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An animal experimental study on pubourethral ligament restoration with platelet rich plasma for the treatment of stress urinary incontinence

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Introduction Minimally invasive methods for injured ligament and tendon restoration have been developed and gained popularity in recent years. Injury and relaxation of the pubourethral ligament (PUL) can lead to stress urinary incontinence (SUI). The aim of this study was to investigate the impact of injecting platelet rich plasma (PRP) into the PUL following its surgical transection resulting in SUI, confirmed by leak point pressure (LPP) measurements pre- and post-intervention in an experimental animal model.

Material and methods Twenty female adult Sprague-Dawley rats were assigned in 2 groups: 1) treatment group with transection of the PUL and application of PRP at the time of transection and at one month follow-up and 2) a control group, with transection of the PUL only. Leak point pressures (LPPs) were measured prior to transection, immediately following the transection and at 1 and 2 months in both groups.

Results The median LPPs for the control group were: LPP – preT: 35.6 (29.8–44.8) cmH_2O, LPP – postT: 14.6 (5.8–19.0) cmH_2O, LPP – 1 month: 27.3 (19.2–33.8) cmH_2O, LPP – 2 months: 29.0 (27.0–34.0) cmH_2O, whereas for the PRP group were: LPP-preT: 40.5 (33.2–46.3) cmH_2O, LPP – postT: 15.7 (3.0–24.0) cmH_2O, LPP – 1month: 31.6 (24.8–37.4) cmH_2O, LPP – 2 months: 36.8 (32.5–45.4) cmH_2O. PRP injections on transected PULs significantly increased LPPs at one month follow-up [31.6 cmH_2O vs. 27.3 cmH_2O, p = .043]. This effect was confirmed at two months [36.8 cmH_2O vs. 29.0 cmH_2O, p <.001].

Conclusions Injection of PRP into transected PULs significantly improved LPPs at one and two months' follow-up. However, further experimental and clinical research is needed to evaluate the safety and efficacy of this treatment, in clinical practice.

Key Words: bladder ‹› incontinence ‹› platelet rich plasma (PRP) ‹› stress urinary incontinence ‹› urethra

INTRODUCTION

The definition of stress urinary incontinence (SUI) is the complaint of involuntary loss of urine on effort or physical exertion, or on sneezing or coughing [1]. The prevalence of SUI increases with age and affects significantly the quality of life [2]. In a large epidemiological study, which included 28,000 women, researchers concluded that urinary leakage is highly prevalent. Seven percent of these women reported significant incontinence and should be considered as potential patients [3].

Urinary continence should be regarded as a complex dynamic process that involves both the bladder and

the urethra. According to the integral theory, the pubourethral ligament (PUL) is the most important anatomical landmark which is associated with SUI when injured [4]. The PUL consists of smooth muscle, elastin, collagen, nerves and blood vessels. This ligament connects the anterior wall of the bladder and the proximal urethra to the symphysis pubis. It is divided into the vaginal and the urethral part. The urethral part 'anterior PUL' inserts into the mid-part of the urethra. The vaginal part, 'posterior PUL', enters the vagina laterally and posteriorly to the urethra, and provides further support [4].

Risk factors associated with SUI, include parity, aging, menopause, estrogen deficiency, obesity, and congenital collagen defects. According to the integral theory structure and function are strongly associated; "Restore the structure, and you will correct the function" [4].

Several surgical techniques are used for the treatment of SUI. Midurethral slings (MUS) are commonly used to correct urethral hypermobility, which is presumed to be secondary to PUL defect. A recent meta-analysis on the outcomes of the currently used surgical procedures for the treatment of female SUI [5], concludes that the MUS are the most popular, most effective and safer treatment for SUI than other surgical procedures. However, the MUS are associated with intra and post-operative complications, such as hemorhage, bladder perforation urethral injuries, pain, voiding dysfunction, urinary tract infections and tape erosion [6].

Urethral bulking agents are a minimally invasive alternative approach for the treatment of SUI, especially in patients suffering from intrinsic sphincter deficiency and the commonly used synthetic ones seem to be safe; although their efficacy remains, to date, suboptimal [7]. Restoration of the anatomy with autologous agents such as fat has been also proposed to limit the potential side effects of synthetic materials; however, one trial in 2001 [8] compared periurethral injection of autologous fat with placebo injection of saline. The trial was terminated due to a death of a participant caused by fat embolism a few days following the injection. There was no statistically significant difference in the outcome measures between the two groups. The poor efficacy and the potentially unsafe use of autologous fat injection have this agent from being used as a treatment for SUI.

A recent Cochrane review concludes that at present, injection therapy cannot be deemed as a first line treatment for women fit for other surgical procedures, due to questionable efficacy [9].

Fibrin adhesives and platelet rich plasma (PRP) have been extensively investigated in the field of tendon restoration over the past decade [10]. PRP consists of numerous growth factors, such as vascular endothelial growth factor (VEGF), insulin growth factor I (IGF-I), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), transforming growth factor beta (TGF-b) and fibroblast growth factor (FGF). These growth factors play a significant role to the pathophysiology of ligament reconstruction [11]. The use of platelet-rich plasma (PRP) injections is rapidly growing in clinical practice. Systematic reviews have concluded that PRP could be considered as an effective tendon therapy, with potentially faster recovery and possibly a reduction in injury, with no reported adverse reactions. PRP is clinically used extensively in orthopedics for musculoskeletal injuries, but also in plastic surgery and dentistry [12].

However, to date, these substances have not been investigated as a potential treatment of female SUI. The adhesive, healing, hemostatic and regenerative abilities of PRP, [13] might result in the treatment of the SUI by restoring the support of the urethra and the bladder neck to the pubic bone. In other words, if this intervention corrects urethral hypermobility, stress incontinence may resolve, with minimal, if any, complications.

With this background, we recently published our hypothesis [14], which refers to the injection of PRP on the PUL, as a possible treatment option for SUI. The aim of this study was to evaluate the efficacy of PRP on urine leak point pressure (LPP) in an animal experimental model with transected pubourethral ligaments.

MATERIAL AND METHODS

Experimental model

Twenty female nulliparous Sprague-Dawley strain rats (12 weeks old) (Hellenic Pasteur Institute, Department of Animal Models for Biomedical Research, Greece) were included. The animals were placed in weather-controlled chambers (temperature 20°C ±1°C, humidity 55 ±5%) under controlled lighting (12 hours light per day) for 15 days to adapt to their new environment. Full nutrient supplementation was ensured with the administration of ELVIZ 510 food pellets. The night before the operation the animals were fasted from food and water.

The animals had the same characteristics (age, parity, body weight, and were grown under the same environmental conditions) and were randomly assigned into two groups using computer-generated randomization. In all animals the PUL was transected. In the first group (control group: 10 animals) no intervention was undertaken following PUL transec-

tion. In the second group (study group: 10 animals), PRP was applied with pipette, immediately after the PUL transection (first phase) and was followed by a second application of PRP at one month follow-up (second phase). According to the wound healing cascade, the cellular and matrix proliferation, is the most significant phase of healing, begins within days of the trauma and lasts from 48 h up to 6 weeks. As there is evidence that the application of PRP accelerates wound healing, the second phase of the experiments was performed in one month [15].
The LPPs were measured 4 times in total in both groups. The first measurement was undertaken before the PUL transection (LPP – preT), the second immediately following the PUL transection and just prior to the first PRP application for the PRP group (LPP – postT) (first phase) and then at one month and just prior to the second PRP application for the PRP group (LPP – 1month) (second phase) and at two months follow-up (LPP – 2 months) (third phase). At each assessment, the LPP was measured five times for each rat and the mean value of these measurements was calculated to confirm the repeatability of the method.

Description of the surgical technique of suprapubic catheter insertion and pubourethral ligament transection

The rats were anaesthetized by an intraperitoneal injection of ketamine hydrochloride 70 mg/kg and of xylazine 6 mg/kg [16]. Our technique was a modification of the techniques described previously by Kefter et al. [17] and Malmgren et al. [18]. A midline suprapubic incision approximately 1 cm long was performed in order to gain access to the peritoneal cavity. The bladder was identified and was held with a pair of atraumatic non-toothed forceps. A 20 G catheter was placed on the bladder dome with a sharp needle as a guide. Following the LPP measurements, the catheter was removed and the incision was closed in layers using 5-0 Vicryl (Ethicon) for the muscle and 3-0 Vicryl for the skin.
The transection of the pubourethral ligament was performed as previously described by Kefter et al. [17] in 2009. A reverse Trendelenburg position was used and the bladder was pulled superiorly and posteriorly to adequately expose and visualize the retropubic space. The PUL was identified and sharply incised (transected) using a size 11 blade. The PRP solution was applied to this region, initially following the PUL-transection and the LPP measurements at the first phase of the experiments, and the same technique was followed at the second phase (1 month). The bladder was held with atraumatic forceps and pulled gently superiorly and posteriorly, whilst the PRP was applied suprapubically (Figures 1, 2). The same technique was followed in the control group. Under anesthesia the bladder was accessed transabdominally and catheterized, but PRP was not injected.

Leak point pressure measurements technique

The LPPs were measured using a water manometer. A 3-way tap with extension lines filled with normal saline (N/S 0.9%) and free of air bubbles was used to connect the manometer (pressure 0 cmH_2O to the level of the bladder) to a fluid bag on one side, and to

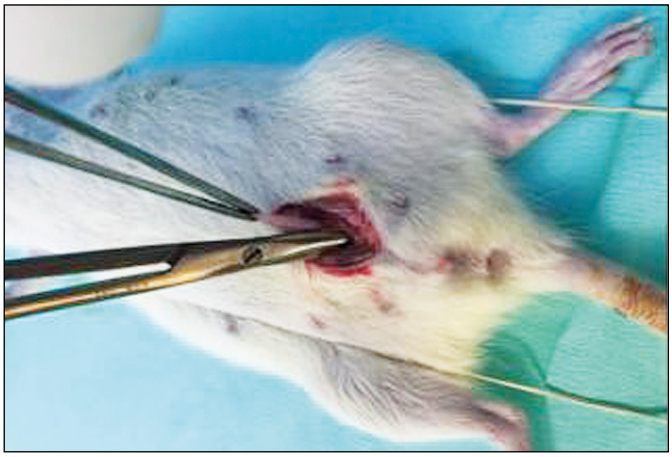

Figure 1. *Transection of the pubourethral ligament. The bladder was held with a pair of atraumatic forceps and gentle traction was applied superiorly and posteriorly, whilst the PUL was transected suprapubically.*

Figure 2. *The bladder was held with a pair of atraumatic forceps, whilst the catheter was inserted through the bladder dome using a sharp needle as a guide.*

the bladder on the other side via a 20 G catheter. The extension line of the manometer was free of fluid. The 3-way tap was then turned so that it was open to the fluid bag (slow flow 1 ml over 3 minutes), to the manometer and to the bladder (all directions), allowing the manometer column to fill with fluid. The fluid level within the manometer column was increasing, as a result of continuous increase of the intravesical pressure, and the infusion of N/S was discontinued at the time of urethral leakage (Figures 3, 4). The leakage of urine occurred at bladder capacity and the pressure at the time of leakage on the manometer scale was documented as LPP, and was marked in centimeters of water (cmH_2O) [19] (Figure 5). This represents the minimal pressure in the bladder that resulted in leakage of urine. We did not apply external pressure.

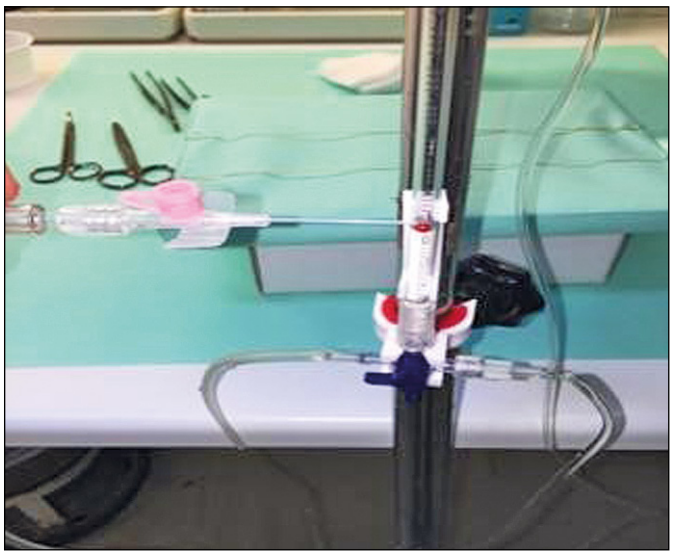

Figure 3. *Water manometer. The bladder was at the level of the manometer and the relative hydrostatic pressure was zero cmH_2O. The red ball of the manometer represents the intravesical pleasure.*

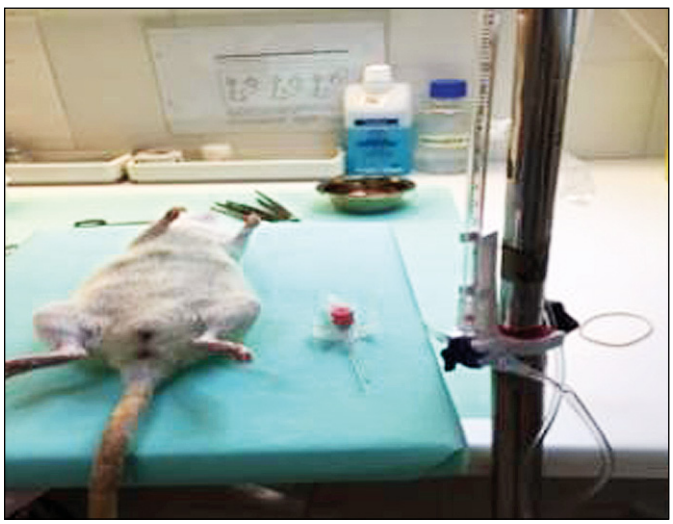

Figure 4. *Water manometer. The bladder was at the level of the manometer and the relative hydrostatic pressure was zero cmH_2O. The red ball of the manometer represents the intravesical pleasure.*

Platelet rich plasma (PRP) preparation

Platelet rich plasma was prepared according to a standardized research protocol for PRP preparation in rats published by Messora et al. [16]. The maximum blood volume that can be safely removed from each rat without fluid replacement, is less than 1.3 ml for a one time sample [20], therefore, 1.2 ml was harvested from each rat with retro-orbital puncture. The blood was collected in 5 ml silicone vacuum tubes with 0.13 ml of 10% sodium citrate and was initially centrifuged at 160 G, for 20 minutes, at room temperature (22°C). Following the centrifugation, two fractions were created. A red fraction at the lower part of the tube (from the red cells) and an upper straw-yellow turbid fraction (serum component). The silicone tube was marked at a point 1 mm below the line dividing the red with the yellow fraction and all the content above this point was retrieved by a pipette and transferred to another 5 ml vacuum tube, in which a line corresponding to 0.13 ml was drawn from the tube's bottom. A second centrifugation was performed for this sample, at 400 G, for 15 minutes, resulting again in two fractions, one above (platelet-poor plasma PPP) and another below the line (PRP). The PPP fraction was pipetted and the leftover PRP fraction was kept in the silicone vacuum tube and was finally activated with 0.07 ml of 10% calcium chloride solution, prior to the application. Autologous PRP was harvested from each rat. Following blood specimen collection, PRP was prepared whilst the rat was in theater for catheterization of the bladder. PRP was injected just before the application. Hence, there were no concerns of a potential immune response.

Statistical analysis

Statistical significance of the difference of the median values between groups was analyzed using the Mann-Whitney U test due to the non normal distribution. Differences were considered significant at $p < 0.05$. The SPSS statistical package was used in this study (IBM Corp. Released 2013 Version 22.0. Armonk, NY: IBM Corp.).

Given the absolute lack of previous studies in this field, we did not perform sample size calculation for

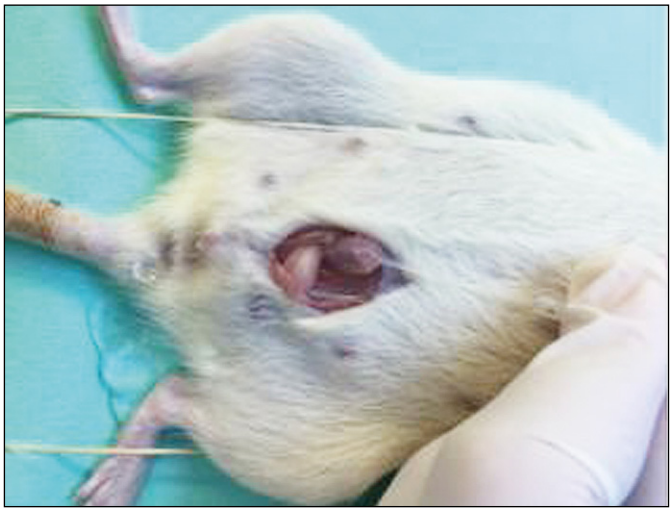

Figure 5. *Water manometer. The bladder was at the level of the manometer, the catheter was inserted to the bladder dome and the pressure at the time of leakage was documented as leak point pressure.*

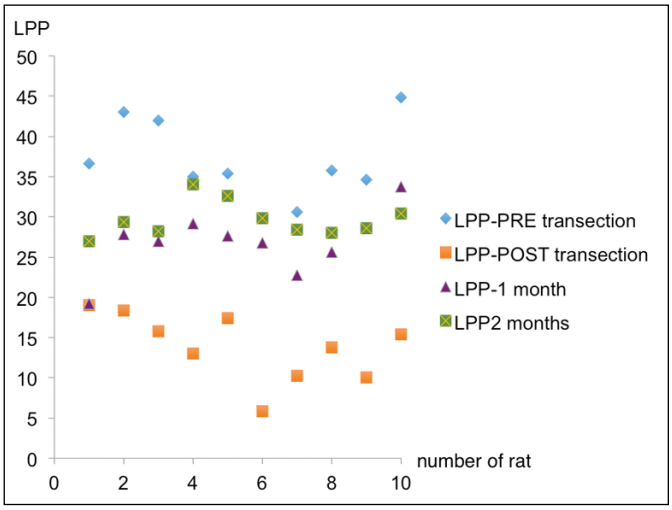

Figure 7. *Control group results. This chart presents the mean leak point pressures in cmH_2O for each rat in the 4 different sets of measurements. Initially before the pubourethral ligament (PUL) transection and following the PUL transection, and later at one and two months follow-up.*

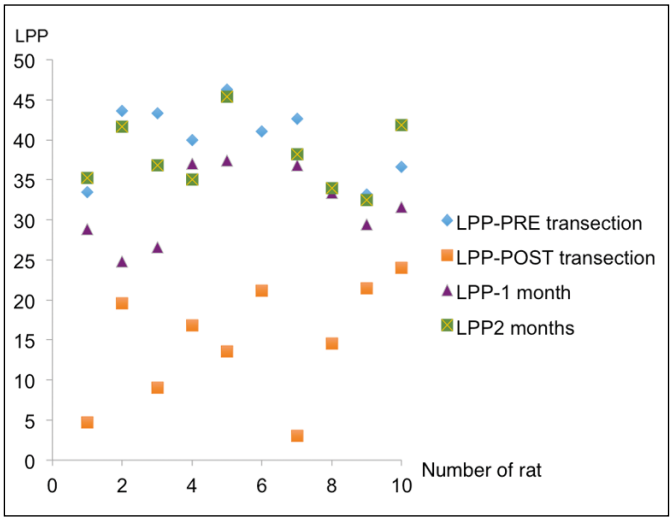

Figure 6. *Platelet rich plasma group results. This chart presents the mean leak point pressures in cmH_2O for each rat in the 4 different sets of measurements. Initially before the pubourethral ligament (PUL) transection and following the PUL transection and later at one and two months follow- up.*

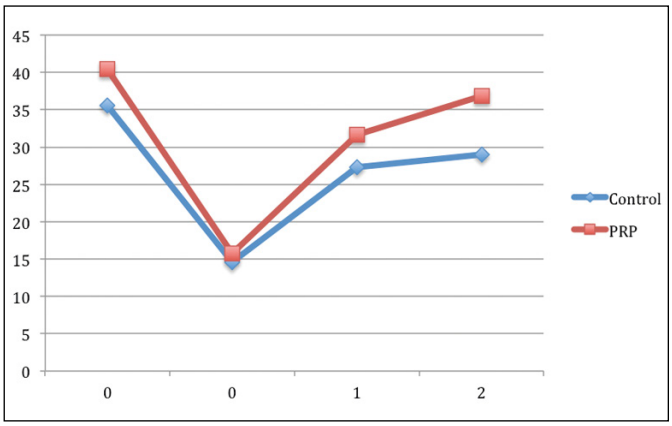

Figure 8. *Median leak point pressures for each group over time at 0 before the pubourethral ligament (PUL) transection, at 0 post PUL transection and at 1 and 2 months.*

our findings and the number of animals that was used was based on the previous study of Kefer et al. [17], also accounting for a potential animal loss of 20%.

RESULTS

Twenty rats were initially included in the study and randomly allocated on a 1:1 ratio into two groups. One rat in the PRP group died 20 min postoperatively. The results of our study are based on the data drawn from the remaining 19 animals. No differences were observed prior to PUL transection and immediately after the transection between the 2 groups.

First phase

The median LPP prior to PUL transection LPP – preT for the control group was 35.6 (29.8–44.8) cmH_2O and for the PRP group 40.5 (33.2–46.3) cmH_2O ($p = 0.353$). The median leak point pressure immediately after the PUL transection (LPP – postT) for the control group was 14.6 (5.8–19.0) cmH_2O and for the PRP group 15.7 (3.0–24.0) cmH_2O ($p = 0.631$). (Figures 6, 7, 8). Following the measurements of the

LPP – postT, PRP was injected only to the rats of the PRP group.

Second phase

At 1-month follow-up the LPPs of the animals that were injected with PRP were significantly higher compared to those of the control group. The median Leak Point Pressure 1 month after the pubourethral ligament transection (LPP – 1month) for the control group was 27.3 (19.2–33.8) cmH_2O, whereas for the PRP group was 31.6 (24.8–37.4) cmH_2O (p = .043). Immediately following the measurements of the LPP at 1 month follow-up, a second application of PRP was performed for the PRP group.

Third phase

The observed differences were still significantly higher at 2-months follow-up. The median leak point pressure 2 months after the pubourethral ligament transection for the control group was 29.0 (27.0–34.0) cmH_2O, whereas for the PRP group 36.8 (32.5–45.4) cmH_2O (p <.001) (Figures 6, 7, 8), (Table 1).

DISCUSSION

Main findings

The findings of our study suggest that PRP may significantly restore LPPs after the transection of the PUL. SUI can have a significant impact on women's quality of life. Application of PRP to a damaged PUL, could be a potentially effective treatment option, for example for women at the postpartum period. This would be a minimally invasive procedure avoiding the risks of major interventions and could potentially be performed under local anaesthesia.
The growth factors found in the PRP, play a significant role in the pathophysiology of ligament healing and reconstruction. Studies have evaluated the efficacy of the PRP in tendon and ligament healing by enhancing the accumulation of mesenchymal stem cells in the area, which in turn enhance the deposition of collagen fibers [21].
Previous studies have shown, that transection of the PUL causes SUI [17, 22, 23]. It has been demonstrated that the LPP of female rats and intact PUL is significantly higher compared to those with an incised PUL (16.3 ±2.74 vs. 36.6 ±8.39 cmH_2O p <0.00001) [24] at 4-days follow-up. The most important finding of the present study is that the local administration of PRP in rats with incised PUL leads to significantly increased LPP measurements compared to the control group, especially at the two-month follow-up.

Table 1. *Results: median pressures in cmH_2O*

Leak point pressure among groups			
	Control	PRP	p-value
LPP – pre	35.6 (29.8–44.8)	40.5 (33.2–46.3)	.353
LPP – post	14.6 (5.8–19.0)	15.7 (3.0–24.0)	.631
LPP – 1 month	27.3 (19.2–33.8)	31.6 (24.8–37.4)	.043
LPP – 2 months	29.0 (27.0–34.0)	36.8 (32.5–45.4)	<.001

Strengths and limitations

Our study evaluated for the first time the impact of PRP application in a transected PUL in an animal experimental model. The experiments were performed under repeatable conditions for higher precision and accuracy. The LPP measurements for each rat were performed under the same conditions of measurement. These conditions include the same measurement technique, the same observer, the same measurement instruments (same manometer and catheters), the same experimental laboratory and the repetition was over the shortest possible period of time. At each assessment the LPP was measured five times for each rat and the mean value of these measurements was calculated in order to achieve higher accuracy.
An iatrogenically transected PUL is not a true representation of the clinical situation of PUL injury where the ligaments are damaged during childbirth. Furthermore, in a clinical setting the type of anaesthesia may affect the urethral sphincter tone and the results may not represent those of non-anaesthetized rats. In addition, during the measurement of the LPPs, the continuous flow of the normal saline that was used, was resulting in continuous increase of the bladder pressure, making it impossible to detect or evaluate the presence or absence of bladder overactivity. Measuring LPP before and after PUL transection would control for such possible confounding. We accepted that the surgical transection of the pubourethral ligament itself is unlikely biologically to have caused overactive bladder that would confound the LPP readings. Also, we did not use flared tip catheter for the bladder catheterization and some leakage of the fluid might have occurred from the point that the catheter was inserted; however we did not notice leakage macroscopically. Another limitation is that because of the second application of PRP, outcomes at 2 months are difficult to interpret as the difference could reflect a long-term effect of

the first treatment, a short-term effect of the second treatment or their combination. Also, the amount of PRP that was applied was arbitrary, according to the maximum harvested material (produced from 1.2 ml of autologous blood) and we have not investigated the effect of the PRP on a group of rats with intact PUL, as well as we did not have a group with intact PUL to follow over time.
Despite these limitations, we believe that this study has provided valuable information regarding the development of PRP treatment for SUI.

Implications for future research

In our study the treatment of PRP started immediately following the PUL injury. Future studies should address further the efficacy and safety of PRP on female SUI in an experimental and clinical setting. This way, we will be able to evaluate its role and effects in humans and further investigate the underlying tissue remodeling and ligament restoration. Also, to date, there is no clear evidence to support the benefit of a repeat application of PRP and the timing that this should be reapplied. The reported number and frequency of the application varies significantly between studies. Studies report outcomes with a single application of PRP, or a repeat application in 6 weeks, even a multiple application every second day until full recovery [25].

Animal models have been previously used to model tissue recovery before introducing various surgical and medical options in clinical practice; hence, it may become a useful precursor for future research. Our findings are rather promising; however, the pathophysiological processes that take place in terms of tissue healing and remodeling have not been yet fully elucidated.

CONCLUSIONS

As pubourethral ligament (PUL) defects have been linked to the pathophysiology of stress urinary incontinence (SUI), the correction of the anatomy of this ligament may lead to the treatment of SUI. The growth factors play a significant role in the process of the restoration of the ligaments and platelet rich plasma (PRP) is an easily prepared solution, rich in growth factors, which can be administered to the damaged ligaments at a low cost. The findings of our study suggest that PRP may significantly improve leak point pressures (LPP) after transection of the PUL. However, further experimental and clinical research is needed to evaluate the safety and efficacy of this treatment, in clinical practice.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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