Revised

Porcine embryo development and inactivation of microorganisms after ultraviolet-C irradiation at 228 nm

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Abstract

It is important to prevent contamination inside the incubator as a method of preventing microbial infections during the embryo culture. In the present study, we examined the effects of ultraviolet-C (UV-C) irradiation, used for microorganism inactivation, on embryo development and the growth of bacteria, including *Escherichia coli* and *Staphylococcus aureus*, and the fungus *Cladosporium cladosporioides*. In the embryo irradiation experiment, we examined the effects of the plastic lid of the culture dish, irradiation distances (10, 20, and 25 cm), and different irradiation wavelengths (228 and 260 nm) during embryo culture for 7 days on the development and quality of porcine *in vitro*-fertilized embryos. None of the embryos cultured in dishes without plastic lids developed into blastocysts after irradiation with 228 nm UV-C. When porcine embryos were cultured in a culture dish with lids, the 228 nm UV-C irradiation decreased blastocyst formation rates of the embryos but not their quality, irrespective of the UV-C irradiation distance. Moreover, irradiation with 260 nm UV-C, even with plastic lids, had more detrimental effects on embryo development than irradiation with 228 nm UV-C. Investigation of the inactivating effects of UV-C irradiation at 228 nm and 260 nm on the growth of the bacteria and fungus showed that 260 nm UV-C reduced the viability to a greater extent than 228 nm UV-C. Moreover, the disinfection efficacy for the bacteria increased when the irradiation duration increased and the distance decreased. In conclusion, porcine embryos can develop into blastocysts without loss of quality even after continuous long-duration irradiation (7 days) with 228 nm UV-C, which can inactivate the growth of bacteria and the tested fungus; however, the development rate of the embryo is reduced.

Keywords: UV-C irradiation, porcine embryos, *Escherichia coli*, *Staphylococcus aureus*, *Cladosporium cladosporioides*

1. Introduction

Pig models are often favored over rodent models due to their clinical relevance and high similarity to human physiology and anatomy, in both genome sequence as well as anatomy and physiology [1]. Embryo production in vitro is an important tool for the generation of valuable pigs, but most ART laboratories use culture media containing antibiotics to minimize the risk of microbial growth, microorganisms occasionally colonize culture dishes containing oocytes and embryos. The exact frequency of these microbial infections is unknown but a review of previous studies indicated that it ranges from 0.35% [2] to 0.69% [3].

It is important to prevent contamination inside the incubator as a method of preventing microbial infections during the embryo culture. Chemical disinfectants, such as ethanol, hypochlorite, chlorhexidine, and ultraviolet (UV) irradiation, have good antimicrobial properties [4-6]. However, the application of chemical disinfectants is prohibited by device manufacturers and typically requires manual execution, limiting their potential applications [7, 8]. Ultraviolet radiation has three different regions depending on the wavelength: UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (< 280 nm) [9]. Of these, UV-C is used in most laboratory bactericidal studies [10]. Moreover, extensive studies on microorganism inactivation using UV light emitting diodes (LEDs) at wavelengths of 254 to 280 nm have been performed thus far [11-13]. Most studies have focused primarily on microbial indicators and very few that target *in vitro* culture of embryos have been conducted [14, 15].

The spectral range of 200 to 230 nm, referred to as far-UV-C, is assumed to be as effective as the 254 nm UV-C irradiation of the widely used mercury vapor lamps, with a much lower risk to humans [16]. Unfortunately, suitable radiation sources are still difficult to obtain, and many properties of far-UV-C radiation have not yet been fully investigated.

Many microbes, including bacteria, viruses, fungi, and spores, are affected by UV-C light [17]. UV light causes damage through the genome of microbes, indirectly impeding transcription and replication and eventually inactivating them [18-21]. The degree of inactivation is proportional to the UV dose received, which in turn is a consequence of its intensity and duration [22], causing less UV-C to reach the target from light sources farther away. Thus, when the distance doubles, only a quarter of the UV-C remains [22, 23]. A previous report has demonstrated that exposure of oocytes to UV irradiation for 10 sec did not affect the viability of nuclear transfer embryos and the production of live calves [24]. However, exposure to UV light for more than 30 sec led to a loss in

membrane integrity, decreased methionine incorporation, altered protein synthesis patterns in bovine oocytes [25], as well as stunted in vitro-fertilized porcine oocytes and damaged mitochondria DNA [15]. On the other hand, limitations of UV-C irradiation related to the penetration of light into an object due to parameters, such as organic matter, can affect the transmittance of the media and restrict its efficacy to the surface of the object [22, 26]. For example, organic materials absorb the penetration and block the reflection of UV-C, which is why surfaces should be cleaned manually to remove organic substances before decontamination [27].

The primary objective of this study was to investigate the development and quality of porcine *in vitro* fertilized embryos after UV-C irradiation at 228 nm and 260 nm, which was used to prevent contamination inside the incubator during embryo culture. The second objective was to compare the effects of UV-C irradiation at 228 and 260 nm with different irradiation durations and distances in the inactivation of representative gram-negative and gram-positive bacteria and a representative fungus, to evaluate the efficacy of UV-C irradiation in preventing microbial contamination inside the incubator during embryo culture.

2. Materials and Methods

2.1 Oocyte collection, *in vitro* maturation (IVM), and *in vitro* fertilization (IVF)

Oocyte collection, IVM, and IVF were performed as described previously [28]. Pig ovaries were collected from the prepubertal gilts at a local slaughterhouse. Cumulus–oocyte complexes were collected from ovaries and cultured in maturation medium for 44 h. The mature oocytes were co-incubated with frozen–thawed ejaculated spermatozoa (1×10^6 cells/mL) for 5 h in porcine fertilization medium (Research Institute for Functional Peptides Co., Yamagata, Japan). After co-incubation, the attached spermatozoa and cumulus cells were gently removed from putative zygotes by mechanical pipetting. The denuded zygotes were transferred to porcine zygote medium (PZM-5; Research Institute for Functional Peptides Co.) and subjected to UV-C irradiation.

2.2 Embryo development after UV-C irradiation

UV-C devices equipped to deliver wavelengths of 228 nm (Model SK-BUVC (228)-0806-DS1CFT; Shikoh Tech Co., Ltd., Hyogo, Japan) with a filter or 260 nm (Model SK-BUVC (260)-0806-DS1CFT; Shikoh Tech Co., Ltd.) were used for UV-C irradiation of culture dishes. The UV-

C device was placed in an incubator above a 4-well Nunc™ culture dish (Thermo Fisher Scientific, Waltham, MA, USA) to irradiate it. As a control, the culture dish containing the embryos was covered with a box made of aluminium foil to prevent UV light from entering and placed in the same incubator. The intensity of the 228 nm and 260 nm wavelength was 1.06 mW/cm^2 and 3.92 mW/cm^2 at 10 cm, and 0.34 mW/cm^2 and 1.22 mW/cm^2 at 20 cm from the dish, respectively. Intensities were measured with a laser power and energy meter (Nova II; Ophir Optronics Solutions Ltd., Saitama, Japan) equipped with a model PD-300-UV photodiode sensor (Ophir Optronics Solutions Ltd.).

After culture in PZM-5 under UV-C irradiation for 3 days, all cleaved embryos were transferred to 500 μL of porcine blastocyst medium (PBM; Research Institute for Functional Peptides Co.) and irradiated for an additional 4 days. Embryos were incubated at 39 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. After irradiation, blastocysts were collected and their quality was assessed.

To evaluate the total cell number and DNA fragmentation in the blastocysts, UV-treated blastocysts were fixed and stained using a combined technique for simultaneous nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL), according to previously described methods [29]. The apoptotic index was calculated by dividing the number of cells containing apoptotic nuclei (labelled by TUNEL) by the total number of cells.

2.3 Microbial inactivation by UV-C irradiation

2.3.1 UV-C irradiation of *Escherichia coli* and *Staphylococcus aureus*

Escherichia coli strain ATCC25922 and *Staphylococcus aureus* strain ATCC25923 were used as model gram-negative and gram-positive microorganisms, respectively, for the disinfection experiments. The bacteria were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37 °C with rotary shaking for 18 h. After overnight culture, the solution was centrifuged at 12,000 rpm for 3 min. The supernatant was discarded, and the bacterial pellet was washed three times with sterilized phosphate buffered saline (PBS, pH 7.4) and suspended in PBS at an initial concentration of 1×10^8 colony forming units (CFU)/mL.

A total of 200 μL of the *E. coli* or *S. aureus* suspension was placed in each well of a disposable 96-well plate (BM Equipment, Tokyo, Japan). The plate was placed under the UV-C device and

irradiated with UV-C light with peak wavelengths of 228 nm and 260 nm, as described previously [30]. Irradiation was performed in a dark room at 25 °C for various periods and distances. After UV-C irradiation, aliquots of the bacterial suspensions were transferred and spread on LB agar and incubated at 37 °C for 24 h. After incubation, the number of colonies was counted, and the survival of the bacterial population was calculated using the following equation:

$$\log \text{survival ratio} = \log (N_t/N_0),$$

where N_0 represents the number of CFU before irradiation, and N_t is the number of CFU after irradiation for time t.

2.3.2 UV-C irradiation of *Cladosporium cladosporioides*

Cladosporium cladosporioides IFM 63149 (Institute of Food Microbiology, Chiba University, Japan) was used to determine the inhibitory activity of UV-C irradiation to a representative fungus. Conidial suspensions (4×10^5 conidia/mL) were prepared as described previously [31]. Aliquots (each 25 µL) of the conidial suspension was placed on potato dextrose agar (PDA) and irradiated with UV-C light at peak wavelengths of 228 nm and 260 nm. After irradiation, the plates were incubated at 25 °C for 2 days. Antifungal activity was expressed as the log survival ratio, using the following equation:

$$\log \text{growth ratio} = \log (N_t/N_0),$$

where N_0 represents the diameter of mycelium without irradiation (mean of measurements made perpendicular to each other), and N_t represents the diameter of mycelium after irradiation for time t.

2.4 Experimental design

2.4.1 Porcine embryos for direct UV-C irradiation

Since the exact frequency of microbial infections is unknown during the culture of embryos, we irradiated porcine embryos with UV-C light throughout the 7 days of the entire culture after IVF.

In the first embryo irradiation experiment, porcine embryos were irradiated with 228 nm UV-C light in a culture dish with or without a plastic lid, at a distance of 20 cm for 7 days, to examine its effect on their development and quality. As a control, embryos were cultured for 7 days without UV-C irradiation.

In the second embryo irradiation experiment, we investigated the effects of different UV-C irradiation distances on the development and quality of the porcine embryos by culturing them in culture dishes with plastic lids and irradiating with 228 nm UV-C at distances of 10, 20, and 25 cm for 7 days. As a control, embryos were cultured for 7 days without UV-C irradiation.

In the third embryo irradiation experiment, we evaluated the effects of different UV-C irradiation wavelengths on the development and quality of embryos. Embryos were irradiated with 228 or 260 nm UV-C for 7 days at a distance of 25 cm. As a control, embryos were cultured for 7 days without UV-C irradiation. Each embryo irradiation experiment was performed five to six times.

2.4.2 Direct UV-C irradiation of *E. coli* and *S. aureus*

In a previous study, it has been demonstrated that 275 nm of LED illumining UV-C light, which is defined as radiation with wavelengths < 280 nm, exhibited high bactericidal activity within 5 min [32]. In this study, therefore, microorganisms were exposed to 228 nm and 260 nm UV-C light for >10 min, respectively, to evaluate the germicidal effect of the UV-C system.

We examined the effects of irradiation distance and time on the inactivation of *E. coli* and *S. aureus* after irradiation with 228 and 260 nm UV-C light. Bacterial suspensions in 96-well plates were irradiated with UV-C at 228 and 260 nm at a distance of 10, 20, 25, and 30 cm for 10, 20, and 30 min. All treatments were performed in a dark room at 25 °C, as described above. Each treatment consisted of three to four independent replicates.

2.4.3 Direct UV-C irradiation of *C. cladosporioides*

Conidial suspensions of *C. cladosporioides* on PDA plates were irradiated with UV-C light at either 228 or 260 nm for 30 min at a distance of 10 cm, based on prior evidence of low fungicidal activity after UV-C irradiation [33, 34]. Each treatment consisted of three independent replicates.

2.5 Statistical analysis

Data for embryo development and antimicrobial inferential analysis were evaluated by analysis of variance (ANOVA), followed by Fisher's protected least significant difference tests using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). Data were subjected to arcsine transformation before statistical analysis. Inferential analysis of the antifungal experiment was

performed using a two-tailed, unpaired Student's *t*-test. Differences with a probability (P) value < 0.05 were considered statistically significant.

3. Results

3.1 Development and quality of embryos after UV-C irradiation

In the first embryo irradiation experiment, when porcine embryos were exposed to UV-C at 228 nm for 7 days in culture dishes without lids, none of the embryos developed into blastocysts (Table 1). However, some embryos developed into blastocysts after UV-C exposure when cultured in dishes with lids. The blastocyst formation rate in this condition was lower ($P < 0.05$) than that of control embryos cultured with lids. There were no differences in the percentage of cleaved embryos, total cell number, and number of apoptotic cells in the resulting blastocysts from embryos cultured in culture dishes with lids, irrespective of UV-C irradiation.

In the second embryo irradiation experiment, the blastocyst formation rates of embryos exposed to UV-C light at 228 nm for 7 days in the culture dishes with lids were lower ($P < 0.05$) than those of control embryos that were not irradiated, irrespective of the UV-C irradiation distance (Table 2). Blastocyst formation rates increased with increasing irradiation distance. However, cleavage rates, total cell numbers, and apoptotic rates in the resulting blastocysts were comparable between the irradiated and non-irradiated groups.

In the third embryo irradiation experiment, the cleavage rate of embryos exposed to UV-C light at 260 nm for 7 days was lower ($P < 0.05$) than that of embryos irradiated at 228 nm and the control embryos (Table 3). In addition, none of the embryos irradiated at 260 nm developed to the blastocyst stage.

3.2 Bactericidal activity of *E. coli* and *S. aureus* after UV-C irradiation

The effects of irradiation distance and time on the inactivation of *E. coli* and *S. aureus* after irradiation at 228 nm and 260 nm were examined. The bactericidal effects of both irradiation wavelengths increased with prolonged irradiation time and shortened irradiation distance in both *E. coli* and *S. aureus* (Fig. 1). The UV-C irradiation of both bacteria at a distance of 10 cm showed the best germicidal effect at both irradiation wavelengths. Moreover, when irradiated with 260 nm UV-C at 10 cm for more than 20 min, the detection limit was lower for both bacteria (Fig. 1B and D).

3.3 Fungal activity of *C. cladosporioides* after UV-C irradiation

The effect of UV-C wavelength on *C. cladosporioides* inactivation after irradiation at a distance of 10 cm for 30 min was examined. Both 228 nm and 260 nm UV-C wavelengths detrimentally affected viability of conidial suspensions. Irradiation with 260 nm UV-C had a higher inactivation efficiency ($P < 0.05$) than irradiation at 228 nm.

4. Discussion

In this study, we observed that porcine embryos could develop to the blastocyst stage even after irradiation with 228 nm UV-C from above, in a culture dish with a lid, for the entire culture period. Moreover, the short period of 228 nm UV-C irradiation inactivated the two bacteria and the fungus. This is a novel study to evaluate the effect of UV-C irradiation on porcine embryo culture.

The UV-C irradiation during the culture period had substantial detrimental effects on embryo development. UV light induces damage to the genomes of bacteria, protozoa, and viruses; breaks bonds; and forms photodimeric lesions in nucleic acids. All these events impede transcription and replication, and ultimately inactivate the microorganisms [18-21]. Direct UV-C damage to nucleic acids occurs at wavelengths absorbed by DNA and RNA in the germicidal UV region between 200 and 300 nm [35, 36]. Furthermore, it has been that UV-C irradiation at MII stage led to subsequent abnormal parthenogenetic activation and no female pronucleus at fertilization [37]. These reports may explain why UV-C irradiation affects the development of porcine embryos. In the present study, when a plastic lid was placed over the dish during embryo culture, it clearly reduced damage to embryo development by UV-C irradiation. Organic materials, such as polystyrene, absorb transmitted UV-C light where the plastic rapidly becomes yellow and gradually becomes brittle when exposed to UV radiation in the presence of air [27, 38]. Discoloration of polystyrene was also observed in the current study during the 7-day exposure to 260 nm UV-C light. These results indicate that the plastic lid could weaken UV-C light and protect the developing embryo from UV-C irradiation.

The UV-C radiation has a short wavelength and high energy, compared to other UV radiation, which allows it to function best in a direct line and over a short distance [22]. However, UV-C irradiation has certain limitations related to the penetration of light into objects. Parameters, such

as the distance and wavelength of exposure, can affect the transmittance of the media, limiting its efficacy to the surface of objects [22]. In the present study, when the embryos cultured in dishes with plastic lids were exposed to 228 nm UV-C light at various distances, the UV-C irradiation decreased blastocyst formation rates of the embryos, but not their quality, irrespective of the UV-C irradiation distance. Moreover, 260 nm UV-C delivered with a higher intensity had a more detrimental impact on embryo development, causing a substantially lower cleavage rate and no blastocyst formation, than at 228 nm UV-C. These results indicate that the amount of inactivation is directly proportional to the UV-C dose received, which in turn is the result of its intensity of exposure [22]. Moreover, blastocyst formation rates increased with increasing exposure distance. Because of the high energy of UV-C radiation, it is bound by the inverse square law, where the propagation of light intensity decreases exponentially with increasing distance from the light source [22]. Embryos in close proximity to the light source are exposed to higher exposure and consequently suffer detrimental effects. These results indicate that irradiation distance is important for embryo viability.

In the present study, we observed that 260 nm UV-C with a higher intensity affected the viability of *E. coli* and *S. aureus* substantially more than did 228 nm UV-C, especially at a distance of 10 cm. Moreover, the disinfection efficiency increased as the irradiation time increased and the irradiation distance decreased. These results suggest a similar trend in embryo irradiation. *S. aureus* required a longer irradiation time than *E. coli*, indicating that gram-positive bacteria may be more resistant than gram-negative bacteria. Ultraviolet light induces the formation of photoproducts owing to the direct absorption of photons by pyrimidine and purine nucleic acid bases [39]. Photoproducts lead to structural distortion of DNA and interrupt RNA transcription and DNA replication, ultimately causing cell mutagenesis or death [40]. The results of the present study support the suggestion of Beauchamp and Lacroix [41] that the lower production of UV photoproducts is due to greater resistance of gram-positive bacteria. These authors observed that another gram-positive bacterium (*Listeria monocytogenes*) produced fewer major photoproducts than *E. coli*.

Irradiation using UV-A of filamentous fungi (e.g., *C. cladosporioides* and *Eupenicillium lapidosum*) results in poor fungicidal activity [33]. Moreover, these organisms are often resistant to UV-C exposure (254 nm) [34]. Therefore, in the current study, we investigated the effect of UV-C wavelength on the inactivation of *C. cladosporioides* by irradiating it at short distances (10 cm)

for a long time (30 min). The results indicate that irradiation at both 228 nm and 260 nm had a fungicidal effect. However, the fungicidal activity at 260 nm irradiation was greater than that at 228 nm irradiation, in which the differences in irradiation effects were similar to those observed in the inactivation of *E. coli* and *S. aureus*.

In conclusion, porcine IVF embryos can develop into blastocysts even after continuous long-duration irradiation (7 days) with 228 nm UV-C, although the development rate of the embryos is reduced. However, even short-duration of UV-C irradiation has been shown to be effective for inactivating bacteria and fungi. When UV-C is used to prevent surface decontamination within incubators during embryo culture, it is intermittent irradiation for short periods. Therefore, surface decontamination in the incubator may be achieved with minimal damage to embryo development by repeated, discontinuous, short-duration irradiation at 228 nm.

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Declaration of interest

The authors declare no conflict of interest.

Author contributions

Q.L. and T.O. conceived the study and wrote the manuscript. Q.L., M.A., A.S. and A.T. performed the majority of experiments. T.O. designed the study, coordinated all of the experiments, and reviewed the manuscript. K.T., N.Y., N.T. and M.N. participated in the laboratorial work and performed the statistical analysis. T.M. revised the manuscript. All authors read and accepted the manuscript.

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Figure legends

Fig. 1. Photo-bactericidal activity in suspensions of *Escherichia coli* (A and B) and *Staphylococcus aureus* (C and D) after irradiation with 228 and 260 nm ultraviolet (UV) light at various distances and for various times. When irradiated with 260 nm UV at 10 cm for more than 20 min, viability was below the detectable levels for both bacteria. The symbols denote: ●: Irradiation distance 10 cm, ■: Irradiation distance 20 cm, ▲: Irradiation distance 25 cm, and ◆: Irradiation distance 30 cm. The data are expressed as mean ± SD (n = 4 in *E. coli* and n = 3 in *S. aureus)*. $^{a\text{-}c}$Values with different superscripts in the same irradiation time are significantly different ($P < 0.05$).

Fig. 2. Photo-antifungal activity against *Cladosporium cladosporioides* after ultraviolet (UV) irradiation at 228 and 260 nm for 30 min at a distance of 10 cm from the fungal suspension. The asterisk (*) denotes a significant difference ($P < 0.05$).

Revised highlighted

Porcine embryo development and inactivation of microorganisms after ultraviolet-C irradiation at 228 nm

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Abstract

It is important to prevent contamination inside the incubator as a method of preventing microbial infections during the embryo culture. In the present study, we examined the effects of ultraviolet-C (UV-C) irradiation, used for microorganism inactivation, on embryo development and the growth of bacteria, including *Escherichia coli* and *Staphylococcus aureus*, and the fungus *Cladosporium cladosporioides*. In the embryo irradiation experiment, we examined the effects of the plastic lid of the culture dish, irradiation distances (10, 20, and 25 cm), and different irradiation wavelengths (228 and 260 nm) during embryo culture for 7 days on the development and quality of porcine *in vitro*-fertilized embryos. None of the embryos cultured in dishes without plastic lids developed into blastocysts after irradiation with 228 nm UV-C. When porcine embryos were cultured in a culture dish with lids, the 228 nm UV-C irradiation decreased blastocyst formation rates of the embryos but not their quality, irrespective of the UV-C irradiation distance. Moreover, irradiation with 260 nm UV-C, even with plastic lids, had more detrimental effects on embryo development than irradiation with 228 nm UV-C. Investigation of the inactivating effects of UV-C irradiation at 228 nm and 260 nm on the growth of the bacteria and fungus showed that 260 nm UV-C reduced the viability to a greater extent than 228 nm UV-C. Moreover, the disinfection efficacy for the bacteria increased when the irradiation duration increased and the distance decreased. In conclusion, porcine embryos can develop into blastocysts without loss of quality even after continuous long-duration irradiation (7 days) with 228 nm UV-C, which can inactivate the growth of bacteria and the tested fungus; however, the development rate of the embryo is reduced.

Keywords: UV-C irradiation, porcine embryos, *Escherichia coli*, *Staphylococcus aureus*, *Cladosporium cladosporioides*

1. Introduction

Pig models are often favored over rodent models due to their clinical relevance and high similarity to human physiology and anatomy, in both genome sequence as well as anatomy and physiology [1]. Embryo production in vitro is an important tool for the generation of valuable pigs, but most ART laboratories use culture media containing antibiotics to minimize the risk of microbial growth, microorganisms occasionally colonize culture dishes containing oocytes and embryos. The exact frequency of these microbial infections is unknown but a review of previous studies indicated that it ranges from 0.35% [2] to 0.69% [3].

It is important to prevent contamination inside the incubator as a method of preventing microbial infections during the embryo culture. Chemical disinfectants, such as ethanol, hypochlorite, chlorhexidine, and ultraviolet (UV) irradiation, have good antimicrobial properties [4-6]. However, the application of chemical disinfectants is prohibited by device manufacturers and typically requires manual execution, limiting their potential applications [7, 8]. Ultraviolet radiation has three different regions depending on the wavelength: UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (< 280 nm) [9]. Of these, UV-C is used in most laboratory bactericidal studies [10]. Moreover, extensive studies on microorganism inactivation using UV light emitting diodes (LEDs) at wavelengths of 254 to 280 nm have been performed thus far [11-13]. Most studies have focused primarily on microbial indicators and very few that target *in vitro* culture of embryos have been conducted [14, 15].

The spectral range of 200 to 230 nm, referred to as far-UV-C, is assumed to be as effective as the 254 nm UV-C irradiation of the widely used mercury vapor lamps, with a much lower risk to humans [16]. Unfortunately, suitable radiation sources are still difficult to obtain, and many properties of far-UV-C radiation have not yet been fully investigated.

Many microbes, including bacteria, viruses, fungi, and spores, are affected by UV-C light [17]. UV light causes damage through the genome of microbes, indirectly impeding transcription and replication and eventually inactivating them [18-21]. The degree of inactivation is proportional to the UV dose received, which in turn is a consequence of its intensity and duration [22], causing less UV-C to reach the target from light sources farther away. Thus, when the distance doubles, only a quarter of the UV-C remains [22, 23]. A previous report has demonstrated that exposure of oocytes to UV irradiation for 10 sec did not affect the viability of nuclear transfer embryos and the production of live calves [24]. However, exposure to UV light for more than 30 sec led to a loss in

membrane integrity, decreased methionine incorporation, altered protein synthesis patterns in bovine oocytes [25], as well as stunted in vitro-fertilized porcine oocytes and damaged mitochondria DNA [15]. On the other hand, limitations of UV-C irradiation related to the penetration of light into an object due to parameters, such as organic matter, can affect the transmittance of the media and restrict its efficacy to the surface of the object [22, 26]. For example, organic materials absorb the penetration and block the reflection of UV-C, which is why surfaces should be cleaned manually to remove organic substances before decontamination [27].

The primary objective of this study was to investigate the development and quality of porcine *in vitro* fertilized embryos after UV-C irradiation at 228 nm and 260 nm, which was used to prevent contamination inside the incubator during embryo culture. The second objective was to compare the effects of UV-C irradiation at 228 and 260 nm with different irradiation durations and distances in the inactivation of representative gram-negative and gram-positive bacteria and a representative fungus, to evaluate the efficacy of UV-C irradiation in preventing microbial contamination inside the incubator during embryo culture.

2. Materials and Methods

2.1 Oocyte collection, *in vitro* maturation (IVM), and *in vitro* fertilization (IVF)

Oocyte collection, IVM, and IVF were performed as described previously [28]. Pig ovaries were collected from the prepubertal gilts at a local slaughterhouse. Cumulus–oocyte complexes were collected from ovaries and cultured in maturation medium for 44 h. The mature oocytes were co-incubated with frozen–thawed ejaculated spermatozoa (1×10^6 cells/mL) for 5 h in porcine fertilization medium (Research Institute for Functional Peptides Co., Yamagata, Japan). After co-incubation, the attached spermatozoa and cumulus cells were gently removed from putative zygotes by mechanical pipetting. The denuded zygotes were transferred to porcine zygote medium (PZM-5; Research Institute for Functional Peptides Co.) and subjected to UV-C irradiation.

2.2 Embryo development after UV-C irradiation

UV-C devices equipped to deliver wavelengths of 228 nm (Model SK-BUVC (228)-0806-DS1CFT; Shikoh Tech Co., Ltd., Hyogo, Japan) with a filter or 260 nm (Model SK-BUVC (260)-0806-DS1CFT; Shikoh Tech Co., Ltd.) were used for UV-C irradiation of culture dishes. The UV-

C device was placed in an incubator above a 4-well Nunc™ culture dish (Thermo Fisher Scientific, Waltham, MA, USA) to irradiate it. As a control, the culture dish containing the embryos was covered with a box made of aluminium foil to prevent UV light from entering and placed in the same incubator. The intensity of the 228 nm and 260 nm wavelength was 1.06 mW/cm^2 and 3.92 mW/cm^2 at 10 cm, and 0.34 mW/cm^2 and 1.22 mW/cm^2 at 20 cm from the dish, respectively. Intensities were measured with a laser power and energy meter (Nova II; Ophir Optronics Solutions Ltd., Saitama, Japan) equipped with a model PD-300-UV photodiode sensor (Ophir Optronics Solutions Ltd.).

After culture in PZM-5 under UV-C irradiation for 3 days, all cleaved embryos were transferred to 500 μL of porcine blastocyst medium (PBM; Research Institute for Functional Peptides Co.) and irradiated for an additional 4 days. Embryos were incubated at 39 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. After irradiation, blastocysts were collected and their quality was assessed.

To evaluate the total cell number and DNA fragmentation in the blastocysts, UV-treated blastocysts were fixed and stained using a combined technique for simultaneous nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL), according to previously described methods [29]. The apoptotic index was calculated by dividing the number of cells containing apoptotic nuclei (labelled by TUNEL) by the total number of cells.

2.3 Microbial inactivation by UV-C irradiation

2.3.1 UV-C irradiation of *Escherichia coli* and *Staphylococcus aureus*

Escherichia coli strain ATCC25922 and *Staphylococcus aureus* strain ATCC25923 were used as model gram-negative and gram-positive microorganisms, respectively, for the disinfection experiments. The bacteria were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37 °C with rotary shaking for 18 h. After overnight culture, the solution was centrifuged at 12,000 rpm for 3 min. The supernatant was discarded, and the bacterial pellet was washed three times with sterilized phosphate buffered saline (PBS, pH 7.4) and suspended in PBS at an initial concentration of 1×10^8 colony forming units (CFU)/mL.

A total of 200 μL of the *E. coli* or *S. aureus* suspension was placed in each well of a disposable 96-well plate (BM Equipment, Tokyo, Japan). The plate was placed under the UV-C device and

irradiated with UV-C light with peak wavelengths of 228 nm and 260 nm, as described previously [30]. Irradiation was performed in a dark room at 25 °C for various periods and distances. After UV-C irradiation, aliquots of the bacterial suspensions were transferred and spread on LB agar and incubated at 37 °C for 24 h. After incubation, the number of colonies was counted, and the survival of the bacterial population was calculated using the following equation:

$$\log \text{survival ratio} = \log (N_t/N_0),$$

where N_0 represents the number of CFU before irradiation, and N_t is the number of CFU after irradiation for time t.

2.3.2 UV-C irradiation of *Cladosporium cladosporioides*

Cladosporium cladosporioides IFM 63149 (Institute of Food Microbiology, Chiba University, Japan) was used to determine the inhibitory activity of UV-C irradiation to a representative fungus. Conidial suspensions (4×10^5 conidia/mL) were prepared as described previously [31]. Aliquots (each 25 µL) of the conidial suspension was placed on potato dextrose agar (PDA) and irradiated with UV-C light at peak wavelengths of 228 nm and 260 nm. After irradiation, the plates were incubated at 25 °C for 2 days. Antifungal activity was expressed as the log survival ratio, using the following equation:

$$\log \text{growth ratio} = \log (N_t/N_0),$$

where N_0 represents the diameter of mycelium without irradiation (mean of measurements made perpendicular to each other), and N_t represents the diameter of mycelium after irradiation for time t.

2.4 Experimental design

2.4.1 Porcine embryos for direct UV-C irradiation

Since the exact frequency of microbial infections is unknown during the culture of embryos, we irradiated porcine embryos with UV-C light throughout the 7 days of the entire culture after IVF.

In the first embryo irradiation experiment, porcine embryos were irradiated with 228 nm UV-C light in a culture dish with or without a plastic lid, at a distance of 20 cm for 7 days, to examine its effect on their development and quality. As a control, embryos were cultured for 7 days without UV-C irradiation.

In the second embryo irradiation experiment, we investigated the effects of different UV-C irradiation distances on the development and quality of the porcine embryos by culturing them in culture dishes with plastic lids and irradiating with 228 nm UV-C at distances of 10, 20, and 25 cm for 7 days. As a control, embryos were cultured for 7 days without UV-C irradiation.

In the third embryo irradiation experiment, we evaluated the effects of different UV-C irradiation wavelengths on the development and quality of embryos. Embryos were irradiated with 228 or 260 nm UV-C for 7 days at a distance of 25 cm. As a control, embryos were cultured for 7 days without UV-C irradiation. Each embryo irradiation experiment was performed five to six times.

2.4.2 Direct UV-C irradiation of *E. coli* and *S. aureus*

In a previous study, it has been demonstrated that 275 nm of LED illumining UV-C light, which is defined as radiation with wavelengths < 280 nm, exhibited high bactericidal activity within 5 min [32]. In this study, therefore, microorganisms were exposed to 228 nm and 260 nm UV-C light for >10 min, respectively, to evaluate the germicidal effect of the UV-C system.

We examined the effects of irradiation distance and time on the inactivation of *E. coli* and *S. aureus* after irradiation with 228 and 260 nm UV-C light. Bacterial suspensions in 96-well plates were irradiated with UV-C at 228 and 260 nm at a distance of 10, 20, 25, and 30 cm for 10, 20, and 30 min. All treatments were performed in a dark room at 25 °C, as described above. Each treatment consisted of three to four independent replicates.

2.4.3 Direct UV-C irradiation of *C. cladosporioides*

Conidial suspensions of *C. cladosporioides* on PDA plates were irradiated with UV-C light at either 228 or 260 nm for 30 min at a distance of 10 cm, based on prior evidence of low fungicidal activity after UV-C irradiation [33, 34]. Each treatment consisted of three independent replicates.

2.5 Statistical analysis

Data for embryo development and antimicrobial inferential analysis were evaluated by analysis of variance (ANOVA), followed by Fisher's protected least significant difference tests using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). Data were subjected to arcsine transformation before statistical analysis. Inferential analysis of the antifungal experiment was

218 performed using a two-tailed, unpaired Student's *t*-test. Differences with a probability (P) value <
219 0.05 were considered statistically significant.
220
221 **3. Results**
222 **3.1 Development and quality of embryos after UV-C irradiation**
223 In the first embryo irradiation experiment, when porcine embryos were exposed to UV-C at
224 228 nm for 7 days in culture dishes without lids, none of the embryos developed into blastocysts
225 (Table 1). However, some embryos developed into blastocysts after UV-C exposure when cultured
226 in dishes with lids. The blastocyst formation rate in this condition was lower ($P < 0.05$) than that
227 of control embryos cultured with lids. There were no differences in the percentage of cleaved
228 embryos, total cell number, and number of apoptotic cells in the resulting blastocysts from embryos
229 cultured in culture dishes with lids, irrespective of UV-C irradiation.
230 In the second embryo irradiation experiment, the blastocyst formation rates of embryos
231 exposed to UV-C light at 228 nm for 7 days in the culture dishes with lids were lower ($P < 0.05$)
232 than those of control embryos that were not irradiated, irrespective of the UV-C irradiation distance
233 (Table 2). Blastocyst formation rates increased with increasing irradiation distance. However,
234 cleavage rates, total cell numbers, and apoptotic rates in the resulting blastocysts were comparable
235 between the irradiated and non-irradiated groups.
236 In the third embryo irradiation experiment, the cleavage rate of embryos exposed to UV-C
237 light at 260 nm for 7 days was lower ($P < 0.05$) than that of embryos irradiated at 228 nm and the
238 control embryos (Table 3). In addition, none of the embryos irradiated at 260 nm developed to the
239 blastocyst stage.
240
241 **3.2 Bactericidal activity of *E. coli* and *S. aureus* after UV-C irradiation**
242 The effects of irradiation distance and time on the inactivation of *E. coli* and *S. aureus* after
243 irradiation at 228 nm and 260 nm were examined. The bactericidal effects of both irradiation
244 wavelengths increased with prolonged irradiation time and shortened irradiation distance in both
245 *E. coli* and *S. aureus* (Fig. 1). The UV-C irradiation of both bacteria at a distance of 10 cm showed
246 the best germicidal effect at both irradiation wavelengths. Moreover, when irradiated with 260 nm
247 UV-C at 10 cm for more than 20 min, the detection limit was lower for both bacteria (Fig. 1B and
248 D).

3.3 Fungal activity of *C. cladosporioides* after UV-C irradiation

The effect of UV-C wavelength on *C. cladosporioides* inactivation after irradiation at a distance of 10 cm for 30 min was examined. Both 228 nm and 260 nm UV-C wavelengths detrimentally affected viability of conidial suspensions. Irradiation with 260 nm UV-C had a higher inactivation efficiency ($P < 0.05$) than irradiation at 228 nm.

4. Discussion

In this study, we observed that porcine embryos could develop to the blastocyst stage even after irradiation with 228 nm UV-C from above, in a culture dish with a lid, for the entire culture period. Moreover, the short period of 228 nm UV-C irradiation inactivated the two bacteria and the fungus. This is a novel study to evaluate the effect of UV-C irradiation on porcine embryo culture.

The UV-C irradiation during the culture period had substantial detrimental effects on embryo development. UV light induces damage to the genomes of bacteria, protozoa, and viruses; breaks bonds; and forms photodimeric lesions in nucleic acids. All these events impede transcription and replication, and ultimately inactivate the microorganisms [18-21]. Direct UV-C damage to nucleic acids occurs at wavelengths absorbed by DNA and RNA in the germicidal UV region between 200 and 300 nm [35, 36]. Furthermore, it has been that UV-C irradiation at MII stage led to subsequent abnormal parthenogenetic activation and no female pronucleus at fertilization [37]. These reports may explain why UV-C irradiation affects the development of porcine embryos. In the present study, when a plastic lid was placed over the dish during embryo culture, it clearly reduced damage to embryo development by UV-C irradiation. Organic materials, such as polystyrene, absorb transmitted UV-C light where the plastic rapidly becomes yellow and gradually becomes brittle when exposed to UV radiation in the presence of air [27, 38]. Discoloration of polystyrene was also observed in the current study during the 7-day exposure to 260 nm UV-C light. These results indicate that the plastic lid could weaken UV-C light and protect the developing embryo from UV-C irradiation.

The UV-C radiation has a short wavelength and high energy, compared to other UV radiation, which allows it to function best in a direct line and over a short distance [22]. However, UV-C irradiation has certain limitations related to the penetration of light into objects. Parameters, such

as the distance and wavelength of exposure, can affect the transmittance of the media, limiting its efficacy to the surface of objects [22]. In the present study, when the embryos cultured in dishes with plastic lids were exposed to 228 nm UV-C light at various distances, the UV-C irradiation decreased blastocyst formation rates of the embryos, but not their quality, irrespective of the UV-C irradiation distance. Moreover, 260 nm UV-C delivered with a higher intensity had a more detrimental impact on embryo development, causing a substantially lower cleavage rate and no blastocyst formation, than at 228 nm UV-C. These results indicate that the amount of inactivation is directly proportional to the UV-C dose received, which in turn is the result of its intensity of exposure [22]. Moreover, blastocyst formation rates increased with increasing exposure distance. Because of the high energy of UV-C radiation, it is bound by the inverse square law, where the propagation of light intensity decreases exponentially with increasing distance from the light source [22]. Embryos in close proximity to the light source are exposed to higher exposure and consequently suffer detrimental effects. These results indicate that irradiation distance is important for embryo viability.

In the present study, we observed that 260 nm UV-C with a higher intensity affected the viability of *E. coli* and *S. aureus* substantially more than did 228 nm UV-C, especially at a distance of 10 cm. Moreover, the disinfection efficiency increased as the irradiation time increased and the irradiation distance decreased. These results suggest a similar trend in embryo irradiation. *S. aureus* required a longer irradiation time than *E. coli*, indicating that gram-positive bacteria may be more resistant than gram-negative bacteria. Ultraviolet light induces the formation of photoproducts owing to the direct absorption of photons by pyrimidine and purine nucleic acid bases [39]. Photoproducts lead to structural distortion of DNA and interrupt RNA transcription and DNA replication, ultimately causing cell mutagenesis or death [40]. The results of the present study support the suggestion of Beauchamp and Lacroix [41] that the lower production of UV photoproducts is due to greater resistance of gram-positive bacteria. These authors observed that another gram-positive bacterium (*Listeria monocytogenes*) produced fewer major photoproducts than *E. coli*.

Irradiation using UV-A of filamentous fungi (e.g., *C. cladosporioides* and *Eupenicillium lapidosum*) results in poor fungicidal activity [33]. Moreover, these organisms are often resistant to UV-C exposure (254 nm) [34]. Therefore, in the current study, we investigated the effect of UV-C wavelength on the inactivation of *C. cladosporioides* by irradiating it at short distances (10 cm)

for a long time (30 min). The results indicate that irradiation at both 228 nm and 260 nm had a fungicidal effect. However, the fungicidal activity at 260 nm irradiation was greater than that at 228 nm irradiation, in which the differences in irradiation effects were similar to those observed in the inactivation of *E. coli* and *S. aureus*.

In conclusion, porcine IVF embryos can develop into blastocysts even after continuous long-duration irradiation (7 days) with 228 nm UV-C, although the development rate of the embryos is reduced. However, even short-duration of UV-C irradiation has been shown to be effective for inactivating bacteria and fungi. When UV-C is used to prevent surface decontamination within incubators during embryo culture, it is intermittent irradiation for short periods. Therefore, surface decontamination in the incubator may be achieved with minimal damage to embryo development by repeated, discontinuous, short-duration irradiation at 228 nm.

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Declaration of interest

The authors declare no conflict of interest.

Author contributions

Q.L. and T.O. conceived the study and wrote the manuscript. Q.L., M.A., A.S. and A.T. performed the majority of experiments. T.O. designed the study, coordinated all of the experiments, and reviewed the manuscript. K.T., N.Y., N.T. and M.N. participated in the laboratorial work and performed the statistical analysis. T.M. revised the manuscript. All authors read and accepted the manuscript.

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Figure legends

Fig. 1. Photo-bactericidal activity in suspensions of *Escherichia coli* (A and B) and *Staphylococcus aureus* (C and D) after irradiation with 228 and 260 nm ultraviolet (UV) light at various distances and for various times. When irradiated with 260 nm UV at 10 cm for more than 20 min, viability was below the detectable levels for both bacteria. The symbols denote: ●: Irradiation distance 10 cm, ■: Irradiation distance 20 cm, ▲: Irradiation distance 25 cm, and ◆: Irradiation distance 30 cm. The data are expressed as mean ± SD (n = 4 in *E. coli* and n = 3 in *S. aureus)*. $^{a\text{-}c}$Values with different superscripts in the same irradiation time are significantly different ($P < 0.05$).

Fig. 2. Photo-antifungal activity against *Cladosporium cladosporioides* after ultraviolet (UV) irradiation at 228 and 260 nm for 30 min at a distance of 10 cm from the fungal suspension. The asterisk (*) denotes a significant difference ($P < 0.05$).

Fig. 1

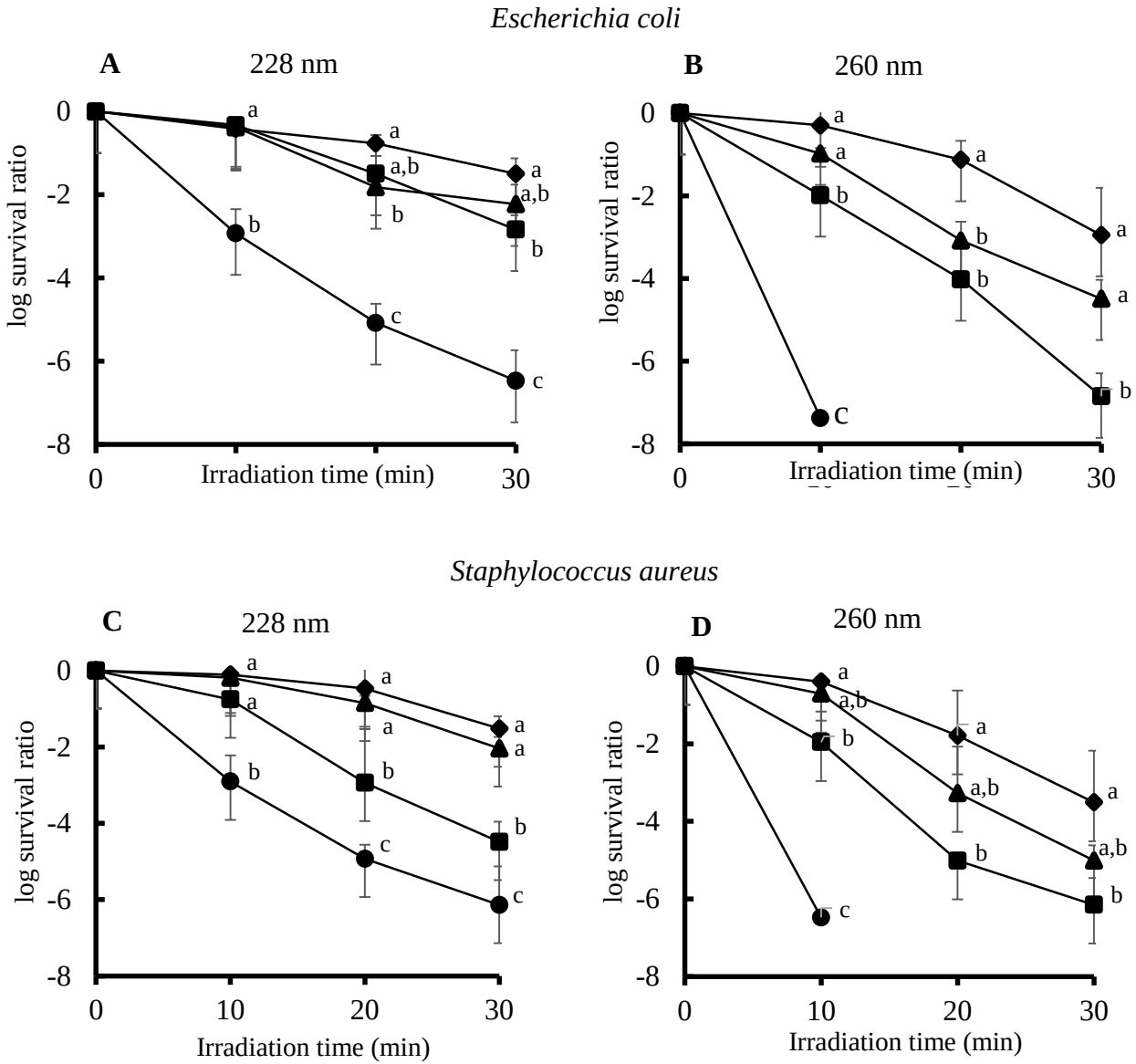

Fig. 2

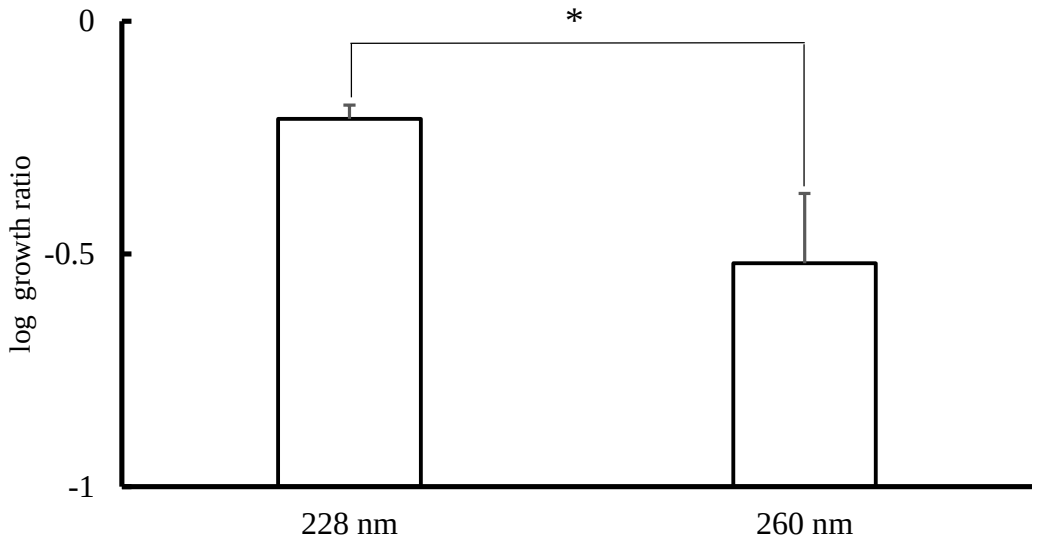

Table 1. Effects of 228 nm ultraviolet (UV) irradiation on the development and quality of porcine embryos*

Light source**	Lid***	No. of embryos examined	No. (%) of embryos		Total cell number of blastocysts	Percentage of apoptotic cells
			Cleaved	Developed to blastocysts		
Control	+	295	264 (89.4 ± 2.2)[a]	63 (21.3 ± 2.6)[a]	64.3 ± 3.9[ab]	3.8 ± 0.6
Control	-	289	251 (86.9 ± 2.6)[a]	32 (11.1 ± 3.0)[b]	73.4 ± 6.1[a]	3.9 ± 0.9
UV irradiation	+	289	249 (86.3 ± 2.1)[a]	36 (12.4 ± 3.0)[b]	55.2 ± 4.5[b]	5.5 ± 1.0
UV irradiation	-	294	115 (39.2 ± 4.8)[b]	0 (0)[c]		

*Six replicate trials were performed. Percentages are expressed as mean ± SEM.

**UV light at 228 nm was used to irradiate a culture dish containing embryos from 20 cm for 7 days. As a control, embryos were cultured for seven days without UV irradiation.

***Embryos were cultured in a culture dish with (+) or without (-) plastic lid during UV irradiation.

[a-b]Values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 2. Effects of 228 nm ultraviolet (UV) irradiation distances on the development and quality of porcine embryos*

Distance**	No. of embryos examined	No. (%) of embryos		Total cell number of blastocysts	Percentage of apoptotic cells
		Cleaved	Developed to blastocysts		
Control	220	185 (84.0 ± 4.2)	36 (16.4 ± 1.2)[a]	71.9 ± 5.5	3.8 ± 0.8
10 cm	222	180 (80.8 ± 5.1)	13 (5.8 ± 1.1)[b]	56.2 ± 7.7	4.3 ± 0.9
20 cm	226	191 (84.5 ± 3.1)	15 (6.7 ± 1.3)[b]	56.3 ± 5.1	4.7 ± 0.8
25 cm	225	195 (86.5 ± 2.3)	25 (11.1 ± 0.6)[c]	64.6 ± 7.0	4.4 ± 0.9

*Five replicate trials were performed. Percentages are expressed as mean ± SEM.

** UV light at 228 nm was used to irradiate a plastic lidded culture dish containing embryos for 7 days from distances of 10, 20, and 25 cm. As a control, embryos were cultured for 7 days without UV irradiation.

[a-c]Values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 3. Effects of wavelengths of ultraviolet (UV) irradiation on the development and quality of porcine embryos*

Wavelengths**	No. of embryos examined	No. (%) of embryos		Total cell number of blastocysts	Percentage of apoptotic cells
		Cleaved	Developed to blastocysts		
Control	234	203 (86.8 ± 2.3)[a]	44 (18.8 ± 1.1)[a]	61.0 ± 2.7	4.7 ± 0.7
228 nm	226	204 (90.2 ± 1.9)[a]	22 (9.7 ± 1.0)[b]	55.9 ± 5.3	6.7 ± 1.1
260 nm	236	10 (4.4 ± 3.4)[b]	0 (0)[c]		

*Five replicate trials were performed. Percentages are expressed as mean ± SEM.

** UV light at 228 or 260 nm was used to irradiate a plastic lidded culture dish with containing embryos from a distance of 25 cm for 7 days. As a control, embryos were cultured for 7 days without UV irradiation.

[a-c]Values with different superscripts in the same column are significantly different ($P < 0.05$).