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Effect of Gossypol on Bull Spermatozoa in Vitro

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Summary. Gossypol acetic acid in a concentration of 1,000 μg/ml solvent is able to immobilize 1 ml of native bull semen (sperm concentration: 8.5 x 10^8/ml; motility rate: 87.4%) within 30 min. After GAA treatment the spermatozoa show severe morphological damage on the membrane system, on the acrosomal complex and on the tubular complex of the end piece. The working mechanism of GAA can be assumed to be inactivation of enzyme activities or in direct reactions with plasma membrane material.

Key words: Gossypol, Bull spermatozoa, Motility, Ultrastructure.

Introduction

Chinese workers discovered that gossypol, a polyphenolic pigment isolated from cotton seed, exerts a remarkable antifertility effect on males of a number of animal species including man [9]. In China the drug has been given to more than 9,000 human volunteers over a period of three years without any overt toxicity; the drug has been reported to be 98.4% effective. This discovery by Chinese workers has aroused considerable interest amongst the students of reproductive biology all over the world to investigate the various aspects of gossypol action. In the recent past a number of reports have been published confirming the antifertility potential of gossypol [2–4, 6, 14]. Recently it has been reported that gossypol inhibits the motility of the spermatozoa in vitro [5, 10, 13]. Although the precise mechanism in the inhibition of sperm motility is not known, it is evidently clear that gossypol inhibits the activities of certain enzymes involved in the metabolic regulation of spermatozoa. The major enzymes reported to be affected by gossypol treatment are ATP'ase [5], lactic dehydrogenase and LDH-X [7, 12]. All these enzymes are associated with the specific morphological compartments of the spermatozoa. The purpose of the present investigation was to examine the morphological changes in these compartments of bull spermatozoa after gossypol treatment by more modern techniques of electron microscopy.

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Material and Methods

Gossypol acetic acid (GAA), obtained from the Rockefeller Foundation, New York, was used in a purity of 98.4%.

Fresh bull semen, collected by means of an artificial vagina was obtained from "Agricultural Station for Artificial Insemination" Klessheim, Salzburg.

The samples of proven motility (progressive motility not less than 80%, and sperm counts not less than 800 million/ml) were used in the present investigations. One ml of native bull semen (1 ml having a spermatozoa concentration of 8.5 x 10^8/ml and a progressive motility of 87.4%) was diluted in 1 ml of 1% Tween 80 made in isotonic tyrode-buffer (pH 7.2) containing 1,000 μg GAA (Fig. 15E). In another set of experiments (Fig. 15D), 1 ml of native semen was mixed with 1 ml of 1% glycerol made in tyrode-buffer, containing 1,000 μg GAA. For controls 1 ml of 1% Tween 80 buffered solution (Fig. 15C), or 1 ml of 1% glycerol buffered suspendant (Fig. 15B) were mixed with equivalent volumes of native semen. One ml of native semen was incubated without any diluting media as an untreated control (Fig. 15A).

All the samples were incubated at 37 °C in a water bath and semen analyses were done in intervals of 5 min. After 30 min of incubation parts of the sample of each group were prepared for electron microscopic investigations. After fixation in 2% glutaraldehyde (buffered in 300 mOsmol cacodylate-Na; pH = 7.2) and 2% osmium tetroxide (buffered in cacodylate-Na) the samples were subdivided into two groups; one group was prepared for fine structure analysis on ultrathin sections. In brief, the samples were brought into small agar tubes and subjected to the following preparation steps as described by Rovan and Simonsberger [11].

In the second group the cell suspensions were dehydrated in ethanol and small volumes of each were mounted on formvarcoated grids. After air drying, the grids were brought into a vacuum device (Balzers 301). The samples were coated with platinum-carbon (eva-

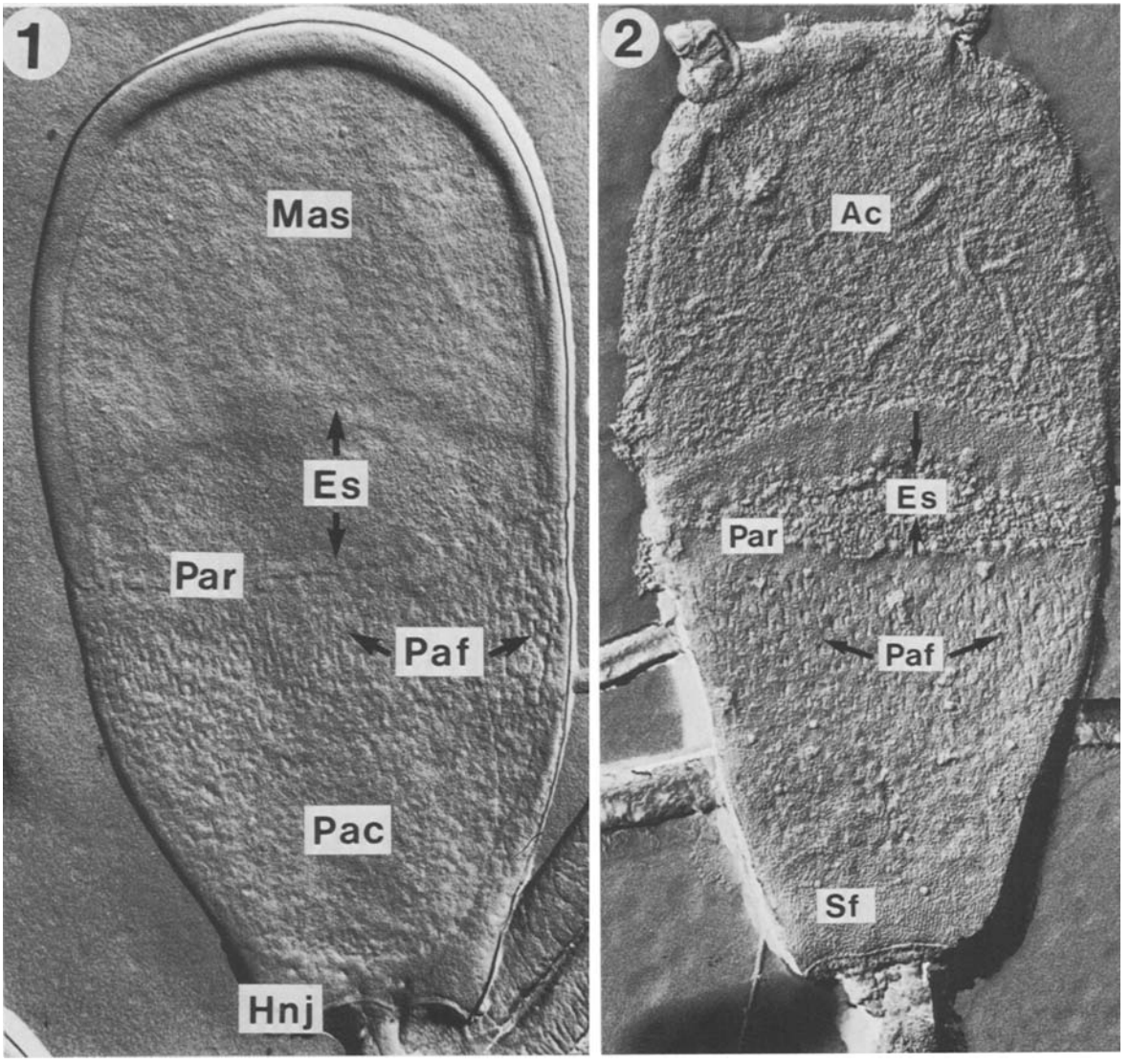


Fig. 1. Head morphology of an untreated control sperm. *Abbreviations* (**Figs. 1 and 2**): *Ac*, Acrosome; *Es*, Equatorial segment; *Hnj*, Head-neck junction; *Mas*, Main acrosomal segment; *Pac*, Post acrosomal cap; *Paf*, Post acrosomal fibrills; *Par*, Post acrosomal ring; *Sf*, Striated fibres. (Magn.: 29,000:1)

Fig. 2. Head fine structure of a GAA treated sperm. The plasma membrane of the acrosomal region is totally disrupted, the outher acrosomal membrane appears folded. The equatorial segment shows the typical hexagonal pattern. (Magn.: 25,110:1)

Fig. 3. Head fine structure of an untreated control sperm. The plasma membrane is closely attached to the acrosome without any foldings or interruptions. (Magn.: 29,000:1) ▶

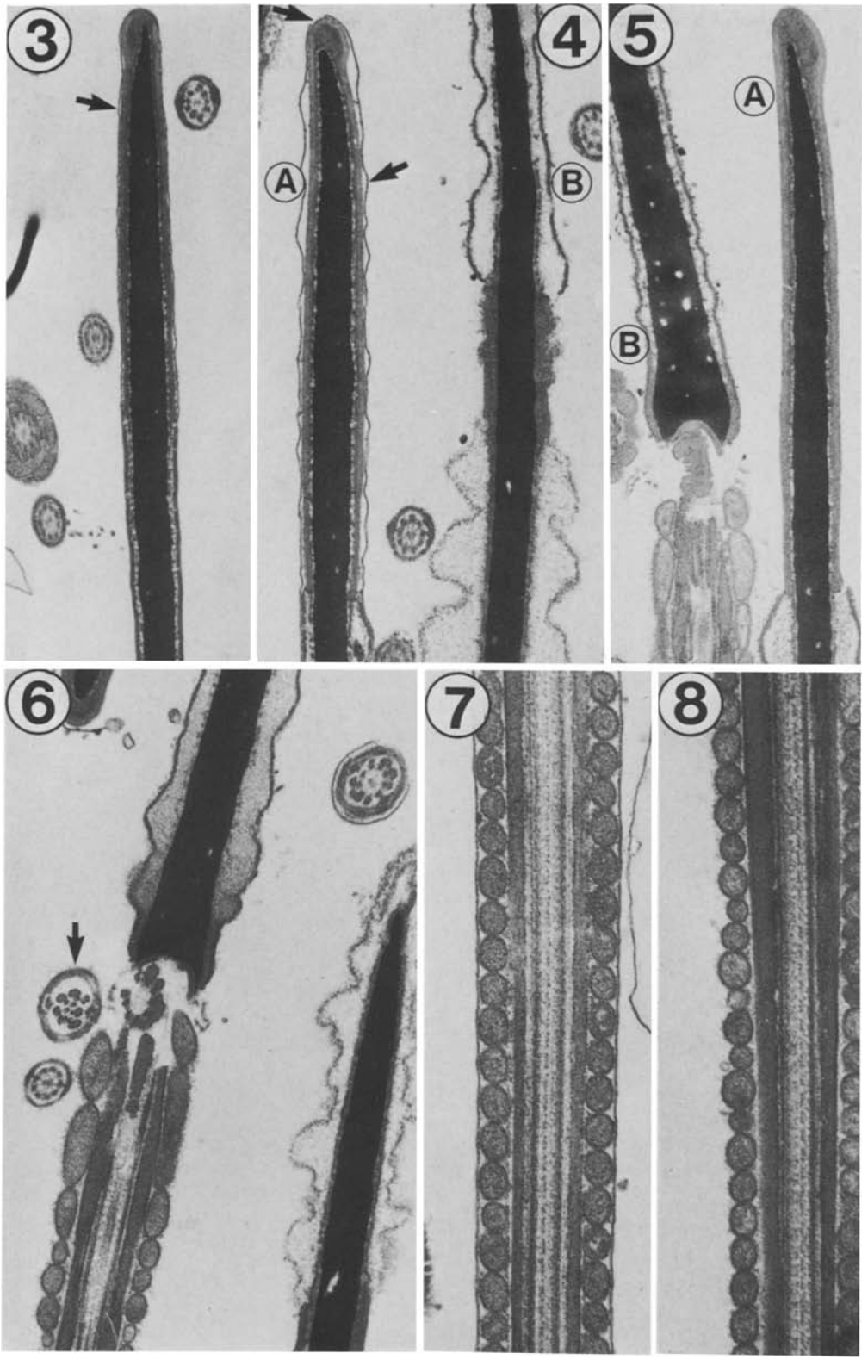
Fig. 4A. The toxic influence of GAA on the head membrane complex is primary indicated by ondulation and partial disruptions (*arrows*) of the plasma membrane (Magn.: 30,000:1). In strong affected sperms (**4B**) the cellular material is either extracted or denaturated, and only small rests of membranes form the conture of the sperm head. (Magn.: 30,000:1)

Fig. 5. In **Fig. 5A** the plasma membrane of GAA treated sperms is frequently detached, and the postacrosomal regions show strong damages. Nevertheless, the acrosome appears more or less unaffected. (Magn.: 29,000:1)

Fig. 6. In correspondence to Fig. 4B and Fig. 5B the acrosomal and the postacrosomal material is either extracted or denaturated preferentially seen in GAA treated sperms. Note the disarrangement of dense fibers and axial microtubules on the cross section of an end piece (*arrow*). (Magn.: 30,000:1)

Fig. 7. The normal arrangement of the mitochondrial sheath of the mid piece of a control sperm. (Magn.: 49,000:1)

Fig. 8. In most of the GAA treated sperms the plasma membrane of the mid piece is lacking, and the mitochondria appear disordered and different in size. (Magn.: 48,000:1)



poration angle: 35 °) and carbon alone (evaporation angle: 90 °) under high vacuum conditions (air pressure: less than 10^{-6} Torr). The thickness of coated metal and carbon layers was standardized by a Quartz Crystal Film Monitor (QSG 201, Balzers). After this procedure the grids were transferred into chromic acid (40% aqueous solution) for 2 h to remove the organic sperm material, and to obtain a purified replica. After washing in several steps with distilled water the metal replicas were mounted on coated grids and examined in TEM Phillips 300.

In comparison to evaluations possible on Scanning EM the preparation as described above and the examination on a TEM guarantees a ten times higher resolution of the cell surface fine structure.

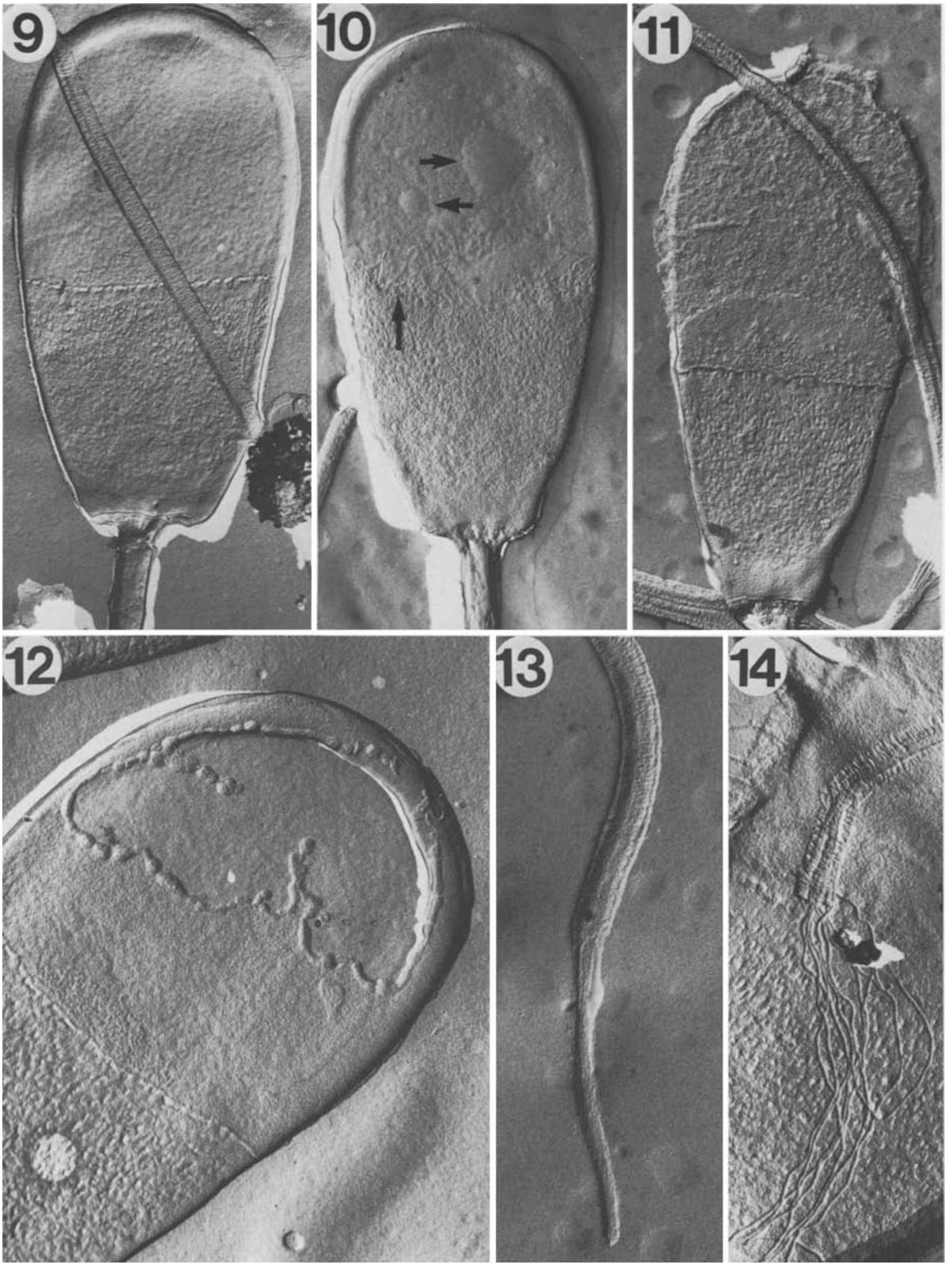


Fig. 9. Normal shaped spermatozoon form the control group incubated with 1 ml of 1% glycerol in tyrode buffer. (Magn.: 8,700:1)

Fig. 10–12. Different steps of membrane damages of GAA treated sperms. **Fig. 10** corresponds to Fig. 4A, and **Fig. 11** to Fig. 2 and Fig. 5A. (Magn.: Fig. 10 and Fig. 11: 8,700:1; Fig. 12: 23,000:1)

Fig. 13. The normal appearance of an end piece of untreated or control sperms. (Magn.: 23,000:1)

Fig. 14. In GAA treated sperms the tubular complex of the end piece is often dissolved in its single fibrilles. (Magn.: 23,000:1)

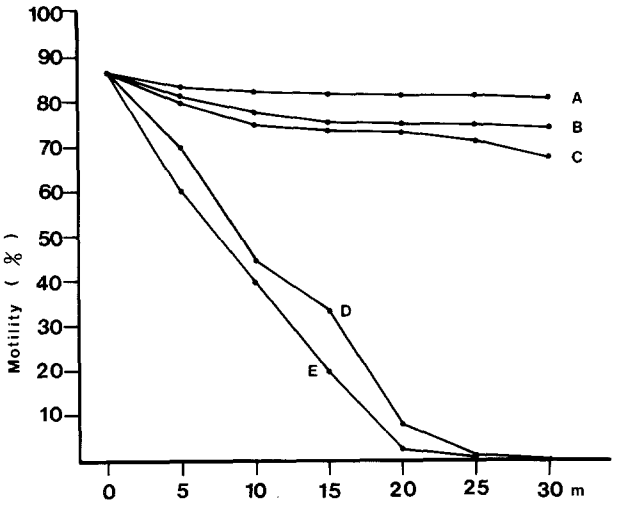


Fig. 15. 1 ml of native bull semen (sperm density of 8.5 x 10^8/ml and progressive motility of 87.4%) was incubated without any diluting media as an untreated control (*A*); 1 ml of native bull semen was incubated with 1 ml of 1% glycerol (*B*) and with 1 ml of 1% Tween 80 buffered solution (*C*) for treated controls. 1 ml of native semen was mixed with 1 ml of 1% glycerol made in tyrode-buffer containing 1,000 µg GAA (*D*) and 1 ml of native semen was diluted in 1 ml 1% Tween 80 made in isotonic tyrode-buffer (pH 7.2) containing 1,000 µg GAA (*E*). All samples were incubated at 37 °C in a water bath and semen analyses were done in intervals of 5 min

Results

The data of native examination (evaluation of motility) are summarized in Fig. 15.

The motility rate of control samples did not differ significantly from untreated controls over a period of 30 min. In contrast the GAA treated sperm suspensions show a remarkable motility decrease within 20 min of incubation (Fig. 15D, E), and no motile sperms were found after 30 min. It is evident that GAA in the concentration used produces a toxic effect on the vitality of bull spermatozoa.

For electron microscopic evaluation 250 single sperms or sperm fractions were checked from all groups, both on surface replicas and ultrathin sections. In the untreated (native) group the morphological analysis indicated 87.6% of counted cells to be normal and 12.4% to be more or less damaged in the arrangement of membrane compartments and organelle architecture. Fig. 1 and Fig. 3 show the typical fine structure of untreated, normal sperms. In controls (treated with the diluting media) no obvious morphological alterations were found (Fig. 9). The percentage relation of unaffected and damaged cells was equivalent to untreated sperms (75.6% normal; 24.4% damaged).

These data correspond to those found in native spermiogram analysis over the incubation period (Fig. 15A–C).

In GAA-treated groups (1,000 µg GAA/ml-diluting media) a high percentage of analysed sperms showed remarkable abnormalities in fine structure (92.1% damaged; 7.9% normal). In brief, the cytoplasmic membrane was found to be ondulated, (Fig. 4, 10) partially broken (Fig. 4, 12) or completely disrupted (Fig. 2, 5, 11) in the head region as well as in the mid-piece of the sperm tail (Fig. 8). On some spermatozoa the acrosomal complex appeared to be dissolved and the acrosomic material exudated like in capacitating or mortified cells (Fig. 4B, 5B and 6).

A similar quantity of these abnormalities was found in both control groups and treated groups and they cannot be attributed to GAA.

No obvious alterations were found in sperm tails, except the separation of the plasma membrane in GAA treated sperms. The arrangement of the mitochondrial matrix, and the configuration of microtubules in the axial complex seemed to be unaffected (Fig. 7). Nevertheless, a distinct number of GAA treated sperms showed a disarrangement of the tubular complex of the tail and end piece (Fig. 6, 14). This defect was never found in untreated or control sperms (Fig. 13). Head/tail separations were not observed [1,9].

Discussion

The data presented in this paper confirm the assumption that GAA in a concentration of 1,000 µg/ml solvent should have a strong immobilizing effect on spermatozoa in vitro.

In summary, the elaborated results show that GAA induces a marked defect in the cellular membrane system. This can be related to reaction of GAA with membrane compounds, or to inactivation of metabolic enzymes. Essential kinetic enzymes, as ATP'ase and LDH, are reported to be partially inactivated by this drug [5, 7, 12].

Furthermore, if the cytoplasmic membrane system is affected, the membrane transport and the membrane electric potential must be affected, too, and the cells have to perish. The extent of cellular necrosis can be related to incubation time of drug media. Therefore it is very difficult to correlate morphological changes of sperm cells of long time in vivo experiments with short running in vitro studies [1, 8, 13].

In a further experiment the working mechanism of GAA on enzymes which are preferentially involved in the kinetic mechanism of mature sperms is to show.

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