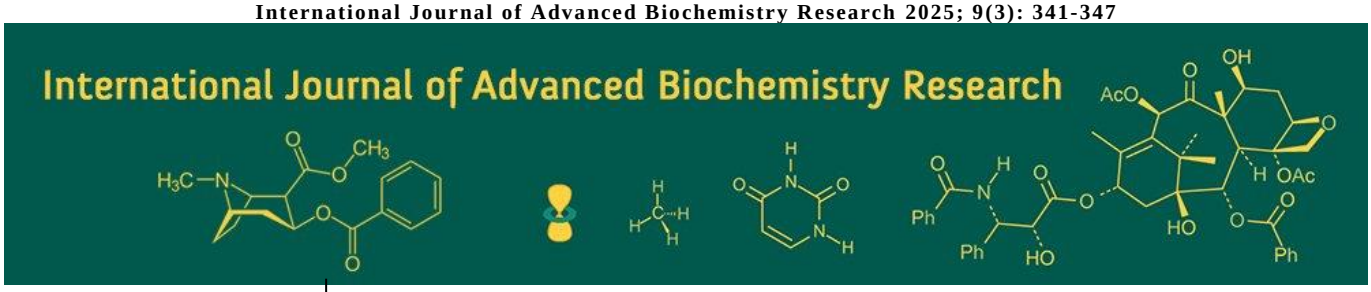

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Cholinergic modulation of contractile responses in caprine urinary bladder smooth muscle: Role of urothelium and atropine sensitivity

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Abstract
The cholinergic system plays a pivotal role in urinary bladder function, primarily facilitating bladder emptying through the activation of muscarinic receptors in smooth muscle. This process can also be modulated by signaling molecules from the urothelium. In this study, we investigated the role of the urothelium in cholinergic-mediated bladder smooth muscle contraction.
Bladder smooth muscle contraction was induced using the muscarinic agonist acetylcholine (ACh). Our results demonstrated that ACh elicited a concentration-dependent contractile response in goat urinary bladder smooth muscle. Notably, the removal of the urothelium (detrusor muscle strips) resulted in a leftward shift of the ACh-induced contractile response curve, leading to a stronger and more pronounced contraction. The muscarinic response was significantly enhanced in the absence of the urothelium compared to intact bladder smooth muscle.
Furthermore, in the presence of atropine, a muscarinic receptor antagonist, ACh-mediated contractions were abolished. Minimal responses were observed in denuded caprine detrusor muscle strips, while no contraction occurred in detrusor muscle strips with an intact urothelium.
Our findings suggest that the caprine urinary bladder exhibits pharmacological properties similar to those of the human bladder, making it a potentially valuable model for evaluating new therapeutic agents for urinary incontinence.

Keywords: Acetylcholine, atropine, caprine detrusor muscle, cholinergic system

Introduction
Urinary incontinence refers to the involuntary leakage of urine, often resulting from an overactive bladder or other underlying conditions such as arthritis, prostatitis, weakened bladder or pelvic floor muscles, or nerve damage caused by diseases like multiple sclerosis, diabetes, or Parkinson's disease. It is more prevalent in older adults, particularly women. With advancements in medicine, there are now more treatment options and management strategies available than ever before. The urinary bladder's inner lining consists of a mucous membrane composed of transitional epithelium, which is continuous with that of the ureters. Beneath this lies the submucosa, a connective tissue layer containing elastic fibers that support the mucous membrane. The most prominent layer of the bladder wall is the muscularis, composed of smooth muscle fibers arranged in an interwoven pattern, collectively known as the detrusor muscle. Detrusor smooth muscle is made up of spindle-shaped cells containing thin actin and thick myosin myofilaments, which are interconnected both electrically and mechanically. Unlike skeletal and cardiac muscles, smooth muscle contraction follows a distinct mechanism. The regulation of bladder function involves multiple neurotransmitters, including acetylcholine, norepinephrine, dopamine, serotonin, excitatory and inhibitory amino acids, adenosine triphosphate, nitric oxide, and neuropeptides. These neurotransmitters play roles in both peripheral and central nervous system control of bladder activity (Andersson, 2013) [6].
Among the various neuronal inputs to the urinary bladder, parasympathetic innervation plays a crucial role in the micturition phase. Acetylcholine, acting on muscarinic M3 receptors, is responsible for the majority of contractile responses in the detrusor muscle across most species (Chess-Williams *et al*., 2001; Matsui *et al*., 2000) [13, 25]. However, the non-adrenergic, non-cholinergic (NANC) neurotransmitter ATP also contributes to

parasympathetically mediated contractions (Burnstock, 2008; D'Agostino *et al*., 2012) [10, 15]. The extent to which the purinergic signaling system influences detrusor contraction, primarily via the P2X1 purinoceptor, varies among species (O'Reilly *et al*., 2001; Vial and Evans, 2000) [29, 45]. In addition to its direct contractile role, ATP exerts paracrine functions, stimulating the release of other neuromodulators from the bladder urothelium (Winder *et al*., 2014) [48]. These substances can modulate bladder function by acting directly on smooth muscle cells, suburothelial cells, afferent or efferent neurons, or by inducing the release of additional transmitters from the urothelium (Birder and Andersson, 2013) [6].

As previously mentioned, acetylcholine plays a crucial role in normal bladder function and can influence signaling at multiple levels of the micturition reflex arc (Kullmann *et al*., 2008) [22]. Consequently, anticholinergic drugs are commonly used to treat overactive bladder (Robinson and Cardozo, 2012) [33]. However, their precise mechanism of action remains unclear. Studies suggest that anticholinergic drugs exert effects during both the voiding and storage phases of micturition. The involvement of the storage phase indicates a potential role for non-neuronal acetylcholine, such as that released from the urothelium or suburothelium (Andersson and Yoshida, 2003) [4]. This raises scientific interest in the interactions between the cholinergic transmitter system and other signaling pathways that may regulate non-neuronal acetylcholine release (Stenqvist *et al*., 2020) [40].

The urothelium and lamina propria, which form the inner lining of the bladder and urethra, exhibit spontaneous phasic contractile activity and respond to neurokinin (Sadananda *et al*., 2008) [34] and muscarinic receptor stimulation (Moro *et al*., 2011) [28]. Although nitric oxide (NO) donors induce relaxation of this tissue, there is no evidence of endogenous NO release (Moro *et al*., 2012) [27]. Crosstalk between the purinergic and cholinergic transmitter systems has been reported, wherein ATP appears to stimulate acetylcholine release, thereby supporting purinergic bladder contractions both *in vivo* and *in vitro* (Stenqvist *et al*., 2017) [41] *in vitro* studies indicate that this atropine-sensitive component of ATP-induced contractions originates from the urothelium (Stenqvist *et al*., 2017) [41]. This finding is further supported by transmitter release studies in both urothelial cells and urinary bladder tissue, suggesting the involvement of the P2Y6 purinoceptor in urothelial acetylcholine release (Silva *et al*., 2015) [38]. However, the specific receptors functionally involved in this interaction remain unidentified, and potential neuronal involvement requires further investigation (Stenqvist *et al*., 2020) [40].

To date, numerous pharmacological compounds have been identified, and a few have been marketed for human use following safety pharmacological studies conducted in laboratory animals. However, in certain safety pharmacology assessments, the use of slaughterhouse specimens instead of live laboratory animals could be highly beneficial, particularly for *in vitro* experiments such as isolated organ bath studies. This approach not only reduces the reliance on laboratory animals but also offers a viable alternative for specific experimental models. In this context, we have previously established the urinary bladder as a prototype smooth muscle model for ruminants, and the role of potassium channel openers in bladder hyperactivity has been well documented (Vijayakumar *et al*., 2007) [46]. The present study was conducted on the goat urinary bladder with the following objectives:

- To investigate the role of the mucous membrane in bladder function and
- To confirm that acetylcholine (ACh)-mediated contractile responses occur via muscarinic receptor activation.

Materials and Methods

Tissue mounting

The entire urinary bladder was collected immediately after slaughter from Black Bengal goats (2.0-2.5 years old) of either sex, weighing between 12.5 and 15.0 kg. The bladder was immediately flushed with Tyrode's solution and transported to the laboratory in chilled Tyrode's solution with the following composition (mM): NaCl, 120; KCl, 5.9; $MgSO_4 \cdot 7H_2O$, 1.2; $NaH_2PO_4 \cdot 2H_2O$, 1.2; $CaCl_2 \cdot 2H_2O$, 2.5; and Glucose, 11.5. Each urinary bladder was weighed and dissected longitudinally along the midline of the ventral surface. Two transverse strips, approximately 8 mm in size, were obtained from the mid-region of the bladder. Additionally, the tissue was cut at the mid-dorsal section, yielding four strips of similar length for further experimentation.

Role of the Urothelium in Cholinergic-Mediated Contractile Responses: Non-Denuded Detrusor Muscle

For isometric contraction studies, urinary bladder smooth muscle with an intact mucous membrane (non-denuded detrusor muscle) was used. A tissue strip measuring approximately 2.5 cm × 8 mm was suspended in an organ bath containing 20 ml of Tyrode's solution, continuously bubbled with oxygen (pH 7.4) and maintained at 37±0.5 °C under a resting tension of 2 g. The tissue was equilibrated for 90 minutes, with washes performed every 15 minutes. Isometric contractions were recorded using a kymograph and fixated with a fixative fluid. To evaluate the cholinergic response, cumulative concentration-related contractions were induced by acetylcholine (ACh) in the range of 10^{-9} to 10^{-4} M, with increments of one log unit. After obtaining the control concentration-response curve, the tissue was allowed to fully relax. Subsequently, the urothelium (mucous membrane) was removed, and the tissue was remounted in the organ bath. Following an equilibration period, the denuded detrusor muscle was exposed to the same ACh concentrations (10^{-9} to 10^{-4} M), and the resulting cumulative contractile responses were recorded.

Antagonistic Effect of Atropine on Cholinergic-Mediated Contractile Responses in Denuded and Non-Denuded Detrusor Muscle

To evaluate the antagonistic effect of atropine (10^{-6} M) on cholinergic-mediated contractions in detrusor muscle with and without urothelium, cumulative concentration-related contractions were induced by acetylcholine (ACh) in the range of 10^{-9} to 10^{-4} M, with increments of one log unit. After obtaining the control concentration-response curve, tissues were treated with atropine (10^{-6} M) before eliciting a second dose-response curve, following complete relaxation of the initial response. In all experiments, tissues were pre-incubated with the cholinergic receptor blocker for 5 minutes prior to the addition of the agonist. Results were expressed as a percentage of the maximal response curve. The inhibitory effect of atropine on ACh-induced contractile

responses was assessed by comparing contractile responses before and after atropine treatment.

Drugs and Chemicals

Acetylcholine chloride (Sigma) and Atropine (Sigma) were used. The drug solutions used were either prepared fresh or were taken from the stock solution (10^{-1} M) stored at 4^0 C in a refrigerator.

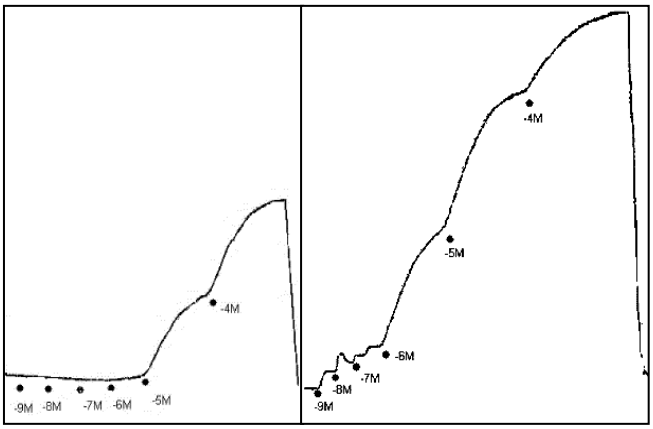
Statistics

Results are presented as the mean±standard error of the mean (SEM) or as means with 95% confidence limits. Statistical significance was determined using Student's t-test, with $p<0.05$ considered statistically significant. The EC_{50} and IC_{50} values, along with their 95% confidence limits, were calculated using regression analysis based on the least squares method. Data analysis was performed using interactive non-linear regression with GraphPad Prism (GraphPad Prism Software, San Diego, CA, USA).

Results

Role of the urothelium on ACh induced concentration-related contractile response

Acetylcholine (ACh) administered cumulatively at concentrations ranging from 10^{-9} M to 10^{-4} M induced a concentration-dependent contractile response in caprine non-denuded detrusor muscle. The threshold concentration required to initiate contraction and the concentration eliciting the maximal response (Emax) were 10^{-6} M and 10^{-4} M, respectively. Following removal of the mucous membrane, isometric contraction studies revealed a 100-fold increase in the contractile response to ACh in denuded tissue compared to non-denuded tissue (Trace 1). In denuded detrusor muscle, the threshold concentration and Emax were found to be 10^{-9} M and 10^{-4} M, respectively. In the presence of the urothelium, ACh-mediated contractile responses at the highest concentration (10^{-4} M) reached an E_{Bmax} of 52.7±3.7% (n=6), whereas the denuded detrusor muscle exhibited a maximal contractile response set at 100% (Table 1). The presence of the urothelium caused a significant ($p<0.001$) leftward shift in the ACh-induced concentration-response curve (Figure 1), with an increase in the mean $LogEC_{50}$ value (4.92 M) compared to the denuded detrusor response (6.06 M). All mean values were compared with 95% confidence limits.



Trace 1: ACh induced concentration-related contractile response Non Denuded (left) and Denuded Detrusor muscle (right).

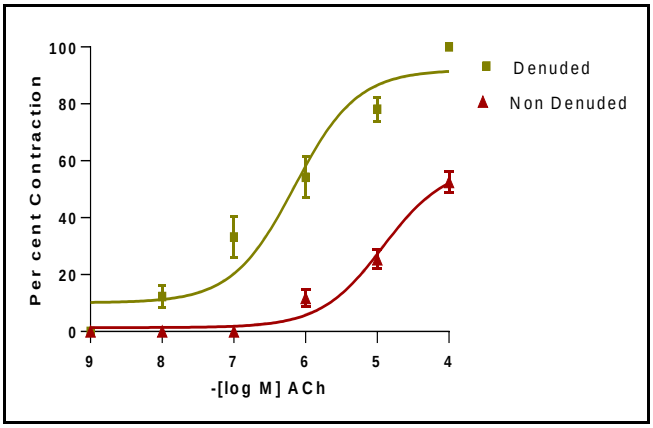


Fig 1: ACh induced concentration-related contractile response Non Denuded and Denuded Detrusor muscle.

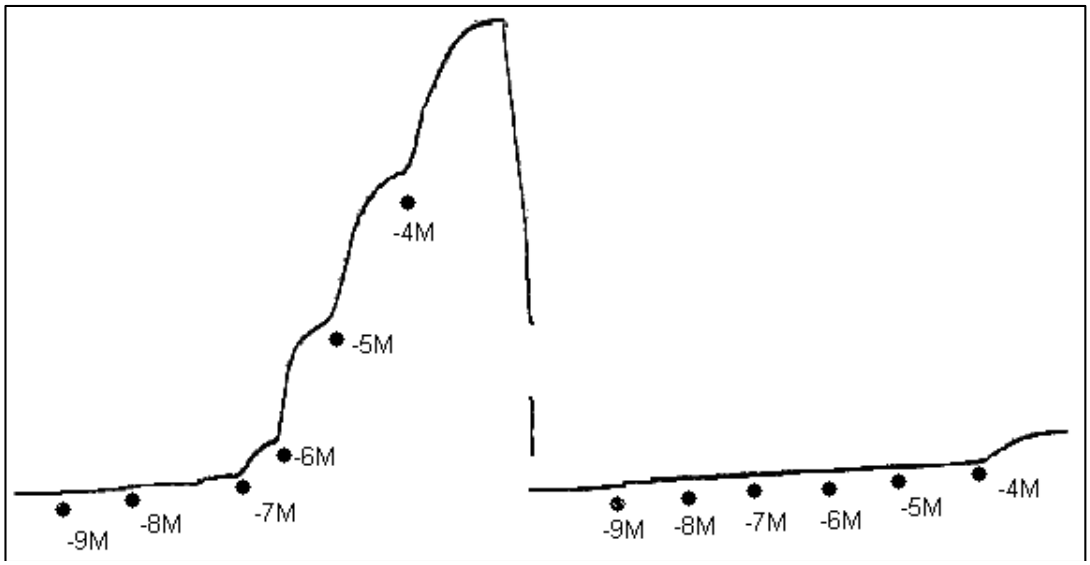
Table 1: ACh-induced dose-related contractile response in Non Denuded Detrusor Muscle *vs* Denuded Detrusor Muscle.

Exp	Response in percent	
	Non Denuded Detrusor Muscle	Denuded Detrusor Muscle
Ach 10^{-9} M	0	4.0±0.9
Ach 10^{-8} M	0	15.7±1.8
Ach 10^{-7} M	0	32.3±6.9
Ach 10^{-6} M	11.8±2.9	54.2±7.2
Ach 10^{-5} M	25.3±3.4	78.2±4.2
Ach 10^{-4} M	52.5±3.7	100

(Non Denuded Detrusor Muscle = 7 Nos.; Denuded Detrusor Muscle = 6 Nos.)

Antagonist effect of Atropine on ACh induced concentration-related contractile responses

Acetylcholine (ACh) administered cumulatively at concentrations ranging from 10^{-9} M to 10^{-4} M induced a concentration-dependent contractile response in goat detrusor muscle. However, in the presence of atropine (10^{-6} M), ACh-induced contractile responses were significantly inhibited (Trace 2). Atropine (10^{-6} M) nearly abolished the contractile response, with an E_{Bmax} of 12.8±3.9% (n = 6) (Table 2). Atropine caused a significant ($p<0.001$) leftward shift in the ACh-induced concentration-response curve, increasing the mean EC_{90} value to 2.6×10^{-4} M compared to the control response (2.5×10^{-6} M). All mean values were compared with 95% confidence limits. Furthermore, in the presence of the urothelium, when tissue strips were pre-incubated with atropine (10^{-6} M), ACh-mediated contractile responses were completely abolished, and the E_{Bmax} value could not be determined. To confirm the cholinergic-mediated contractile response, the strips were thoroughly washed and subjected to isometric contraction studies. The results demonstrated a 100-fold increase in the mean EC_{50} value of ACh in denuded tissue compared to non-denuded tissue.



Trace 2: Impact of atropine (10^{-6} M) on ACh induced concentration-related contractile response in Denuded Detrusor muscle.

Table 2: Antagonist effect of atropine (10^{-6} M) on ACh-induced dose-related contractile response in goat urinary bladder.

Exp	Response in percent	
	Control	Atropine 10^{-6} M
Ach 10^{-9} M	2.9±1.5	0±0
Ach 10^{-8} M	6.5±2.4	0±0
Ach 10^{-7} M	15.4±3.0	1.3±0.8
Ach 10^{-6} M	41.5±3.0	2.8±1.1
Ach 10^{-5} M	73.7±1.7	3.7±2.0
Ach 10^{-4} M	100	12.8±3.1

(Control = 20 Nos.; Atropine = 6 Nos.)

Discussion

Bladder urothelial cells have been demonstrated to function as "sensor-transducers," capable of both releasing and responding to various signaling molecules (Birder and Andersson, 2013) [6]. These cells actively detect chemical, mechanical, and thermal stimuli, generating appropriate cellular responses (Chai *et al*., 2016) [42]. Urothelial cells release a range of signaling molecules, including ATP, acetylcholine, substance P, nerve growth factor, nitric oxide, antiproliferative factor, and cytokines (Sui *et al*., 2014; de Groat, 2013) [7, 16]. Additionally, urothelial cells express multiple classes of transducer receptors, including cholinergic (nicotinic and muscarinic), adrenergic (α and β), transient receptor potential channels (TRPV1, TRPV2, TRPV4, TRPM8), purinergic (P2X, P2Y), adenosine (A2a, A2b, and A3), and estrogen (ERα, ERβ) receptors (de Groat, 2013; Birder *et al*., 2002) [16, 9]. Among the mechanosensitive proteins expressed by urothelial cells are amiloride-sensitive ion channels (Du *et al*., 2007) [17] and Piezo1, which has been implicated in stretch-evoked Ca^{2+} influx and ATP release in urothelial cell cultures (Miyamoto *et al*., 2014) [26]. These mechanosensitive receptors respond to mechanical stretch by increasing intracellular calcium levels, leading to the subsequent release of ATP. The ATP released by urothelial cells can, in turn, signal neighboring urothelial cells, contributing to bladder sensory and functional regulation (Sun and Chai, 2006) [42].

Involvement of Urothelium in Caprine Bladder Smooth Muscle Contractility

In the present study, we examined the role of the urothelium in modulating cholinergic-mediated contractile responses in caprine bladder smooth muscle. Our findings indicate that the presence of the urothelium significantly attenuates cholinergic-mediated contractions compared to bladder smooth muscle devoid of urothelium. This attenuation may be attributed to the release of relaxing molecules from the urothelium upon acetylcholine stimulation.

These findings align with previous studies demonstrating the inhibitory influence of urothelial-derived factors on bladder contractility. For instance, adenosine A1 receptors are known to be expressed in the uroepithelium of the bladder (Yu *et al*., 2006) [12], and adenosine-induced relaxation has been primarily attributed to P1A1 receptor activation in normal rat bladders (Vesela *et al*., 2011) [44]. Additionally, the impact of the mucosa on bladder strip contractility has been reported in various species. Maggi *et al*. (1987) [24] showed that guinea pig bladder strips lacking the mucosa exhibited enhanced contractility in response to substance P compared to full-thickness bladder strips, while responses to electrical field stimulation (EFS), histamine, or KCl remained unaffected by the presence or absence of the mucosa. Similarly, Pinna *et al*. (1992) [31] reported that bradykinin-induced contractions were significantly reduced in rat bladder strips when the mucosa was intact.

Further supporting these observations, Levin *et al*. (1995) [23] found that the feline bladder mucosa exerted an inhibitory effect on detrusor smooth muscle contractions. Likewise, studies in porcine bladder tissue suggested that the mucosa contributes to smooth muscle relaxation, hypothesizing that this effect originates from the urothelium or potentially other cellular components within the mucosal layer (Hawthorn *et al*., 2000) [19]. Our findings reinforce the concept that the urothelium plays a critical role in modulating bladder smooth muscle function by releasing bioactive factors that influence detrusor contractility.

Understanding this urothelial contribution could provide valuable insights into bladder physiology and potential therapeutic targets for conditions such as overactive bladder and urinary incontinence.

The Role of the Detrusor Muscle and Muscarinic Receptors in Bladder Function

Beyond the urothelium, the bladder wall primarily consists of the detrusor muscle, which plays a crucial role in bladder function. The ability of the detrusor muscle to contract over

a wide range of lengths and to relax appropriately is essential for the bladder's filling and micturition processes. These functions are regulated by intricate neural and hormonal control systems, and the detrusor muscle contains multiple receptor types and signaling pathways that mediate its activity.

Acetylcholine is the principal neurotransmitter governing bladder voiding, primarily acting through muscarinic receptors on smooth muscle cells (Wein, 2001) [47]. The distribution of muscarinic receptors within the bladder is not uniform; receptor density and sensitivity to muscarinic stimulation are highest in the bladder dome and lowest in the base. This gradient ensures efficient bladder emptying by optimizing contractile responses in different bladder regions.

Pharmacological, biochemical, and genetic studies have demonstrated that muscarinic receptors in the urinary bladder are heterogeneous in nature. At least three pharmacologically distinct muscarinic receptor subtypes—M1, M2, and M3—have been identified in the bladder (Akici *et al*., 2000) [1]. These subtypes play distinct roles in modulating detrusor contractility, with M3 receptors primarily mediating contraction, while M2 receptors contribute to contractile responses under certain physiological and pathological conditions. Understanding the distribution and function of muscarinic receptors in the bladder is essential for developing targeted pharmacotherapies for disorders such as overactive bladder and urinary incontinence.

Acetylcholine-Mediated Contractile Response and Atropine Sensitivity in Caprine Bladder Smooth Muscle

Our study demonstrated that acetylcholine (ACh) induces a concentration-dependent contractile response in caprine detrusor muscle strips that lack the urothelium. These findings align with previous studies, where cholinergic muscarinic receptor agonists and electrical stimulation of intrinsic cholinergic nerves induced contractions in detrusor strips from healthy human bladders. Furthermore, contractile responses to muscarinic stimuli can be completely abolished by atropine in humans (Kinder and Mundy, 1985) [21], primates (Craggs *et al*., 1986) [14], pigs, and rabbits (Sibley, 1984) [37].

Consistent with these observations, we found that the acetylcholine-mediated contractile response in caprine detrusor muscle was significantly attenuated in the presence of atropine. This confirms that the contractile response is mediated primarily via atropine-sensitive cholinergic mechanisms. Previous research has identified muscarinic receptor subtypes (M1, M2, and M3) in the human bladder, with M3 receptors predominating in detrusor muscle. In the sheep ureterovesical junction, contractions are mediated through M1, M2, and M3 receptor subtypes (Rivera *et al*., 1992) [32]. Notably, co-activation of M2 receptors may enhance M3-mediated responses by inhibiting adenylate cyclase, suppressing sympathetically mediated detrusor relaxation, inactivating K+ channels, or activating nonspecific cation channels (Sellers and Chess-Williams, 2012) [36].

Stimulation of M3 receptors by ACh leads to phosphoinositol hydrolysis, which in turn releases intracellular Ca^{2+} and facilitates Ca^{2+} influx through L-type calcium channels. This process enhances contractile sensitivity via myosin light chain phosphatase inhibition through Rho kinase activation (Andersson, 2002) [3].

Potential Non-Cholinergic Mechanisms in Caprine Bladder Contraction

Interestingly, despite atropine significantly reducing contractile responses, minimal ACh-induced contractions were still observed in caprine bladder smooth muscle. This suggests the involvement of a non-cholinergic system, possibly purinergic signaling, in ACh-mediated contractile responses.

Previous studies support this hypothesis. Atropine-resistant contractions have been observed in dysfunctional bladders, and these responses are not due to differential muscle sensitivity to ATP or cholinergic agonists (Bayliss *et al*., 1999) [5]. Additionally, Sjögren *et al*. (1982) [39] reported atropine-resistant contractions in human bladder smooth muscle stimulated transmurally. Several studies propose that these responses originate from non-cholinergic nerves (Henderson & Roepke, 1934; Ambache & Zar, 1970; Dumsday, 1971; Burnstock *et al*., 1972) [20, 2, 18, 10].

Atropine-resistant, nerve-mediated contractions occur in human detrusor muscle primarily from bladders with detrusor overactivity—whether idiopathic, neuropathic, or obstructive (Bayliss *et al*., 1999) [5]—as well as in the detrusor muscle of small animals. These residual contractions can be abolished by pre-treatment with α, β-methylene ATP (ABMA), which desensitizes ionotropic purinergic receptors, suggesting that ATP acts as a co-transmitter alongside acetylcholine. Additionally, ATP release from nerves is independent of acetylcholine release (Pakzad *et al*., 2016) [30], highlighting the potential for therapeutic strategies targeting ATP release to manage overactive bladder conditions.

However, atropine-resistant ACh-mediated contractile responses may occur through multiple mechanisms and vary across species and pathological conditions. While some studies attribute these responses to ATP-mediated purinergic signaling (Sjögren *et al*., 1982) [39], others suggest direct smooth muscle stimulation due to increased excitability of the smooth muscle membrane (Tagliani *et al*., 1997) [43]. Regardless of the underlying cause, alterations in detrusor muscle function in abnormal bladders present potential targets for drug development aimed at modulating bladder contractility.

Future Directions and Study Limitations

Our study suggests that an interaction between purinergic and cholinergic transmitter systems may exist at multiple levels within the micturition reflex arc. Further research is required to fully characterize this functional relationship.

Several limitations should be acknowledged

Sex-Based Differences: Our study was conducted exclusively on male caprine specimens. Male-specific organs, such as the penis and prostate, may influence bladder smooth muscle contraction, and these effects were not accounted for in our study.

Additional Influencing Factors: Various other physiological and pathological factors may contribute to bladder smooth muscle contraction, requiring further investigation.

Species-Specific Variations: While our findings align with data from human and animal studies, species-specific differences in bladder contractility mechanisms warrant further comparative research.

Future experiments should address these limitations by incorporating female specimens, exploring additional molecular pathways, and conducting comparative studies across different species.

Conclusion

Our findings demonstrate that acetylcholine-mediated contractile responses in caprine bladder smooth muscle occur via both atropine-sensitive and atropine-resistant pathways. Additionally, the presence of urothelium significantly modulates cholinergic-mediated contractile responses, likely through the release of relaxing factors.

Moreover, our results align with previous studies on human urinary bladder smooth muscle, suggesting that the caprine bladder may serve as a relevant model for studying bladder physiology. Further investigations into caprine bladder smooth muscle function could enhance our understanding of urinary incontinence mechanisms and contribute to the development of more effective therapeutic strategies for human bladder dysfunction.

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