**Original article**

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

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

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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decided that pentoxifylline used for in vivo purpose proposed Insufficient Medical Service and pentoxifylline was thus withdrawn from the French materia medica.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 phosphodiesterease inhibitor, for the replacement of pentoxifylline.

Methods. – Sixteen frozen-thawed epididymal or testicular samples displaying no or very poor spontaneous motility (<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.

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**Mots clés :**

Papavérrine
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 épididymaires. 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 utilisée 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 papavérrine, un autre inhibiteur de phosphodiésterase 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 (<5 % de mobilité globale) ont été soumis à la pentoxifylline (3.6 mM) et à la papavérrine (93 μM). Par ailleurs, un test de toxicité en
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) program, 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 eases 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 (MEASA) 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., ≤ 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
sperm counted and a suspension of $10 \times 10^6$ was prepared and further incubated for 2 hours to allow capacitation before adding to the fertilisation droplet.

For PV treatment, capacitated sperm was exposed for 30 minutes to 1/1200 PV (93 μM). This sperm solution was used for fertilization. Fertilization was done in droplets under oil (10 μL of sperm solution was added to the 30 μL fertilization drop). Cumulus Oocyte Complexes (COCs) from the left oviducts were fertilized with PV-treated sperm; COCs from the right oviducts were fertilized with control sperm. Fertilization was allowed for three hours. After washing, the zygotes were cultured in control conditions (M16 medium) for eight days to get an indication of their capacity to hatch and to survive upon hatching.Embryo development was scored on day 2 (two-cell stage), day 5 (expanded blastocyst stage), and day 6 (blastocyst).

2.5. Statistical analysis

The Wilcoxon sign rank test was used to compare sperm motility between each scoring day. This nonparametric test allows comparison of repeated measurements on a single sample by testing the hypothesis $H_0$: mean = 0. A $p$-value < 0.05 was considered significant and thus $H_0$ was rejected.

3. Results

3.1. Protocol I: comparison between PF and PV on sperm motility

Table 1 shows the results observed. Both PF and PV solutions allowed marked improvement of total spontaneous sperm motility (respectively 23% and 27%, vs 1% for spontaneous motility, $p < 0.001$). In two cases, PF was more efficient than PV. In three cases, PF and PV exhibited the same results. In the other 11 cases, PV treatment was more efficient than PF treatment. Mean total motility was significantly higher in the PV group than in the PF group (27% vs 23%, $p < 0.05$).

3.2. Protocol II: MEA

For MEA, No Effect Level (NOEL) was usually considered if both the two-cell stage on day 2 and the blastocyst rate on day 6 were $\geq 80\%$. In our experiment, as day 2 two-cell stage rate were 100% for both the control group and the PV group, and as day 6 blastocyst rates were also 100% for both groups (Table 2), PV was considered safe. Embryo scoring on day 5 was performed to confirm the intrinsic quality of the embryos but was not used for decision making.

3.3. Protocol III: Mouse IVF Assay

For mouse IVF, NOEL was considered if the two-cell stage on day 2 was $\geq 80\%$ and if the blastocyst rate on day 6 was $\geq 75\%$. In our experiment, the two-cell stage rates on day 2 were 100% and 93% for the control and the PV group, respectively. Blastocyst rates on day 6 were 96% and 88% for the control and the PV group, respectively (Table 3). Embryo scoring on day 5 was performed to confirm the intrinsic quality of the embryos but was not used for decision making. PV was thus considered as safe.

4. Discussion

The effects of PF on in vivo [1] and in vitro [2–5] sperm motility were used in the 1990s to improve the results obtained after Intra Uterine Insemination [6], In Vitro Fertilization [7] and in cases of acrosome reaction insufficiency [8]. Tasdemir et al. demonstrated that PF could initiate motility in testicular spermatozoa [14] and suggested that it may be used to differentiate live testicular spermatozoa during ICSI and therefore improve fertilization and pregnancy rates. First pregnancies and births were then quickly described after use of PF before ICSI to initiate motility in initially immotile frozen-thawed epididymal and testicular spermatozoa [9,15]; these results were confirmed thereafter by other authors [16]. PF treatment was then more widely performed in ICSI programs as random injections of immotile spermatozoa give poor results [10].

Table 2
Assessment of safety of papaverine (PV). Mouse Embryo Assay.

<table>
<thead>
<tr>
<th>Type of Spermatozoa</th>
<th>Spontaneous motility (%)</th>
<th>Motility after PF treatment (%)</th>
<th>Motility after PV treatment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>100</td>
<td>10/10</td>
</tr>
<tr>
<td>PV-treated mouse embryos</td>
<td>10/10</td>
<td>100</td>
<td>10/10</td>
</tr>
</tbody>
</table>

One-cell mouse embryos were incubated with 93 μM PV for 30 minutes. No difference was observed in embryo development on exposure to PV compared to the control group. Satisfactory testing requires a cleavage and blastocyst rate $\geq 75\%$.

Table 3
Assessment of safety of papaverine (PV). In Vitro Fertilization (IVF) of mouse oocytes using PV-treated mouse spermatozoa.

<table>
<thead>
<tr>
<th>Type of Spermatozoa</th>
<th>Spontaneous motility (%)</th>
<th>Motility after PF treatment (%)</th>
<th>Motility after PV treatment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12/12</td>
<td>100</td>
<td>14/14</td>
</tr>
<tr>
<td>PV group</td>
<td>14/15</td>
<td>93</td>
<td>13/14</td>
</tr>
</tbody>
</table>

Mouse embryo development observed after IVF using either untreated spermatozoa (control) or PV-treated spermatozoa (PV group). No significant difference was observed in embryo development on exposure to PV. For mouse IVF, NOEL was considered if the two-cell stage on day 2 was $\geq 80\%$ and if the blastocyst rate on day 6 was $\geq 75\%$. 

Table 1
Effect of pentoxifylline (PF) and papaverine (PV) on total motility of frozen-thawed spermatozoa obtained surgically.

<table>
<thead>
<tr>
<th>Type of Spermatozoa</th>
<th>Spontaneous motility (%)</th>
<th>Motility after PF treatment (%)</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>PV-treated mouse embryos</td>
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</table>

One-cell mouse embryos were incubated with 93 μM PV for 30 minutes. No difference was observed in embryo development on exposure to PV compared to the control group. Satisfactory testing requires a cleavage and blastocyst rate $\geq 75\%$.

TESE: Testicular Sperm Extraction; MESA: Microsurgical Epididymal Sperm Aspiration.

a $p < 0.001$.
b $p < 0.05$. 

References [1–5,7–9,13–16].
Toreental® has been withdrawn from the French materia medica since 2011 [12] and PF manufactured by Sigma is prohibited for medical purposes. 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 aim of this study was to propose another IPDE available in an injectable form to replace it. We chose to study papaverine (Papaverine Serb®), Serb®, France), another IPDE-enhancing intracellular cAMP and cGMP also allowing energy liberation. We compared (Table 1) the efficiency of PF and PV on epididymal or testicular frozen-thawed samples displaying poor sperm motility (≤ 5% total motility, mean total motility = 1%). In all cases, both PF and PV treatments demonstrated motility improvement. Mean motility observed after PV treatment was higher than mean motility obtained after PF treatment (27% vs 23%, P < 0.05). Interestingly, in this study we used a 40-fold lower PV concentration (93 µM PV vs 3.6 mM PF).

During a Hypo-Osmotic Swelling Test (HOST), sperm is incubated in hypo-osmolar medium. Sperm with a functional membrane undergo swelling of the cytoplasmic space and the sperm tail fibers curl [17]. Therefore, a HOST was proposed to select viable sperm before ICSI [18,19]. However, tail curling observed during a HOST is not as easy to distinguish from the initiation of motility induced by PF treatment. Mangoli et al. [20] observed better fertilization rates after PF treatment of testicular biopsies than after a HOST (52% vs 41%, respectively, P < 0.005) and obtained better pregnancy rates in the PF group (32% vs 16%, P < 0.005).

In vitro culture of immotile spermatozoa at 37 °C in an atmosphere of 5% CO2 for 2–3 h or more before ICSI has been proposed to enhance or initiate sperm motility [11,21,22]. However, some authors did not observe any motility initiation when only immotile testicular spermatozoa were retrieved [23]. Moreover, the optimal incubation times seem to be relatively long, between 48–72 h post-sperm retrieval, making testicular sperm in vitro maturation less easy than with motility stimulants.

Theophylline, another IPDE and sperm motility inducer, was investigated in a prospective study using a ready-to-use solution [24]. The time for identification and isolation of motile sperm after thawing was significantly faster in the Theophylline group than in the control group. However, little information was given about the percentages of motile sperm with and without this compound. The authors indicated that only three patients of 41 had only immotile sperm before cryostorage including one patient with no motile sperm even after Theophylline incubation. Last, Theophylline safety was not assessed. However, these authors observed significantly higher rates of fertilization (79.9%), blastulation (63.9%) and pregnancy (53.9%) after use of Theophylline compared to control cases (respectively 63.3%, 46.8% and 23.8%).

To our knowledge, no precise study has been conducted to assess caffeine or other sperm-motility stimulant on immotile frozen-thawed epididymal or testicular spermatozoa. However, caffeine has revealed deleterious effects on blastocyst development [25], and has been shown to impair embryonic development in humans [26].

Tourneay et al. [27] demonstrated PF treatment of sperm was not toxic for mouse embryos obtained using treated sperm. Nevertheless, these authors also demonstrated that PF was directly toxic on one-cell mouse embryos. We want to emphasize, as did Yovich et al. [28], that PF-treated sperm is always washed after incubation before being injected in oocytes and that the embryo-toxicity reported by Tourneay et al. does not correspond to human PF-ICSI protocols. The MEA widely used to assess the safety of any batch of media used in ART human protocols was used in this study to assess the safety of PV on one-cell mouse embryos (Protocol II). As the blastocyst rate observed after PV treatment on mouse embryos was the same as the blastocyst rate observed for the controls (100% blastocyst rate in both groups) (Table 2), we considered that no toxic effect of PV was observed.

This safety was confirmed in Protocol III when IVF was performed on mouse oocytes using PV-treated spermatozoa. Indeed, the blastocyst rate observed in PV group was > 75% (Table 3) as is the case for non-toxic agents.

Finally, the present report answers to a recent article [29] that proposed PV as a very promising candidate to enhance sperm motility but that requested experiments to establish safety and clinical effectiveness of this compound in IVF programs.

5. Conclusion

Papaverine, an IPDE-enhancing intracellular cGMP and cAMP levels, is proposed for the first time to safely induce sperm motility in cases of very poor motility to be able to use mobilized spermatozoa for ICSI. These results allow us to propose papaverine as a replacement of pentoxifylline, which has been withdrawn from the French materia medica.

Disclosure of interest

The authors declare that they have no competing interest.

References


