

Erythropoietin Increases *In Vitro* Motility and Vitality of Human Spermatozoa

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Abstract. *Background/Aim:* Erythropoietin and its receptor are expressed in the male reproductive system. Initial studies have shown that erythropoietin affects the motility of spermatozoa. The aim of the present study was to investigate the *in vitro* effect of erythropoietin in the motility and vitality of human spermatozoa. *Materials and Methods:* Forty-three semen samples, obtained after 2-4 days of abstinence from sex, were analyzed and processed using density gradient centrifugation. Aliquots containing one million of spermatozoa were treated with either erythropoietin, at concentrations of 10 and 100 mIU/ μ l or standard culture medium for one hour. *Results:* Progressive motility and vitality of spermatozoa significantly increased following treatment with erythropoietin. The effect was not dose-dependent. *Conclusion:* The supplementation of culture medium with erythropoietin improves sperm processing in terms of vitality and motility. Future research should focus on the effects of erythropoietin on sperm capacitation as well as on the signal transduction pathways activated by erythropoietin and its receptor in spermatozoa.

Erythropoietin (EPO) is a highly glycosylated peptide produced mainly by the adult kidneys and fetal liver. The EPO gene encodes a protein of 193 amino acids and following cleavage the circulating protein consists of 165 amino acids with a molecular weight of 30.4 KDa (1-3). EPO has a half-life of 7-8 h and its actions are mediated by a high-affinity transmembrane receptor (EPOR) (4). Binding of EPO to the EPOR induces a dimerization and/or reorientation of EPOR to

a dimeric structure. The dominant signal transduction pathway activated by EPOR is the Jak/STAT (5-13).

EPO is the main regulator of erythropoiesis and its actions on bone marrow erythroid progenitor cells have been extensively studied (1, 4, 8-10). Despite this, the detection of EPOR as well as the expression of EPO in other tissues and cells has revised the conventional notion on its biological functions. Among the sites where EPO and EPOR are expressed is the male reproductive system. EPO expression has been detected in the mouse epididymis (14), in Sertoli and peritubular myoid cells of rats (15). In rat Leydig cells, two receptors with different affinity to recombinant human EPO (rHuEPO) have been detected (16). In humans, EPO expression has been found in the testicular germ cell line (17) and can be measured in seminal plasma (18), while EPOR has been detected in human spermatozoa (19). In human Leydig cells, rHuEPO can directly stimulate testosterone production, an effect that seems to be independent of gonadotropin secretion (20). Akman *et al.*, have managed to reduce the gonadotoxic effects of doxorubicin in adult male rats by administering a long-acting EPO analogue. The group found that following EPO analogue administration, the oxidative stress was reduced and the sperm motility and viability were improved (21).

Motility of spermatozoa is a crucial parameter of basic semen analysis and is related to natural conception but also to the successful outcome of assisted reproduction techniques (ART) (22-23). Sperm processing during ART aims to obtain an aliquot with a high percentage of vital and motile spermatozoa by removing immotile spermatozoa, somatic cells and seminal plasma. The use of sperm processing media that can support and enhance the motility and vitality of the selected spermatozoa is of significant importance. Therefore, the supplementation of sperm processing media with factors that can enhance motility and vitality can only be desirable.

In this study, we decided to investigate the *in vitro* effects of an EPO analogue on the motility and vitality of human spermatozoa selected after density gradient centrifugation and washing.

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Materials and Methods

The study was conducted in the Laboratory of Physiology, Faculty of Medicine, School of Health Sciences, Democritus University of Thrace, Alexandroupolis, Greece, as part of the research project “Study of the effects of growth factors on the motility and vitality of human spermatozoa” (MIS 5049528), during the period May 2020 – February 2021. The study was approved by the Ethics Committee of Democritus University of Thrace (ΔΠΘ/ΕΗΔΕ/58931/203).

Forty-three volunteers gave semen samples after 2-4 days of sexual abstinence. All of them signed an informed consent after receiving information about the study. The exclusion criteria were: i) severe astheno -, oligo- or teratozoospermia, ii) previous surgical interventions in the reproductive system or iii) receiving drugs with a potential impact on semen parameters. Each semen sample was subjected to basic semen analysis according to WHO guidelines (24), followed by sperm processing using a density gradient and washing. The pellet was suspended in Quinn’s Sperm Washing Medium (SAGE In Vitro Fertilization, Inc., Trumbull, CT, USA) and sperm concentration as well as progressive motility were assessed. Subsequently, two aliquots, each with one million progressively motile spermatozoa were taken. The volume of each aliquot was finalized at 300 µl. One aliquot was incubated at 37°C for 1 h in culture medium (control) and the second aliquot was incubated, under the same conditions, in the culture medium supplemented with 10 or 100 mIU/µl of epoetin alpha (Binocrit, Sandoz GmbH, Langkampfen, Austria). Therefore, there were two treatment groups: the one with the low and the other with the high concentration of EPO. Each experimental had its own paired control group as different volunteers participated in the low and high treatment groups. Immediately after 1 h incubation, the motility and vitality of the spermatozoa were assessed.

All operations were performed by the same person, under a laminar flow chamber (ESCO Class II, Type A2). Spermatozoa concentration was assessed using Improved Neubauer hemacytometer (Poly-Optik GmbH, Bad Blankenburg, Germany). For the motility assessment we used a NIKON Eclipse E200 (Nikon, Japan) microscope equipped with phase contrast lenses and a heating stage. For the sperm vitality assessment, we used eosin/nigrosin staining (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Sperm aliquots were incubated in a Hera cell 150 (Thermo Electron Co, Langensfeld, Germany). Discontinuous density gradients were prepared using the density gradient system Sil-Select (Fertipro NV, Beernem, Belgium).

Statistical analysis was conducted using Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). Wilcoxon matched pairs test was used for the comparisons between controls and treatment groups. Mann-Whitney *U*-test was used for the comparisons between the groups of the low and high concentration of EPO. The significant level was set at 0.05. The values in the text and Tables are presented as mean±standard deviation.

Results

Twenty-one volunteers, 18 to 45 years old, gave semen samples for the experiments with the low concentration of EPO (10 mIU/µl). Treatment with EPO significantly improved sperm’s progressive motility, non-progressive motility and vitality compared to controls (Table I). Namely, the progressive motility with EPO was 10.048±6.078% and the vitality was 11.238±6.300% higher compared to that of the untreated sperm.

Table I. Motility and vitality improvement of spermatozoa treated with 10 mIU/µl EPO. Values show mean±standard deviation (N=21).

	EPO 10 mIU/µl	Control	p-Value
Progressive motility	47.524±13.303	37.476±12.339	0.000
Non-progressive motility	13.762±4.036	10.000±4.528	0.001
Immotile	39.429±12.600	52.048±11.280	0.000
Vitality	70.905±7.569	59.667±9.441	0.000

Table II. Motility and vitality improvement of spermatozoa treated with 100 mIU/µl EPO. Values show mean±standard deviation (N=22).

	EPO 100 mIU/µl	Control	p-Value
Progressive motility	40.045±8.437	27.364±10.060	0.000
Non progressive motility	10.455±6.139	8.500±3.609	0.281
Immotile	49.500±8.146	64.136±9.280	0.000
Vitality	71.227±6.747	59.136±6.777	0.000

For the experiments with the high concentration of EPO (100 mIU/µl), semen samples from 22 volunteers, aged 19 to 51 years old, were used. Again, EPO significantly improved the progressive motility and vitality compared to controls (Table II). Non-progressive motility, although higher with EPO, was not significantly different from that of the untreated sperm. EPO improved the progressive motility by 12.682±4.110% and the vitality by 12.091±4.492%.

In order to compare the effects of the low vs. the high concentration of EPO, the differences in progressive motility and vitality between the controls and the two groups of EPO were extracted. Although with the high concentration of EPO there was a trend for greater improvement in both progressive motility and vitality, the statistical analysis showed that this was not significant (progressive motility: *U*=167.500, *p*=0.123; vitality: *U*=229.000, *p*=0.971).

Discussion

The detection of EPO protein and its receptor in the male reproductive system has put forward the question of its physiological role in reproductive functions. Early *in vivo* studies have shown that EPO has a stimulatory effect in Leydig cells leading to an increased testosterone production in rats and humans (20, 25). In nephrectomized rats, EPO administration can alleviate steroidogenesis, spermatogenesis, epididymal sperm maturation and fertility (26). The experiments of Akman *et al.* in adult rats have shown that *in vivo* administration of an EPO analogue protects spermatogenesis from the known gonadotoxic effects of Doxorubicin that leads to the formation of free radicals,

suggesting that EPO exerts antioxidant properties (21). In this study, the adult rats treated with EPO analogue had sperm with improved motility and vitality compared to rats treated with Doxorubicin only (21). In another experimental study using adult rats with testicular torsion/detorsion, *in vivo* administration of an EPO analogue prevented testicular damage and reduced oxidative markers again, suggesting that EPO may be acting as a direct and/or indirect antioxidant (27). On the other hand, the EPO gene transfer or EPO administration in male rabbits had no significant effects on sperm concentration, motility or vitality (28).

The expression of EPOR in human spermatozoa as well as the presence of EPO protein in semen plasma triggers questions on their role in the ejaculated sperm and their possible effects in sperm motility and vitality after *in vitro* treatment. The results of the present study show that EPO, as a supplement in the sperm-processing medium, can increase the progressive motility and vitality of human spermatozoa after 1 h. In our experiments, we used washed spermatozoa thus we removed the native EPO present in semen plasma which allowed us to test the EPO effects by comparing spermatozoa incubated in two doses of EPO against spermatozoa incubated in EPO-free medium. These effects were not dose-dependent, at least not on the two doses used in the present study. In the only other similar study published, Tug *et al.* have examined the effects of EPO on sperm motility by incubating human spermatozoa in various concentrations of EPO: 0.1, 1, 10, 100 mIU/ml for 4 h (29). They found improvement in the sperm's progressive motility at all concentrations except the lowest one (0.1 mIU/ml) (29). Comparing our results with those of Tug *et al.* (29) we should first note that the positive effect of EPO on motility is confirmed, except that they realized there is a minimum concentration that can act on sperm's motility. Secondly, the differences in the design of the two studies possibly are behind the different results regarding the dose dependence; Tug *et al.* (29) used EPO at concentrations from 0.1 to 100 mIU/ml and incubated spermatozoa for four hours while in our study we used higher concentrations of EPO (10 mIU/ μ l and 100 mIU/ μ l) and the incubation time was 1 h. It is worth to mention that we decided on a 1-h sperm incubation in EPO in an attempt to mimic the conditions of human-assisted reproduction, where sperm processing is usually performed within 1 h after the delivery of the semen sample (24).

In our study, as already mentioned, an increase in spermatozoa vitality was observed following incubation with EPO. To our knowledge, this is the first report showing this effect in human sperm. The underlying mechanisms responsible for the effects of EPO on the motility and vitality of ejaculated spermatozoa are not known. It could be hypothesized that the binding of EPO to its receptor located in the plasma membrane of spermatozoa activates signaling

pathways resulting in the protection of spermatozoa from reactive oxygen species and apoptosis or the enhancement of mitochondrial function, thus, resulting in better motility. Especially for the sperm's motility, it is not known whether EPO has an effect as an antiapoptotic/antioxidant agent or it acts on the sperm's motility *per se*. The antiapoptotic effect of EPO through activation of its receptor has been shown in erythroid precursor cells (30-31) and there are indications of antioxidant effects from previous studies (21, 27). The study of EPOR in the plasma membrane of spermatozoa and the signal transduction pathways activated by the binding of EPO to EPOR could shed some light into the mechanism behind increased sperm motility and vitality. Currently, there is no knowledge of either the precise location of EPOR in the cell membrane of spermatozoa or the downstream signaling pathways. The investigation of this topic could contribute, not only to a clear understanding of the mechanisms behind the improvement of motility and vitality following an *in vitro* treatment with EPO, but also to a better understanding of the physiology of spermatozoa.

In conclusion, the present study showed that treatment of ejaculated spermatozoa with EPO can significantly improve their progressive motility, confirming the findings of a previous study (29). It also showed, for the first time, that treatment with EPO can significantly increase sperm's vitality *in vitro*. According to our results, these effects are not dose-dependent. To our opinion, future research should focus on the EPOR expressed on the cell membrane of spermatozoa and the signal transduction pathways involved in their motility and vitality. The effects of EPO on the induction of capacitation and acrosome reaction are two other topics for future research as they are both critical for fertilization.

Conflicts of Interest

The Authors declare no potential conflicts of interest in relation to this study.

Authors' Contributions

B A was involved in the study's conception and design, data analysis and writing of the manuscript. A T K performed the experiments, data acquisition and analysis. C M contributed substantially to data acquisition and revision of the manuscript.

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