THE EFFECTS OF COLCHICINE ON SPERMATOGENESIS IN NITELLA

F. RUDOLF TURNER

From the Cell Research Institute, The University of Texas at Austin, Austin, Texas 78712. Dr. Turner's present address is Department of Botany, Indiana University, Bloomington, Indiana 47401

ABSTRACT

Treatment of Nitella antheridia with colchicine results in various sperm abnormalities, depending upon duration of exposure and subsequent recovery. Early effects of treatment include disappearance of spindle fibers and a cessation of ordered cell wall formation in dividing cells. Sperm released from antheridia treated for 24 hr and allowed to recover for 4–5 days possess branched flagella. After a recovery period of 6–10 days the sperm appear normal; however, following longer recovery periods, the sperm exhibit variations in size and number of flagella. Branched flagella contain a variety of microtubule patterns ranging from branches containing a single microtubule to flagella with an excess of microtubules. Spermatids which differentiate in the presence of colchicine lack flagella and a microtubular sheath. Nuclear contents undergo condensation stages; however, the nucleus as a whole does not undergo the orderly elongation and coiling characteristic of untreated Nitella spermatids. Long-term colchicine treatment followed by a recovery period produces atypical microtubules and microtubular aggregations in the spermatid. The results indicate that colchicine affects not only polymerization of microtubule subunits but also factors responsible for their ordered spatial relationships in the cell. The presence of microtubules is a prerequisite for normal morphological changes during spermiogenesis.

INTRODUCTION

Colchicine and its derivative Colcemid are effective inhibitors of mitosis in a large number of plant and animal systems (2, 9, 10, 14, 16). At the ultrastructural level these agents have been shown to affect the microtubules of the spindle, causing a reversible dissolution or disorientation of the spindle fibers (5, 19, 21). Colchicine and related compounds have also been shown to have a similar effect on other types of microtubular systems in a wide variety of cell types (19, 21, 28, 29, 34–36).

Numerous functions have been postulated for microtubules on the basis of their form and distribution in a wide variety of cell types. Among these functions, the involvement of microtubules in the development and maintenance of cell shape is becoming increasingly apparent (27–30, to mention a few). The present investigation was undertaken to study the effects of colchicine on several different types of microtubular systems in a single organism and to gain a better understanding of the role of microtubules in differentiation. The antheridia of Nitella proved to be suitable material for this study because of the ease with which they can be treated and the synchrony exhibited by the cells within a single antheridium. Observations were made on the effects of colchicine on mitosis in the antheridal filament cells prior to spermiogenesis and on the formation of flagella and the microtubular sheath in the spermatid.
The normal course of spermatogenesis in *Nitella* has been described at the ultrastructural level (31).

**MATERIALS AND METHODS**

Male plants of the dioecious charophytes *Nitella missouriensis* (Allen) and *Nitella mirabilis* Nordst. ex J. Gr. bearing antheridia in various stages of development were cultured in the presence of colchicine1 (1.25–2.50 × 10⁻³ M) for varying periods of time. After treatment, the plants were either fixed or washed with water and allowed to develop normally.

For light microscopic study, sperm escaping from mature antheridia were collected daily, spread out on a microscope slide, and fixed with OsO₄ vapors. After air drying, these preparations were mounted in immersion oil (nD = 1.464) and examined with phase-contrast optics.

For electron microscopy, antheridia were collected at intervals and processed as described previously (31). The primary fixative in the earlier study consisted of 4% glutaraldehyde buffered with 0.05 M sodium cacodylate (pH 7.2) (24). In later studies this was replaced with a fixative consisting of 4% glutaraldehyde and 0.7% picric acid, buffered to pH 7.2 with sodium cacodylate, which was found to give better penetration of the antheridial cells. Fixation was carried out at room temperature for 2–8 hr.

Following a rinse in buffer, the antheridia were postfixed for 2 hr at 4°C in 1% osmium tetroxide buffered with 0.05 M sodium cacodylate (pH 7.2), stained overnight with 0.5% aqueous uranyl acetate, and embedded in a mixture of Epon and Araldite (Mixture No. 1 of Mollenhauer[17]). 1µ thick plastic sections were examined with a phase-contrast microscope, and antheridia containing the desired stages were selected for study with an RCA EMU-3F electron microscope. Additional observations were made on preparations of whole sperm shadowed with platinum or negatively stained with 2% phosphotungstic acid (PTA), pH 7.3, or 1% aqueous ammonium molybdate.

**RESULTS**

For *N. missouriensis*, the most consistent results were obtained with a 2.5 × 10⁻³ M (0.1%) colchicine solution. This concentration proved to be toxic for *N. mirabilis*, necessitating the use of a lower concentration (1.25 × 10⁻³ M). Both species exhibited similar responses to colchicine treatment and showed no significant differences at the ultrastructural level. Some degree of variability in the results was experienced due to differences in the physiological condition of the plants at the time of treatment. The results presented here represent the cumulative observations of a series of experiments carried out over a period of several years.

**Morphology of Sperm Released after Treatment**

*Nitella* sperm collected for the first 3 days after a 24 hr exposure to colchicine appear normal in all respects. A majority of the sperm collected 4 and 5 days after treatment, however, show pronounced bifurcations or branches along the flagella. Although readily seen with phase-contrast optics, these branches can be seen more clearly in material prepared for electron microscopy (Fig. 1). The flagellar branches do not appear to be restricted to any particular location along the flagella, and they vary considerably in length and diameter. Usually a given flagellum will bear a single branch, although multiple branches are occasionally observed. Other flagella may be completely devoid of branches.

Following a recovery period of 6–10 days, the sperm appear normal. Sperm maturing after a recovery period longer than 10 days exhibit a large variation in size and in the number of flagella on a single sperm (Fig. 2). The number of excess flagella on an individual sperm is not necessarily a multiple of the normal complement of two and may range from one to as many as four extra.

**Short Term Colchicine Treatment**

In dividing antheridial filament cells fixed immediately after 24 hr colchicine treatment, there is a complete loss of spindle microtubules (Fig. 3). The spindle region contains numerous ribosomes and small vesicles but is devoid of other organelles. In many instances the chromosomes show evidence of clumping (Fig. 3). These pre-spermatid antheridial filament cells frequently are multinucleate, and many contain partial cell walls indicating the cessation of wall formation in the presence of colchicine (Fig. 5).

After a recovery period of 1 or more days following colchicine treatment for 24 hr, typical mitotic figures containing oriented spindle microtubules are again seen (Fig. 4). Cytoplasmic microtubules reappear in interphase cells. Many of the centrioles remain in clusters during the recovery period (Fig. 21), and excess centrioles are eventually carried into the spermatids (Fig. 22) where they give rise to excess flagella (Figs. 2, 23). Other

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1 Sigma Chemical Co., St. Louis, Mo., Matheson Coleman & Bell, East Rutherford, N. J.

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FIGURE 1 An electron micrograph of a Nitella sperm from a colchicine-treated antheridium showing two branches (arrows) on one flagellum. The second flagellum, only partially included in the micrograph, is normal. For a description of normal sperm morphology, see Turner (30). Platinum shadowed preparation. C 1, R 5.* × 5000.

FIGURE 2 The anterior end of a sperm possessing two excess flagella in addition to the normal complement of two. The flagella (f₁–f₄) are covered by a normal scale coat. Platinum shadowed preparation. C 1, R 12. × 10,000.

* The designation C₁, R₁ will be used to designate the duration of exposure to colchicine and the subsequent recovery period, respectively. Units are given in days.
Figure 3 A portion of an antheridial filament cell illustrating the effects of colchicine on mitosis. Note the clumped chromosomes and the absence of spindle fibers. C 1, R 0. × 10,000.

Figure 4 A division figure in an antheridial filament cell which has been treated with colchicine and then allowed to recover. The chromosomes are distinct and oriented spindle fibers (arrow) are present. C 1, R 1. × 24,000.
abnormalities seen in the treated cells, notably incomplete cell walls and walls formed obliquely to the long axis of the antheridal filament, tend to persist during the recovery period (Fig. 6). This unequal cytokinesis is at least partially responsible for the variation in size of the mature sperm released from treated antheridia.

Both shadowed (Figs. 1, 7) and negatively stained (Fig. 8) preparations of sperm released 4-5 days after treatment confirm the light microscopic observations of branched flagella. Microtubules can be distinguished in the main axis of the flagellum and in the branches in negatively stained preparations; however, many of the fine structural details are obscured by the heavy scale coat surrounding the flagellum (Fig. 8). In some instances the microtubules of a branch can be followed into the main axis of the flagellum (Fig. 8). At present there is no reason to believe that the microtubules themselves branch, but rather it appears as though the spatial orientation of the microtubules in the axial complex becomes disrupted, causing one or more of them to follow a different path. A related type of disruption may occasionally be seen along the flagellar shaft when several of the microtubules loop back on themselves forming a distended area in the flagellum (Fig. 7).

Thin sections of spermatids with branched flagella reveal cross-sections of flagella and branches with a wide variety of microtubule patterns (Fig. 11). These range all the way from branches containing a single microtubule to flagella possessing an excess number of microtubules in addition to the normal "9 + 2" configuration. Regardless of the type of abnormality, all flagella and branches are covered by a normal-appearing scale coat. The few longitudinal sections which have been obtained, passing through the fork of a branch, do not provide any evidence that the microtubules themselves branch. Rather they also indicate that some of the microtubules diverge away from the axial filament complex of the flagellum to form a branch (Figs. 9, 10).

In the course of this investigation, no evidence has been found to show that the structure of the centriole or basal body is in any way affected by colchicine treatment. Abnormalities induced in the flagella are restricted to regions distal to the basal bodies.

Long Term Colchicine Treatment

A limited number of observations have been made on antheridia which have been subjected to colchicine treatment for an extended period of time. Spermatids which have been allowed to develop in colchicine for 1 wk or longer with no subsequent recovery appear to be completely devoid of microtubules. Many of the later stage spermatids are highly asymmetric with long processes extending out from the surface of the protoplast (Figs. 12, 13). These extensions lack microtubules but are covered by a typical scale coat. Several micrographs show electron-opaque material (filibrils?) regularly disposed in regions where one would ordinarily expect to find the microtubular sheath in untreated spermatids (Figs. 14, 15).

During these long-term treatments, the nuclear contents undergo condensation stages similar to those seen in untreated spermatids. The characteristic form changes of the nucleus which occur during these stages of spermiogenesis in untreated material, however, do not accompany nuclear condensation in the presence of colchicine. The orderly elongation and coiling of the nucleus seen in normal spermatids is replaced by apparently random changes in the shape of the nucleus, resulting in highly pleomorphic nuclei during late spermatid stages in the treated material (Figs. 12, 13).

After long term colchicine treatment, followed by a recovery period, microtubules are again present in various patterns and locations in the spermatids. Portions of the microtubular sheath containing regularly spaced microtubules are often seen (Fig. 16). In addition, clusters of microtubules are frequently found in the body of the spermatid and in cellular extensions (Figs. 17-20). In many of the clusters, a large portion of the microtubules appear to be associated with membrane-like structures of uncertain derivation (Figs. 17, 18).

Of particular interest in the long term colchicine-treated antheridia is the appearance of doublet and triplet microtubules in the spermatids (Figs. 19, 20). These atypical microtubules may be present as isolated structures or may occur in association with other microtubules. Several triplets have been found containing what appear to be only partially formed microtubules (Fig. 19).
Figure 5 An antheridial filament cell undergoing division at the time of treatment. Individual or groups of chromosomes are enclosed by separate membranes, and a portion of an incomplete cell wall (arrow) lies near the center of the cell. C 1, R 0. × 11,500.

Figure 6 Small portion of an antheridial filament fixed after colchicine treatment and subsequent recovery. Note characteristic disoriented and incomplete cell walls which cause gross differences in adjacent cells. C 1, R 1. × 8000.
DISCUSSION

Delay and Carpentier (8) have reported on the action of colchicine on Chara, an alga closely related to Nitella. They demonstrated that the antheridial filament cells attained varying degrees of polyploidy following treatment and that the sperm differentiating from these cells exhibited numerous abnormalities including extra flagella and variation in size. These investigators apparently did not observe branched flagella in their preparations.

Mitosis in the antheridial filament cells of Nitella is similar to animal cell mitosis with respect to the mitotic apparatus and behavior of the centrioles (31). The additional feature of cell wall formation makes cytokinesis in this system more comparable to cell division in plants. The effects of colchicine observed in dividing antheridial filament cells are consistent with those reported for several different animal and plant systems (5, 19, 21, 32). Under the conditions employed in the present investigation, the loss of spindle microtubules is complete. The action of colchicine on the spindle is reversible as evidenced by the reappearance of ordered spindle microtubules following removal of colchicine.

The interruption of cell plate formation and the appearance of disoriented cell walls during colchicine treatment and subsequent recovery has been noted by several authors (19, 32, 33). There is a substantial amount of evidence to support the idea that microtubules are involved in the orderly deposition of cell wall material as originally proposed by Ledbetter and Porter (15, 7, 12, 20, 35). Treatment of wheat seedlings with colchicine (19) results in cell wall malformations which coincide with the disappearance of cell wall-associated microtubules, further supporting the work of Green (11) relating microtubules to wall formation and the loss of orientation of wall synthesis to the presence of colchicine.

As a consequence of the formation of malformed and disoriented cell walls in the antheridial filaments, many daughter cells receive unequal proportions of the various cell organelles following cytokinesis. Some of these inequalities may be carried through the remainder of spermatogenesis and manifest themselves as abnormalities, such as variations in size and number of flagella, in the mature sperm. These types of abnormalities first appear in the free sperm 11 days after treating the antheridia with colchicine. From this it is inferred that spermiogenesis, as measured from the last antheridial filament cell division, occupies a period of 10-11 days. Employing similar tech-
FIGURE 8 A negatively stained preparation of a small portion of a branched flagellum showing two microtubules diverging from the axial filament complex to form the branch. The amorphous material surrounding the microtubules is residue from the scale coat. × 88,000.

FIGURES 9–10 Longitudinal sections of branched flagella showing variations in the course of microtubules into the branches. Fig. 9, × 29,000; Fig. 10, × 39,000.
Figure 11  Representative transverse sections of flagella and flagellar branches illustrating the wide variety of microtubule patterns occurring in spermatids exposed to colchicine during flagellar formation. 

a-e, g, h, × 107,000; f, × 87,000; i, × 66,000.

Techniques, Delay and Carpentier (8) found a period of 9 days for Chara vulgaris.

Present evidence indicates that colchicine affects microtubules by binding directly to the macromolecular subunits, preventing their assembly into microtubules (3, 4, 25). The disappearance of spindle and cytoplasmic microtubules in the presence of colchicine can be explained if it is assumed that an equilibrium exists between this type of microtubule and its unpolymerized subunit (13). Any complexing of the subunits with colchicine would shift this equilibrium in a direction away from the formed microtubule.

The microtubules comprising the flagella and the microtubular sheath are much less labile than spindle fibers and cytoplasmic microtubules. Once formed, the former are relatively stable and are unaffected by agents such as colchicine. This is consistent with the findings of other investigators who have used various criteria, such as stability in the presence of colchicine, to categorize microtubules into different groups (1, 6).
**Figure 12** Longitudinal section through a portion of antherial filament containing spermatids which have developed for 11 days in the presence of colchicine. Note highly irregular nuclei which have undergone condensation during treatment, and the numerous cytoplasmic extensions. C 11, R 0. X 8500.

**Figure 13** A single spermatid enlarged to show details mentioned in the preceding figure. No microtubules have been found in these cells. C 11, R 0. X 21,500.
The appearance of branched flagella following colchicine treatment was unexpected. Because of the 4–5 day delay between the time of treatment and the appearance of branches and the fact that the continued presence of colchicine completely blocks flagella formation, it is assumed that these cells were undergoing ciliogenesis at the time of treatment. In this instance the colchicine apparently affects not only the polymerization of microtubule subunits but also affects factors responsible for their ordered spatial relationship in the flagellum. A somewhat analogous situation was reported by Tilney (28) who showed that the microtubules forming the axopodia of Actinosphaerium lose their regular spacing shortly after being exposed to colchicine. The inhibition of ciliogenesis by colchicine and Colcemid has been reported by others (22, 26); however, at present the formation of branched flagella appears to be a unique feature of Nitella.

Also unexpected was the appearance of doublet and triplet microtubules, as well as arrays of single microtubules, in spermatids after a brief recovery period following prolonged colchicine treatment. Microtubule precursors apparently accumulate during the colchicine block, probably in localized areas of the spermatids, where they polymerize to form microtubules when the colchicines is removed. It has been demonstrated that concentrations of colchicine sufficient to inhibit ciliogenesis need not inhibit RNA or protein synthesis (22). The formation of doublet and triplet microtubules may reflect differences in the subunits or in the local environment of the subunits at the time of polymerization. For a discussion of possible factors controlling the distribution and specific ordering of microtubules, the reader is referred to Tilney (27).

The membrane-like structures sometimes found associated with microtubules in spermatids following recovery from long-term treatment appear to be in direct contact with these microtubules. The membrane-like structures do not exhibit a typical unit membrane structure and do not appear to bear any direct relationship to other organelles of the cell. To date no evidence has been found to further clarify the nature of these structures or to indicate their origin.
Figures 17-20  Small portions of spermatids which have been allowed to recover following long term colchicine treatment. Figs. 17 and 18 illustrate clusters of microtubules associated with membrane-like structures. Many of the microtubules show a very regular distribution with respect to these structures. Figs. 19 and 20 show doublet and triplet microtubules which have formed during the recovery period. In Fig. 19, two microtubules appear to be linked by an incomplete portion of a third (arrow). C 9, R 2.

Fig. 17, x 95,000; Fig. 18, x 55,000; Fig. 19, x 108,000; Fig. 20, x 126,000.

The organized material which is occasionally found in treated spermatids, in regions normally occupied by the microtubular sheath in untreated plants, may represent microtubular material in an altered form. Several investigators have reported on the apparent transformation of microtubules into fibrillar elements in the presence of colchicine (23, 34, 36). It is conceivable that altered micro-
FIGURE 21 A small portion of an antheridial filament cell from a treated plant which has been allowed to recover. In close proximity to the nucleus is a cluster of five centrioles, two of which appear to be joined at their proximal ends (arrow). C 3, R 6. × 36,000.

FIGURE 22 A portion of a treated spermatid containing three centrioles. The extra centriole is linked to the other two by a bifurcation of the striated fiber. C 1, R 15. × 48,000.

FIGURE 23 Transverse section through the anterior end of a late spermatid treated 16 days prior to fixation. Two excess flagella and a portion of an extra microtubular sheath are present. C 1, R 16. × 55,000.

tubular material, perhaps in the form of fibrils, is ordinarily masked by the dense cytoplasm of the spermatid and is only occasionally recognizable in favorable sections.

Several additional features of the response of this system to colchicine may be noted. The results of long-term treatment indicate that although an organized microtubular sheath is not necessary for nuclear condensation in the spermatid, it is necessary for the normal elongation and coiling of the nucleus. The orderly arrangement of mitochondria, nucleus, and plastids is absent in spermatids which have developed in the presence of colchicine. These observations indicate that the microtubular sheath functions primarily as a cytoskeletal system upon which the remaining cell organelles are arranged. Similarly, development of the motility apparatus is inhibited; however, numerous cellular extensions, devoid of microtubules, may be formed.

Extra centrioles which are incorporated into the maturing spermatid eventually give rise to excess flagella. The basal bodies derived from these centrioles are linked by extra striated fibers, the function of which is presently unknown (31). The formation of these fibers is apparently the result of an interaction between the basal bodies and is one example of how an experimental system such as this might be used to study interactions between organelles.

The differentiation of a highly specialized cell type involves the participation of, and the interaction among, a number of cell organelles. When some portion of this closely integrated process of cell differentiation is blocked or otherwise altered, the results of this disruption manifest themselves as abnormalities in the final product. In his review on plant microtubules, Newcomb (18) has discussed evidence relating microtubules to the formation of the cell plate and the ordered deposition of cell wall material. Some of the experimental systems reviewed show an apparent interaction between microtubules and vesicles derived from the Golgi apparatus and/or the endoplasmic reticulum which is disrupted by agents affecting the microtubules.

Utilizing the plant alkaloid colchicine to inhibit specifically microtubule formation, the present study has shown that in this system also microtubules are necessary for directed cell plate formation in prespermatid antheridial filament cells. It has demonstrated that microtubules contribute to the segregation of organelles and to the development of form in the Nitella spermatid much as they do in the sea urchin embryo (30). Microtubules may also be necessary for maintenance of sperm shape as they are retained in the mature sperm (31).
The use of similar techniques on a variety of differentiating systems should prove of value in the elucidation of microtubule function. The controlled induction of specific modifications in specialized cells through the use of chemical agents such as colchicine could also prove useful in the study of the functioning of other cell organelles. Thus, a study of branched flagella may lead to a better understanding of flagellar motion, while a study of sperm with excess flagella might shed light on the problem of flagellar coordination.

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