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Influence of Cholesterol and Hydrogen Peroxide, Alone and in Combination, on Sperm Morphology

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We studied the effects of increased cholesterol on the morphology of ejaculated human spermatozoa by manipulating cholesterol content of the medium. The results, surprisingly, showed an increase of head and neck abnormalities similar to that caused by hydrogen peroxide, a known source of oxidative damage. The highest rate was observed in samples exposed to both substances, showing that cholesterol did not protect spermatozoa from the damaging action of H_2O_2, and maybe even exacerbated it. We could hypothesize that conferring excessive rigidity to sperm cell membrane by cholesterol beyond a certain point is no longer beneficial to the integrity of the membrane, and may actually predispose it to new types of damage. The observed increased rate of sperm decapitation could indicate that the reduced fluidity of cell membrane, combined with the active motility of ejaculate spermatozoa, could lead to breakage in the neck region as a focal point of mechanical strain.

Key words: Spermatozoa, oxidative damage, cholesterol, morphological defects

Introduction

Sperm cells have a characteristic pattern of changes in the membrane cholesterol content: it increases during epididymal storage to ensure stability [8], remains relatively high in the ejaculate, and then decreases during capacitation to prepare the cell membrane for acrosome reaction and gamete fusion [3]. Despite the importance of this dynamics, still little is known about the effects of cholesterol membrane content on sperm structure and function. Some authors have found higher levels of cholesterol in seminal plasma of patients with teratozoospermia than in men with normal sperm morphology [4], while others have reported a positive association between the

percentage of morphologically normal spermatozoa and the amount of cholesterol in seminal plasma [1]. These contradictions may be due to the complexity of processes associated with the natural secretion and regulation of cholesterol, and may be resolved by experiments directly exposing sperm cells to different concentrations of cholesterol. So far, the influence of higher cholesterol content in the medium has been investigated by researchers trying to improve cryopreservation of spermatozoa of farm animals. Cholesterol addition has been shown to increase viability and motility of cryopreserved spermatozoa by improving their membrane integrity and preventing apoptosis [10]. However, little is known about the effects of cholesterol on sperm cells outside the context of cryodamage. Among the questions that should be addressed are whether cholesterol would have a similar protective action in physiological conditions, and whether it could partially prevent the harmful effects of other agents. A major source of damage to sperm cells are reactive oxygen species such as hydrogen peroxide [7]. The aim of the present study was to evaluate the morphology of human spermatozoa treated with cholesterol, hydrogen peroxide, and a combination of the two.

Materials and Methods

Cells used in this study were ejaculate spermatozoa provided by eight healthy volunteers aged 20-40. Informed consent was obtained from all volunteers. Fresh semen samples were incubated at 37°C for 30 minutes in order to achieve semen liquefaction. Sperm count, motility and morphology were determined. Only normozoospermic samples (according to WHO criteria, and Kruger strict criteria) were used for further investigation, therefor two of the volunteers' samples were no longer used in the study. The remaining semen samples were diluted with gamete buffer (COOK Medical, USA) to a concentration of 2 million cells/mL and divided into four groups. The first group was used as a control and was incubated at 37°C for 3.30h in gamete buffer. Spermatozoa in the second group were incubated for 30 min at 37°C in gamete buffer, and then H_2O_2 was added to 400 μM and incubation continued for another 3h in order to induce oxidative stress. Spermatozoa in the third group were incubated with cyclodextrin cholesterol (Sigma-Aldrich, Germany) added to a ratio of 2 mg per 120 million spermatozoa [6], for 3.30h. Spermatozoa in group 4 were treated with cyclodextrin cholesterol for 30 min., and then H_2O_2 was added to 400 μM and incubation continued for another 3h. Total incubation time for all samples was 3.30h. After incubation all samples were centrifuged for 10 min at 1400 rpm, and diluted in fresh gamete buffer. Spermatozoa were stained as described before [5], with slight modifications. Briefly, they were fixed on slides with cold ethanol for 5 min, air-dried, stained with 0.4% buffered stock solution of Giemsa (Sigma-Aldrich, Germany) diluted 1:20 with distilled water for 15 min, rinsed with distilled water and air-dried before observation. For each slide, 2×100 spermatozoa were counted and their morphology was evaluated. Based on their morphological features, sperm cells were subdivided into four categories: normal, with head or neck abnormalities, with middle piece abnormalities, and with abnormalities of the tail distally of the middle piece. In cases of multiple abnormalities, only the predominant defect was taken into account.

Results and Discussion

The proportions of the four morphological categories of sperm cells in untreated and treated samples are shown in **Table 1**. In the control group, no major changes in morphology were observed after the incubation (**Fig. 1A**). In all other groups, the tail distally of the neck seemed largely unaffected by the treatments, but abnormalities in the head and neck regions were more common (**Fig. 1B, C, D**). In cholesterol-treated samples, the proportions of abnormally sized heads (too large or two small) were increased, and spermatozoa with head detached from the tail, a very severe defect of the neck, were often observed (**Fig. 2**). In most cases the detached head and tail were visible in a single vision field which is indicative that the detachment occurred during the spermatozoa manipulations (**Fig. 3**). To our knowledge, such effects have not been previously reported. They could be a direct result of cholesterol's ability to change thickness, rigidity, compressibility, and curvature of biological membranes [11]. In spermatozoa these effects could be more substantial due to membrane composition specifics, namely significant amount of poly-unsaturated fatty acids (PUFA). In model membrane systems cholesterol leads to the segregation of phospholipids containing PUFAs' resulting in the formation of disorganized membrane regions and rearrangement of the lipid rafts [9]. Presence of cholesterol in sperm cell membranes in concentrations exceeding the physiological could improve cryopreservation survivability [6] but according to our results, prolonged exposure and incubation leads to generation of significant morphological defects and membrane stress points that result in decreased cell stability.

Table 1. Proportions of different morphological categories of sperm cells (in percentages) in control samples and samples treated with cholesterol, H_2O_2 and cholesterol plus H_2O_2, respectively.

	Normal morphology	**Abnormal head or neck**	**Abnormal middle piece**	**Abnormal distal tail**
Control	45%	25%	18%	12%
Cholesterol	35%	33%	19%	13%
H_2O_2	33%	36%	19%	12%
Cholesterol + H_2O_2	17%	57%	17%	9%

In the group treated with H_2O_2, the results were predictable. PUFAs are particularly sensitive to oxidative stress due to the double bonds. Lipid peroxidation leads to loss of membrane integrity and morphological abnormalities with a strict dose dependent trend [2]. H_2O_2 damage is usually associated with damage to the middle piece and loss of motility, while our results demonstrate increased morphological abnormalities in the head and neck, which could be explained by the higher H_2O_2 concentrations used. Compared to the cholesterol group, the H_2O_2 group had more misshaped heads and necks, and fewer enlarged heads and decapitated spermatozoa.

The group treated with both cholesterol and H_2O_2 showed the least amount of normal spermatozoa (17%), and the largest percentage of spermatozoa with head and neck defects (47%) (**Table 1, Fig. 2D**). Results indicate that the added cholesterol is unable to protect sperm cell membranes from oxidative stress at high H_2O_2 concentrations, and furthermore, leads to increased damage and subsequent changes in morphology. This could be caused either by the cholesterol induced segregation of PUFAs and a resulting massive oxidative damage to highly disorganized membrane domains that are not present in untreated spermatozoa, or cholesterol itself becomes a target of H_2O_2 which leads to the formation of cholesterol-hydroperoxyde and intensification of oxidative damage. Further research is needed to determine the cause of the observed damage aggravation.

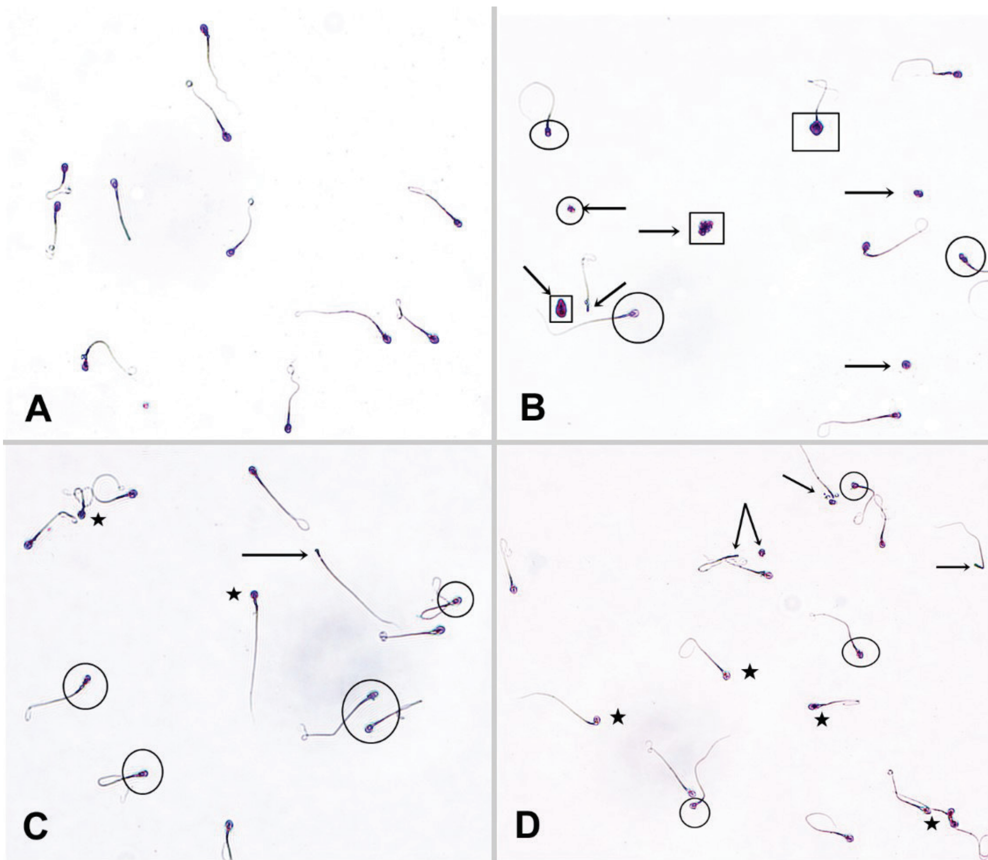

Fig. 1. A – control group, B – cholesterol treated spermatozoa, C – H_2O_2 treated spermatozoa, D – spermatozoa treated with both cholesterol and H_2O_2. Designations: → severed head/tail; ○ small head; □ large head; ★ abnormal morphology. Original magnification 400×.

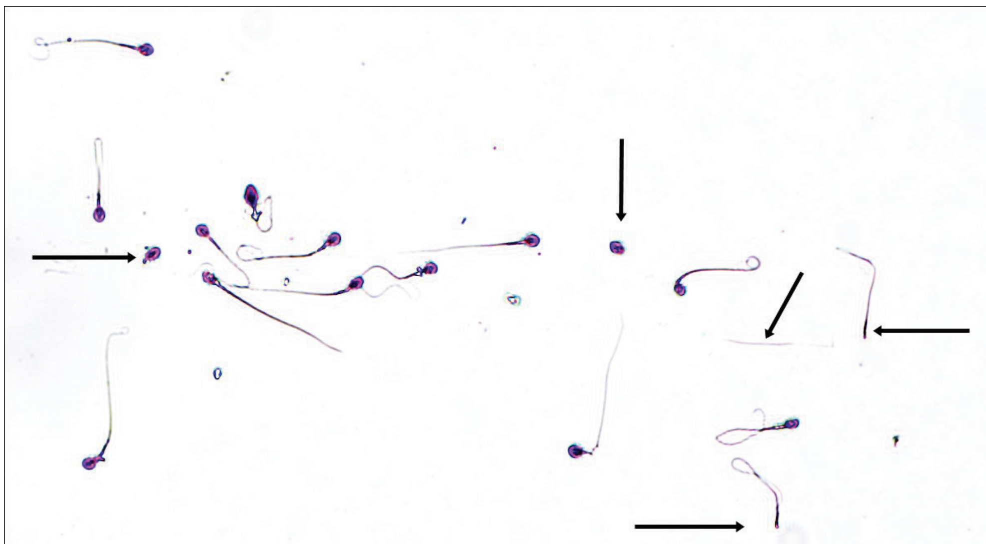

Fig. 2. Abnormal morphology and spermatozoa breakages in cholesterol treated samples. Designations and original magnification as in **Fig. 1**.

Conclusions

While cholesterol may be protective for sperm cells during cryopreservation, under physiological conditions it causes morphological abnormalities at a rate comparable with that of the known damaging agent H_2O_2, and provides no protection against H_2O_2 damage.

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