

# Ultrastructural Changes in *Cryptosporidium parvum* Oocysts by Gamma Irradiation

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**Abstract:** *Cryptosporidium parvum* is known as one of the most highly resistant parasites to gamma irradiation. To morphologically have an insight on the radioresistance of this parasite, ultrastructural changes in *C. parvum* sporozoites were observed after gamma irradiation using various doses (1, 5, 10, and 25 kGy) following a range of post-irradiation incubation times (10 kGy for 6, 12, 24, 48, 72, and 96 hr). The ultrastructures of *C. parvum* oocysts changed remarkably after a 10-kGy irradiation. Nuclear membrane changes and degranulation of dense granules were observed with high doses over 10 kGy, and morphological changes in micronemes and rhoptries were observed with very high doses over 25 kGy. Oocyst walls were not affected by irradiation, whereas the internal structures of sporozoites degenerated completely 96 hr post-irradiation using a dose of 10 kGy. From this study, morphological evidence of radioresistance of *C. parvum* has been supplemented.

**Key words:** *Cryptosporidium parvum*, ultrastructure, gamma irradiation

## INTRODUCTION

*Cryptosporidium parvum* is an obligate intracellular protozoan that infects a wide range of vertebrates, including humans and animals [1]. Most infections are acquired from water or food contaminated with infectious oocysts [2,3]. Previous studies have found that *C. parvum* exhibits the highest known resistance to gamma irradiation among parasites [4,5]. The excystation rate of *C. parvum* oocysts that receive a 2-kGy dose of gamma irradiation is the same as that for non-irradiated oocysts, and this rate decreases by only 50% in oocysts that receive a 20-kGy dose [4]. It has been suggested that gamma irradiation at 50 kGy is necessary to completely eliminate *C. parvum* infectivity in mice [5]. This is the highest reported resistance to irradiation among parasites. In a recent report, the infectivity of *C. parvum* oocysts in HCT-8 cells was reduced under 3 log<sub>10</sub> by 10 kGy irradiation [6]. Since the dose of *C. parvum* required for infection is known to be quite low [7], even 0.1% of oocysts that maintain infectivity still pose a threat to the host. It was

recently shown that DNA damage of *C. parvum* oocysts is induced by gamma irradiation in a dose-dependent manner, and that DNA damaged from a 10-kGy dose peaks at 6 hr post-irradiation and rejoins thereafter [8]. In the present study, we examined the morphological changes in *C. parvum* after gamma irradiation to morphologically characterize the radioresistance of this organism.

## MATERIALS AND METHODS

### Animal care and *C. parvum* oocyst preparation

Specific pathogen-free C57BL/6J female mice (8-9 weeks old) were purchased from Daehan BioLink Co. (Eumsung, Republic of Korea) and housed at constant temperature under controlled illumination. Mice were orally infected with  $2 \times 10^6$  *C. parvum* oocysts (KKU isolate) after inducing immunosuppression by providing dexamethasone phosphate disodium salt (Sigma, St. Louis, Missouri, USA) in drinking water ad libitum at a dose of 10 mg/ml [9]. Mouse feces were collected from the wire-bottom cages, and oocysts were purified as described [10]. Purified oocysts were maintained at 4°C for less than 2 weeks in filtered (0.22 µm) distilled water. The animal study was approved by the Animal Care and Use Committee of Konkuk University.

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### Gamma irradiation of *C. parvum* oocysts

A 1.5-ml microcentrifuge tube containing  $2 \times 10^7$  purified *C. parvum* oocysts in 1 ml filtered (0.22  $\mu\text{m}$ ) distilled water was immersed in a 50-ml tube filled with distilled water to induce backscattering and reduce the temperature increase caused by absorption of high-dose radiation energy. Irradiation was performed at room temperature (20°C) for 2 hr with a  $^{60}\text{Co}$  IR221 High Performance Tote Irradiator (MDS Nordion, Ottawa, Canada). *C. parvum* oocysts were irradiated at various doses (1, 5, 10, and 25 kGy), and 1 group of oocysts was incubated for various times (0.5, 1, 6, 12, 24, 48, 72, and 96 hr) after irradiation at 10 kGy. Oocysts in the control group were maintained at room temperature during mock irradiation at 0 kGy. The temperature of the control and irradiated sample tubes was the same before and immediately after irradiation (20°C  $\pm$  0.3°C).

### Transmission electron microscopy

*C. parvum* oocysts were fixed with 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 2 hr and post-fixed with 1% phosphate-buffered osmium tetroxide for 1 hr. Oocysts were dehydrated in an ethanol series (30% to 100%) and propylene oxide and then embedded in Epon (Polybed 812). Ultrathin sections (90- $\mu\text{m}$ ) were prepared with an ultramicrotome (Leica, Wetzlar, Germany) and were stained with 2% uranyl acetate in 50% methanol and lead citrate. Sections were examined using a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

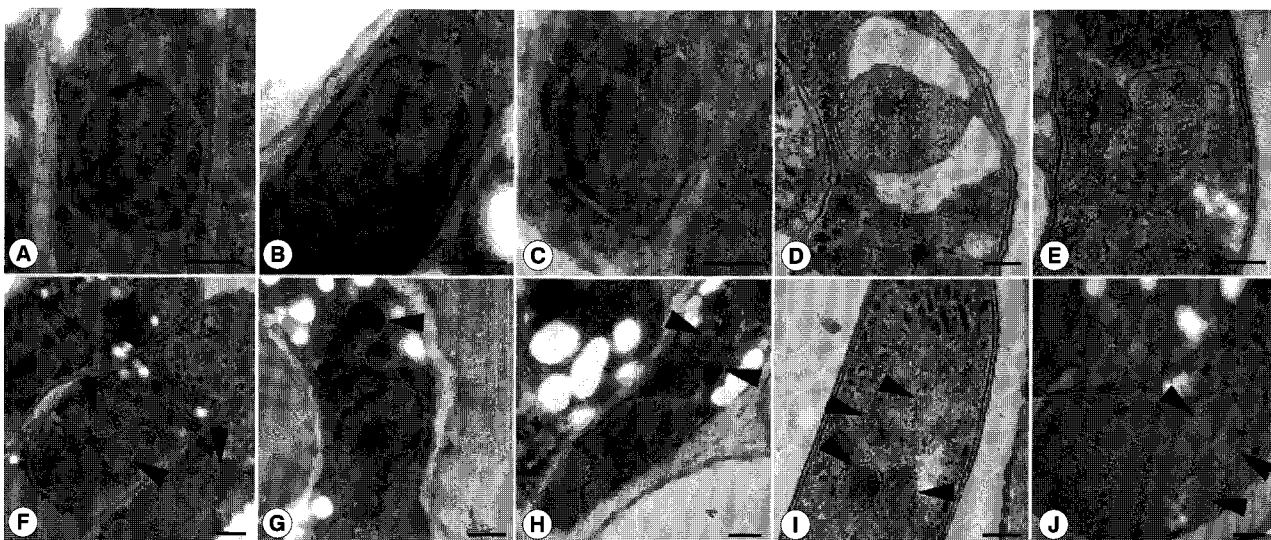
## RESULTS

### Radiation dose-dependent morphological changes in *C. parvum* oocysts

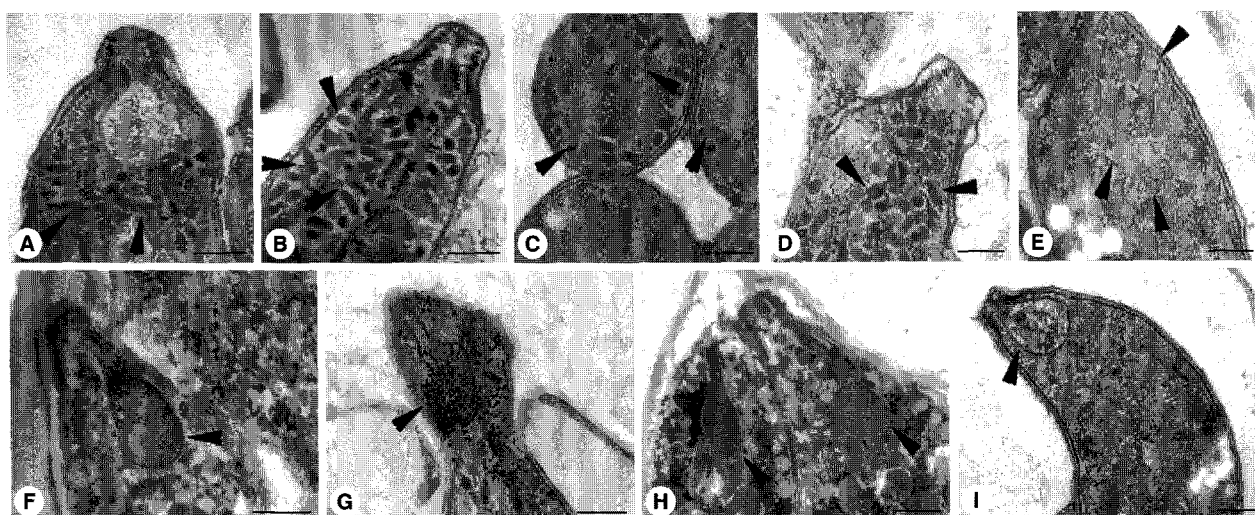
The nuclei of non-irradiated *C. parvum* sporozoites had peripheral condensed chromatin and well-demarcated, widely spaced nuclear membranes (Fig. 1A). Plastid-like organelles located anterior to the nuclei were observed (Fig. 1A). The nucleus of *C. parvum* irradiated at 1 kGy was the same (Fig. 1B). However, peripheral chromatin began to disappear starting at 5 kGy (Fig. 1C), and the nuclear membrane started to separate from the cytoplasm, progressing to a wide empty space between the cytoplasm and nucleus at 10 kGy (Fig. 1D). At 25 kGy, nuclei were shrunken and had irregular membrane boundaries (Fig. 1E).

The dense granules in non-irradiated *C. parvum* sporozoites were located anterior to the nucleus but posterior to micronemes and had a well-demarcated, homogenous, round shape (Fig. 1F). Morphological changes in dense granules were not prominent at lower than 5 kGy irradiation (Fig. 1G, H), but dense granules became pale and appeared degranulated at greater than 10 kGy irradiation (Fig. 1I, J).

Micronemes in non-irradiated sporozoites were spherical or rod-shaped and were located under the conoid and alongside the rhoptry (Fig. 2A). The micronemes were unchanged at lower than 10 kGy irradiation (Fig. 2B-D). However, they manifested as swelled and pale in electrodensity at 25 kGy (Fig. 2E).



**Fig. 1.** Changes in nuclear (A-E) and dense granule (F-J) morphology of *C. parvum* sporozoites following gamma irradiation. (A, F) non-irradiated control; (B, G) 1 kGy; (C, H) 5 kGy; (D, I) 10 kGy; (E, J) 25 kGy. Bar=0.2  $\mu\text{m}$ . Asterisk in A; plastid-like organelle. Arrowheads; dense granules.



**Fig. 2.** Changes in microneme (A-E) and rhoptry (F-I) morphology of *C. parvum* sporozoites following gamma irradiation. (A, F) non-irradiated control; (B) 1 kGy; (C, G) 5 kGy; (D, H) 10 kGy; (E, I) 25 kGy. Bar=0.2  $\mu$ m. Arrowheads; micronemes (A-E) and dense granules (F-I).



**Fig. 3.** Changes in the oocyst wall of *C. parvum* following gamma irradiation. (A) nonirradiated control; (B) 1 kGy; (C) 5 kGy; (D) 10 kGy; (E) 25 kGy. Bar=0.2  $\mu$ m.

The rhoptry in non-irradiated sporozoites was pouch-shaped and opened to the conoid, which is the anterior-most structure (Fig. 2F).

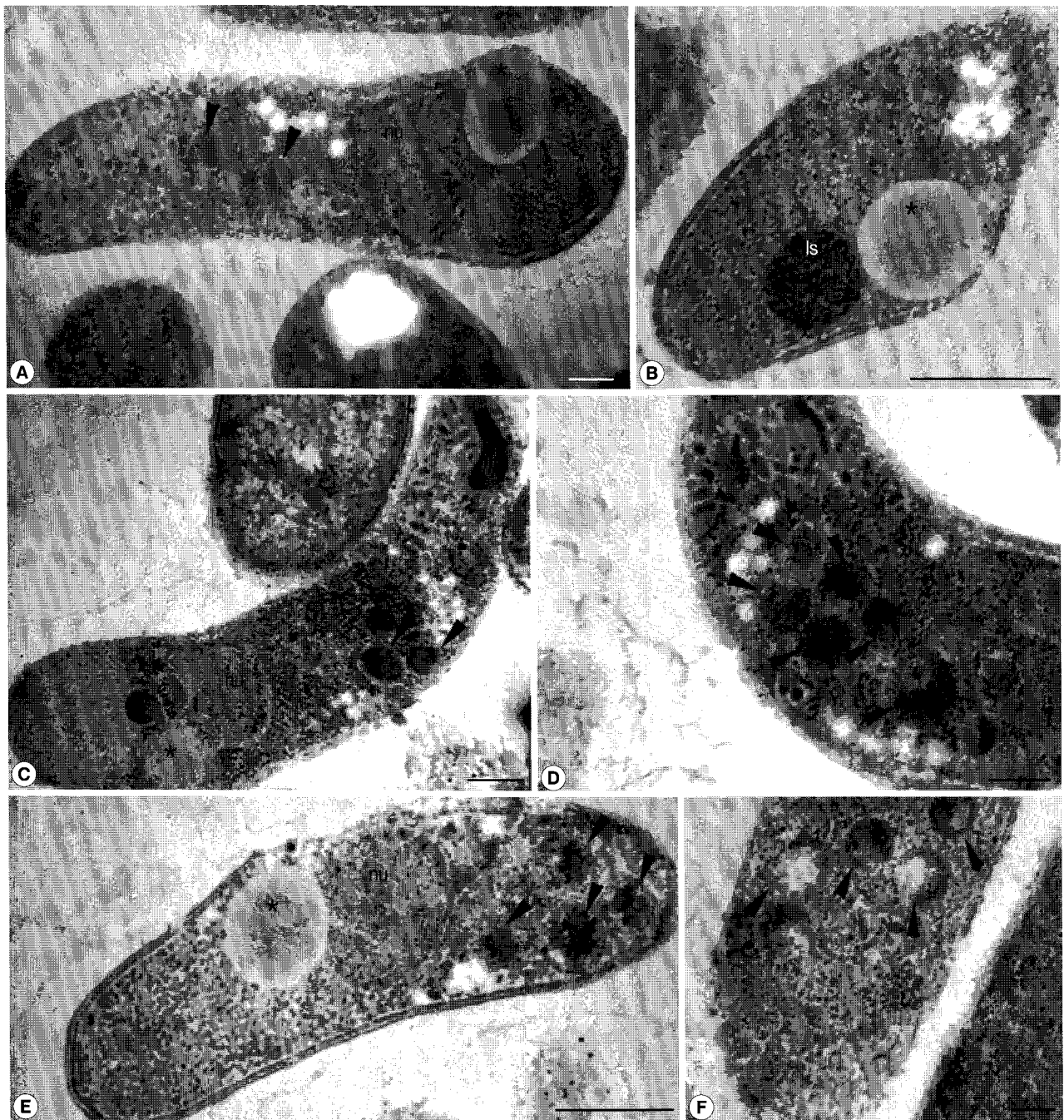
The morphology of the rhoptry was not significantly changed at up to 10 kGy irradiation (Fig. 2G, I), although the neck portion was shorter at 10 kGy and the inside appeared empty (Fig. 2I). The oocyst wall structure in non-irradiated *C. parvum* showed 3 layers, i.e., outer electrodense, middle electrolucent, and inner electrodense layers. The outside of the outer electrodense layer was coated with small particulate structures (Fig. 3A). The oocyst wall was not affected by gamma irradiation at up to 25 kGy (Fig. 3B-E).

#### Time-dependent morphological changes of *C. parvum* sporozoites after 10 kGy irradiation

To observe time-dependent morphological changes, *C. parvum* oocysts were irradiated at 10 kGy, and ultrathin sections were prepared from 6 to 96 hr after irradiation. After irradiation,

large homogenous vacuoles appeared near the nucleus in most sporozoites from 6 to 96 hr (Figs. 4-5). Dense granules were unchanged at 6 hr post-irradiation (Fig. 4A, B) compared to non-irradiated controls. Granules with empty pale centers appeared at 12 hr post-irradiation (Fig. 4C, D), with most granules having this empty appearance thereafter (Figs. 4E, F; Fig. 5).

Peripheral chromatin and the nucleolus disappeared in a very short time, at 6 hr (Fig. 4A), and eventually the nuclear membrane could not be differentiated from the cytoplasm (72-96 hr, Fig. 5D-H). Some sporozoites showed secondary lysosome-like bodies at 6 hr that were dark, granular, and round-shaped (Fig. 4B), as well as nuclei that were dark black and lamellated at 72 hr (Fig. 5E). The crystalloid body in the posterior part of the sporozoites maintained its original structure until 72 hr (Fig. 5E). Most of the sporozoites had no nucleus or cytoplasmic organelles 96 hr post-irradiation, but large homogenous vacuoles remained until 96 hr (Fig. 5F-H).



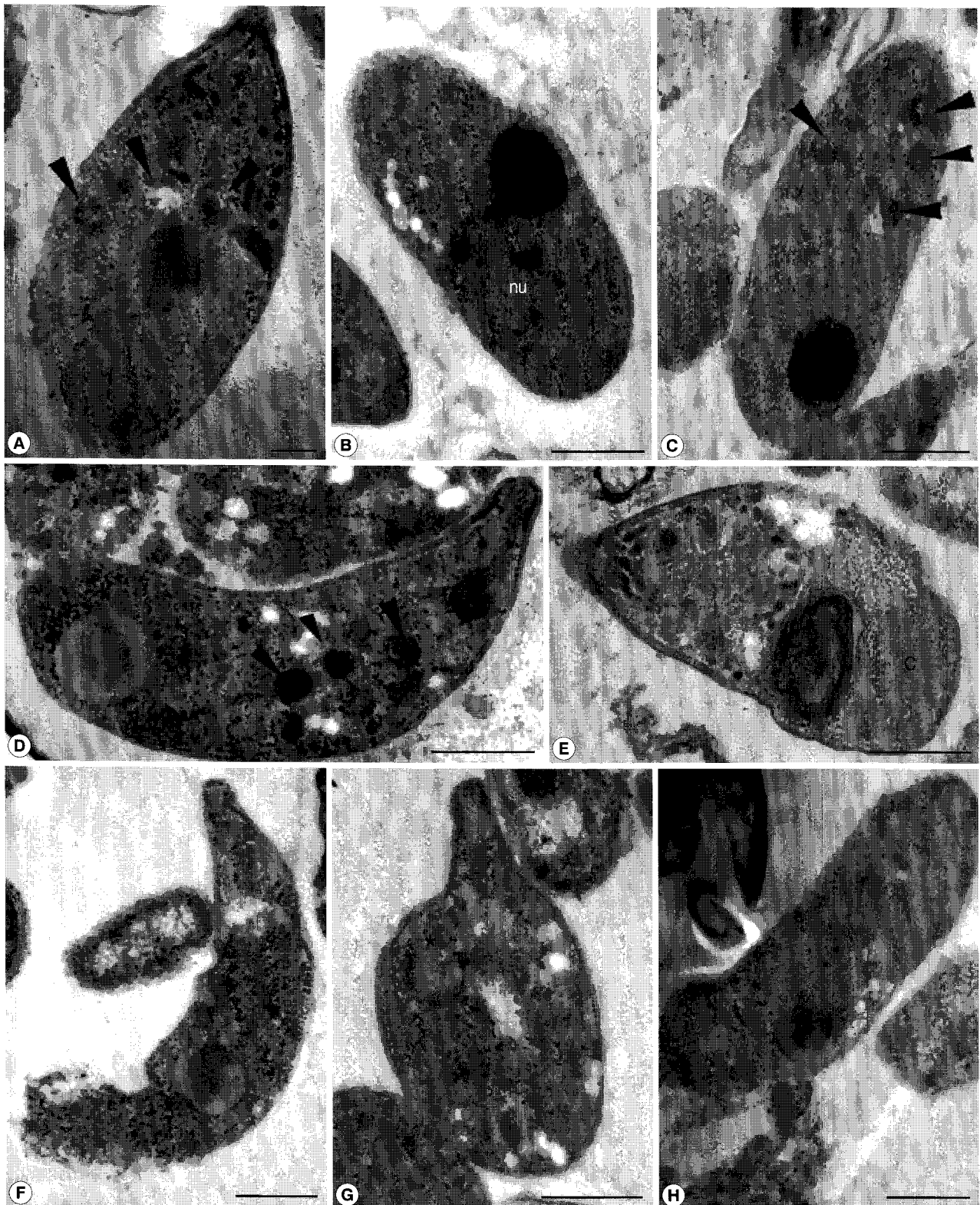
**Fig. 4.** Time-dependent morphological changes in *C. parvum* sporozoites 6 to 24 hr after irradiation at 10 kGy. (A-B) 6 hr; (C-D) 12 hr; (E-F) 24 hr. Bar=0.5  $\mu$ m. Arrowheads; dense granules. Asterisks; large homogenous vacuoles. ls, secondary lysosome-like body; nu, nucleus.

## DISCUSSION

Nuclear membranes are the principal targets of radiation [11,12]. Dilatation of the perinuclear space has been observed in a wide variety of cells following different types of irradiation [13-16]. Low doses of irradiation may induce a rapid and re-

versible swelling of nuclei in a variety of cells [13]. In our study, most of the internal structures in *C. parvum* sporozoites were unchanged by irradiation using doses up to 5 kGy. However, some of the sporozoites showed nuclear changes, such as loss of peripheral chromatin, at 5 kGy. Severe morphological changes in internal structures of *C. parvum* sporozoites mostly oc-





**Fig. 5.** Time-dependent morphological changes in *C. parvum* sporozoites 48 to 96 hr after irradiation at 10 kGy. (A-C) 48 hr; (D-E) 72 hr; (F-H) 96 hr. Bar=0.5  $\mu$ m. Arrowheads; dense granules. Asterisks; large homogenous vacuoles. c, crystalloid body; nu, nucleus.

curred with more than 10 kGy irradiation. Changes in dense granules were first observed at 10 kGy, and most of the internal contents appeared to become degranulated, so that dense granular membranes could be distinguished clearly. Micronemes seemed to be more resistant to gamma irradiation than any other internal organelles, because morphological changes were not prominent until at least 25 kGy were used. In a previous study, rod-like micronemes in non-irradiated normal sporozoites became round following loss of the quasi-helical arrangement of internal proteins within them [17]. Our observations suggest that this protein arrangement in micronemes may be affected by only high doses of irradiation.

Mitochondria have long been considered to be direct intracellular targets of ionizing radiation, and structural and functional alterations of mitochondria are commonly observed in irradiated cells [18,19]. However, the absence of mitochondria in *C. parvum* may be one of the reasons for the radioresistance of this organism [20].

From the previous studies, it has been known that, following ionizing radiation, the number and total volume of lysosomes increase in the cells, and elevated lysosomal enzyme activity can be detected biochemically [21-24]. These findings suggest that cell death is a consequence of the unregulated release of digestive enzymes from lysosomes after irradiation. In our study, we did not find microbodies, such as primary lysosomes in the sporozoites; however, at 10 kGy, we observed newly formed bodies that looked like secondary lysosomes seen in mammalian cells [25]. The present study also showed that the crystalloid bodies retained their original structure until 72 hr post-irradiation at 10 kGy, although the function of these structures is not known.

The oocyst wall is composed of 2 electrodense layers (50 nm thick) separated by a thin electroluscent space [26]. The surface of *C. parvum* oocysts, known as the glycocalyx, has uniformly distributed aggregates that is antigenic [26]. We found that the oocyst wall structure was also highly resistant to gamma irradiation up to 25 kGy.

Interestingly, large vacuoles were formed in the cytoplasm of sporozoites that were irradiated at 10 kGy. These newly formed vacuoles were always located adjacent to the nucleus and retained their appearance until 96 hr, when all other internal structures had degenerated. The role of these produced vacuoles after irradiation of *C. parvum* is unclear. However, it is clear that these vacuoles formed as a consequence of exposure to radiation. Further studies are necessary to identify the nature of

these vacuoles in *C. parvum*.

In a previous study using the comet assay, rejoining of damaged DNA in *C. parvum* after 10 kGy irradiation started at 6 hr post-irradiation and was completed at 72 hr, when the DNA appeared the same as non-irradiated *C. parvum* DNA [8]. In contrast, we found that morphological recovery of damaged sporozoites after 10 kGy irradiation did not occur. Therefore, these findings suggest that DNA rejoining does not certainly mean morphological restoration as well.

In conclusion, the ultrastructure of *C. parvum* oocysts was markedly changed following gamma irradiation. Nuclear membrane changes and degranulation of dense granules were observed at 10 kGy or more, and morphological changes in micronemes and rhoptry were observed at 25 kGy or more. From this study, morphological evidence of radioresistance of *C. parvum* has been supplemented.

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