Dependence of damage within 10 min to myocardial cells by a photodynamic reaction with a high concentration of talaporfin sodium outside cells *in vitro* on parameters of laser irradiation

Emiyu Ogawa^{a,*}, Arisa Ito^a, Tsunenori Arai^a

^aSchool of Fundamental Science and Technology,

Graduate School of Science and Technology, Keio University, 3-14-1 Hiyoshi

Kohoku-ku, Yokohama, 223-8522, Japan

*Corresponding author:

Emiyu Ogawa, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

Tel.: +81 45 563 1151; Fax: +81 45 566 1587

E-mail: e.ogawa@appi.keio.ac.jp (E. Ogawa)

arisa.i@arai.appi.keio.ac.jp (A. Ito)

tsunearai@appi.keio.ac.jp (T. Arai)

1 **Introduction**

2 Radiofrequency catheter ablation is widely used to block abnormal electrical propagation in
3 the heart as a treatment for tachyarrhythmia, of which there are approximately 520,000 cases
4 per year worldwide (1, 2). Severe complications, such as cardiac tamponade, pulmonary vein
5 stenosis, phrenic nerve injury, and esophageal fistula, are caused by thermal injury of the
6 myocardium and surrounding organs (3, 4). Asymptomatic cerebral infarction is caused by
7 thrombus formation due to heat generation (5, 6). Even though cooling abilities of catheters
8 have improved, the above complications have not been resolved (7). Previously, we proposed
9 a non-thermal method that can be applicable for tachyarrhythmia treatment. This method is
10 based on a photodynamic reaction with a high concentration of photosensitizer outside cells:
11 a photodynamic reaction with a short time interval to laser irradiation (8-11). We have carried
12 out fundamental laboratory studies and animal studies of the photodynamic reaction with a
13 high concentration of talaporfin sodium outside cells (12-17), but a response within
14 approximately 20 min has not been reported. Catheter ablation for tachyarrhythmia requires
15 an immediate response of the myocardial electrical conduction because an
16 electrophysiological diagnosis is used to judge when to end the treatment during
17 tachyarrhythmia treatment (18).

1 We investigated the immediate occurrence of irreparable severe damage to
2 myocardial cells up to 10 min after a photodynamic reaction with a high concentration of
3 photosensitizer outside cells. We measured the time to irreparable severe damage occurrence
4 and the parameters that govern this response time using various talaporfin sodium
5 concentrations, irradiances, and radiant exposures. Severe myocardial damage occurrence
6 was defined by particular changes in Ca^{2+} concentration, measured by fluorescence
7 observation as well as morphological changes. We discussed the necrosis occurrence in the
8 clinical situation by the obtained parameter dependences.

9

10 **Materials and methods**

11 **Preparation of myocardial cells, photosensitizer solutions, and a solution of the Fluo-4**

12 **AM molecular probe**

13 Primary rat myocardial cells (Primary Cell Company Ltd., Hokkaido, Japan) at 2.0×10^5
14 cells/ml were cultured in 35-mm Φ culture dishes (AGC Techno Glass, Chiba, Japan) with
15 immersed cover glasses (Matsunami Glass, Osaka, Japan) to allow the cells to adhere. Cells
16 were cultured for 2–3 days, as described in our previous report (13). Talaporfin sodium
17 (molecular weight, 799.69; Meiji Seika Pharma, Tokyo, Japan) was used as the

1 photosensitizer. Talaporfin sodium, a chlorin photosensitizer, is water-soluble and has an
2 absorbance peak of 664 ± 10 nm in the Q band (19). Talaporfin sodium is used clinically for
3 early stage lung cancer and malignant glioma in Japan (20, 21). Here, talaporfin sodium was
4 dissolved in minimal essential medium (MEM; Invitrogen) with 10% FBS, and the
5 concentration was varied from 10 to 30 $\mu\text{g/ml}$. Intracellular Ca^{2+} concentration was
6 identified using Fluo-4 AM. Fifty microliters of dimethyl sulfoxide (DMSO) (Wako Pure
7 Chemical Industries, Tokyo, Japan) was used to pre-dissolve 50 μg of Fluo-4 AM. MEM
8 with 10% FBS (1.5 ml) was used to dilute the Fluo-4 AM solution with 7.5 μl DMSO. The
9 final concentration of Fluo-4 AM was 5 μM , with a Ca^{2+} concentration of 2.2 mM. The
10 absorption wavelength range of Fluo-4 AM was 450–600 nm. The talaporfin sodium solet
11 and Q bands were 404 and 664 nm, respectively. We selected the center wavelengths of the
12 excitation lasers to use for the photodynamic reaction (663 nm) and fluorescence
13 measurement (488 nm). Talaporfin sodium absorption was not affected by the fluorescence
14 measurement.

15

16 **Intracellular Ca^{2+} observation using confocal microscopy**

17 The flow of ions through ion channels creates an action potential and leads to the contraction

1 of myocardial cells (22). The mechanical and electrical activity of a myocardial cell can be
2 evaluated by measuring the change in intracellular Ca^{2+} , which exhibits a large gradient
3 between the inside and outside of the cell (23). Changes in intracellular Ca^{2+} over time can be
4 observed using the Fluo-4 AM probe. A Nipkow-type confocal unit (CSU-X1; Yokogawa,
5 Tokyo, Japan) was mounted on a near-infrared upright differential interference microscope
6 (BX51WI-FL-IRDIC; Olympus, Tokyo, Japan). An Argon-ion laser of wavelength 488 nm
7 (National Laser, Salt Lake City, UT, USA) was used for the excitation of Fluo-4 AM. An
8 electron-multiplying CCD camera (DU897; Andor Technology, Belfast, UK) was used to
9 obtain Fluo-4 AM fluorescence images. A near-infrared cooled CCD camera (Relora-XR;
10 QImaging, Surrey, Canada) was used to observe myocardial cell morphology. The optical
11 microscopy system is shown in Fig. 1. A red diode laser of wavelength 663 nm (Sony, Tokyo,
12 Japan) was connected to the microscope to excite the talaporfin sodium. A shutter
13 (Shiguma-koki, Tokyo, Japan) was used to control the irradiation time to vary the radiant
14 exposure. The irradiation area was $5.1 \times 10^{-3} \text{ mm}^2$ when a water immersion objective lens
15 (Olympus) was used for observation. A power meter (Coherent, Santa Clara, CA, USA)
16 measured the emitted laser power from the objective lens to vary the laser irradiance from 30
17 to 290 mW/cm^2 . It was confirmed using photosensitizer fluorescence measurements that this

1 irradiance induced a continuous photodynamic reaction with a high concentration of
2 photosensitizer outside myocardial cells without oxygen depletion, as described in our
3 previous study (13).

4 <Figure 1>

5 **Changes in intracellular Ca^{2+} concentration during and after the photodynamic**
6 **reaction with a high photosensitizer concentration outside myocardial cells**

7 Myocardial cells were incubated for 20 min with 1.5 ml of prepared Fluo-4 AM solution, at
8 room temperature and in the dark. Photosensitizer solution (2.7 ml) was added after
9 incubation and removal of the Fluo-4 AM solution. This corresponded to a depth of 2.8 mm
10 in a 35-mm Φ Petri dish. Healthy myocardial cells, which contracted periodically, were
11 selected using bright-field observation. Fluo-4 AM fluorescence images were obtained
12 before, during and after the photodynamic reaction with a high concentration of
13 photosensitizer outside myocardial cells that was induced by radiant exposure of 10–40
14 J/cm^2 at a wavelength of 663 nm. Fluorescence observation at a 400-ms frame rate
15 continued up to 10 min after initiation of laser irradiation. The maximum contact time with
16 talaporfin sodium was up to 13 min, thus, it could be judged to be a photodynamic reaction
17 with a high concentration of photosensitizer outside myocardial cells, as shown in our

1 previous study (12).

2 Myocardial cell morphology before and after the photodynamic reaction with a
3 high concentration of photosensitizer outside cells was observed using bright-field
4 microscopy. The integrated Fluo-4 AM intensity of a single cell was measured to obtain the
5 intracellular Ca^{2+} concentration, and several cells were observed in each image. Necrosis
6 was confirmed by morphological observation. Many blebs were formed after the
7 photodynamic reaction with a high concentration of photosensitizer outside myocardial
8 cells; the myocardial cell membrane was ruptured; and the cytoplasm had leaked to the
9 outside. We confirmed irreparable severe damage with membrane rupture after the
10 photodynamic reaction with a high concentration of photosensitizer outside myocardial
11 cells. A preliminary experiment using caffeine was performed to confirm that any Ca^{2+}
12 increase from the sarcoplasmic reticulum could be ignored in comparing great increasing
13 from the outside the cells by the photodynamic reaction with a high concentration of
14 photosensitizer outside cells.

15 SPSS v 22 (IBM, Armonk, New York, NY, USA) was used to carry out Student's t-tests.

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1 **Results**

2 **Irreparable severe damage of myocardial cells by a photodynamic reaction with a high** 3 **concentration of photosensitizer outside myocardial cells**

4 Figure 2 shows fluorescence images of Fluo-4 AM before and during the reaction, from $t=0$
5 s up to $t=137$ s ($t=0$ corresponds to the start of laser irradiation). The same figure also shows
6 post-reaction fluorescence images up to $t=247$ s, confirming that extracellular damage had
7 occurred. Figure 2a shows the periodic fluorescence intensity change of a healthy
8 myocardial cell, which was confirmed under bright-field microscopy to have periodic
9 contractions before laser irradiation. The fluorescence intensity had a peak of width 2 s and
10 interval 4 s. This fluorescence intensity change interval was matched to the myocardial cell
11 contraction observed under bright-field microscopy. A clump of six cells was seen in the
12 observation field, as shown in Fig. 2. Figure 2b shows that, during the photodynamic
13 reaction with a high concentration of photosensitizer outside myocardial cells, the
14 intracellular Ca^{2+} concentration increased after the stoppage of the periodic fluorescence
15 intensity change as shown in Fig. 2a. Some blebs started to form as inflating balloons
16 approximately 2 min after the beginning of laser irradiation around the cell membrane as
17 shown in Fig. 2b. Figure 2c (following on time-wise from Fig. 2b) shows more bleb

1 formation after laser irradiation ($t=177$ s). The fluorescent intensity decreased as the
2 cytoplasm leaked from the damaged myocardial cells because of the necrosis that occurred
3 after laser irradiation as shown in Fig. 2c ($t=247$ s). Approximately 10 blebs were
4 recognized 177 s after the photodynamic reaction, corresponding to 1.6 blebs/cell on
5 average.

6 <Figure 2>

7 Figure 3 shows a typical example of the change in fluorescence intensity caused by the
8 photodynamic reaction. The change was induced by a radiant exposure of 40 J/cm^2 and a
9 photosensitizer concentration of $10 \text{ }\mu\text{g/ml}$. A periodic change in fluorescence was observed
10 before the photodynamic reaction with a high concentration of photosensitizer outside
11 myocardial cells. The intracellular Ca^{2+} concentration increased during laser irradiation for
12 the photodynamic reaction with a high concentration of photosensitizer outside myocardial
13 cells, and then decreased after a plateau peak. We confirmed that myocardial necrosis had
14 already occurred at 610 s using morphological observations; however, it is possible that
15 necrosis was induced before the observation at 610 s. The black arrow in Fig. 3 denotes the
16 occurrence of irreparable myocardial cell damage, which we think corresponds to the leakage

1 of the cytoplasm resulting from cell membrane rupture. We calculated the average time of the
2 occurrence of irreparable myocardial cell damage after initiation of laser irradiation.

3 <Figure 3>

4 **Response time of the irreparable severe damage induced by various photosensitizer** 5 **concentrations, radiant exposures, and irradiances**

6 The response time of the irreparable severe damage to myocardial cells, after the onset of
7 laser irradiation in the photodynamic reaction with a high concentration of photosensitizer
8 outside cells, is presented in Figs. 4 and 5. The response time decreased with increasing
9 photosensitizer concentration (Fig. 4) and increasing irradiance (Fig. 5). The shortest time
10 before damage occurrence was 209 s, at a concentration of 30 $\mu\text{g/ml}$ photosensitizer and at 40
11 J/cm^2 radiant exposure. However, radiant exposure had no significant effect on the time of
12 damage occurrence at a given photosensitizer concentration. The damage response time
13 decreased significantly by increasing the photosensitizer concentration or the irradiance.

14 <Figure 4>

15 <Figure 5>

16 **Discussion**

17 **Oxygen conditions of the photodynamic reaction in our experimental setup**

1 We previously reported that oxygen is the rate-controlling factor in our particular
2 photodynamic reaction with a high concentration of photosensitizer outside myocardial cells
3 *in vitro* because there were $\sim 10^5$ more photosensitizer molecules in the well of the 96 well
4 plate than in experiments on the intracellular photodynamic reaction (13). We confirmed the
5 photodynamic reaction continuity using 96 well plate *in vitro* system under controlled
6 condition (13). We think we can confirm the photodynamic reaction continuity in the present
7 setup because the oxygen situation could be considered as follows. The ratio between the
8 total solution volume and the reaction volume may be the most important factor. The
9 diameter of the system was 6.7 mm in the previous study and 35 mm Φ in the present setup.
10 The depth of the photosensitizer solution was 2.8 mm in both cases, meaning the solution
11 volume was $2.7 \times 10^3 \text{ mm}^3$ in this study, compared with 100 mm^3 in the previous work. The
12 laser-irradiated volume was $5.1 \times 10^{-3} \text{ mm}^3$ in this study and 100 mm^3 in the previous study.
13 The ratio of the reaction volume to the solution volume was 1.2% in this study and 100% in
14 the previous study. In the present study, 98.8% of the solution volume was non-reactive,
15 therefore, we had a huge oxygen reserve that quickly diffused into the reaction volume. We
16 previously showed that about 70% of dissolved oxygen could be consumed with laser
17 irradiation of 0.5 J/cm^2 , in a 96-well plate (13). We assume that the dissolved oxygen in

1 $2.7 \times 10^3 \text{ mm}^3$ is enough to satisfy oxygen consumption in a reactive volume ratio of 1.2%.
2 We suggest that the photodynamic reaction in the present study continued during the
3 excitation laser irradiation without oxygen depletion, so we think this setup could possibly be
4 applied to the parameter study.

5

6 **Observation method of immediate damage and bleb formation using Fluo-4 AM**

7 Using Fluo-4 AM, we clearly observed changes in intracellular Ca^{2+} concentration as well as
8 morphological changes such as bleb formation. There are no reports of bleb formation when
9 using Fluo-4 AM to mark the inside of the myocardial cell membrane but not the surface.
10 Compared with fluorescent membrane dyes, Fluo-4 AM clearly showed the bleb formation,
11 according to the Ca^{2+} distribution. At the same time, with the membrane rupture as the
12 present study by the photodynamic reaction with a high concentration of photosensitizer
13 outside myocardial cells, we think we could evaluate the cytoplasm leak by the intensity
14 decreasing, which may be induced necrosis occurrence by the irreparable severe damage.

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1 **Response time of severe damage depends on rate of singlet oxygen production**

2 We found that different radiant exposures did not significantly affect the damage response
3 time. The general principle of laser–tissue interaction is that the treatment effect is in
4 proportion to radiant exposure, assuming that there is constant laser–tissue interaction
5 efficiency over interaction threshold (24). In the general understanding of the photodynamic
6 reaction using 2nd generation photosensitizer such as talaporfin sodium, singlet oxygen, the
7 product of the type II reaction, is the major factor in cytotoxicity (25). Induction of
8 photocytotoxicity by singlet oxygen is determined by conditions such as irradiance, radiant
9 exposure, photosensitizer concentration, and oxygen concentration. The rate-controlling
10 factor is the collision reaction between the photosensitizer, oxygen and a photon (25).
11 Oxygen depletion might not have occurred in the present study; thus, the photodynamic
12 reaction might have continued until the end of the irradiation. Therefore, the rate of the
13 photodynamic reaction could be determined by the laser irradiance and photosensitizer
14 concentration. We believe that the response time to myocardial cell damage may have
15 depended on the rate of singlet oxygen production; that is, the rate of progression of the
16 photodynamic reaction.

17

1 **Applicability of the photodynamic reaction with a high concentration of photosensitizer**
2 **outside myocardial cells for arrhythmia treatment**

3 Figure 5 shows the response time to myocardial cell damage induced by various irradiances
4 with 20 µg/ml talaporfin sodium and 20 J/cm² radiant exposure. A previous study reported
5 that talaporfin sodium concentration in human plasma decreased from 27 to 20 µg/ml as the
6 time after injection increased from 0.2 to 6 h (19). We varied the photosensitizer
7 concentration from 10 to 30 µg/ml to cover this range, which we believe is comparable with
8 the concentration range used clinically.

9 There are three necessary elements of the photodynamic reaction: laser irradiation,
10 photosensitizer, and oxygen. Since oxygen depletion did not occur and the photosensitizer
11 concentration was limited, we focused on laser irradiation conditions. We found that our
12 photodynamic reaction with a high concentration of photosensitizer outside myocardial cells
13 induced cell death within a few minutes, and the time to damage decreased with increased
14 irradiance, but was unaffected by total radiant exposure.

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1 **Limitations**

2 Ca^{2+} measurement does not correspond exactly to membrane potential. The response time for
3 myocardial cell damage does not correspond exactly with the actual occurrence of necrosis.
4 However, we adopted this criterion for simplicity and to ensure that necrosis had occurred.
5 We also used rat primary myocardial cells that had different characteristics from human
6 myocardial cells. In clinical procedures, an acute electrophysiological response does not
7 always indicate necrosis.

8

9

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2 **Figure Captions**

3 **Figure 1** Optical microscopy design used to measure temporal intracellular Ca^{2+} changes and
4 cell morphological changes during photodynamic reaction with high concentration of
5 photosensitizer outside cells.

6 **Figure 2** Intracellular Ca^{2+} changes before and during the photodynamic reaction with a high
7 concentration of photosensitizer outside cells; from $t=0$ s to $t=131$ s and after the reaction
8 until $t=247$ s. (a) Periodic changes in the fluorescence intensity of a healthy myocardial cell
9 before laser irradiation. The fluorescence intensity had a peak of width 2 s and interval 4 s.
10 This fluorescence intensity change interval was matched to the myocardial cell contraction
11 observed under bright-field microscopy. (b) Increasing intracellular Ca^{2+} concentration
12 during the photodynamic reaction with a high concentration of photosensitizer outside cells
13 after the periodic fluorescence intensity change stops. (c) Membrane blebs showing
14 decreasing fluorescent intensity as the cytoplasm leaked from the damaged cells, caused by
15 the necrosis that occurred after laser irradiation.

16 **Figure 3** Typical examples of fluorescence intensity changes during the photodynamic
17 reaction with a high concentration of photosensitizer outside the cells. The black arrow

denotes the occurrence of irreparable myocardial cell damage, which we think corresponds to the leakage of the cytoplasm resulting from cell membrane rupture.

Figure 4 Response time of damage occurrence after initiation of laser irradiation, at various photosensitizer concentrations and radiant exposures. SD and P value calculations are shown for 40 J/cm^2 , indicated with †. Irradiance, 290 mW/cm^2 ; *P < 0.05 relations were found between $10\text{--}20 \text{ }\mu\text{g/ml}$ and $10\text{--}30 \text{ }\mu\text{g/ml}$ in the talaporfin sodium concentration. SPSS Statistics (version 22; IBM Corp,) was used to perform a Student's t-test. N indicates the number of cells that induced damage within 10 min of observation in the 23 cells that were treated and observed. The number, N, of 40 J/cm^2 radiant exposures was 5 at $10 \text{ }\mu\text{g/ml}$, 11 at $20 \text{ }\mu\text{g/ml}$ and $30 \text{ }\mu\text{g/ml}$ in the talaporfin sodium concentration. N=2-18 in the other plots.

Figure 5 Damage response time at various irradiances. Photosensitizer concentration: $20 \text{ }\mu\text{g/ml}$. Radiant exposure: 20 J/cm^2 . Data at 290 mW/cm^2 irradiance is also plotted in Figure 5. *P < 0.05 relations were found between $30\text{--}120 \text{ mW/cm}^2$ and $30\text{--}293 \text{ mW/cm}^2$. SPSS Statistics (IBM) was used to perform Student's t-tests. N indicates the number of cells that induced damage within 10 min of observation in the 30 cells in the field of view. N=22 in the case of the 30 mW/cm^2 irradiance, N=27 in the case of 60 mW/cm^2 , N=23 in the case of 120 mW/cm^2 , and N=18 in the case of the 290 mW/cm^2 .

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.