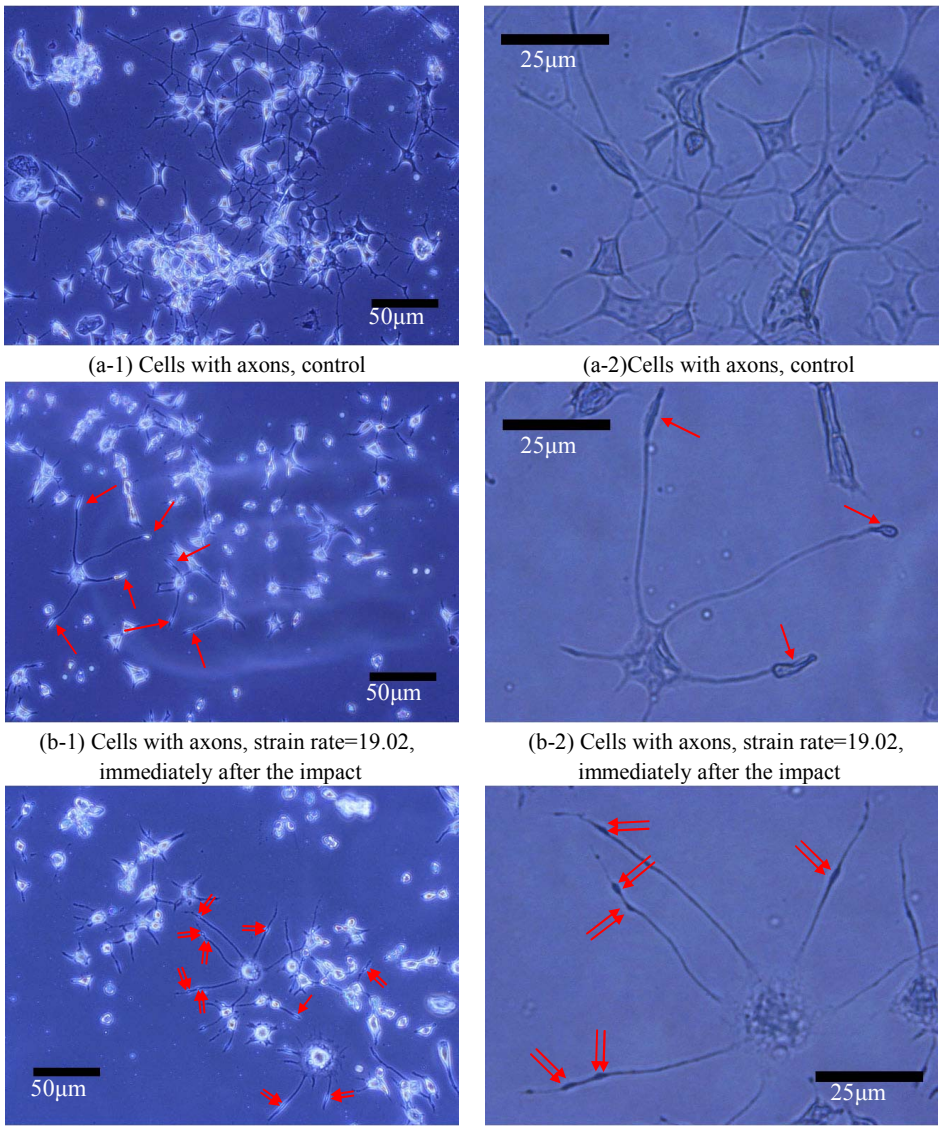
For the two results obtained from 3000 and 5000 G (correspond to 0.035% and 0.065% in the strain, and to 6.67 and 13.02(1/s) in the strain rate), mortality of cells with axons was not significantly higher than in cells without axons. When the input acceleration was larger than 6000 G, the strain was larger than 0.15%, and the strain rate was larger than 13.11 (1/s), mortality of cells with axons was significantly higher than in cells without axons (Figs. 5a, 5b, and 5c; $*p<0.05$).

Therefore, it appeared that mortality of cells increased as the strain rate increased, and cells with axons had an increased mortality than cells without axons when the strain rate was larger than 13.11 (1/s).

3.4 Morphological change

In order to observe damage to axons, cells with axons were observed with a phase-contrast microscope. Phase-contrast images of cells with axons on the dish are shown in Fig.6.

Phase-contrast images of cells with axons before the experiments (control) are shown in Figs. 6a-1 and 6a-2. Cells extending their axons and a network creates with these axons can be observed. Cells and their axons attached to the substrate. Terminal swellings or the beadings of axons are not observed at this stage. Phase-contrast images of cells immediately after the impact with the strain rate of 19.02 (1/s) are shown in Figs. 6b-1 and 6b-2; terminal swellings of axons are indicated with single arrows in the figures. Terminal swellings can be observed when the terminals of axons detach from the substrate. Since beading occurs in the later stages of damage, beading can not be observed yet at this stage. Phase-contrast images of cells 4 h after the impact with the strain rate of 19.02 (1/s) are shown in Figs. 6c-1 and 6c-2; beadings in the damaged regions are indicated with double arrows in the figures. Although beadings can not be observed in the images immediately after the impact as shown in Figs. 6b-1 and 6b-2, beadings can be clearly observed in the images taken after 4 h as shown in Figs. 6c-1 and 6c-2.



(a-1) Cells with axons, control

(a-2)Cells with axons, control

(b-1) Cells with axons, strain rate=19.02, immediately after the impact

(b-2) Cells with axons, strain rate=19.02, immediately after the impact

(c-1) Cells with axons, strain rate=19.02, 4 h after the impact

(c-2) Cells with axons, strain rate=19.02, 4 h after the impact

Fig. 6 Phase-contrast images of PC12 cells, control (a-1, a-2), immediately after the impact (b-1, b-2) and 4 h after the impact(c-1,c-2). For a clearer view, (a-1), (b-1), and (c-1) are enlarged in (a-2), (b-2), and (c-2), respectively. Terminal swellings are indicated with single arrows and beadings are indicated with double arrows.

4. Discussion

In the preliminary experiment of this study, the cytotoxicity and mortality of PC12 cells were not measured when the input acceleration was less than 1000 G, so the experiment was performed with more powerful acceleration (3000-10000 G). A strong correlation between the input acceleration and cytotoxicity (and mortality) of cells was not observed. The acceleration generated in the head was less than 1500 G, so the input acceleration of this study had a considerably large value. Since the mass of cell is very small, the force that cell receives from the input acceleration becomes very small. Therefore, it is not suitable to evaluate the tolerance of cells to acceleration, although it is realistic and effective to use acceleration and the duration of acceleration when evaluating tolerance of humans. Therefore, no conclusion about the relationship between the input acceleration and the tolerance of cells should be drawn from the present study.

In this study, the strain at the bottom of dish was from 0.035% to 0.201%.The dish was not distorted as expected even though the input acceleration was very powerful, because the cultured dish was made from acrylic with high stiffness. It is reported a strain of larger than 10% caused damage to axons [8], however, the strain obtained from the impact experiment was too small to damage the cell, so a poor correlation between the strain and cytotoxicity (and mortality) of cells was observed in this study.

The strain rate of the dish caused by the input acceleration ranged from 6.67 (1/s) to 19.02 (1/s). The duration of the strain became shorter because the duration of the input acceleration was short, so the strain rate became larger even though the strain at the bottom of dish was small. It was reported a strain rate of larger than 10 (1/s) caused damage to axons [3] [5] [7], therefore, the strain rate obtained from the impact experiment was large enough to damage the cell. LaPlaca *et al.* [7] gave a load to the cultured cells in which the strain was 50% and the strain rate was 30 (1/s), and observed approximately 20% cell mortality. When mortality of cells in this study was 20%, the strain was 0.2% and the strain rate was 19.02 (1/s). Thus, a high mortality of cells can be obtained if the strain rate is large even if strain is small. Therefore, the strain rate seemed to be the most appropriate to evaluate the cytotoxicity and mortality of cells.

The cytotoxicity and mortality of PC12 cells, with and without axons, increased as the strain rate increased. When the strain rate was 6.67 (1/s) and 13.02 (1/s), the cytotoxicity and mortality of cells with axons was not significantly higher than in cells without axons. When the strain rate was larger than 13.11 (1/s), the cytotoxicity and mortality of cells with axons was significantly higher than in cells without axons. It was reported a strain rate of larger than 10 (1/s) causes damage to axons [3] [5] [7], therefore, significant differences in the cytotoxicity and mortality of cells with and without axons was not observed when the strain rate was 6.67 (1/s). The cytotoxicity (and mortality) of cells was measured when the strain rate was 13.02 (1/s); however, only 2 results were obtained from the impact experiments, since cells after the impact were not collected successfully. Thus, the cytotoxicity (and mortality) of cells with axons was not significantly higher than cells without axons.

Morphological changes of axons caused by damage, as proposed by Nakayama *et al.* [9] were observed. Axonal terminal swellings were observed immediately after the impact, and axonal beadings were observed 4 h after the impact, so both the early and latter stages of axonal damage were confirmed.

5. Conclusion

In this study, in order to study the influence of the axonal damage on cell damage, an impact experiment with huge acceleration was performed on PC12 cell line. In order to evaluate damage to axon, the impact experiments were performed on cells with and without axons. The strain at the bottom of cultured dish was measured, and the strain rate was calculated. The cytotoxicity and mortality of PC12 cells were evaluated by the input acceleration, strain and strain rate. As a result, the strain rate seemed to be the most appropriate to evaluate the cytotoxicity and mortality of cells. The cytotoxicity and mortality of cells increased as the strain rate increased, and cells with axons were more easily damaged and had an increased mortality than cells without axons when the strain rate was larger than 13.11 (1/s). These data suggest that the presence of axons increased the cytotoxicity and mortality of cells.

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