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A transmission electron microscopy study on effects of a modified Glutaraldehyde fixation on *Acanthamoeba castellanii*

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Abstract. Transmission electron microscopy (TEM) can provide high resolution imaging of biological specimens. The study is to establish the effects of a modified glutaraldehyde (GA) compare to the standard GA fixation on *Acanthamoeba castellanii* from TEM perspectives and thus provide precise and accurate information on the ultrastructure studies of the parasite. By increasing the contrast, the ultrastructures of the parasite were more evident. The TEM images were obtained from parasites fixed with the modified GA and the standard GA and then the area of the nucleus and the gray values of the image of the nucleus of the parasites were measured. The mean areas of the nucleus were found to be significantly reduced in the standard GA fixed parasites (12210.4 nm^2) compared to the modified GA fixed parasites (8676.3 nm^2) ($p<0.05$). The mean gray values of the image were significantly reduced from 2024 in the standard GA fixed parasites (2024) to the modified GA fixed parasites (1636) ($p<0.05$). The study shows that the modified GA produced significantly better contrast on TEM images of the *A. castellanii* compared to the standard GA. This was because the modified GA generated more free water molecules during fixation and the uptake of modified GA by the nucleus of the parasite organizing all protein constituents in the cell into a more closely packed configuration than that of the standard GA. With such properties, the modified GA is a better fixative providing better images for ultrastructures of the parasite.

INTRODUCTION

The detailed understanding and characterization of the cell structure often requires analysis by electron microscopy. Light microscopy offers limited analysis and is often too broad in resolution to distinguish the intimate subcellular structures. Studies of biomembranes and membranous organelles require electron microscopy, as the resolving power of light microscopes is often insufficient to analyze subtle changes in topological and structural details (Christoph *et al.*, 2001).

Resolution in the electron beam is normally limited by contrast and not of its resolving power. Contrast of images is determined by the nature and extent of interactions between the electron beam and the specimen. Contrast is defined as the relative difference in intensity between an image point and its surroundings (Tian-Hu, 1995). Contrast can also be defined as the relative difference to the gray value of the image. This gray value is sometimes referred to as the intensity of a pixel of the image (Paul, 1993). The amount of contrast that occurs at any particular specimen point is dependant on the specimen's density and overall thickness and is relatively independent of the atomic number, chemical composition or other specimen properties (Christoph *et al.*, 2001). Hence, as mass thickness increases, the probability of contrast increases. However, contrast may be increased by the

preferential addition of materials of high atomic number during specimen preparation (Tian-Hu, 1995).

For the study of ultrastructure in biological tissues, chemical fixatives such as pH-buffered glutaraldehyde (GA) are commonly used as part of the preparative procedures. It is commonly assumed that during such chemical fixation, there is a reasonable uniform penetration of such a fixative-buffer combination into the tissue samples (Bullock, 1984). The introduction of GA in electron microscopy gave a more rapidly penetrating fixative with insolubilized proteins contents. GA has fairly small molecules, each with aldehyde groups, separated by a flexible chain of 3 methylene bridges. The potential for cross-linking is obviously much greater than with formaldehyde because it can occur through both the –CHO groups and over variable distances (John, 2000). All these –CHO groups will combine with any protein nitrogen with which they come into contact. The rate of GA penetration is dependant primarilly on the type of the specimen tissue, temperature ambience and the concentration (Hayat, 1981).

The modified GA (6% GA, 0.4% H_2O_2) has been reported to preserve enzymatic activity of cells (Peracchia & Mittler, 1972). The effectiveness of this mixture has been presumed to depend on the activity of reaction products of H_2O_2 and GA. A reaction between H_2O_2 and double bonds of ±, ^{2} unsaturated aldehydes may give rise to epoxy groups. Epoxides are known to be unstable compounds that are capable of reacting with amino and hydroxyl groups (Byard *et al.*, 1986). Thus the addition of epoxy groups to the unsaturated polymers of GA enhances their reactivity with tissues components. The improved GA fixation with H_2O_2 may increase available O_2 during fixation. Some evidence indicates that in GA and protein reaction, pyridine compounds are formed and that intermediate products in this reaction rapidly use O_2 under conditions of fixation (Hayat, 1981).

Transmission electron microscopy (TEM) is essential for viewing internal structures of protozoa cell because most parasitic protozoa in human are less than 50 µm in size. An electron microscopy study of the ultrastructure of trophoizoites and cystes of *A. castellanii* has been undertaken by Bowers & Korn (1968). In addition, morphogical changes during encystment and excystment of *A. castellanii* have been examined by Chambers & Thompson (1972).

In recent years, the use of fast freeze fixation followed by freeze substitution on *A. castellanii* trophozoite stage was shown to be most satisfactory for the ultrastructure cellular components due to the rapidity of the fixation. Nevertheless, this study require special equipments and cryoprotectivity (Gonzales-Robles *et al.*, 2001). This study investigates the use of the modified GA in the ultrstructure preservation of the cellular components of trophozoite stage of *A. castellanii*.

MATERIALS AND METHODS

Parasites Culture

Acanthamoeba castellanii was originally obtained in 2004 through the courtesy of Prof. Mulkit Singh of the National University of Singapore. The parasite was cultured and then cryopreserved in liquid nitrogen. For the study, the parasites were retrieved from liquid nitrogen and cultured in 4% Mycological peptone (Oxoid, UK) in 25 mm^2 culture flask at room temperature.

Fixations

The standard GA contains 2.5% of GA in 0.1N phosphate buffer (Timothy *et al.*, 2000). The modified GA contains 6% GA and 0.4% H_2O_2 in 0.1N phosphate buffer (Hayat, 1981).

Parasite Preparation

When the parasite density was high, they were dislodged from the culture flask by placing it on ice for 2 to 5 minutes. The parasites were then harvested and washed twice with sterile PBS, pH 7.2. The parasites were then counted, using Neubauer Counting Chamber and adjusted to 1.4 X

10^4 cells per test. The parasites were then fixed overnight with the modified GA and the standard GA in separate micro-centrifuge tubes. Specimens were then washed twice in 0.1N phosphate buffer, resuspended in 10% albumin solution and centrifuged at 8 000 rpm for 10 minutes. The resultant sediment of parasites was then fixed in the modified GA (as test) and the standard GA (as control) for another 24 hours. Each solidified sample was cut using single edged blade, and the block containing parasites was further fixed in 1% osmium tetraoxide (O_SO_4), dehydrated in graded solutions of 50%, 70%, 90% and 100% ethanol for 5 minutes each, followed by infiltration with epoxy resin and embedded in polyethylene capsules. The blocks were was then trimmed and cut to 90 nm ultra thin sections, followed by mounting on 200 mesh thin bar copper grids (Agar). Then the sections were double stained in uranyl acetate (2%) and lead citrate (1%) (Timothy *et al.*, 2000). Each specimen was examined at magnification of 4 400X, 11 000X, and 25 000X by using Technai G2 TEM at an accelerating voltage of 80 Kv.

Measurement of the contrast of nucleus of *Acanthamoeba castellanii*

For the determination of effect of the modified GA in the preparation of specimen for TEM, we focused on areas of the nucleus for better analysis of the contrast of the parasites. The area of nucleus and the gray value of the image of the nucleus of the parasites were measured by Megaview II Soft Imaging System software. The area of nucleus and the gray value of the image of the nucleus of the parasites of the standard GA fixation are as the control batch and the modified GA fixation parasites are as the test batch. The mean value area of the nucleus and the gray value of the image of the nucleus of 40 parasites each from the control and test batches respectively were analyzed by unpaired *t* test statistical software SPSS.

RESULTS

Effects of the modified GA on nucleus of *A. castellanii*

The images of the nucleus using the standard GA are as in (B), (D) and (F) while the images of nucleus using the modified GA are as in (A), (C), (E). Clearly, the images (A), (C), (E) are showing better contrast compared to the images of (B), (D), (E). The mean area of the nucleus was found to be significantly reduced from 12210.4 nm^2 in the standard GA fixed parasites to 8676.3 nm^2 ($p < 0.05$) (Table 1) of the modified GA fixed parasites, a 29% reduction mean area of the nucleus. The mean gray values of the image were significantly reduced from 2024 in the standard GA fixed parasites to 1636 ($p < 0.05$) (Table 1) of the modified GA fixed parasites, a 19 % reduction mean gray values of the image. The study shows that the modified GA presented significantly better contrast on TEM images of the *A. castellanii*.

Further more, organelles in the parasite's cell such as the golgi apparatus and mitochondria are also more clearly defined in these in the modified GA (Figure 1, A) compared to the standard GA (Figure 1, B). More heterochromatin is observed in the modified GA (Figure 2, C) as compared to that in the standard GA (Figure 2, D). Mitochondria in the modified GA (Figure 3, E) shows the outer and inner membrane more clearly compared to the standard GA's mitochondria (Figure 3, F).

Table 1. Mean values of area of nucleus & gray values of Modified and Standard GA of *Acanthamoeba castellanii*

Acanthamoeba castellanii	Modified GA	Standard GA
Mean area of nucleus (nm^2)	8676.3	12210.4
Mean gray values	1636	2024

(A) (B)

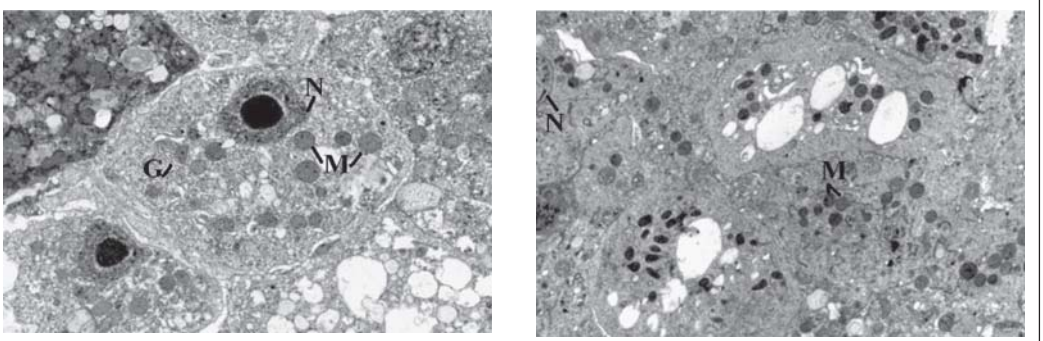

Figure 1. The TEM images of the parasies fixed with the modified GA (A) & with standard GA (B). Bar ▬▬ 2 000 nm (4 400X). N = nucleus membrane, G = Golgi Apparatus, M = mitochondria

(C) (D)

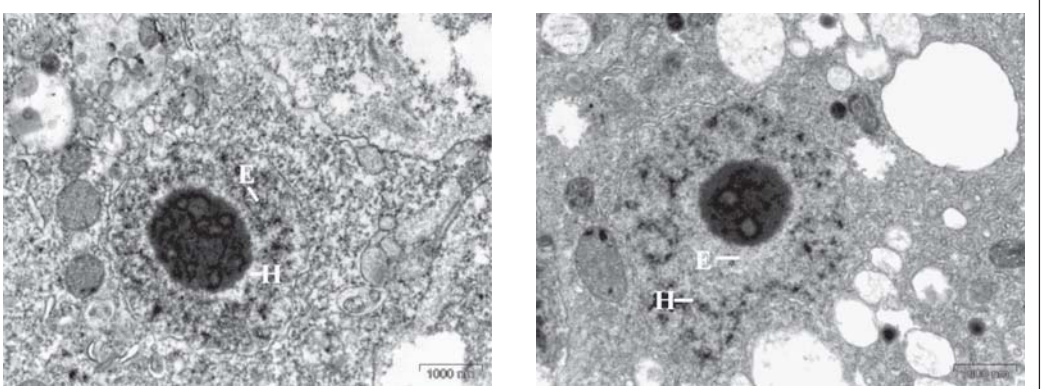

Figure 2. The TEM images of the nucleus of the parasites fixed with the modified GA (C) & with standard GA (D). Bar ▬▬ 1 000 nm (11 000X). H = heterochromatin, E = euchromatin

(E) (F)

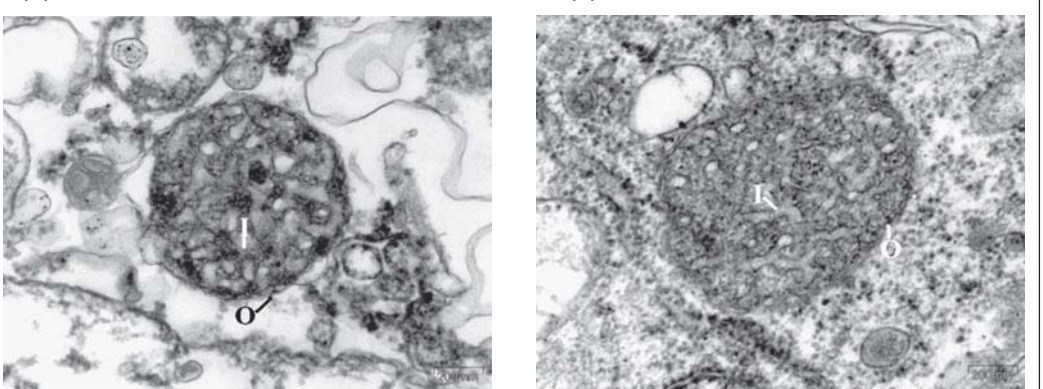

Figure 3. The TEM images of the mitochondria of the parasites fixed with the modified GA (E) & with standard GA (F).
Bar ▬▬ 200 (25 000X). I = inner membrane, O = outer membrane

DISCUSSION

The study shows that the modified GA offers significantly better contrast on TEM images of *A. castellanii* compared to the standard GA. This is because the modified GA is a superior fixative compared to the standard GA. Its superiority is displayed by the data showing the modified GA significantly reduced the area of the nucleus of the parasite compared to that of the standard GA ($p<0.05$). One explanation is that the proteins of the nucleus undergo a mechanism of cross-linking with the modified GA. This mechanism generates more free water molecules compared to the standard GA, similar to the findings of Jearanaikoon & Abraham-Peskir (2005). These free water molecules are dehydrated by the series of ethanol used in the sample processing protocol. In addition, the shrinking of the nucleus is caused by the uptake of the modified GA by the nucleus of the parasite organizing all protein constituents in the cell into a more closely packed configuration than that of the standard GA. The more closely packed and more organized the configurations in the cells are, the more contrast to the ultrastructure of the parasites during electron microscopy examination, this is comparable to the findings of Jearanaikoon & Abraham-Peskir (2005).

Secondly, the modified GA reacts faster to the protein groups of the parasite compared to the standard GA. This is proven by the higher presence of heterochromatin observed in the nucleus of the modified GA compared to that in the standard GA. The abundance of heterochromatin in the nucleus of the parasite shows that the parasite has been fixed closer to its temporary inactive stage (Harry, 1996). This is due to the procedure of freezing the cultured medium that causes the parasites to dislodge from the culture flask. This is in contrast with the standard GA, where there is more euchromatin observed in the nucleus of the parasite. The more euchromatin observed the more active the parasites are (Harry, 1996). Hence, the modified GA fixes the parasite closer to its acquired conditions.

Thirdly, the morphology of the chromatin is more clearly defined in the modified GA compared to the standard GA. The other organelles in the parasite's cell such as golgi apparatus and mitochondria are also more clearly defined in the modified GA. This because the matrix cell in the modified GA is relatively lesser than to the standard GA.

The modified GA is therefore is a better fixative compared to the standard GA especially in the TEM molecular studies of *Acanthamoeba castellanii*. It is able to react faster, penetrate deeper into the cells, produce increased contrast, immobilize cell contents closer to acquired conditions, better preserve the cytoplasmic organelles, and offer more protection during the sectioning and embedding processes. Further detailed studies must be done on modified GA because this study did not focus on the chemical extraction i.e enzymes and effects on other type of tissues.

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