

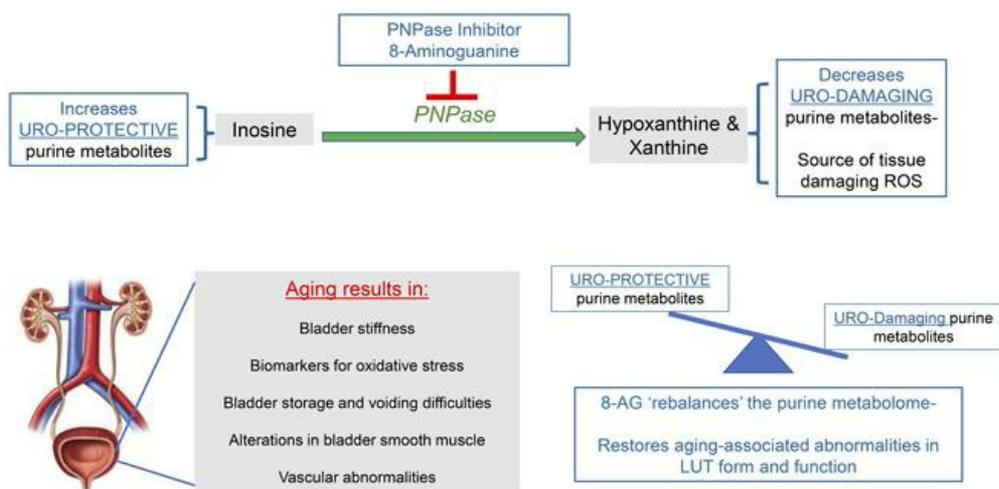
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Purine Nucleoside Phosphorylase Inhibition Ameliorates Age-Associated Lower Urinary Tract Dysfunctions

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ABSTRACT

In the aging population, lower urinary tract (LUT) dysfunction is common and often leads to storage and voiding difficulties classified into overlapping symptom syndromes. Despite prevalence and consequences of these syndromes, LUT disorders continue to be undertreated simply because there are few therapeutic options. LUT function and structure were assessed in aged (>25 months) male and female Fischer 344 rats randomized to oral treatment with a purine nucleoside phosphorylase (PNPase inhibitor) 8-aminoguanine (8-AG) for 6 weeks or vehicle. The bladders of aged rats exhibited multiple abnormalities: tactile insensitivity, vascular remodeling, reduced collagen-fiber tortuosity, increased bladder stiffness, abnormal smooth muscle morphology, swelling of mitochondria and increases in uro-damaging purine metabolites. Treatment of aged rats with 8-AG restored all evaluated histological, ultrastructural and physiological abnormalities toward that of a younger state. 8-AG, is an effective treatment that ameliorates key age-related structural and physiologic bladder abnormalities. Because PNPase inhibition blocks metabolism of inosine to hypoxanthine and guanosine to guanine, likely uro-protective effects of 8-AG are mediated by increased bladder levels of uro-protective inosine and guanosine and reductions in uro-damaging hypoxanthine and xanthine. These findings demonstrate 8-AG has translational potential for treating age-associated LUT dysfunctions and resultant syndromes in humans.

INTRODUCTION

The effects of aging on the lower urinary tract (LUT) are complex. (1-3) Multiple bladder components become dysfunctional with age including the mucosal, muscular, stromal and neural elements. Pathological features arise from: 1) vascular alterations leading to ischemia and associated reperfusion injury); 2) mucosal alterations characterized by increases in mucosal permeability and loss of mucosal cells which disrupts cell-cell and cell-interstitium communications; 3) decreased bladder sensation; and 4) the inability of the urinary bladder musculature to exhibit normal compliance (low pressure) during filling and storage and normal contractility during emptying. These pathological differences interact and converge to produce LUT symptoms and signs including bladder overactivity, urgency, nocturia, impaired bladder contractility and urinary incontinence during filling/storage, a diminished force of urinary stream and increased residual urine during emptying.(4-7) These symptoms are often grouped together into overlapping syndromes, such as overactive bladder (OAB) and underactive bladder (UAB), with each set designated as a particular named “LUT dysfunction” (LUTD) or symptom syndrome. LUTDs are common in the elderly and frail. Indeed, the demographics of LUTDs suggest a steep increase in their occurrence and underlying causative etiologies beginning in the fifth decade in both genders, increasing until death and affecting at least 30% of the post-50-year old population. (5, 6)

Despite the prevalence and impact of LUTDs on quality of life in humans, LUTDs continue to be undertreated simply because there are few successful therapeutic options, especially minimally invasive ones, and no preventative strategies nor known modulating interventions that reverse physiologic differences. Emerging evidence, however, suggests that alterations in purine nucleoside phosphorylase (PNPase) levels reflects the participation of oxidative injury and cellular damage.(8, 9) Unlike other treatments, such as allopurinol, which targets only hypoxanthine metabolism, blocking PNPase can increase levels of ‘uro-protective’ precursors (e.g., inosine and guanosine)(10-12) while simultaneously decreasing levels of ‘uro-toxic’ products (e.g., hypoxanthine and xanthine).(13) Complex diseases require pleiotropic medicines to ameliorate the underlying multiple mechanisms that give rise to the pathophysiology. Inasmuch as reactive oxygen species (ROS) are involved in the pathophysiology of aging-related bladder dysfunction and because ROS are produced by multiple pathways, age-related bladder dysfunction does indeed fall into the category of a complex disease

that will require pleiotropic drugs to manage. Here we report, for the first time, on our recently developed strategy to correct and reverse age-related differences in bladder dysfunction.

RESULTS

Metabolic-cage studies in untreated, aged (>25 months) versus young adult Fischer 344 rats revealed decreases in voiding frequency (Figure 1A) and increases in the inter-void interval (Figure 1B) as well as voided volume (Figure 1C) in aged rats. Also, aged rats demonstrated decreased von Frey sensitivity(14, 15) to tactile (mechanical) stimuli, both abdominal (visceral; Figure 1D) and cutaneous (hindpaw; Figure 1E). The von Frey test is a non-invasive behavioral method which uses a series of calibrated von Frey filaments to assess the sensitivity to mechanical stimuli (i.e., response to pressure stimuli) at various anatomical locations. These differences suggest an age-associated deterioration of tactile sensitivity and are suggested to be indicative of sensory decline. Notably, in aged rats treated for six weeks with oral 8-aminoguanine (8-AG), both voiding behavior and tactile sensitivity were similar to those observed in young rats.

The findings illustrated in Figures 1A-1D are consistent with the clinical syndrome designated as UAB. To further investigate whether the aged Fischer 344 rat is an animal model of UAB and whether 8-AG reverses UAB, we sought to develop a more decisive test to diagnose UAB *in vivo* in rats. It is well-known in cardiac physiology that cardiac output is a direct function of preload, i.e., end diastolic volume; thus, a better *in vivo* estimate of cardiac contractility is afforded by normalizing cardiac output to end diastolic volume. (16) We considered that a similar approach would be useful to assess bladder contractility in rats *in vivo*. Thus, we devised a method to assess bladder activity corrected for bladder “preload”, an approach that yields an experimental bladder contractile index (EBCI). In this regard, for a given voiding event, the cumulative urine volume (ml) was plotted as a function of time (sec). Each plot was fitted to a straight line using non-linear regression analysis (without a priori constraints) in GraphPad Prism. The experimental bladder contractility index (EBCI) was calculated by dividing the best-fit slope provided by the regression analysis by the total voided volume. We observed that for all voiding events, the volume-time relationship was linear (with most R^2 -values between 0.95 and 0.99) from the initiation through the completion of voiding (see Figures 2A, 2B and 2C). Therefore, the slope of the best-fit line was used as the most accurate estimate of urine flow rate during a given voiding event. Although urine flow rate is indicative of bladder emptying efficiency, this parameter would be dependent on the initial volume of urine in the bladder (i.e., bladder preload). This is because bladder stretching activates micturition reflexes which are initiated by local factors released by the bladder cellular elements. To

control for preload, for each voiding event we divided the urine flow rate by total voided urine to give an EBCI. This calculated EBCI was highly reproducible. As shown in Figure 2D, the EBCI in aged rats was significantly lower compared with young rats, a finding consistent with UAB in aged Fischer 344 rats. Importantly, the EBCI in aged rats treated with 8-AG was similar to that of untreated young rats.

Bladders perfused with fluorescent beads showed that young bladders contained mostly straight vessels throughout the tissue (see white arrows in representative image Figure 3A). In contrast, aged bladders (representative image Figure 3B) displayed only tortuous vessels (red arrows) and exhibited areas of decreased perfusion that appeared ischemic (severe tortuosity obstructs blood flow and bead injection shows perfusion).(17) Chronic treatment of aged rats with 8-AG (representative image Figure 3C) reduced the number of tortuous vessels, and the tissue no longer appeared ischemic, resembling young tissue. Prior to imaging, bladders were cleared using the CUBIC method(18) and images acquired by ribbon-scanning confocal microscopy.(19) In addition, as shown in Figure 3D, compared to younger rats, untreated aged rats showed a significant decrease in bladder blood flow (measured using a Doppler flowmeter).(20) In contrast, there was no significant difference in bladder blood flow in young versus 8-AG treated old rats (Figure 3D).

As illustrated in Figure 4A-4D, aging was associated with alterations in the expression of key proteins in the bladder mucosa. For example, there was a trend toward decreased mitofusin 2 (MFN2). MFN2 normally facilitates removal of damaged mitochondria and is important for cellular viability by contributing to the maintenance of the mitochondrial network(21). There was also increased dynamin-related protein 1 (DRP-1). DRP-1 is critical for establishing mitochondrial morphology, and overexpression of DRP-1 has been linked to abnormal mitochondrial dynamics and overproduction of defective mitochondria in mammalian cells(22). In the aged rat, Parkin was overexpressed in the bladder mucosa. Parkin has a multitude of functions including removal of damaged mitochondria under conditions of oxidative stress(23). Finally, in aged rats caspase 3 expression was elevated in the bladder mucosa. Caspase 3 is a protein that promotes apoptosis.(24) With 8-AG treatment, these biochemical abnormalities in the bladder mucosa were restored to levels similar to that of the younger state.

In all bladders, the passive response of bladder wall specimens to mechanical loading under prescribed stretch was measured as a surrogate for the pressure/volume relationship seen during bladder filling(25),

(representative graph from n=12 rats, Figure 5E). In all bladders the mechanical reaction to mild stretch, was a compliant response ('soft phase') in which large changes in the extension of the bladder wall caused little change in stress. Upon further stretch, a noncompliant response ('stiff phase') was reached (Figure 5D), in which small changes in the extension of the bladder wall caused a large change in stress. The stretch at which this steep increase in stiffness occurred (the critical stretch, indicated by an asterisk in Figure 5D) depended on the tortuosity of the collagen fibers in the detrusor layer. In this regard, the tortuosity enabled bladder filling at low mechanical loads (or pressure) because fibers contributed little to load bearing until they were straightened ('recruited'). As increasing numbers of fibers were recruited, the bladder rapidly stiffened. For the aged bladder, collagen fibers in the detrusor layer were recruited early (Figure 5B) relative to the young bladder at the same stretch, (Figure 5A). This early recruitment was caused by the diminished collagen-fiber tortuosity in the aged bladder and resulted in a leftward shift of the bladder's stress-stretch relationship (Figure 5E), manifested as a substantial reduction in the stretch at which the stiff phase for bladder occurred in aged relative to young rats (Figures 5E). Notably, after 8-AG treatment, the aged bladder regained the collagen-fiber tortuosity in the detrusor layer (Figure 5C) and this manifested as recovery of the prolonged soft or compliant regime (i.e., rightward shift of the stress-stretch relationship) seen in young bladders (Figure 5E). Correspondingly, the critical stretch for the treated aged bladder recovered, approaching that of the young bladder (Figure 5F), also seen as a rightward shift of the stress-stretch relationship (Figure 5E).

As a result of the premature stiffening in the detrusor, the untreated aged bladder never expanded sufficiently to effectively recruit collagen fibers in the lamina propria layer, even at physiological levels of bladder stretch (Figure 6B). In contrast, the collagen fibers within the lamina propria layers of both the young and treated aged bladders were able to contribute to load bearing at physiological loading levels (Figures 6A, 6C). Taken together, these data support the conclusion that treatment of older rats with 8-AG, restored collagen fiber tortuosity in the detrusor layer (Figure 5C), corresponding to a larger fraction of highly tortuous fibers which would allow increased filling at a lower pressure. This resulted in a more extensible (i.e., compliant) bladder closer to a normal state. In addition, bladders from aged rats also exhibited a significant increase in bladder wall thickness (Figure 6D) which was restored to that of a younger state by 8-AG treatment.

Aging was also associated with significant morphological differences in the urinary bladder smooth muscle showing a separation and degeneration of smooth muscle cells (compare Figure 7A to Figure 7B). Further, this structural pathology was accompanied by swelling and disruption of the smooth muscle mitochondria (compare Figure 7D to Figure 7E). With 8-AG treatment, smooth muscle structural anomalies (Figure 7C), including mitochondrial alterations (Figure 7F), were reversed.

In addition, aging was associated with a trend toward decreased expression of the smooth muscle marker α -smooth muscle actin (Figure 8D) and significant increases within detrusor smooth muscle in both the cellular senescence marker p16 (Figure 8A) and in catalase activity (Figure 8B), which plays a role in defenses against ROS in aging.(26) We also observed a significant increase with aging in both cleaved caspase 3 (Figure 8C) and cleaved PARP (Figure 8E)(27), both of which are involved in senescence and programmed cell death. Moreover, 8-AG restored the expression of the senescence marker p16 (Figure 8A), catalase activity (Figure 8B), cleaved caspase 3 (Figure 8C), and cleaved PARP (Figure 8E) to levels similar to the younger state.

PNPase transforms inosine and guanosine to their respective bases (i.e., inosine into hypoxanthine and guanosine into guanine). (8, 9, 28) In untreated aged rats, urinary levels of endogenous 8-AG were undetectable, but were restored to younger levels following oral 8-AG treatment (Figure 9A). Further, in untreated aged rats, urinary hypoxanthine levels were higher compared with young rats, and oral 8-AG treatment reduced urinary hypoxanthine to levels comparable to younger rats (Figure 9B). Aged rats exhibit a trend toward decreased urinary levels of guanosine (protective role in age-related diseases)(29), yet urinary guanosine levels were similar in 8-AG-treated aged rats compared with young rats (Figure 9C).

DISCUSSION

Here, we report results comparing urinary bladder form and function in young versus old versus 8-AG-treated old rats. Our findings demonstrate that untreated older rats, compared to young rats, exhibit: 1) bladder filling/storage and emptying dysfunction; 2) decreases in tactile sensitivity to mechanical stimuli; 3) abnormal bladder vascular remodeling and reduced bladder blood flow; 4) abnormalities in smooth muscle morphology and mitochondrial structure; 5) reduced collagen fiber tortuosity; 6) decreased bladder compliance; 7) increased urinary levels of 'uro-damaging' hypoxanthine; and 8) decreased urinary levels of 8-AG. Importantly, all LUT outcome measures were similar in young rats versus older rats treated with 8-AG, an endogenous and potent inhibitor of PNPase. These findings demonstrate that the PNPase inhibitor, 8-AG, has strong translational potential for treatment of age-associated LUT dysfunctions in humans.

PNPase, not to be confused with PNPT1, is an enzyme that is expressed in most tissues.(28) This enzyme belongs to the family of glycosyltransferases, is expressed in both bacteria and mammals and is one of the key enzymes involved in the purine salvage pathway.(30, 31) PNPase transforms inosine and guanosine to their respective bases (i.e., inosine into hypoxanthine and guanosine into guanine).(8, 9, 28) Patients with a complete lack of PNPase activity have a decreased T-cell function resulting in a disorder of the immune system termed immunodeficiency.(32) Thus, this enzyme is involved in cell metabolism of nucleosides and nucleotides and helps maintain immune function.

Although PNPase is a critically important enzyme in the purine salvage pathway, there are reasons to hypothesize that partially inhibiting PNPase might protect the LUT from injury. In this regard, both inosine and guanosine exert beneficial anti-inflammatory and tissue-protective effects in various target organ systems including the LUT. For example, administration of exogenous inosine exerts protective effects on the urinary bladder following experimentally induced obstruction or spinal cord injury.(10, 11) The mechanism of action may involve stimulation of adenosine receptors and/or prevention of oxidative damage via scavenging of free radicals and peroxynitrite. Guanosine is also tissue-protective in a number of animal models of injury or degenerative diseases. In contrast to guanosine and inosine, elevated levels of inosine's downstream metabolite *hypoxanthine* over time may exhibit harmful effects due to *production of ROS* when metabolized by xanthine oxidase to xanthine. The increased metabolism of hypoxanthine is linked to inflammatory and other disorders including

ischemia.(13) For example, a shift in purine catabolism with enhanced accumulation of (potentially injurious) hypoxanthine may play a role in declining myocardial tolerance to ischemia with aging.(33) Increased oxidative damage by ROS is deleterious to cells and plays a key role in progression of a number of diseases. Because hypoxanthine is a potential free radical generator, hypoxanthine has also been used as an indicator of hypoxic conditions. Not surprisingly, treatments that inhibit oxidation of hypoxanthine suppress inflammatory cytokines and oxidative stress in a number of disorders. Because PNPase inhibition blocks the metabolism of inosine to hypoxanthine and guanosine to guanine, likely the uro-protective effects of PNPase inhibitors in general, and 8-aminoguanine in particular, are mediated by increases in bladder levels of inosine and guanosine (uro-protective purines) and reductions in bladder levels of hypoxanthine (uro-damaging purine and ROS generator). However, it should be noted that in addition to hypoxanthine-associated ROS expression, 8-AG may exert beneficial effect on bladder function by multiple pathways including those that impact immune function and inflammation. Unlike other treatments such as allopurinol (which only targets hypoxanthine metabolism), blocking PNPase should increase 'uro-protective' precursors (inosine and guanosine) while simultaneously decreasing levels of 'uro-toxic' hypoxanthine.

Despite the ability of PNPase inhibitors to increase inosine and guanosine while decreasing hypoxanthine, we did not anticipate that 8-AG would have such wide-ranging beneficial effects on molecular, cellular and functional abnormalities in the bladder. Inosine, guanosine and hypoxanthine have not been previously shown to affect microvascular architecture, reorganize collagen fibers or reverse mitochondrial abnormalities. Also, the wide-ranging effects of 8-AG in aging are surprising. Our studies were performed in rats near the end of their life span and who had already developed severe bladder pathologies that were unlikely to be reversed by any treatment. Yet 8-AG treatment for only 6 weeks completely or partially reversed all of the measured molecular, cellular and functional bladder abnormalities associated with aging. This is indeed a novel and important finding. The efficacy of 8-AG to reverse age-related decrements in bladder form and function may be due to pleiotropic effects associated with blocking PNPase.

Although 8-aminoguanine was quite effective with regard to reversing age-related bladder pathologies, one must be cognizant of potential toxicities associated with this strategy. PNPase deficiency, inherited as an autosomal recessive disorder, leads to build up of deoxy-guanosine triphosphate which is toxic to dividing cells.

However, even though PNPase is ubiquitously expressed, the deficiency of this enzyme specifically affects T-cells which predisposes patients to repeated and persistent infection.(34) In addition, infants with PNPase deficiency have been shown to grow more slowly as compared to healthy infants.(35) Thus, given this background one would expect that inhibiting PNPase would be too toxic to treat bladder disorders. However, studies show that partial inhibition of PNPase is safe. For example, heterozygous parents with only one normal PNPase gene have $1/4^{\text{th}}$ to $1/2^{\text{th}}$ the normal activity of PNPase yet are not affected by the deficiency. Moreover, nearly complete inhibition of PNPase with forodesine is necessary to suppress the purine salvage pathway.(36, 37)

The LUT is particularly susceptible to the negative effects of age (and intercurrent morbidities) with increased prevalence of storage and voiding symptoms with aging.(1-3, 5, 6) Thus, it was imperative that we evaluate bladder function in vivo. In this regard, a recent study compared a number of functional LUT measurements in rats assessed by metabolism-cage experiments versus urodynamic measurements; the results were indistinguishable.(38) The main advantage of this approach was that key variables could be assessed in conscious rats. Another advantage was that measurements could be performed without causing cellular damage (caused by catheterization) so that subsequent ultrastructural, biochemical and molecular studies could be reliably performed.

Our findings that both bladder filling/storage and emptying are significantly impaired with age is consistent with clinical reports of underactive bladder in older patients.(2, 39-41) In addition, our findings of an increase in inter-void interval as well as voided volume are in line with findings using aged rodents.(42) This suggests that aging may be in part associated with a change in the structure or function of smooth muscle, and/or a decrease in sensitivity to differences in bladder volume. We compared bladder structure and function in young adult versus aged rats. Since studies were not conducted on animals with a range of ages, in the present study differences with respect to bladder function and cellular state between young adult and aged rats could have been due to either aging per se or, alternatively, to maturation processes. Indeed, it would be highly informative to evaluate bladder function and structure throughout the lifespan of Fischer 344 rats.

In the present study, we captured in vivo voiding events in conscious rats with second-by-second time resolution. This acquisition of fine-granular data allowed us to explore in detail the relationship between voided

volume and time in conscious rats. We were impressed by the remarkably linear relationship between cumulative voided volume and time and exploited this to develop a novel approach for assessing voiding efficiency, i.e. the experimental bladder contractility index (EBCI). The EBCI provides an index that reflects bladder voiding efficiency (as measured by the slope of the volume-time relationship assessed by regression analysis) normalized to bladder preload (as estimated by the total void volume). Since the vigor of bladder emptying is driven by volume (stretch)-sensitive neural reflexes and release of autocrine/paracrine factors, the EBCI provides an estimate of bladder efficiency that can be compared among rats with different initial bladder preloads. This methodology provided supporting evidence that in conscious, aged rats voiding efficiency is indeed reduced, a finding consistent with clinical observations in older patients, and that 8-AG corrects age-related reductions in voiding efficiency.

While data on aging and bladder filling sensation is limited, studies suggest that alterations in both bladder afferent structure and function with advanced age can impair urinary bladder storage and voiding. Age-associated differences in sensation may be due to anatomical and functional alterations in peripheral nerves in addition to decreased blood flow to nerve endings and other risk factors (such as nerve damage from chronic diseases such as diabetes).(43) In general, the elderly are less sensitive to mechanical stimuli and exhibit reduced ability to detect vibration, touch and pressure. (44-46) Our findings of age-associated deficits in both abdominal (visceral) and tactile (cutaneous) sensory functions support this view. While the underlying mechanism for these differences may involve a number of factors, studies in animals have shown that increased oxidative stress (47) may be a contributing factor to aging of the peripheral nervous system.(48) Our findings of a reversal of tactile and abdominal sensory function with 8-AG (which decreases damaging purine metabolites such as hypoxanthine a source of ROS) supports a role for oxidative damage.

Older patients exhibit a number of alterations with age such as decreased smooth muscle cell function and increased fibrosis or stiffening of the bladder (decreased compliance) that can lead to an increase in urinary urgency and/or inability to empty the bladder well. For example, an ultrastructure study focusing on the bladder detrusor by Elbadawi et al showed that patients with geriatric voiding dysfunction (e.g., impaired contractility) exhibited distinctive smooth muscle cell atrophy and degeneration.(49) Consistent with findings in older adults, we show that aged rat detrusor smooth muscle exhibit structural alterations including smooth muscle

degeneration, swelling and disruption of the mitochondria and abnormalities of a number of key proteins associated with mitochondrial quality control. At the cellular level, mitochondria are considered the powerhouse of organelles, generating 95% of all cellular energy and are major players in energy production, intracellular communication and are associated with a number of age-related diseases.(47, 50-52) Mitochondrial decline is known to be one of the key hallmarks of aging and age-related disorders. In this regard, mitochondrial dysfunction has been shown to be associated with increased oxidative damage and differences in mitochondrial morphology and function.(53, 54) In further support of the critical role that mitochondria play in aging are studies that show that increasing mitochondrial function or decreasing mitochondrial ROS production extends the health span in multiple species.

Aging-related alterations in the extracellular matrix (ECM) may also impact the function of various cell types in the bladder wall. Despite having different etiologies, most chronic fibrotic disorders are associated with a persistent production of similar factors including ROS that stimulate excessive tissue remodeling (e.g., ECM production), which progressively destroys the organ's architecture and in turn, its function.(55, 56) As the bladder fills, the coordinated recruitment of collagen fibers across both the smooth muscle and lamina propria layers, essential for the elasticity of the bladder wall, is lost during aging. Further, this impacts the ability of the urothelium to sense changes in mechanical deformation occurring during a micturition cycle and release mediators that may influence sensation. Both collagen and elastin networks compose the majority of the ECM of most organs, including the skin and also provide structural support, and there is limited evidence that increased collagen fiber accumulation may occur in bladders of older patients. (49, 57) However, our study focused on mechanisms underlying how aging can impact collagen architecture, which in turn influences functional properties of the bladder. Not only is the amount of these fibers important for proper bladder function, but also their orientation, conformation and recruitment during bladder filling. For example, collagen type III fibers (found in the walls of distensible organs and blood vessels) display specific orientations depending on bladder volume as well as the specific location of the fibers within the bladder wall.(58) When the bladder is quiescent and empty, fibers appear as loose (wavy) networks with random orientation. During bladder expansion, fibers straighten in the direction of the applied force and appear long and thin lying parallel to the urothelium and the smooth muscle. This

arrangement likely allows maximal bladder storage without imposing stress on the bladder wall, thus assuring adequate bladder compliance and minimizing bladder injury.

Our findings reveal that aging leads to decreased recruitment of collagen fibers during bladder stretch which is likely to correlate with increased bladder stiffness and these differences are 'restored' to that of a younger state by 8-AG suggesting a link between oxidative stress and changes in ECM stiffness. In aging bladders, changes in the smooth muscle/collagen ratio and possibly ECM composition likely impair the orientation, conformation and even recruitment of collagen fibers. Our group has previously shown, using a combination of biaxial stretch and multiphoton imaging, that a coordinated recruitment of collagen across the lamina propria and detrusor layers is essential for normal elasticity of the bladder wall. (25) As in our prior work, we found that wall compliance can be lost in the aging bladder by premature recruitment of collagen fibers. These fibers effectively arrest further expansion of the bladder. This suggests an altered tension/stretch may be imposed on the urothelial cells and/or smooth muscle as the bladder fills. This modified mechanical stress on the urothelium and smooth muscle likely alters 'mechanical sensing' and impairs paracrine communication within the bladder wall and thus may contribute to bladder symptoms reported in aged adults.

Elevated levels of hypoxanthine may exhibit harmful effects to the LUT due to the production of ROS when hypoxanthine is metabolized by xanthine oxidase to xanthine and then to uric acid. It is known that hypoxanthine, via transporters ENT1 and 2, (59, 60) is efficiently transported across cell membranes thus increased urinary hypoxanthine can gain access to underlying tissues leading to cellular and organ damage. Our findings reveal that urinary hypoxanthine levels are elevated in aged rat bladder as well as in urines from a limited number of patients with LUTDs as compared to controls without underlying urologic disorders (unpublished observations). Thus, though ROS can have physiologic roles, sustained ROS levels (for example in aging and age-related disorders) is likely to result in tissue injury due to oxidative damage and mitochondrial dysfunction- ultimately resulting in diminished sensation, cellular loss/damage and excessive deposition of collagen fibers/loss of elasticity.

Symptoms common in this group of older patients include poor bladder contraction during emptying with decreased stream and residual urine (underactive bladder or UAB), overactive bladder during filling/storage (often coexisting with UAB during emptying), and varying types of urinary incontinence or loss of bladder control

(stress, urgency, mixed or spontaneous incontinence), arising from a combination of bladder and urethral dysfunction. The demographics of these disorders suggest a steep increase in occurrence of these symptoms and their underlying causative etiologies beginning in the fifth decade in both genders, increasing until death, and effecting at least 30% in aggregate of the post-50-year-old population. While patients and health care providers may consider these conditions a normal part of aging, clearly LUTDs are not normal and impair quality of life and burden healthcare resources.(61)

Treatments for LUTDs include behavioral therapy and pelvic floor exercises, anti-muscarinics, α -adrenoceptor agonists, electrical stimulation and interventional therapies (e.g., periurethral bulking agents, suburethral support tapes and artificial urinary sphincters for stress urinary incontinence, onabotulinum toxin-A injections into the bladder wall for overactivity).(62-65) However, these treatments, which are largely unsuccessful, are frequently not adequate to control or reverse the symptoms, and many have a high incidence of adverse events. For example, drugs such as the antimuscarinics have been associated with the development of cognitive deficiencies in the elderly, intra-detrusor onabotulinum toxin-A can result in urinary retention and artificial urethral sphincter implantation carries a high incidence of infection or malfunction.(66) Thus, despite the prevalence and consequences of LUTDs, many of these conditions continue to be undertreated. Clearly, treatment of LUT dysfunction is an unmet medical need with limited to no available options. Perhaps, most importantly, no current nor historic therapies are known to prevent the advent of the above noted histologic or physiologic abnormalities, so the promise of an effective treatment that would avert the consequences of age and injury related bladder dysfunction is extremely enticing.

CONCLUSIONS

It is clear from the above discussion that: 1) age-related LUTDs are prevalent and, given the aging of a substantial segment of every society are increasingly so; and 2) there is an unmet need for effective and safe treatments for LUTDs. While efficacy in comparison to other purine scavengers is not known, our long-term rat studies (unpublished) with 8-AG demonstrated lack of toxicity to the heart, liver, kidney, brain and adrenal gland as assessed by histological examination of these tissues following 40 days of 8-AG treatment. In this context, our previous finding that 8-AG treatment is safe and our current findings that 8-AG ameliorates the molecular,

cellular and functional abnormalities in the aging bladder are particularly significant; perhaps offering hope to millions who suffer from life-altering LUTDS. These findings may indicate broad field differences throughout the urinary tract which could portend a parallel extensive symptoms-based responsiveness in a variety of urinary syndromes that are highly prevalent and have significant impact on quality of life.

MATERIALS AND METHODS

Animals. This study employed male and female young, yet mature (3 mo) and aged (25-30 mo) Fischer 344 rats (Charles River; Wilmington, MA and the NIA rodent colony). Aged rats were treated with oral 8-aminoguanine (8-AG; 5 mg/kg/day for 6 weeks in drinking water, Toronto Research Chemicals, Toronto, Ontario Canada) versus a control (untreated) group. We noted no differences between 8-AG or control rats in either food or water intake, body weight or overall behavior. We also observed no difference between males versus females tested and as we did not observe a robust difference in signal between the two, we combined the data.

Voiding Analysis. Young, aged and 8-AG-treated aged rats were placed in metabolic cages (24 hr; once per week) for the duration of the study and an average of all measurements were obtained for each rat. The light cycle was from 7:00 AM to 7:00 PM, and food and water were provided ad libitum. Voided urine was collected in cups attached to force displacement transducers (Grass Technologies, Warwick, RI) connected to a computer (Windaq data acquisition software DATAQ Instruments Inc., Akron, OH). Data were averaged for 24 h and also analyzed for 12-h periods during the day (7:00 AM—7:00 PM) and night (7:00 PM—7:00 AM). Voiding frequency (voids per hour), inter-void interval, and volume per void were analyzed. Voiding frequency was calculated as the number of voiding events per hour during 24 hr and during the 12-hr day and 12-hr night periods. Volume per void, which defines bladder capacity, was calculated as an average of the voids occurring during these periods. Voiding efficiency was estimated for a given voiding event by plotting the relationship between cumulative void volume (ml) and time (seconds). Each plot was fitted to a straight line using non-linear regression analysis (without a priori constraints) in GraphPad Prism. The experimental bladder contractility index (EBCI) was calculated by dividing the best-fit slope provided by the regression analysis by the total voided volume. Although the total voided volume does not take into account residual urine in the bladder, it does provide a readily captured estimate of the bladder “preload”.

Von Frey Testing: Forty-eight hr after the metabolic cage studies, tactile sensitivity was measured once per week for the duration of the study (an average of all measurements were obtained for each rat) using von Frey nylon filaments (Stoelting Co., Wood Dale, IL, USA) applied to the suprapubic and plantar hindpaw.(14, 15) Von Frey thresholds have been used for pain sensitization as well as age-associated differences in sensitivity to mechanical or pressure stimuli. The withdrawal threshold (using up-down method) was determined, with

positive withdrawal responses defined as sharp retractions, licking/scratching or vocalizations and the threshold was defined as the force (grams) represented by the von Frey filament which elicited the positive response.

Vascular Alterations: Bladders that had been perfused transcardially with PBS buffer containing 20 nm yellow-green fluorescent beads (THERMO FISHER™, F8787) to highlight the vasculature were fixed then cleared by removing lipids and dissolving light-absorbing chromophores within the tissue.(18) Volumetric data were acquired using a Caliber ID RS-G4 ribbon scanning confocal microscope (Caliber ID, Rochester NY), which can acquire large-area images at high (350 nm) resolution using a high NA 1.0, 20x long working distance (8mm) lens (CFI190 20xc GLyc (NIKON™ Inc, Tokyo, Japan)), [uses a high nA (1.0) long working distance (8 mm) with low magnification (20x)]. (19) This affords the advantage of interrogating whole tissues in a three-dimensional environment without the need to section. Real time blood perfusion (1 mm³ tissue) was accomplished using a BLF22D laser Doppler flowmeter (Transonic Systems, Inc) with a surface probe (TypeS-APLPHS) applied to the serosal surface of the bladder (apex and neck) wall using Doppler light shift from moving RBCs to analyze flow by the Bonner algorithm. This method gives robust, non-invasive microvascular flow signals in the bladder wall of anesthetized rats.

Bi-axial Stretch Combined with Multi-Photon (MPM) Imaging: Planar biaxial mechanical testing coupled with multiphoton microscopy was performed on bladder wall specimens to assess bladder mechanical function while simultaneously observing changes in the collagen microstructure.(25) Methods followed those in (25). Briefly, within 2 hours of harvest, intact bladders (n=9 including 3 aged, 3 aged treated and 3 young) were cut open longitudinally and trimmed into 6±1 mm x 6±1 mm square specimens for mechanical testing with sides aligned in the situ longitudinal and circumferential directions. Samples were then positioned in our custom-designed biaxial testing system and mechanically tested. Local stretch was calculated using fiducial markers on the sample, defined as the ratio of the distance between markers in the sample when loaded and unloaded. Stress was calculated based on the load measurement and initial cross-section area perpendicular to the loading direction. During testing, samples were immersed in Hank's buffer salt solution (HBSS) without calcium and with added EDTA (0.5 mM), nifedipine (5µM; Sigma) and thapsigargin (1µM; Tocris Biosciences) to inhibit smooth muscle cell contraction.

A multiphoton microscope (Olympus FV1000 MPE) equipped with a Coherent Chameleon Ti:Sapphire pulsed Laser was used to image the undulated (tortuous or wavy) collagen fibers in the mounted samples without staining or fixation during loading (i.e., stretch) as per our previously published methods. (25) Stacks of 2D planar images were generated by imaging sequentially across the wall thickness. To avoid tissue damage while obtaining a large range of stretch, loading was stopped at the stretch where collagen fibers were visibly straightened (heretofore termed recruited). This was defined as the maximum stretch. Fiber tortuosity was measured by tracing collagen fibers across the 2D slices (Filament function in Imaris, Bitplane, Switzerland). (25, 67) (Fiber arc length (s) and cord length (L) were determined for each fiber tracing and used to calculate the tortuosity. The tortuosity of an undulated or wavy fiber is therefore greater than one and approaches one as the fiber becomes fully straightened.

Transmission electron microscopy (TEM): Urinary bladder sections from each group (young, aged, aged + 8-AG treatment) was fixed in cold 2.5% glutaraldehyde in 0.01 M PBS. The specimens were rinsed in PBS, post-fixed in 1% osmium tetroxide with 1% potassium ferricyanide, rinsed in PBS, dehydrated through a graded series of ethanol and propylene oxide solutions and embedded in Poly/Bed® 812 (Luft formulations). Semi-thin (300 nm) sections were cut on a Leica Reichart Ultracut, stained with 0.5% Toluidine Blue in 1% sodium borate and examined under the light microscope. Ultrathin sections (65 nm) were stained with uranyl acetate and Reynold's lead citrate and examined on a JEOL 1400 transmission electron microscope with a side mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA).

Western immunoblotting: Bladder preparations were homogenized using Lysing Matrix D in a FastPrep 24 instrument (MP Biomedicals, Solon, OH) in HBSS (5 mM KCl, 0.3 mM KH_2PO_4 , 138 mM NaCl, 4 mM NaHCO_3 , 0.3 mM Na_2HCO_3 , 0.3 mM Na_2HPO_4 , 5.6 mM glucose and 10 mM HEPES, pH 7.4) containing complete protease inhibitor cocktail (1 tablet/10 ml, Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, 1:100). After centrifugation (13000 rpm; 15 min at 4°C), the membrane protein fraction was prepared by suspending the membrane pellets in lysis buffer containing 0.3 M NaCl, 50 mM Tris-HCl (pH 7.6) and 0.5% Triton X-100 and the same concentration of protease inhibitors as above. The suspensions were incubated on ice and centrifuged (13000 rpm; 15 min at 4°C). The protein concentrations of the combined supernatants were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). After denaturation (100°C for

5 min) in the presence of Laemmli sample buffer, lysate from each sample was separated on a 4-15% TGX Stain-Free SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA). As a reliable loading control, total protein measurement per sample was determined using Bio-Rad Stain Free SDS-PAGE gel technology. UV-activated protein fluorescence was imaged on a ChemiDoc MP (Bio-Rad). After proteins were transferred to polyvinylidene fluoride membranes, the membranes were incubated in 5% (w/v) dried milk dissolved in TBS-T (20 mM Trizma, 137 mM NaCl, 0.1% Tween-20, pH 7.6), rinsed with TBS-T, and incubated overnight at 4°C with primary antibody (including α -smooth muscle actin (Novex 701457), senescent marker p16 (Abcam AB51243), mitofusin 2 (MFN2, Abcam AB56889), DRP-1 (dynamin-related protein 1, Cell Signaling 8570), Parkin (Cell Signaling 4211), caspase 3 (Cell Signaling, cleaved 9664, total 9665), PARP (Cell Signaling 9542), diluted in TBS-T containing 5% (w/v) milk. After washing in TBS-T, the membranes were incubated with secondary antibody (Sheep anti-mouse HRP; Southern Biotech, Birmingham, AL or Donkey anti-rabbit HRP; Advansta, San Jose, CA) for 1 hour in 5% (w/v) Milk TBS-T, washed, and incubated in WesternBright Quantum (Advansta, Menlo Park, CA) and then imaged on a ChemiDoc MP (BioRad). The volume (intensity) of each protein species was determined and normalized to total protein using Image Lab software (Bio-Rad).

Purine Metabolome Measurement. Urine samples were diluted 1 to 30 with water, and heavy isotope internal standards were added to each sample. Purines were separated by reversed-phase ultra-performance liquid chromatography (Waters UPLC BEH C18 column, 1.7 μ m beads; 2.1 x 150 mm; Milford, MA) and quantified by selected reaction monitoring using a triple quadrupole mass spectrometer (TSQ Quantum-Ultra; ThermoFisher Scientific, San Jose, CA) with a heated electrospray ionization source. The mobile phase was a linear gradient flow rate (300 μ L/min) of 1% acetic acid in water (pH, 3; mobile phase A) and 100% methanol (mobile phase B) and was delivered with a Waters Acquity ultra-performance liquid chromatographic system. The gradient (A/B) settings were: from 0 to 2 minutes, 99.6%/0.4%; from 2 to 3 minutes, to 98.0%/2.0%; from 3 to 4 minutes, to 85.0%/15.0%; from 4 to 6.5 minutes, to 99.6%/0.4%. The instrument parameters were: sample tray temperature, 10°C; column temperature, 50°C; ion spray voltage, 4.0 kilovolts; ion transfer tube temperature, 350°C; source vaporization temperature, 320°C; Q2 CID gas, argon at 1.5 mTorr; sheath gas, nitrogen at 60 psi; auxiliary gas, nitrogen at 35 psi; Q1/Q3 width, 0.7/0.7 units full-width half-maximum; scan width, 0.6 units; scan time, 0.01 seconds. The following transitions (selected reaction monitoring) were obtained: guanosine

(284→152 m/z, RT = 3.10 min); $^{13}\text{C}10^{15}\text{N}_5$ -guanosine (299 → 162 m/z, RT= 3.10 min); hypoxanthine (137 → 119 m/z, RT = 1.86 min); $^{13}\text{C}_5$ -hypoxanthine (142 → 124 m/z, RT = 1.86 min); aminoguanine (167 → 150 m/z, RT = 1.50 min); $^{13}\text{C}_2^{15}\text{N}$ -aminoguanine (170 → 153 m/z, RT = 1.50 min).

Statistics: Data were analyzed in GraphPad Prism 6 (GraphPad, La Jolla, CA) using Student's t-test (1 tailed) and one-way ANOVA followed by appropriate post-hoc tests. $P < 0.05$ was considered significant. Results are expressed as means \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; **** $P < 0.0001$.

Study Approval: The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all procedures. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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Acquiring Data: AWJ, FC, MG-S, AMW, DB-S

Providing Reagents and Developed Methodologies: LAB, SCW, AJK, AMW, DB-S, AMR, DN, AJW, EKJ

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Data Interpretation: LAB, SCW, AMR, AJW, RRD, EKJ

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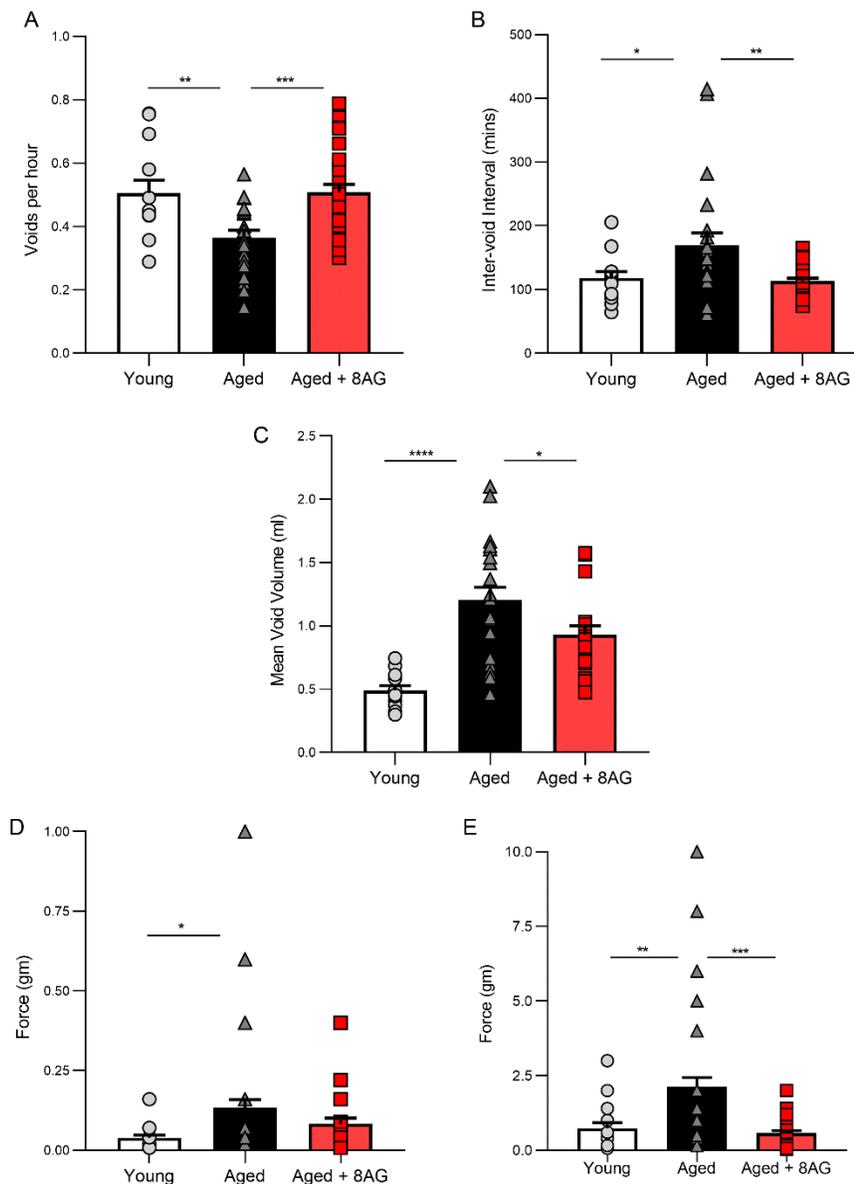


Figure 1A-1E: 8-Aminoguanine (8-AG) attenuates age-related differences in bladder function. 8-AG decreases voiding frequency (Figure 1A; n= Young 13; Aged 21; Aged+8-AG 30) and increases both the inter-void interval (Figure 1B; n= Young 13; Aged 22; Aged+8-AG 30) and voided volume (Figure 1C; n=Young 13; Aged 21; Aged +8-AG 22). In addition, aging decreases abdominal (Figure 1D; n=Young 18; Aged 58; Aged+8-AG 35) and cutaneous (Figure 1E; n=Young 13; Aged 21; Aged+8-AG 23) responses to tactile mechanical stimuli, which is restored to a younger state by 8-aminoguanine (8-AG) treatment. Values represent means and SEMs. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.

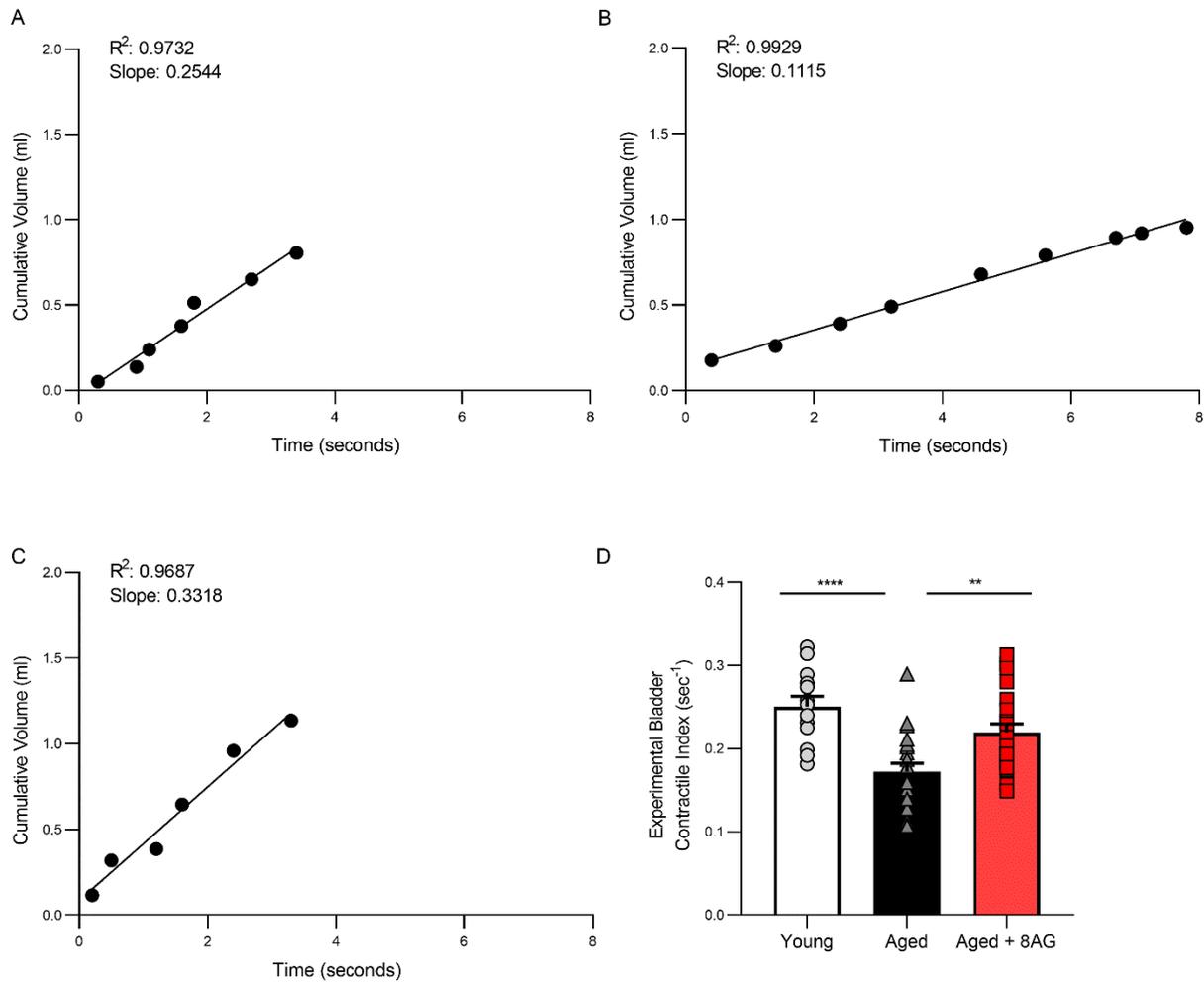


Figure 2A-2D: 8-Aminoguanine (8-AG) attenuates age-related differences in experimental bladder contractile index (EBCI). 8-AG also attenuates age-related decreases in EBCI (Figure 2D; n=Young 13; Aged 21; Aged+8-AG 23). Figure 2A-C are representative examples of volume-time relationship plots for young (Figure 2A), aged (Figure 2B) and aged rats treated with 8-AG (Figure 2C). Values represent means and SEMs. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.

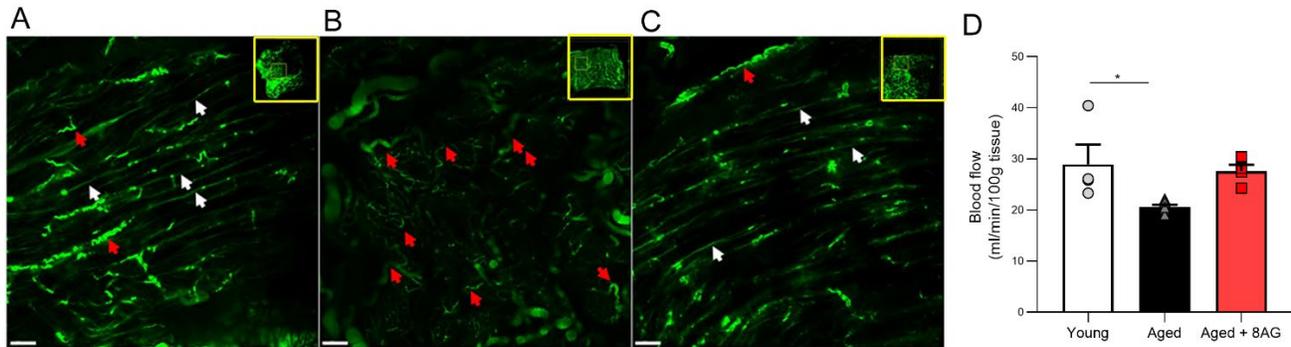


Figure 3A-3D: Ribbon-scanning confocal microscopy coupled with perfusion of the bladder with fluorescent beads shows increased vascular tortuosity with appearance of reduced perfusion in aged bladders. Figure 3B is a representative image (from $n =$ Young 3; Aged 3 and Aged+8-AG 3 rats) showing age-associated differences compared with young bladders (Figure 3A) which demonstrates mostly straight vessels throughout the tissue. The aged bladder treated with 8-aminoguanine (8-AG, Figure 3C) reduces vessel tortuosity and the tissue no longer appears ischemic, resembling young tissue. Sample size $n=9$ rats total. Panel insets display the reconstruction of the complete bladder with voxels of $0.4 \times 0.4 \times 10$ mm. Yellow boxes display the location of the high magnification images. Prior to imaging, bladders were cleared using the CUBIC method and images were acquired by ribbon-scanning confocal microscope. Scale bars are 200 microns. Figure 3D ($n =$ young 4; aged 6; aged+8-AG 4) shows doppler flowmeter measurements revealing a significant decrease in bladder blood flow in aged compared to young rats; blood flow defects in the old bladder are reversed to a younger state 29 with 8-AG treatment. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.

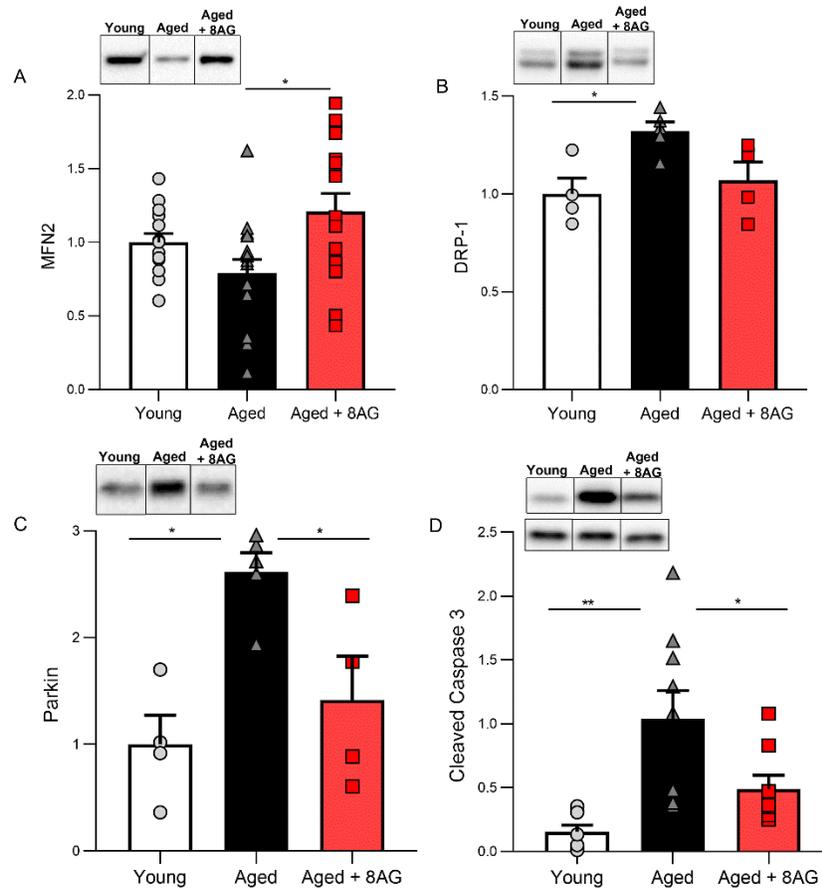


Figure 4A-4D: Western immunoblotting revealed significant aging-associated alterations in proteins linked to mitochondrial dynamics and quality control within the bladder mucosa. These include mitofusin 2 (MFN2, Figure 4A; n= Young 14; Aged 16; Aged+8AG 16), a protein involved in mitochondrial fusion; Dynamin-related protein (DRP-1, Figure 4B; n=Young 4; Aged 5; Aged+8AG 4), which is involved in mitochondrial fission, parkin (Figure 4C; n=Young 4; Aged 5; Aged+8AG 4), which plays a role in mitophagy and cleaved caspase 3 (Figure 4D; n=Young 8; Aged 9; Aged+8AG 8), which is activated upon initiation of apoptosis. In all cases, treatment with 8-aminoguanine (8-AG) restored changes similar to a younger state. Representative immunoblotting is inset within each graph. Cleaved caspase 3 (top of inset) is normalized to total caspase 3 (bottom of inset). All samples were run on the same blot but representative samples were not contiguous. Values represent means and SEMs. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.

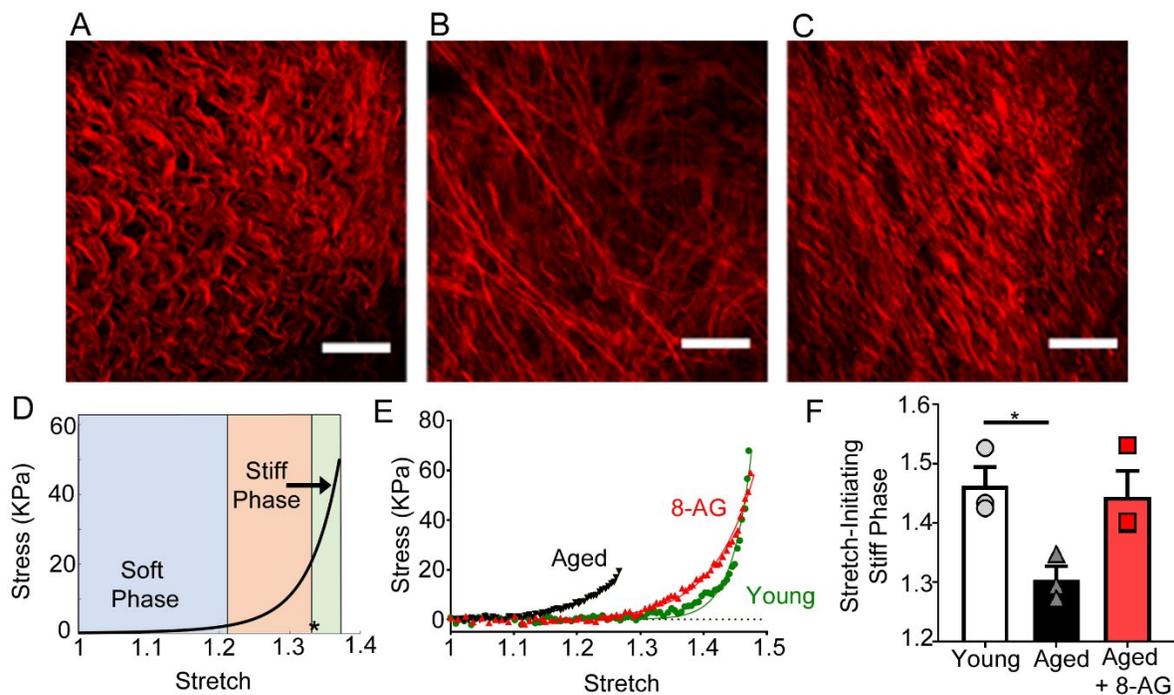


Figure 5A-5E: Concurrent imaging and mechanical testing of bladder collagen fibers in the detrusor layer of young versus aged bladder. The collagen fibers in young bladder are highly tortuous (representative Figure 5A; occurred in 3/3 rats) enabling large stretch with very little change in load (stress). This soft phase of the stress-stretch curve is followed by a transition to a stiff phase at higher stretch, (Figure 5D; asterisk indicates onset of stiff phase or critical stretch). There is a substantial decrease in tortuosity in the detrusor layer of aged bladders (representative Figure 5B, occurred in 3/3 rats) compared to young bladders leading to a premature recruitment of collagen fibers. The early recruitment leads to shortening of the soft phase and early shift to the stiff phase in aged versus young bladders (best fit graph with individual data points plotted Figure 5E; n=3 each for Young, Aged, Aged+8-AG). This trend was reversed after 8-aminoguanine (8-AG) treatment resulting in partial recovery in tortuosity (representative Figure 5C, occurred in 3/3 rats), rightward shift of stress-stretch curve towards the young bladder curve (Figure 5E), and recovery in critical stretch for onset of stiff phase (Figure 5F). Values represent means and SEMs, Scale bars are 100 mm. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.

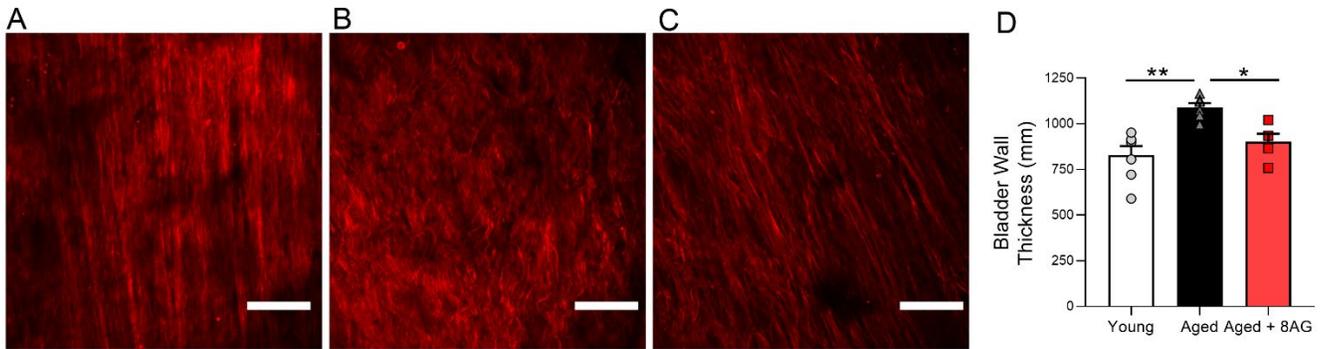


Figure 6A-D: Concurrent multi-photon imaging and biomechanical testing of bladder collagen fibers in lamina propria of young versus aged rat bladder. The collagen fibers in the lamina propria of young bladder are recruited (straightened) under physiological loading levels (representative panel Figure 6A; occurred in 3/3 rats). In contrast, the aged bladder did not expand sufficiently to recruit collagen fibers, even at physiological loads (representative panel Figure 6B; occurred in 3/3 rats). Collagen fibers in the bladders of aged animals treated with 8-AG were able to be recruited (straightened) at physiological loading levels (Figure 6C; occurred in 3/3 rats). Aged bladders also exhibit increased wall thickness as compared to younger rat bladders. Bladder wall thickness in aged rats was restored toward a younger state with 8-AG treatment (Figure 6D; n=Young 4; Aged 4; Aged+8AG 4). Values represent means and SEMs. Scale bars are 100 μ m. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.

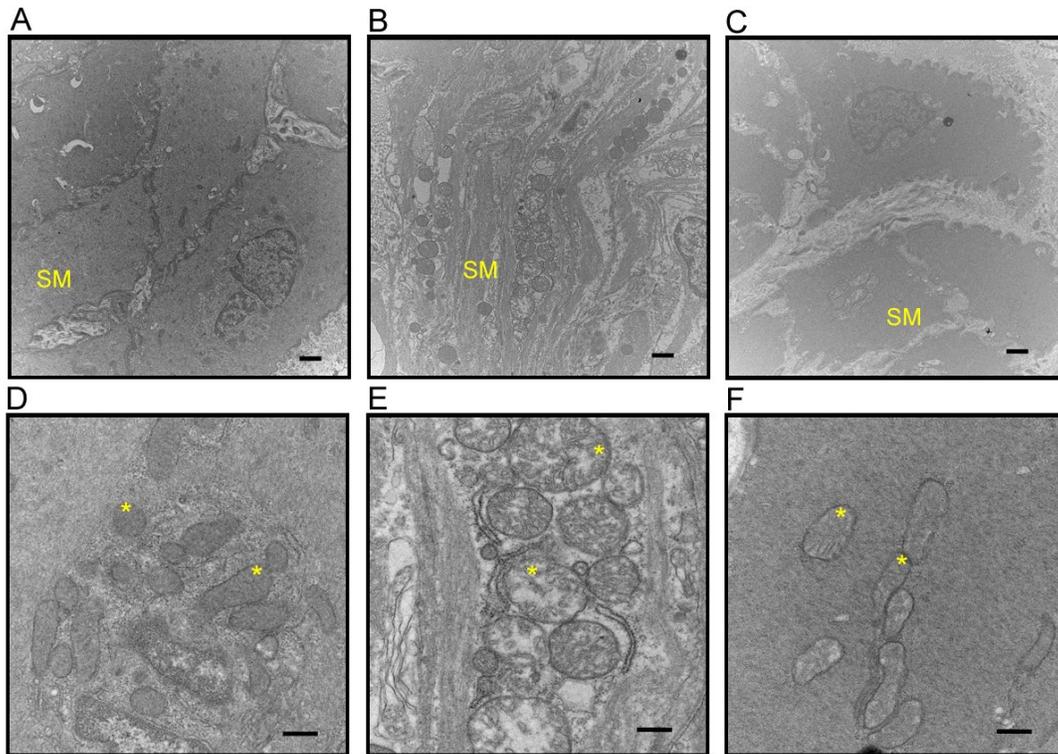


Figure 7A-7F: Representative transmission electron microscopy images of bladder smooth muscle in young rats, aged rats and aged rats ($n=3$ each) treated with 8-aminoguanine (8-AG). These images reveal abnormal detrusor smooth muscle (SM) morphology in aged rats (Figure 7B, which includes separation and degeneration of cells as compared to young rats (Figure 7A; 3/3 rats). However, the abnormal morphology in aged rats is restored to a younger state by 8-AG treatment. Figure 7D-7F depicts higher magnification transmission electron microscopy images revealing significant swelling and disruption of smooth muscle mitochondria (* denote mitochondria) in aged bladders (Figure 7E; 3/3 rats) compared with young bladders (Figure 7D; 3/3 rats); these anomalies were restored to a younger state by 8-AG treatment (Figure 7F; 3/3 rats). Scale bars are 1mm and magnification 15,000 (Panels A-C) and scale bars 600nm and magnification 30,000 (Panels D-F).

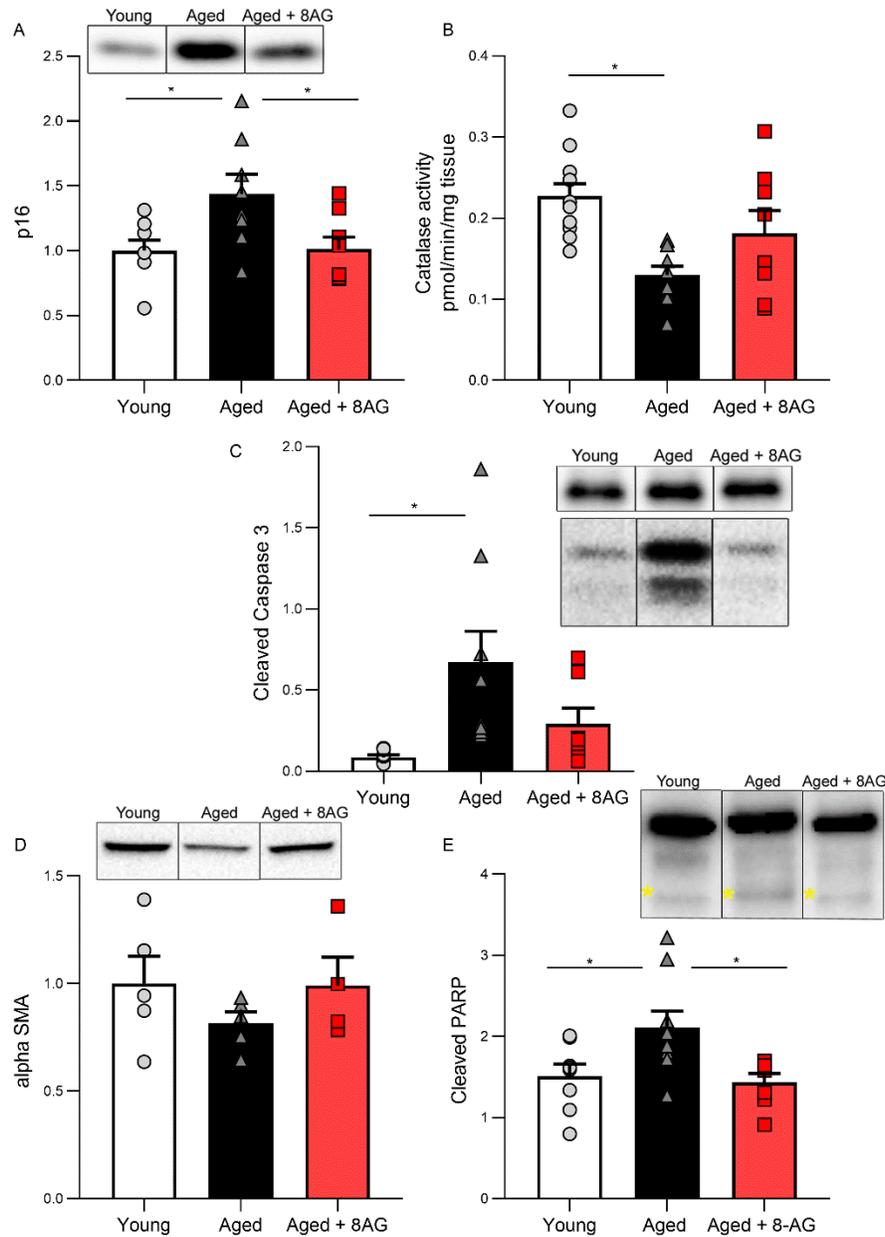


Figure 8A-8E: Aged bladder detrusor exhibited alterations in various biomarkers. These include senescent biomarker p16 (Figure 8A; n=Young 8; Aged 8; Aged+8AG 8), catalase activity (Figure 8B; n=Young 11; Aged 10; Aged+8AG 8) and cleaved caspase 3 (cleavage product shown in lower panel (17-19kDa), compared to uncleaved caspase 3 in upper panel, 35kDa, n=Young 8; Aged 9; Aged+8AG 7). Aged bladder detrusor smooth muscle exhibit alterations in alpha-smooth muscle actin (Figure 8D; n=Young 5; Aged 5; Aged+8AG 4). Significant differences were determined in PARP activation, as indicated by cleavage product at 89kDa (lower band,

31 yellow asterisks), compared to uncleaved PARP (upper band, 116kDa). (Figure 8E; n=Young 8; Aged 9; Aged +8AG 7). 8-AG treated in aged rats restored these biomarkers to that of a younger state. Representative immunoblotting is inset within each graph. All samples were run on the same blot but representative samples were not contiguous. Values represent means and SEMs. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.

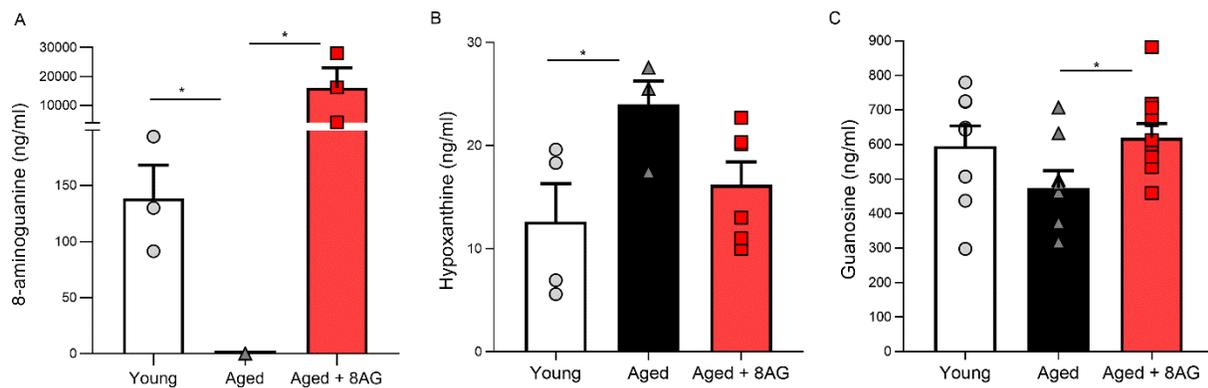


Figure 9A-9C: Purine metabolome measurements in young, aged and aged rats treated with 8-aminoguanine. In aged rats endogenous urinary 8-aminoguanine (8-AG) is below assay detection limits (Figure 9A; n=Young 3; Aged 4; Aged+8AG 3); yet aged rats have higher urinary hypoxanthine levels (Figure 9B; n=Young 4; Aged 4; Aged+8AG 6); both of these abnormalities are restored to younger levels with 8-AG treatment. In addition, guanosine levels (Figure 9C; n=Young 8; Aged 8; Aged+8AG 10) are altered with age and recovered with 8-AG treatment. Ordinary one-way ANOVA was used to evaluate significance which was considered at $p < 0.05$.