INTRODUCTION

Progressive motility and vitality are two critical parameters for the fertilization ability of spermatozoa. Both of them are positively associated with fertilization ability of spermatozoa. Here, the effects of IGF-I and NGFβ on PM and vitality of human spermatozoa were investigated.

Methods: Forty-three volunteers gave semen samples after 2-3 days of sexual abstinence. Each sample was processed with density gradient centrifugation and sperm washing. The pellet was divided into 3 aliquots. An aliquot containing one million of progressively motile spermatozoa was incubated for an hour (37°C) in standard culture medium (control group), and two aliquots with the same number of progressively motile spermatozoa were incubated in medium supplemented with IGF-I or NGFβ. Two concentrations of IGF-I (100 ng/ml and 1000 ng/ml) and NGFβ (0.5 ng/ml and 5 ng/ml) were tested.

Results: Both growth factors significantly increased PM and vitality in comparison with control either at the low or the high concentration. IGF-I seemed to be more effective than NGFβ. The effects did not seem to be dose dependent with the exception of the effect of IGF-I on vitality.

Conclusions: The enhancement of PM and vitality of human spermatozoa by IGF-I and NGFβ opens new ways for the improvement of sperm processing. Further research is needed to determine the most effective concentrations.

KEYWORDS
IGF-I, motility, NGFβ, spermatozoa, vitality
organisms and tissues. It acts as mitogen, it mimics the effects of insulin, it promotes survival, increase and development of cells, and therefore, it is implicated in many physiological and pathological functions. The bioavailability of IGF-I is controlled by IGF binding proteins (IGFBPs) and their proteases (IGFBP proteases). IGF-I acts through three receptors: IGFR-I, insulin receptor (with lower affinity than insulin), and a hybrid receptor IGFR-I–insulin receptor. IGFR-I is a transmembrane tyrosine kinase receptor, usually activating the signal transduction pathways of Akt, mTOR, MAPK, and GSK3β. In humans, IGF-I has been found to express in Sertoli cells, primary spermatocytes, and weakly in Leydig cells. It is also present in seminal plasma. IGFR-I is expressed in germinal epithelium, Sertoli, and Leydig cells as well as in secondary spermatocytes and at the early stages of spermatids. In mature human spermatozoa, IGFR-I is present in the equatorial region and the acrosome. Studies in animal species have documented the expression of IGFR-I in rat Leydig cells and in mature spermatozoa, namely in the acrosomal region of bull spermatozoa and in the acrosomal region, equatorial region, middle piece and the tail of rabbit’s spermatozoa.

IGF-I seems to be indispensable for the normal development and function of male reproductive organs. The fact that adult male mice homozygous for a mutation of the IGF-I gene have reduced size of testis and substandard development of the other reproductive organs, reduced testosterone levels due to insufficient development of Leydig cells and diminished sperm production. The importance of IGF-I for the normal function of male reproductive organs is further supported by findings, showing that seminal plasma IGF-I concentrations are significantly correlated with sperm concentration and the percentage of morphologically normal spermatozoa, being lower in infertile patients. Testis and/or epididymis are thought to be the main source of IGF-I in the ejaculate as vasectomized patients have reduced levels of IGF-I in seminal plasma. The presence of IGFR-I in mature spermatozoa indicates a direct effect of IGF-I on their function. A number of studies, either in animal species or in humans, provided evidence that IGF-I affects motility and also acts as antioxidant-protecting sperm membranes. NGF belongs to the group of neurotrophins. It is a protein of 26 kDa coming from proNGF that is a larger protein of 130 KDa. ProNGF consists of three proteins: NGFα, NGFβ and NGFγ in a ratio 2:1:2. NGF is known for its effects on the development, survival and mitotic activity of neuronal cells, but it is also known that it affects other types of cells. NGF binds at least in two types of receptors: the receptor of tropomyosin kinase A (TrkA) and the low affinity NGF receptor (LNGFR/p75NTR). In 1988, it was reported the expression of NGF in the testis and epididymis of mouse and rat. Subsequent studies confirmed the initial findings. Namely, in mouse, NGF is expressed in Leydig, peritubular myoid, and Sertoli cells, whereas TrkA is present in non-germ cells and p75 is expressed in Sertoli and peritubular cells. In adult rats, NGF is expressed in Leydig cells, seminiferous tubules, and germinal cells at all stages, whereas TrkA is present in elongated spermatids, spermatozoa, seminiferous tubules, and p75 in Sertoli, Leydig cells, seminiferous tubules, pachytene spermatocytes, and elongated spermatids. NGF and its receptors were also identified in Leydig cells, Sertoli cells, spermatogonia, caudal epididymis, and seminal vesicles of the adult Japanese monkey. In golden hamsters, NGF was found in Leydig cells, spermatocytes, and elongated spermatids, whereas p75 had an ubiquitous distribution in testis and TrkA was expressed in Sertoli cells. In bovines, NGF was detected in ejaculated sperm and TrkA was found in the acrosomal cap, nucleus, and tail of ejaculated spermatozoa.

The first study on the expression of NGF and its receptors in human reproductive organs reported that in fetal testis, Sertoli and interstitial cells are sites of NGF expression and peritubular cells are sites of p75 expression. Later, the presence of NGF protein was confirmed in Leydig cells of adult and fetal testis as well as the expression of TrkA and p75. In another study, NGF protein was detected in seminal plasma and mRNA for TrkA in human spermatozoa with the levels of both NGF protein in seminal plasma and TrkA mRNA in spermatozoa being lower in oligoasthenozoospermic than in fertile men. Based on the presence of NGF and its receptors in testis and spermatozoa as well as initial experimental studies, several investigators proposed that NGF affects motility and vitality. Especially in golden hamsters, NGF seems to induce acrosome reaction.

Taking into consideration these studies, we decided to investigate the effects of IGF-I and NGF on progressive motility and vitality of human spermatozoa, when the growth factors are added on the culture medium during sperm processing.

2 MATERIALS AND METHODS

This was a controlled experimental study conducted in the Laboratory of Reproductive Physiology-IVF, Faculty of Medicine, School of Health Sciences, Democritus University of Thrace, Greece, during 2020, in the context of the research project “Study of the effects of growth factors on the motility and vitality of human spermatozoa” (MIS 5049528). The study was approved by the Ethics Committee of Democritus University of Thrace.

Forty-three volunteers gave semen samples by masturbation after two or three days of sexual abstinence. Each volunteer signed an informed consent after having received detailed information on the study. The exclusion criteria for volunteers’ recruitment were as follows: severe oligo-, astheno-, or terato-zoospermia, recent disease, or taking medications with a potential impact on semen. In each sample, basic semen analysis was performed according to WHO and the semen sample was processed with density gradient centrifugation and sperm washing with the standard culture medium. After sperm washing, the
pellet was resuspended in standard culture medium at a total volume of 300 μl and the total sperm count as well as progressive motility was evaluated. Then, an aliquot containing 1 million of spermatozoa with progressive motility was incubated for one hour in standard culture medium (control group) and two aliquots with the same number of progressively motile spermatozoa were incubated in standard culture medium supplemented with IGF-I or NGFβ. The final volume of each aliquot was 300μl. The aliquots were incubated in a HeraCell 150i (Thermo Scientific) at 37°C and 0% CO₂. Following incubation, progressive motility and vitality were assessed. Two different concentrations of IGF-I and NGFβ were tested: 100 ng/ml and 1000 ng/ml for IGF-I; 0.5 ng/ml and 5 ng/ml for NGFβ. Therefore, the experimental procedure was consisted of two phases (Figure 1).

In phase A, there were three groups: control group, where 1 million progressively motile spermatozoa were incubated in standard culture medium; IGF-I low group, where 1 million progressively motile spermatozoa were incubated in standard culture medium supplemented with 100 ng/ml IGF-I; NGFβ low group, where 1 million progressively motile spermatozoa were incubated in standard culture medium supplemented with 0.5 ng/ml NGFβ.

In phase B, the control group had the same characteristics as in Phase A; IGF-I high group, where 1 million progressively motile spermatozoa were incubated in standard culture medium supplemented with 1000 ng/ml IGF-I; NGFβ high group, where 1 million progressively motile spermatozoa were incubated in standard culture medium supplemented with 5 ng/ml NGFβ.

In all experiments, spermatozoa were incubated for one hour in 37°C. All experiments were conducted by the same person. Concentration was assessed with improved Neubauer hemocytometer, and motility with the use of a Nikon E200 microscope equipped with a heating stage at 37°C and phase contrast lenses, vitality with eosin/nigrosin test.

Quinn’s sperm washing medium (SAGE In Vitro Fertilization, Inc) served as standard culture medium. IGF-I and NGFβ were purchased from PeproTech (Rocky Hill). Eosin and nigrosin were purchased from Sigma-Aldrich Chemie Gmbh (Taufkirchen).

Statistical analysis was conducted in Statistica 6.0 (StatSoft Inc). The use of non-parametric tests was chosen as some of the variables did not follow the normal distribution, which was assessed with both Shapiro-Wilk and Kolmogorov-Smirnov tests for normality.

In both Phase A and Phase B experiments, Wilcoxon matched pairs test was used for the comparisons between the control (standard culture medium) and experimental groups (culture medium supplemented with IGF-I or NGFβ). For the comparison between the experiments of Phase A and Phase B, the Mann-Whitney U test was used since the experiments were performed independently from each other. Differences were considered significant at values of P < .05. The values, in the text, are presented as mean±standard deviation.

**FIGURE 1** The design of the study [Colour figure can be viewed at wileyonlinelibrary.com]
3 | RESULTS

3.1 | Phase A

Twenty-one volunteers donated fresh semen samples with satisfactory values to run the experiments. The spermiogram values before the experiments are presented in Table 1. The age of volunteers varied between 18 and 45 years old (32.29 ± 9.34).

The incubation of human spermatozoa for one hour with the low concentrations of IGF-I and NGFβ significantly increased progressive motility and vitality in comparison with control (Table 2). The vitality in IGF-I was slightly higher than in NGFβ, close to a statistically significant level (P = .058). On the other hand, progressive motility in IGF-I was significantly higher than in NGFβ (P < .001).

3.2 | Phase B

In Phase B, twenty-two volunteers with an age between 19 and 61 years old (28.06 ± 12.78) donated semen samples. The main characteristics of semen samples are presented in Table 3.

The incubation in the high concentrations of IGF-I and NGFβ significantly increased both progressive motility and vitality in comparison with control (Table 2). The vitality in IGF-I was slightly higher than in NGFβ, close to a statistically significant level (P = .058). On the other hand, progressive motility in IGF-I was significantly higher than in NGFβ (P < .001).

3.3 | Comparison between Phase A and Phase B

In order to compare the effects of the low vs the high concentrations of IGF-I and NGFβ, the differences in progressive motility and vitality between the control group and the two groups of growth factors were extracted in Phase A and B. Although there was a trend for higher improvement of progressive motility with the high concentrations of IGF-I, the statistical analysis showed it was not significant. Regarding vitality, the high concentration of IGF-I resulted in a statistically significant improvement compared to the low concentration of IGF-I (P = .009). The high concentration of NGFβ showed a slight improvement of both progressive motility and vitality compared to the low concentration, but the differences were not statistically significant (Table 5).

4 | DISCUSSION

The worldwide spread and the ever-increasing use of assisted reproduction techniques have brought to the fore the need for more efficient methods of sperm processing. In this context, several substances have been tested as supplements to sperm processing media in order to improve motility and vitality of human spermatozoa. Caffeine, pentoxifylline, and 2-deoxyadenosine have shown to enhance motility, but there is also evidence they have detrimental effects on embryonic development.

We chose a different approach: to study the in vitro effect of growth factors on the motility and vitality of human sperm, which are present in the male reproductive system and semen. We also chose to test their effects during a short time period (one hour) as sperm processing is usually performed within one hour after delivery of the semen sample. The concentrations tested in the present study were determined in the basis of previous reports and preliminary experiments.

The results of the present study showed that both IGF-I and NGFβ improve in vitro progressive motility and vitality of human spermatozoa. These results are in agreement with previous studies in spermatozoa of animal species and humans.

Henricks et al showed that IGF-I increases motility in bull spermatozoa. In rabbit spermatozoa, IGF-I increased motility and vitality.
vitality. In equine spermatozoa, IGF-I also increased motility. In pig spermatozoa, which were frozen and then thawed, it was found that the addition of IGF-I, after thawing, did not improve motility but reduced the oxidative stress. Selvaraju et al showed that the addition of IGF-I to buffalo spermatozoa increased the acrosome reaction. In frozen buffalo spermatozoa, motility was significantly increased after thawing and incubation with 100 ng/ml IGF-I. However, relevant studies with human spermatozoa have given conflicting results. Thus, Sanchez-Luengo et al reported that incubation of human spermatozoa with 25 μg/ml IGF-I significantly increased their activation. On the contrary, Miao et al found that incubation of human spermatozoa with 50 ng/ml IGF-I reduced certain motility parameters. It is possible that this negative effect is explained by the low concentration of IGF-I they used.

The effects of NGF on sperm parameters have also studied in several animal species and humans. Studies with hamsters have shown that the incubation of spermatozoa with NGF improves motility and acrosomal reaction. In bovine spermatozoa, it was shown that incubation with NGF improves vitality. Saeednia et al found that in frozen-thawed semen samples from asthenozoospermic patients, the treatment with 0.5 ng/ml NGF significantly increased motility, vitality and decreased DNA fragmentation, but the treatment with 1 or 5 ng/ml had no significant effects. Lin et al (2015) tested three different concentrations of NGF (0.1μmol/L, 1μmol/L, 10μmol/L) on human sperm motility. They found that the two higher concentrations (1μmol/L and 10μmol/L) can significantly improve motility, and in particular, they increased the percentage of grade A motile spermatozoa. This effect was time-dependent showing an increase of the relative number of grade A motile spermatozoa up to 40 minutes of incubation time.

In Phase A experiments, IGF-I improved progressive motility more than NGFβ. In Phase B experiments, IGF-I gave better results than NGFβ in both parameters: progressive motility and vitality. The effect of both growth factors does not seem to be dose-dependent, at least in those concentrations tested in the present study, with the exception of the effect of IGF-I on vitality where the high concentration gave significantly higher results than the low one.

The favorable effects of IGF-I and NGFβ on motility and vitality can be a useful tool in assisted reproduction, especially in IUI and conventional IVF where adequate motility and vitality are critical factors for fertilization outcome. IVF experiments in rabbits where spermatozoa were treated with a protein complex containing IGF-I gave encouraging results. Similarly, encouraging results were reported by Selvaraju et al in buffalo IVF cycles where spermatozoa were treated with IGF-I (100 ng/ml); the cleavage rate in the IGF-I treated group was significantly higher than in the control. Regarding NGFβ, a recent study showed that supplementation of IVF medium with NGFβ improved embryonic cleavage rates and hatching rates of blastocysts in bovines. Although in this study, not only the spermatozoa but also the oocytes were exposed in NGFβ during fertilization process and the investigators concluded that NGFβ acts directly on the oocyte, the results clearly show that NGFβ in IVF medium can improve the IVF outcome.

In conclusion, the present study showed that the treatment with IGF-I or NGFβ can improve in vitro motility and vitality of human spermatozoa. IGF-I seems to be more effective than NGFβ, at least with the concentrations used in this study. According to the present results, the effects do not seem to be dose dependent with the exception of the effect of IGF-I on vitality. However, to our opinion, future research should focus on the effects of different concentrations of these growth factors in order to determine the most effective ones by testing more concentrations. The induction of capacitation and acrosome reaction are two other points for future research as they are necessary steps for successful fertilization. The duration of incubation is another interesting point for future research. Although one hour is a short incubation

### TABLE 3 Spermiogram values of the volunteers before sperm preparation in Phase B experiments

|                      | Mean ±SD  | Minimum | Maximum |
|----------------------|-----------|---------|---------|
| Total sperm count (X10⁶) | 93.59 ± 100.32 | 4.00 | 442.00 |
| Progressive motility (%) | 39.64 ± 9.62 | 19.00 | 58.00 |
| Nonprogressive motility (%) | 15.23 ± 8.78 | 5.00 | 42.00 |
| Immotility (%) | 44.68 ± 9.51 | 27.00 | 63.00 |
| Vitality (%) | 72.27 ± 6.67 | 60.00 | 86.00 |

Abbreviation: SD, Standard deviation. N = 22.

### TABLE 4 Motility and vitality values in Phase B experiments

|                      | CONTROL | IGF-I (1000 ng/ml) | NGFβ (5 ng/ml) |
|----------------------|---------|-------------------|----------------|
| Progressive motility (%) | 27.36 ± 10.06 | 38.00 ± 10.05 | 31.73 ± 9.15 |
| Nonprogressive motility (%) | 8.50 ± 3.61 | 10.32 ± 2.68 a | 11.50 ± 3.88 a |
| Immotility (%) | 64.14 ± 9.28 | 51.68 ± 10.81 | 56.32 ± 10.90 |
| Vitality (%) | 59.14 ± 6.78 | 70.09 ± 6.66 | 63.86 ± 6.24 |

Note: The values are expressed as mean ± standard deviation. Pairwise comparisons (Wilcoxon matched pairs test, P < .01) showed there were statistically significant differences in all cases except those denoted by the same letter. In all comparisons, P < .001 except the comparisons regarding non progressive motility between IGF-I and control as well as between NGFβ and control where P < .05.
period, it is useful to investigate whether a shorter incubation with IGF-I or NGFβ can effectively promote progressive motility and vitality of human spermatozoa.

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DISCLOSURES

Byron Asimakopoulos, Aggeliki Tiptiri-Kourpeti, and Chryssa Metallinou declare that they have no conflict of interest. All procedures followed in this study were in accordance with the ethical standards of the Ethical Committee of Democritus University of Thrace and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained by all volunteers who donated semen samples.

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| TABLE 5 | Comparisons of the improvement in progressive motility and vitality between the low and the high concentrations of IGF-I and NGFβ |
|-------------|----------------------------------------------------------------------------------------------------------------------------------|
| Parameter                                           | Two sided pre-test | Z adjusted | Two sided pre-test | Z adjusted |
| IGF-I (100 ng/ml)                                    | Two sided test     |            | IGF-I (1000 ng/ml)  |            |
| Improvement of Progressive motility (%)              |                     |            |                     |            |
| 0.107                                                | 10.636 ± 3.983     | -1.621     | 10.955 ± 4.029     | -2.934     |
| Improvement of Vitality (%)                          |                     |            |                     |            |
| 0.009                                                | 6.714 ± 6.528      | -2.394     | 6.574 ± 6.528      | -2.594     |

Note: The improvement was calculated by subtracting the respective values of the control experiments from the values of the experiments with the growth factors. The values are expressed as mean ± standard deviation. The comparisons were performed with Mann-Whitney U test.
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