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Original article

Papaverine as a replacement for pentoxifylline to select thawed testicular or epididymal spermatozoa before ICSI

La papavérine pour remplacer la pentoxifylline et sélectionner les spermatozoïdes testiculaires ou épididymaires viables congelés avant ICSI

P. Terriou [a,*,b], E. Hans [a,b], R. Cortvrindt [c], C. Avon [a,b], O. Charles [a,b], J. Salzmann [a,b], P. Lazdunski [a,b], C. Giorgetti [a,b]

[a] *Institut de médecine de la reproduction, 6, rue François-Rocca, 13008 Marseille, France*
[b] *Laboratoire ALPHABIO, 6, rue François-Rocca, 13008 Marseille, France*
[c] *BVBA, Martelaarslaan 349, 9000 Gent, Belgium*

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ABSTRACT

Objectives. – Pentoxifylline has been used to improve sperm motility in Assisted Reproductive Technology mainly by initiating sperm motility in immotile spermatozoa samples obtained surgically. Indeed, as Intracytoplasmic Sperm Injection leads to very poor results when using immotile gametes, pentoxifylline gives better results by easing the selection of viable sperm mobilized after incubation. In 2011, the French Haute Autorité de santé decided that pentoxifylline used for in vivo purpose proposed Insufficient Medical Service and pentoxifylline was thus withdrawn from the French materia medica. We here assessed the efficacy on spermatozoa motility and the safety of papaverine, another phosphodiesterase inhibitor, for the replacement of pentoxifylline.
Methods. – Sixteen frozen-thawed epididymal or testicular samples displaying no or very poor spontaneous motility ($\leq$ 5% total motility) were subjected to both pentoxifylline (3.6 mM) and papaverine (93 μM). A duplicate Mouse Embryo Assay and an In Vitro Fertilization Mouse Assay in duplo were used to discard any toxic effect of papaverine.
Results. – Papaverine gave better results than pentoxifylline (mean total motility: 27% vs 23%, $P < 0.05$). No Effect Level were observed in the two different Mouse Embryo Assays performed.
Conclusion. – Papaverine is a useful tool to replace pentoxifylline in ICSI programs to select viable spermatozoa in frozen-thawed sperm samples displaying no or very poor motility.

Mots clés :
Papavérine
Pentoxifylline
Spermatozoïdes
Testiculaires
Épididymaires
ICSI

RÉSUMÉ

Objectifs. – La pentoxifylline a été utilisée pour initier la mobilité de spermatozoïdes tous immobiles, notamment ceux obtenus lors de biopsies testiculaires ou de ponctions épidydimaires. En effet, dans ce cas, il est difficile de différencier les vivants des morts, l'injection intracytoplasmique se fait au hasard et les résultats sont médiocres. La pentoxifylline, en initiant la mobilité chez certains d'entre eux, permet donc d'obtenir de meilleurs résultats. En 2011, la Haute Autorité de santé ayant estimé que la pentoxifylline utlisée in vivo rendait un service médical insuffisant, cette dernière a été retirée du marché français. Pour remplacer la pentoxifylline, nous évaluons ici l'efficacité et la sécurité de la papavérine, un autre inhibiteur de phosphodiestérase agissant sur la mobilité des spermatozoïdes.
Méthodes. – Seize échantillons de spermatozoïdes testiculaires ou épididymaires congelés présentant une mobilité spontanée nulle ou très faible après décongélation ($\leq$ 5 % de mobilité globale) ont été soumis à la pentoxifylline (3,6 mM) et à la papavérine (93 μM). Par ailleurs, un test de toxicité en

* Corresponding author.
E-mail address: pterriou@aol.com (P. Terriou).

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duplicate sur embyons de souris et un test de fécondation in vitro en duplo également sur embryons de souris ont été réalisés pour écarter un éventuel effet toxique de la papavérine.
Résultats. – La papavérine a permis d'obtenir de meilleurs résultats que ceux obtenus avec la pentoxifylline (moblité globale moyenne: 27 % vs 23 %, $p < 0{,}05$). Les deux tests de toxicité réalisés chez la souris ont montré l'absence d'effet toxique de la papavérine.
Conclusion. – La papavérine peut remplacer la pentoxifylline avant ICSI pour sélectionner les spermatozoïdes vivants au sein d'échantillons de gamètes testiculaires ou épididymaires décongelés spontanément immobiles.

1. Introduction

Pentoxifylline (PF) is an inhibitor of 3′,5′-nucleotidase phosphodiesterase (IPDE) which increases intracellular cAMP and improves human spermatozoa motility both in vivo [1] and in vitro [2,3], hyperactivation [4] and mucus penetration [5]. This compound was introduced to improve the results of Assisted Reproductive Technology (ART) programs, especially Intra-Uterine Insemination [6], In Vitro Fertilization [7] and in cases of acrosome reaction insufficiency [8]. Nowadays, PF is mostly used before performing IntraCytoplasmic Sperm Injection (ICSI) in case of immotile epididymal or testicular spermatozoa [9]. Indeed, random injections of immotile spermatozoa give unsatisfactory results [10]. Injection of initially immotile spermatozoa displaying a very poor motility after several hours of incubation allows fertilization [11] but this procedure is difficult and time consuming. Better results are obtained with PF, which easies the selection viable spermatozoa before ICSI.

The PF medicinal product used in human materia medica in cases of chronic arterial occlusion (Torental®, Sanofi-Aventis, France) has been withdrawn from the french pharmacies because of Insufficient Medical Service (in vivo) since 2011 [12]. Moreover, PF manufactured by Sigma is prohibited for clinical use. Nevertheless, the French Agence de BioMédecine included in 2013 the selection of live spermatozoa before ICSI using IPDE into the official list of allowed technics used for the amelioration of ART procedures [13]. The goal of this study was to replace PF by another IPDE, papaverine (PV), used in human materia medica because of its antispasmodic activity and in cases of erectile dysfunction. PV activity on immotile spermatozoa was thus compared to that of PF (non-inferiority study) and its safety was assessed using two different Mouse Embryo Assays.

2. Methods

2.1. Patients

From January 2013 to December 2014, our patients displaying obstructive azoospermia underwent Microsurgical Epididymal Sperm Aspiration (MESA) and patients displaying non-obstructive azoospermia without testicular atrophy underwent TEsticular Sperm Extraction (TESE). The recovered spermatozoa were then frozen, one of the obtained straws being afterwards assessed for post-thaw motility. Before freezing, all patients gave informed consent. The thawing test was not performed in cases where there were less than five available straws in order to give optimal chances of success with further ICSI procedures.

Sixteen azoospermic patients displaying a poor post-thaw motility (i.e. $\leq 5\%$ total motility) were included in this study: thirteen had non-obstructive azoospermia, three had obstructive azoospermia.

2.2. Protocol I: comparison of the effects of PF and PV on human immotile spermatozoa

After thawing, straws were divided into three equal parts for:

- a conventional post-thaw test without any motility enhancer;
- a PF-test (10 minutes incubation in PF solution);
- and a PV-test (10 minutes incubation in PV solution).

PF (Torental®, 100 mg injectable solution, Sanofi-Aventis, France) was diluted 1/10 vol in culture medium (BM1®, Eurobio, France) in order to obtain a 7.2-mM concentration. Sperm preparations were then diluted v/v in this mother solution (final PF concentration in the sperm preparation: 3.6 mM) [9].

PV (Papaverine Serb®, 40 mg injectable solution, Serb, France) was first diluted in sodium chloride at an initial concentration of 1/100 PV. This initial solution was then diluted 1/6 in embryo culture media (BM1®). Finally, sperm preparations were diluted v/v in the 1/600 PV solution. The final PV concentration in the sperm preparation was 1/1200 (93 μM).

2.3. Protocol II: Mouse Embryo Assay (MEA)

Male and female mice (B6CBAF1) were taken from an in-house breeding program with C57bl/6JOaHsd females and CBAcaOlaHsd males, both obtained from Harlan Laboratories (The Netherlands) and housed according to international standards with free access to water and food. Female mice (24–26 days old) for oocyte and zygote donation were superovulated. Mice were injected i.p. at 7PM, with Folligon® (2,5 IU, Intervet, Belgium) and 48 hours later with Chorulon® (2.5 IU, Intervet, Belgium). Mice for zygote retrieval were mated overnight. Oocytes for IVF and zygotes for MEA were retrieved the next day, respectively at 10AM and 2PM. Zygotes were retrieved from plug-positive females in M2 medium (Sigma, Belgium), pooled and randomly distributed in groups of 10. The MEA was performed in duplicate (2×10 for the assay control and 2×10 for the test condition). Zygotes of the test condition were exposed for 30 min to 1/600 PV in M2 medium and subsequently cultured in the control condition (M16, Sigma) in an open, oil-free culture system.

Embryo scoring was performed on day 2 (two-cell stage), day 5 (expanded blastocyst) and day 6, and was always done at 11 AM, in line with mouse embryo development.

2.4. Protocol III: mouse IVF in duplo (two-mouse, intra-mouse comparison)

To ensure good oocyte/zygote quality a mild superovulation protocol was used. A mean of 15 oocytes/zygotes per mice were retrieved.

Epididymides were collected from 2 fertile proven stud males. In each, two cuts were made and sperm was allowed to swim out for 10 minutes. Sperm at the edge of the dish was collected, motile