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Title: New therapeutic approach with Extracellular vesicles from stem cells for intestinal cystitis/bladder pain syndrome

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ABSTRACT

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a debilitating chronic disorder characterized by suprapubic pain and urinary symptoms such as urgency, nocturia, and frequency. The prevalence of IC/BPS is increasing as diagnostic criteria become more comprehensive. Conventional pharmacotherapy against IC/BPS has shown suboptimal effects, and consequently, patients with end-stage IC/BPS are subjected to surgery. The novel treatment strategies should have two main functions, anti-inflammatory action and the regeneration of glycosaminoglycan and urothelium layers. Stem cell therapy has been shown to have dual functions. Mesenchymal stem cells (MSCs) are a promising therapeutic option for IC/BPS, but they come with several shortcomings, such as immune activation and tumorigenicity. MSC-derived extracellular vesicles (MSC-EVs) hold numerous therapeutic cargos and are thus a viable cell-free therapeutic option. In this review, we provide a brief overview of IC/BPS pathophysiology and limitations of the MSC-based therapies. Then we provide a detailed explanation and discussion of therapeutic applications of EVs in IC/BPS as well as the possible mechanisms. We believe our review will give an insight into the strengths and drawbacks of EV-mediated IC/BPS therapy and will provide a basis for further development.

INTRODUCTION

Interstitial cystitis/bladder pain (IC/BPS) syndrome is a debilitating pain syndrome that presents with a wide range of symptoms including pelvic pain, urinary frequency, urgency, and cystoscopy findings, such as Hunner's lesion or glomerulation following hydrodistension. However, its diagnosis depends on the exclusion of other overlapping disorders. The prevalence of IC/BPS was long known to be higher in females (1). However, recent reports have demonstrated a higher prevalence in males (2, 3). Although no consensus exists on the actual pathophysiology of IC/BPS, numerous theories have emerged including mast cell infiltration, inflammation, glycosaminoglycan layer/urothelial dysfunction, and autoimmune dysregulation (4, 5).

Urothelium, a special form of epithelial tissue that lines the urinary tract walls, including the proximal urethra and urinary bladder, serves as a vital barrier against pathogens, toxins, and wastes (6, 7). The urothelium, which is made up of uroplakins (UPs) complexes, forms the urine-blood barrier and is supported by a thick pseudostratified transitional epithelium (multi-layered) and an asymmetric and fully differentiated superficial membrane (umbrella) (8-10). UPs are categorized into four subtypes: UP1a, UP1b, UP2, and UP3, which are implicated in the urothelium's permeability. These proteins coalesce together to form crystalline plaques on the bladder lumen's surface (10, 11). Proliferation or hyperplasia is a defensive reaction to any damage to the urothelium to rebuild the urine-blood barrier (12, 13). The main obstacles to bladder tissue regeneration are the scarcity of adequate tissue sources and senescence-associated primary cultures of bladder cells for several passages (14). For example, some trials using gastrointestinal tract-derived tissue for bladder regeneration showed inefficiency due to tumor formation, recurrent infection, metabolic abnormalities, and stone formation

(15). Through the present comprehensive review article, we want to report risk and benefit of stem cell therapy for IC/BPS. In addition, we would introduce new approach with EVs from stem cell in order to push the limit of the stem cell therapy.

CONTENTS

There are several drugs and surgical methods which are recommended for IC/BPS therapy by global societies, such as the American Urological Association (AUA), the European Society for the Study of IC (ESSIC), and the Society of Interstitial Cystitis of Japan (SICJ) (16). Unfortunately, the current conventional drugs and alternative surgical interventions do not guarantee complete recovery and are associated with harmful side effects (16, 17).

Because of its chronic nature and high prevalence, bladder dysfunction is an attractive target for stem cell therapy. Numerous preclinical trials for bladder dysfunction, such as detrusor underactivity, stress urinary incontinence, overactive bladder, and IC/BPS, have been established, although clinical investigations in patients are still sparse (18).

Stem cell-based bladder dysfunction therapy includes several mechanisms, such as anti-inflammation, anti-fibrosis, urothelium regeneration, anti-oxidant, anti-apoptosis, and modulation of specific signaling pathways including Wnt and AKT/mTOR pathways (19).

Therefore, stem cell-based therapy has garnered attention as a robust alternative option. Mesenchymal stem cells (MSCs) have a proven record of therapeutic efficacy in human clinical trials and have been effective in a wide range of pre-clinical studies of tissue regeneration in various immunologic and degenerative diseases (20-22). MSCs secrete paracrine factors, which are the key mediators of MSC-associated therapeutic activities. Most, if not all, of the MSCs' paracrine activities, are mediated by extracellular vesicles (EVs), which are 50–1000 nm in diameter and secreted by all cell types (23, 24). EVs pass through biological barriers such as the blood-brain barrier (25) and synovial membranes (26). It is evidenced that EVs are carriers of the exogenous RNAs, such as siRNA (27), miRNA (28), and modified miRNAs (29), which could be functional molecules in vitro and in vivo. In addition, previous reports demonstrated the immunostimulatory or immunosuppressive capacities of EVs based on their target and cellular source (30). The immunomodulatory capacities of EVs could be beneficial for the treatment of inflammatory, autoimmune, and hypersensitivity diseases (31). Small EVs (sEVs), of 50 to 200 nm in diameter, isolated from MSC culture supernatants that are maintained under various culture conditions, have proved to be therapeutically effective in various preclinical models (23, 32).

Pathophysiology of IC/BPS

The aetiology of IC/BPS is perplexing (33). Nevertheless, numerous hypotheses endeavor to explain IC/BPS pathogenicity. Bladder epithelial damage, mast cell activation, neuroinflammation, suppression of tight junction protein, afferent nerve plasticity, infection, abnormal urothelial signaling, destruction of the superficial urothelial glycosaminoglycan

(GAG) layer, and psychological factors have been reported as the etiological factors that lead to IC/BPS as illustrated in Figure 1 (16, 34, 35). A commonly acknowledged postulation of IC/BPS pathophysiology proposes that the chronic inflammatory state is induced by early damage or defect in the mucosal membrane of the bladder. Urothelium, a unique type of epithelium, is composed of polysaccharides (chondroitin sulfate and hyaluronic acid) in its outer layers and glycoproteins in the deeper layers (36). The injured urothelium is the main culprit for the impaired barrier function, which allows urine solutes, such as potassium ions, to seep into the suburothelium, leading to neuronal and muscle cells depolarization and inflammation-related damage, urgency, and pain (37, 38).

The bladder pain is aggravating, especially, during the bladder filling process. Besides its barrier function, urothelium is also implicated in sensory transduction through sensing physiological and chemical signals in the bladder wall and releasing signaling molecules (39). Urothelium-mediated signal transduction is not fully characterized; however, urothelial cells can produce substances P, acetylcholine, and ATP, which are involved in the activation of the bladder afferent neurons (40).

Chronic inflammation possibly plays a key role in IC/BPS pathogenesis. In bladder biopsies of some patients with bladder pain, mast cells, leucocytes, and lymphocytes were found infiltrating the bladder wall and suburothelial layers, along with increased vasculature and thickening of the bladder wall. The clinical observations demonstrated subsequent chronic pain in patients with frequent reports of urinary tract infections (UTIs). UTIs are among the exacerbating factors of IC/BPS that commence at an early age and progress to IC/BPS in adulthood (41). In IC/BPS patients, the proliferating mast cells in the bladder wall have been linked to inflammation, allergic responses, and bladder hypersensitization (42, 43).

Of note, a link has been shown between the incidence of clinical IC/BPS and autoimmune

diseases (44). Autoantibodies, which are involved in the autoimmunity mechanism, showed an adverse action on the bladder urothelium, connective tissues, and smooth muscles (44). Further, a nationwide study recently reported IC/BPS in patients with primary Sjögren's syndrome (45). Taken together, there is a strong link between autoimmune disorders and IC/BPS pathogenicity, as evidenced by the chronic inflammation and the presence of autoantibodies.

Limitations of stem cell therapy

Before discussing stem cell therapy limitations, we first need to briefly address the beneficial effects of stem cells in the treatment of various diseases, especially IC/BPS. Basically, MSC-based therapies are attributed to their intertwined roles including suppressing inflammation by releasing cytokines, supporting healing by expressing growth factors, altering host immune responses by secreting immunomodulatory factors, augmenting responses from endogenous repair cells, and acting as mature functional cells such as bone cells (46). Stem cell-mediated IC/BPS therapy is ascribed to various mechanisms, including their direct differentiation into the main bladder cells, including urothelium and smooth muscles (SMCs), their transplantation via several routes, and the activation of the vital signaling pathways that are involved in bladder regeneration, such as mitogen activated protein kinases, AKT, Wnt-GSK3β/β-catenin, and mTOR signaling pathways (16, 47-50). The possible application of stem cells in treatment of the bladder diseases at the preclinical level is also proved (18). However, Clinicaltrials.gov currently shows no ongoing stem cell treatment clinical trials in IC/BPS (18). On the other hand, the main concern over stem cell therapy is the lack of safety proof. It is difficult to implant foreign living cells into a sophisticated structure like the

human body. Due to the vision loss in patients with macular degeneration, an age-related eye condition, after the injection of autologous stem cells at a U.S. clinic (51), there has been a growing concern over the safety of unproven stem cell therapies are used. The risk of post-transplantation tumorgenicity is associated with the donor's age, growth modulation by the recipient tissues (52), and the dysfunction of the patient immune system due to long-term chemotherapy (53). Of note, long-term in vitro culture of MSCs could lead to unfavorable consequences, such as chromosomal abnormalities, senescence, and genetic instabilities, which negatively influence the engraftment (52, 54). Moreover, investigations in rodents and dogs demonstrated that intravenously injected MSCs are trapped in the pulmonary capillaries and large populations of MSCs are largely cleared, but some get through to the damaged target tissue (55-58). In addition, stem cell engraftment led to immune reaction-associated stress that resulted in unfavorable outcomes, such as cellular necrosis and differentiation anomalies (59).

Specifically, stem cell therapy holds numerous limitations and challenges that hinder its clinical application in bladder disease therapies, such as the controversies over the transplantation route and the dose of the cells, the undefined mechanism of action of several stem cell-mediated bladder disease therapies, and the in vivo IC/BPS models used for verification of stem cell effects lack the reproducibility and needs further careful authentications (16, 60).

Furthermore, when stem cells are introduced to target cells, the therapeutic effectiveness of MSCs may not correspond with engraftment, differentiation, or cell fusion (61). Overall, MSCs therapeutic actions are mediated via the paracrine effect, which is involved in tissue repair, and not replacement-based therapy (62). In this prime, numerous studies demonstrated that MSC-conditioned culture medium produced therapeutic effects similar to cell delivery in

rodent models of various disease models (63, 64), which has been supported by genomics data showing that MSCs secrete a huge array of bioactive proteins (65, 66).

Overcoming the hurdles with stem cell-derived EVs

MSCs application in urologic regenerative medicine has been widely studied due to their multilineage differentiation capacity (67, 68). However, the potential MSCs tissue regeneration mechanism is by their paracrine action via the released soluble factors including growth factors, cytokines, and chemokines rather than MSC differentiation and structural interaction with host tissue (69, 70). The paracrine effect is dependent on the transfer of proteins, bioactive lipids, and genetic material such as mRNA, miRNAs, and other non-coding RNA. The paracrine impact of stem cells is also mediated via secreted EVs (71). MSCs can release a wide spectrum of soluble factors such as secretomes into the culture medium, which are less immunogenic and tumorigenic (72, 73). MSC paracrine activity could be classified into various activities, such as anti-apoptotic, anti-inflammatory, angiogenic, immunosuppressive, and immunomodulating impacts (74).

The specific compositions of MSC-derived EVs (MSC-EVs) differ according to tissue source and in vitro cell stimulation approach (72); EVs isolation procedures include ultracentrifugation, filtration, immunoaffinity, precipitation, size exclusion, and microfluidic devices (75, 76). The basic criteria for EVs isolation and characterization are based on the recommendations by Minimal information for studies of extracellular vesicles 2018 (MISEV2018) (77). However, the recent EVs isolation techniques are limited by lack of reproducibility and variation in the quality of the EVs produced, and therefore need further

considerations (78). Broadly, EVs could be categorized as "exosomes" or "microvesicles" (24). The term "exosome" commonly refers to a specific class of sEVs (sEVs) formed by the endosomal system (79), which vary from the "ectosomes" (microvesicles and microparticles) that emerge from the plasma membrane (80) or other similarly sized EVs with an undefined biogenesis pathway (81). "Small" in the term sEVs indicates a population range of approximately 50–200 nm in diameter. The production of sEVs is currently thought to be one of the mediators of MSC therapeutic properties (23). In numerous in vitro functional assays and relevant pre-clinical disease models, sEVs derived from in vitro MSC cells have been reported to have therapeutic activities that imitate those of MSCs (82, 83). Furthermore, MSC-EVs convey a large amount of verified therapeutic agents into target cells, such as nucleic acids, proteins, miRNA, and lipids, to modulate numerous biological functions (84). MSC-EV-associated tissue regeneration is majorly attributed to the MSC-EVs capacity to enhance the proliferation process and suppression of the apoptotic changes (85, 86). The prominent role of MSC-EVs in maintaining the immune hemostasis is attributed to the modulation of immune cell fate and inhibition of uncontrolled inflammation (85, 87). One of the key mechanisms of MSC-EVs in tissue regeneration is stimulation of the angiogenesis via activation of various signaling pathways (88, 89). The MSC-EVs urinary bladder wall diseases therapeutic activities are mediated via the bladder tissues regeneration and suppression of inflammation, which helps to prevent disease development and recurrence (90). Besides the possible effects of the purified EVs in the treatment of bladder diseases, we will also explain examples of the roles of stem cell conditioned medium (CM) or co-culture platforms in bladder disease therapy that represent the paracrine action of stem cells in which EVs are potently involved. In 2014, Adamowicz et al. demonstrated the regenerative capacity of MSC-derived conditioned media (MSC-CM) in the bladder wall when administered

intravesical in IC patients, which was attributed to its high content of cytokines, growth, and trophic factors that possess immunomodulatory, anti-inflammatory, and angiogenic activities (91). In 2018, Xie et al. demonstrated the potency of umbilical cord-derived mesenchymal stem cells (UC-MSCs) to alleviate the inflammatory-associated changes, enhance the proliferation, and block the apoptosis when co-cultured within human urothelial cells, SV-HUC-1 cells that were pretreated with TNF-α (92). Interestingly, the application of siRNA targeting EGF in the UC-MSCs abolished the anti-inflammatory activity of UC-MSCs when co-cultured with TNF-α-exposed SV-HUC-1 cells. The authors showed that increased AKT and mTOR, as well as significant decrease in the protein expression level of the cleaved caspase-3, are involved in the therapeutic activity of UC-MSCs against the in vitro interstitial cystitis model using TNF-α-treated SV-HUC-1 cells (92).

Hypoxia is implicated in the activation of the inflammatory events and the consequent fibrosis of the bladder smooth muscles (93). In this regard, a study by Wiafe and colleagues detected the in vitro upregulation of hypoxia-associated genes, TNF-α, IL-1β, IL-6, HIF3α, VEGF, TGF-β1, and αSMA, in 3% oxygen tension-exposed bladder SMCs and then tested the effects of direct and indirect co-culture with bone marrow-derived MSCs (BM-MSCs) in reducing hypoxia-related changes (94). Direct co-culture is reliant on cell-to-cell interaction, whereas indirect co-culture (based on trans-well system) is cell-to-cell interaction independent. Interestingly, both co-culture methods led to a significant downregulation of TGF-β1 and IL-6, which are associated with fibrosis and pro-inflammation, respectively. Moreover, the significant increase in the expression level of the potent anti-fibrotic cytokine, IL-10, and the marked decrease in the expression level of αSMA, collagen I and III transcripts, and the total collagen proteins were demonstrated upon co-culture with BM-MSCs. Taken together, MSC-EVs were evidenced to be effective IC therapies via several

mechanisms, including delivery of therapeutic miRNA and growth factors, anti-inflammation, anti-fibrosis, and modulation of key signaling pathways (**Figure 2**).

In 2017, Lv et al. affirmed the cross-link between miR-214 inhibition and the enhancement of epithelial-mesenchymal transition that leads to fibrosis of the bladder wall and subsequent interstitial cystitis in postmenopausal women, which is mediated via the upregulation of Mitofusin 2 (Mfn2) (95). Accordingly, another study confirmed the protective capacity of exosomes derived from miR-214-enriched BM-MSC, which were cultured under hypoxic conditions against the oxidative damage in the cardiac stem cells (96). This effect is mediated via the suppression of calcium/calmodulin-dependent protein kinase II (CaMKII). Taken together, this study paves the way for revisiting the effect of miR-214 as an attractive candidate in exosome-mediated IC/BPS therapy. Various MSC-EVs showed anti-inflammatory (97), anti-fibrotic (98, 99), immunomodulatory (100) functions via modulation of a wide range of miRNAs, which need to be tested in IC/BPS therapy. In addition, there is a scarcity of studies that show the impact of stem cell derived EVs in IC/BPS therapy, which paves the way for further studies and clinical trials as well. A brief comparison between MSC and EVs in cystitis therapy is summarized in Table 1.

CONCLUSIONS

IC/BPS is a complicated chronic illness with unclear etiology. There are a variety of IC/BPS therapeutic options and surgical interventions, however, they are associated with detrimental side effects and do not guarantee a complete recovery. MSCs have shown unique therapeutic

activities and have been considered for the treatment of bladder diseases. However, stem cells engraftments for balder tissue regeneration face major challenges such as immune reaction, low survival rate, and tumorigenicity, which limit their clinical application. To overcome these constraints, numerous scientific works have demonstrated the efficiency of paracrine mechanisms of MSCs in the treatment of a wide range of diseases, which is represented in the secretion of a diverse range of growth factors, miRNAs, proteins, cytokines, and chemokines. EV secretion is considered the main mediator of MSCs paracrine mechanism. Here, we highlighted the advantages of the application of EVs as a cell-free platform over the direct use of stem cells in cystitis therapy. We explained how EVs contribute to cystitis treatment via the enhanced proliferation, anti-inflammatory, anti-fibrotic, and immunomodulatory functions. Furthermore, we showed the role of EVs as cargos for therapeutic molecules such as miRNAs and their role in the alleviation of cystitis. However, further investigations into miRNA carried by EVs in IC/BPS therapy are needed. Moreover, further in-depth comparative studies on MSCs and their EVs in IC/BPS treatment are required for the clarification of the effectiveness and efficacy of EVs over the parent MSCs before the clinical applications. We believe further improvements in separation, characterization, engineering, and efficacy evaluation of MSC-EVs are essential for deriving high quality EVs with utmost activity for IC/BPS therapy in the future.

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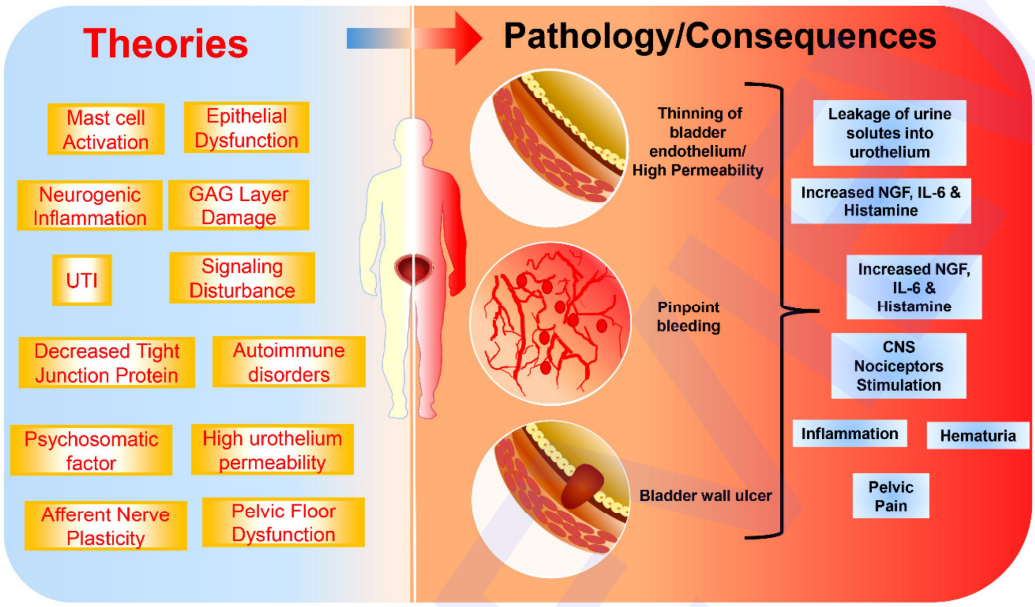
CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Fig. 1. IC/BPS Pathophysiology



GAG: glycosaminoglycan, UTI: urinary tract infections, NGF: nerve growth factor, IL-6: interleukin-6, CNS: Central nervous system

Fig. 2. MSC-EVs mechanisms in IC/BPS therapy. A schematic diagram summarizing MSC-EV separation methods and their potential modes of action in IC/BPS therapy. Parts of this figure were created using Servier Medical Art (https://smart.servier.com), licensed under a Creative Commons Attribution 3.0.

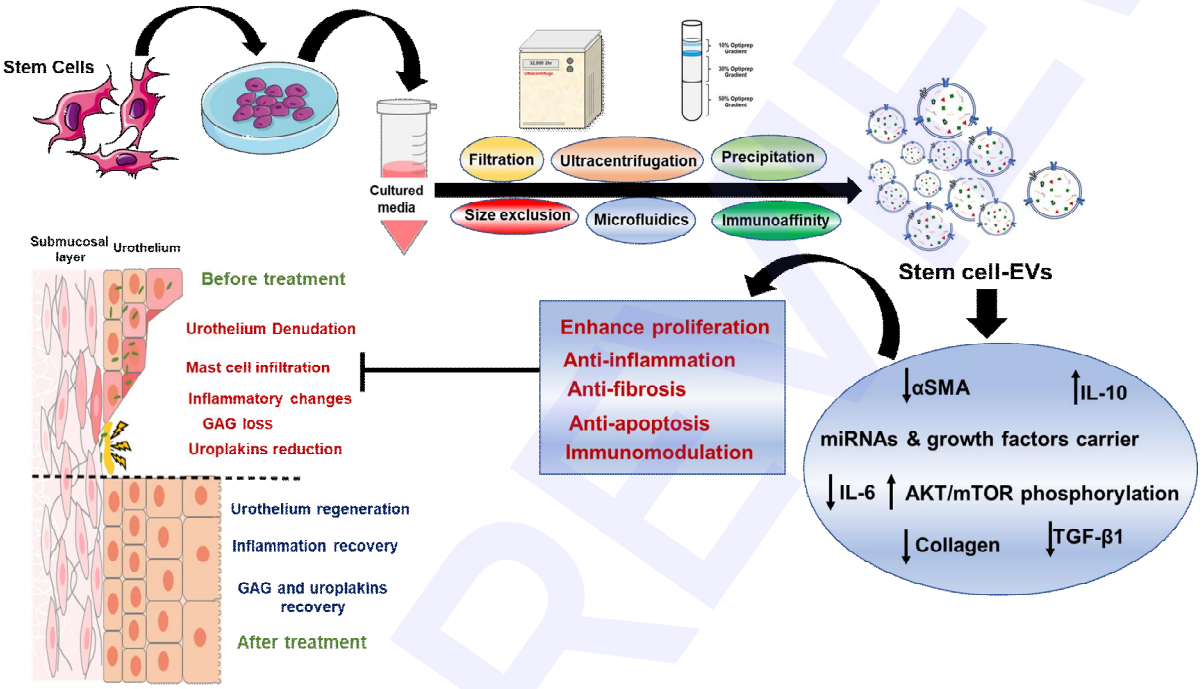


Table 1. Comparison of stem cell therapy and EVs therapy.

Item	Stem cell therapy	EVs therapy
Source	Primary tissues of various body organs	Cultured media
Interstitial cystitis therapy mechanisms	-Cell replacement via their differentiation into urothelium and muscle layers -Migration to injured site -Anti-inflammation -Anti-fibrosis -Modulation of signaling pathways	-Delivery of therapeutic molecules (miRNA and growth factors). -Enhance proliferation -Anti-fibrosis -Anti-apoptosis -Immunomodulation
Pros	-Availability -Easily isolated and expanded -Multilineage differentiation -Unique immunological properties	-Cell-free platform -Carrier of the cell therapeutic molecules -Easily engineered. -Easily stored
Cons	-Post-transplantation tumorigenicity -Donor-dependent quality -Genetic instability and chromosomal abnormalities -Senescence -Short-term survival at the injured site -Engraftment failure	-Costly -Irreproducible and inefficient separation methods -Heterogenicity -EVs characterization Difficulties -Scarcity of EVs specific markers

AKT: serine/threonine kinase, mTOR: mechanistic target of rapamycin kinase, TGF-β: transforming growth factor- β

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Fig. 1.

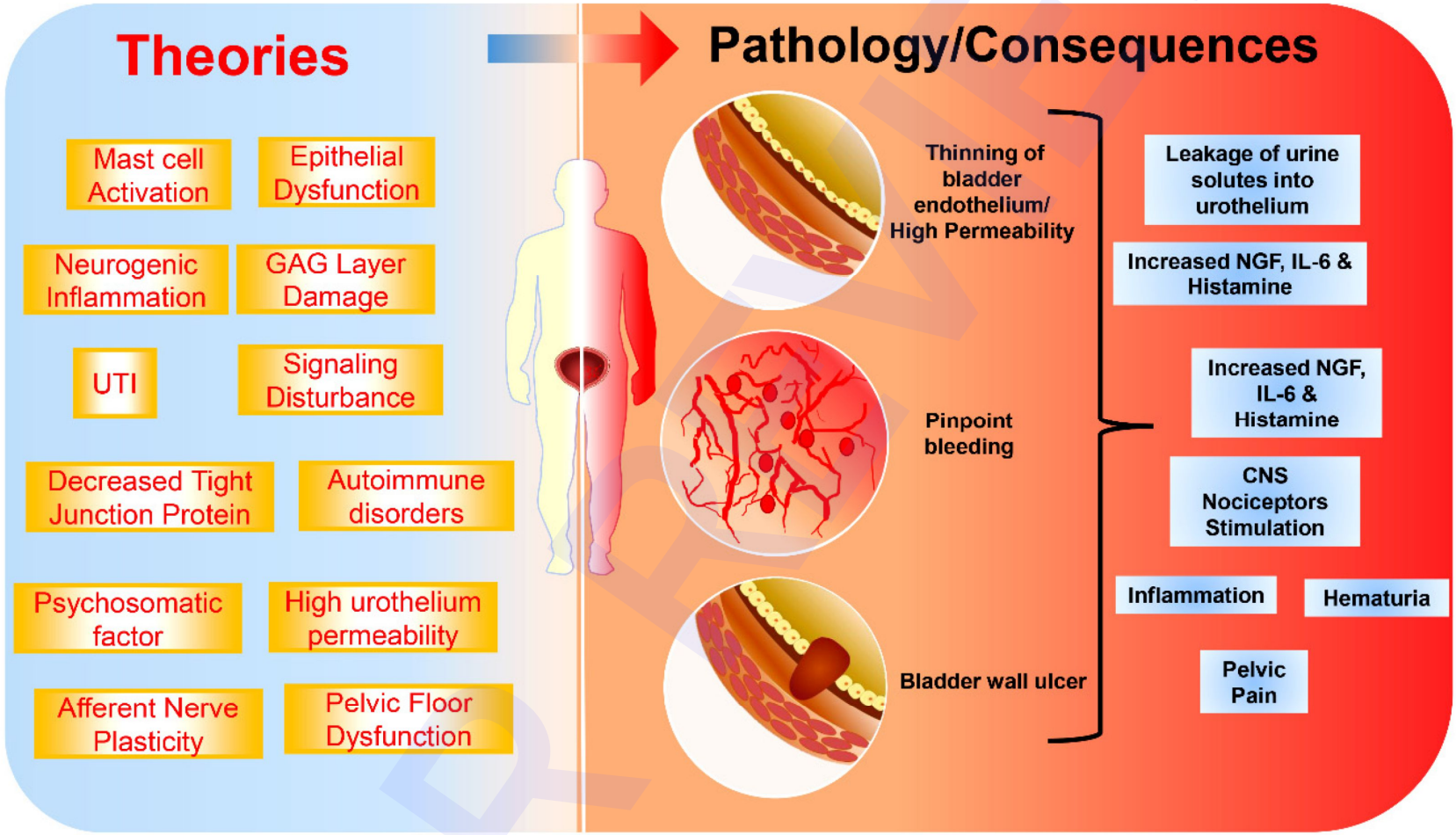
Theories
Mast cell Activation
Epithelial Dysfunction
Neurogenic Inflammation
GAG Layer Damage
UTI
Signaling Disturbance
Decreased Tight Junction Protein
Autoimmune disorders
Psychosomatic factor
High urothelium permeability
Afferent Nerve Plasticity
Pelvic Floor Dysfunction
Pathology/Consequences
Thinning of bladder endothelium/ High Permeability
Pinpoint bleeding
Bladder wall ulcer
Leakage of urine solutes into urothelium
Increased NGF, IL-6 & Histamine
Increased NGF, IL-6 & Histamine
CNS Nociceptors Stimulation
Inflammation
Hematuria
Pelvic Pain

Fig. 2.