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Aladár Aujeszky Doctoral Program of Theoretical Veterinary Sciences

Biochemical background of Feline Idiopathic Cystitis

Ph.D. thesis

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List of abbreviations

$^1O_2$	singlet oxygen
8-OHdG	8-hydroxy-2-deoxy guanosine
ACKR3	atypical chemokine receptor 3
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUM	asymmetric unit membrane
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCK-8	cell counting kit-8
CRF	corticotropin releasing factor
DAPI	diamidino phenylindole
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetra acetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FBS	foetal bovine serum
FIC	feline idiopathic cystitis
FITC	fluorescein isothiocyanate
FLUTD	feline lower urinary tract dysfunction
GAG	glucosaminoglycan
GRP78	glucose-regulated protein 78
gp130	130 kD membrane glycoprotein
H_2O_2	hydrogen peroxide
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HOCl	hypochlorous acid
HPAA	hypothalamic-pituitary-adrenal axis
IC	interstitial cystitis

IL	interleukin
JAK	Janus kinase
LDH	lactate dehydrogenase
LO•	alkoxyl radical
LOO•	peroxyl radical
LOOH	lipid hydroperoxide
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
MEM	minimal essential medium
MEMO	multimodal environmental modification
M-PER	mammalian protein extraction reagent
NE	norepinephrine
NO	nitrogen monoxide
$O_2•^-$	superoxide anion radical
O_3	ozone
OH•	hydroxyl radical
$ONOO^-$	peroxynitrite
PCA	principal component analysis
PBS	phosphate buffered saline
PC	protein carbonyl
R^2	Pearson correlation coefficient
RNS	reactive nitrogen species
ROS	reactive oxygen species
SDF-1	stromal cell derived factor 1
SNS	sympathetic nervous system
SP	substance P
TBA	thiobarbituric acid
TER	transepithelial electrical resistance
TNF	tumor necrosis factor
UTI	urinary tract infection

1. Summary

Feline idiopathic cystitis (FIC) is one of the most common disorders in feline veterinary praxis with different combinations of symptoms such as hematuria, stranguria, dysuria, periuria, pain and hypersensitivity during urination. In case of the occurrence of petechial hemorrhages of bladder submucosa of symptomatic cats detectable by cystoscopy, the disease is referred as interstitial cystitis. This name also reflects the similarities in symptoms and pathogenesis to human interstitial cystitis (IC). The exact cause and pathogenesis of the disease are widely studied; however not yet fully understood. FIC is considered as a multifactorial disease, based on a complex interaction between the urinary bladder, adrenal glands, nervous- and immune system and the environment. It is strongly suggested that stress may have an important role in the history of FIC, so the examination of this factor is essential to have a more accurate understanding of the development of the disease. Therefore, the first main goal of the present thesis was to establish a novel, well-characterized primary uroepithelial cell culture from feline origin which contains differentiated urothelial cells and could serve as a proper tool for studying the biochemical background of FIC *in vitro,* especially focusing on the pathological role of the main regulatory stress hormone norepinephrine (NE) in the pathomechanism of FIC.

The uroepithelial cells were gained from the bladder of a euthanized cat, being excised and washed followed by overnight incubation at 4°C in dispase containing medium. The epithelial cells were separated from the underlying connective tissue, resuspended and digested in trypsin-EDTA solution, and incubated at 37°C for 30 min. Thereafter, following multistep centrifugation, the cells were seeded on collagen-coated culture dishes and cultured for 6 days. The cell cultures were characterized by Giemsa and immunofluorescence staining. For proving the epithelial origin of the cultured cells eFluor conjugated pan-cytokeratin specific antibody binding was used, and to determine the rate of differentiation the uroepithelial cells were labelled with specific fluorescein isothiocyanate (FITC) conjugated uroepithel-binding anti-uroplakin antibodies. Pan-cytokeratin positivity was detected from the first day after plating; however, uroplakin positivity appeared only 6 days after plating which shows that the cultured uroepithelial cells need few days to differentiate.

Further, the other aim of the present study was to examine the molecular effects of acute and chronic intermittent NE-triggered stress on the established cell culture which served as an *in vitro* model for studying the neurohumoral and biochemical background of FIC. Therefore, an acute, short term (1h) and a pulsative (3x1h) NE treatment were applied at the concentration of 10, 100 and 1000 µM. After the acute NE exposure, increased metabolic activity of the cells, further, elevated extracellular concentration of the pro-inflammatory

cytokine interleukin-6 (IL-6) and pro-inflammatory chemokine stromal cell derived factor (SDF-1) were measured, suggesting the direct pro-inflammatory role of NE in the stress response of the uroepithelial cells. In addition, the modulatory action of the NE in the oxidative status of the cultured cells was also examined, and elevated protein carbonyl (PC) level was measured; however, the concentration of malondialdehyde (MDA), glucose-regulated-protein 78 (GRP78) and 8-hydroxy-2-deoxy guanosine (8-OHdG) were not increased. These results suggest that the acute NE exposure can induce oxidative stress by the oxidative damage of proteins without triggering lipid peroxidation, endoplasmic reticulum stress or DNA damage. Regarding the barrier function of the uroepithelial cells after the short term NE treatment, it was found that NE was able to decrease the glycosaminoglycan (GAG) concentration, the abundance of the tight junction protein claudin-4 and also the transepithelial electrical resistance (TER) values of the cultured cells, suggesting that an acute NE challenge might diminish the barrier integrity of uroepithelial cells.

As the recurrence of symptoms in FIC-affected cats in response to repeated stress is very common, the investigation of the molecular effects of the chronically elevated NE is essential for a deeper understanding of the pathogenesis of the illness. Hence, in the second trial the effects of intermittent stress were mimicked by 3x1 h NE exposure of the cultured uroepithelial cells. Regarding our results, the response of the repeatedly stressed cells was similar to the consequences of acute exposure: 3x1 h NE exposure resulted in elevated extracellular SDF-1 production, however in contrast to the acute treatment, IL-6 concentrations remained unchanged suggesting that SDF-1 might be more involved in disease progression than IL-6 in case of chronic FIC cases. Further, the pulsative NE treatment induced oxidative stress by increasing H_2O_2 production and it was also able to disturb the barrier integrity of the uroepithelial monolayer by decreasing the GAG concentration, the claudin-4 content and the TER values of the cell cultures, moreover, compared to acute treatment, lower NE concentrations were already capable to reduce GAG levels and TER values suggesting that the cultured uroepithelial cells are more sensitive to repeated exposure to NE than to a single acute treatment.

Based on the results of the present PhD thesis it can be concluded that short term NE exposure mimicking acute stress can provoke an inflammatory response and decrease the barrier integrity of cultured feline uroepithelial cells. Hence, it is highly expected that stress-associated NE release may play an important mediatory role in the pathogenesis of FIC. Furthermore, also the chronic recurrent NE exposure resulted molecular changes in inflammatory response, however a different inflammatory cytokine profile was detected compared to acute stress exposure. The oxidative status and barrier function of the cultured cells were also altered by intermittent NE treatment. According to these results it can be concluded that NE may play a key role in the pathogenesis of the disease in both acute and

chronic recurrent cases, and that the presence of the elevated NE concentrations in the body of the FIC affected cats may have a direct impact on the changes in the bladder that are involved in the development of disease symptoms. Information provided by the present study contribute to the better understanding of the pathogenesis and biochemical background of FIC; further, the newly established primary uroepithelial cell culture from feline origin can serve as a useful tool for investigating not only the pathomechanism of FIC, but also for examining other urinary tract disorders in cats.

Összefoglalás

A macskák idiopatikus húgyhólyaggyulladása (FIC) az egyik leggyakoribb egészségügyi probléma, amivel a macskatartók felkeresik állatorvosaikat. A betegség során különböző tünetek együttesen fordulnak elő, mint például hematuria, stranguria, dysuria, periuria, fájdalom és vizelés közbeni túlérzékenység. Amennyiben cisztoszkópiás vizsgálattal pontszerű vérzések láthatók a tüneteket mutató macskák hólyagjának szubmukózájában, a betegséget intersticiális cisztitisznek nevezik. Ez az elnevezés tükrözi a tünetek és a patogenezis hasonlóságát az emberi intersticiális cisztitisszel (IC). A FIC pontos oka és kórfejlődése széles körűen tanulmányozott, és bár még nem teljesen ismert, elmondható, hogy egy komplex multifaktoriális kórkép, amelynek kialakulása során a húgyhólyag, a mellékvesék, az ideg- és immunrendszer, valamint a környezet közötti összetett kölcsönhatás játszik alapvető szerepet. Korábbi szakirodalmi adatok alapján valószínűsíthető, hogy a stressznek kiemelt jelentősége lehet a FIC kialakulásában, ezért ennek a tényezőnek a vizsgálata elengedhetetlen a betegség kórfejlődésének pontosabb megértéséhez. Ezért a jelen dolgozat egyik fő célja egy új, jól jellemzett, differenciált húgyhólyaghámsejteket tartalmazó, macska eredetű primer húgyhólyaghámsejt-tenyészet létrehozása volt, amely megfelelő modellként szolgálhat a FIC biokémiai hátterének *in vitro* vizsgálatára, különös tekintettel a fő szabályozó stresszhormon, a noradrenalin (NE), FIC patomechanizmusában betöltött kóroktani szerepére.

A húgyhólyaghámsejteket egy előzőleg végleges elaltatásra kerülő, és a tulajdonos által tudományos célra felajánlott macska hólyagjából nyertük. A hólyagot laparotómiát követően kiemeltük, megtisztítottuk és egy éjszakán keresztül diszpáztartalmú tápfolyadékban emésztettük 4^{o}C-on. A hámsejteket egy steril sejtkaparó segítségével elválasztottuk a kötőszöveti rétegtől, majd a sejtszuszpenziót 30 percen keresztül tripszin-EDTA tartalmú tápfolyadékban 37^{o}C-on történő folyamatos kevertetés mellett tovább emésztettük. Ezután többlépcsős centrifugálást követően a sejteket kollagénnel bevont tenyésztőedényekre raktuk le, és 6 napig tenyésztettük. A sejttenyészeteket Giemsa- és immunfluoreszcens festéssel jellemeztük. A tenyésztett sejtek epiteliális eredetének bizonyítására eFluor-ral jelölt pán-citokeratin ellenanyagot alkalmaztunk, a differenciálódás mértékének meghatározására pedig az uroepitél sejteket specifikus, fluoreszcein-izotiocianáttal (FITC) konjugált uroplakin III. ellenanyaggal mutattuk ki. A pán-citokeratin-pozitivitás már a lerakást követő első napon kimutatható volt; az uroplakin-pozitivitás azonban csak 6 nappal a lerakás után jelentkezett, ami arra utal, hogy a tenyésztett húgyhólyaghámsejteknek szükségük van néhány napra a differenciálódáshoz.

Jelen PhD tanulmány másik fő célja a noradrenalin (NE) által kiváltott akut és krónikus intermittáló stressz molekuláris hatásainak vizsgálata a létrehozott húgyhólyaghámsejt-tenyészeten, amely *in vitro* modellként szolgált a FIC neurohumorális és biokémiai hátterének tanulmányozásához. Ennek érdekében akut (1 óra) és pulzáló (3x1 óra) NE kezeléseket alkalmaztunk 10, 100 és 1000 µmol/l koncentrációban. Az akut NE expozíciót követően a sejtek fokozott metabolikus aktivitását, továbbá emelkedett extracelluláris pro-inflammatorikus citokin, interleukin-6 (IL-6) és a pro-inflammatorikus kemokin, stromal cell derived factor 1 (SDF-1) koncentrációkat mértünk, ami az NE közvetlen gyulladáskeltő szerepére utal az uroepitél sejtek stresszválaszában. Ezen kívül vizsgáltuk az NE moduláló hatását a tenyésztett sejtek oxidatív állapotára is, melynek során emelkedett fehérje-karbonil (PC) koncentrációt mértünk; a malondialdehid (MDA), a glükóz-szabályozott 78-as fehérje (GRP78) és a 8-hidoxi-2-dezoxiguanozin (8-OHdG) koncentrációja azonban nem mutatott szignifikáns emelkedést. Ezek az eredmények arra utalnak, hogy az akut NE-kezelés a fehérjék oxidatív károsodása révén oxidatív stresszt idézhet elő anélkül, hogy lipidperoxidációt, endoplazmatikus retikulum stresszt vagy DNS károsodást váltana ki. Vizsgáltuk továbbá az NE uroepiteliális sejtek barrierfunkciójában betöltött szerepét, és eredményeink alapján elmondható, hogy az akut NE-kezelés képes volt csökkenteni a tápfolyadék glükozaminoglikán (GAG) koncentrációját, a húgyhólyaghámsejtek *tight junction* sejtkapcsolatainak klaudin-4 fehérjemennyiségét és a tenyésztett sejtek transzepiteliális elektromos ellenállásának (TER) értékét, utalva arra, hogy az akut NE kezelés csökkentheti az uroepiteliális sejtek barrierintegritását.

Mivel a FIC-ben szenvedő macskáknál a tünetek kiújulása ismétlődő stressz hatására nagyon gyakori, a krónikus intermittáló, emelkedett NE koncentráció molekuláris hatásainak vizsgálata elengedhetetlen a betegség kórfejlődésének pontosabb megértéséhez. Ezért a harmadik kísérletben az időszakosan visszatérő stressz hatásait a tenyésztett húgyhólyaghámsejtek 3x1 órás NE kezelésével modelleztük. Eredményeinket tekintve az ismételt stresszhatásnak kitett sejtek molekuláris válasza hasonló volt az akut kezelés következményeihez: a 3x1 órás NE kezelés megnövekedett extracelluláris SDF-1 termelést eredményezett, azonban az akut kezeléssel ellentétben az IL-6 koncentrációja változatlan maradt. Ez arra utal, hogy a FIC krónikus eseteiben az SDF-1 fontosabb szerepet tölthet be a betegség progressziójában, mint az IL-6. Ezen kívül a pulzáló NE-kezelés oxidatív stresszt idézett elő a H_2O_2-termelés fokozásával, és csökkentette a húgyhólyaghámsejt-tenyészet barrierintegritását is, melyet a tenyészetek GAG-koncentrációjának, klaudin-4-tartalmának és TER-értékeinek csökkenése tükrözött. Elmondható továbbá, hogy ezekben a vizsgálatokban az akut kezeléssel összehasonlítva már az alacsonyabb NE-koncentrációk is csökkentették a GAG-koncentrációt és a TER-értékeket, ami arra enged következteti, hogy a tenyésztett uroepitéll sejtek érzékenyebbek az ismételt NE-expozícióra, mint az egyszeri akut kezelésre.

A jelen doktori értekezés eredményeit összefoglalva megállapítható, hogy az akut stresszt imitáló 1 órás NE kezelés gyulladásos választ és oxidatív stresszt idézett elő, valamint csökkentette a tenyésztett húgyhólyaghámsejtek barrierintegritását. Ezen eredmények alapján elmondható, hogy a stresszel összefüggő NE-felszabadulás fontos közvetítő szerepet játszhat a FIC kórfejlődésében. Továbbá, a krónikus, ismétlődő NE kezelés is molekuláris változásokat eredményezett a sejtek gyulladásos válaszában, habár az akut stressz-expozícióhoz képest eltérő gyulladásos citokinprofil volt kimutatható; valamint az alkalmazott pulzáló NE kezelés a húgyhólyaghámsejtek oxidatív állapotát és barrierfunkcióját is befolyásolta. Ezen eredmények alapján megállapítható, hogy a húgyhólyaghámsejtek képtelenek voltak alkalmazkodni az NE tartós jelenlétéhez, ezáltal igazolva, hogy az NE kulcsszerepet játszhat a betegség kórfejlődésében mind az akut, mind a krónikusan visszatérő esetekben is. Továbbá elmondható, hogy a FIC-ben érintett macskák szervezetében mérhető emelkedett NE koncentráció jelenléte közvetlen hatással lehet a hólyagban bekövetkező, tünetekben megnyilvánuló változások kialakulására. A jelen PhD tanulmány által szolgáltatott új tudományos eredmények hozzájárulnak a FIC patogenezisének és biokémiai hátterének pontosabb megértéséhez; valamint az újonnan létrehozott, macska eredetű primer húgyhólyaghámsejt-tenyészet a jövőben hasznos modellként szolgálhat nemcsak a FIC patomechanizmusának, hanem a macskák egyéb húgyúti rendellenességeinek *in vitro* vizsgálatához is.

2. Introduction and literature overview

Feline lower urinary tract dysfunction (FLUTD) is one of the most common diseases in domestic cats (*Felis silvestris catus*), with symptoms including variable combinations of hematuria, stranguria, dysuria, periuria, pain and hypersensitivity during urination. Differential diagnosis of the clinical signs may include urolithiasis, urinary tract infection, neoplasia or parasites, but there are some cases when no specific underlying cause can be diagnosed by clinical evaluation, so the disease is often referred to as feline idiopathic cystitis (FIC). The possible cause(s) and the pathogenesis of the disorder are widely studied; FIC should be considered as a complex, multifactorial disease. It is strongly suggested that stress may have an important role in the history of FIC, thus the examination of this factor is essential to gain a deeper knowledge concerning the development of the disease (Chew et al., 2013). FIC is very similar to the human interstitial cystitis (IC) in both its symptoms and development, therefore the examination of its pathomechanism is important not only in the veterinary, but in the human medicine as well, further, it is supposed that FIC could also serve as a useful animal model of IC (Buffington et al., 1996; Lavelle et al., 2000).

2.1. The structure of the bladder

The bladders of cats with FIC undergo both functional and anatomical changes compared to healthy animals; however, in order to understand these changes, it is necessary to have an overview of the structure of the bladder.

The urinary system is made up of the kidneys, ureters, bladder and urethra. The urinary bladder (*vesica urinaria*) is a mucous-lined, muscular-walled organ whose function is to collect and temporarily store the urine. Urine removes harmful or unnecessary molecules and residues excreted by the body, which makes its medium hypertonic. For this reason, a special epithelium called the uroepithelium, which covers the inner wall of the bladder, the ureter, the upper third of the urethra and the renal pelvis, plays a particularly important role in protecting the urinary tract (Röhlich et al., 2014). This special epithelium is made up of 3 layers: a layer of basal cells attached to the connective tissue, then an 1-2 cell layers thick intermediate layer of transitional cells, and finally the top layer of large, spreading, characteristically shaped cells called umbrella cells with an asymmetrical apical plasma membrane (Apodaca, 2004; Wu et al., 1994) (**Fig.1**.), containing uroplakins as special uroepithelial differentiation products. Uroplakins are stored in cytoplasmic vesicles connected to the cell membrane and, in addition to their barrier role, they also play a role in the bladder's volumetric capacity (Wu & Sun, 1993; Yu et al., 1990). According to our recent

knowledge, four types of uroplakins exist: Ia., Ib., II., and III., from which uroplakin III. can be detected only in uroepithelial cells of the urinary bladder, ureter, and renal pelvis (Kaufmann et al., 2000). Therefore, uroplakin III. is a suitable marker to characterize differentiated uroepithelial cells in cell cultures (Truschel et al., 1999).

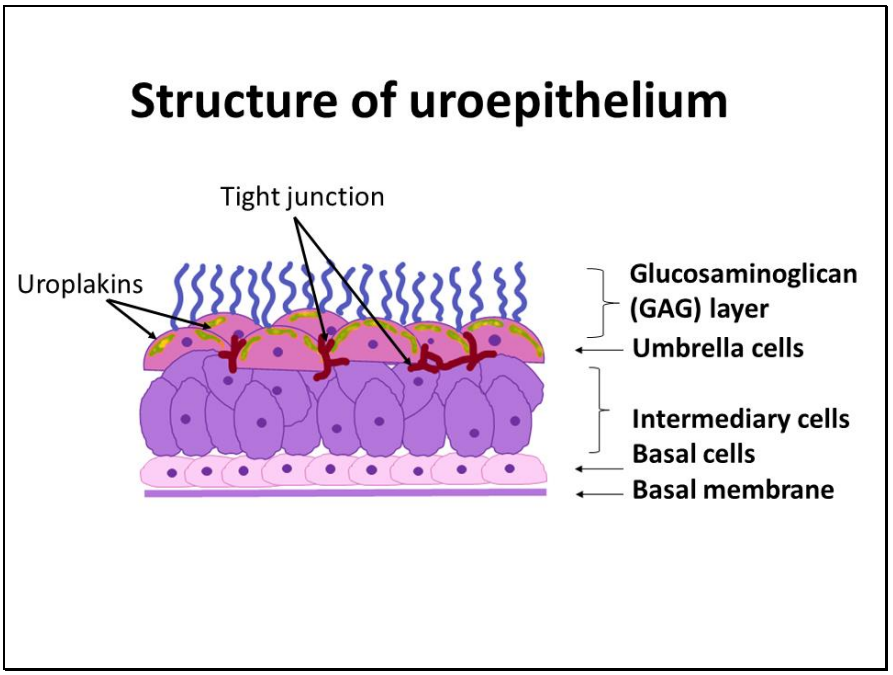

Fig.1.: The structure of uroepithelium (source: own editing)

The umbrella cells are connected by tight junctions, which provide an effective barrier against ion-, ammonia-, bacteria-, and urea-flux, and make the uroepithelium suitable to form a protective barrier between the urine and blood flow. These tight junctions consist of cytoplasmic proteins, cytoskeletal elements and transmembrane proteins. The most characteristic proteins of tight junctions are zona occludens, occludin, and claudins, having great importance for the maintenance of the epithelial barrier integrity. Claudins are members of a multigene protein family with 24 molecules, and they are able to form ion channels and to regulate the membrane permeability depending on the size, charge, and electrical resistance of the ions. Claudin-4, -8, and -12 can be found in tight junctions of the umbrella cells (Acharya et al., 2004; González-Mariscal et al., 2003; Tsukita et al., 2001). On the apical surface of the upper layer of umbrella cells described above, a layer of glucosaminoglycans (GAGs) can be found, which, in addition to its barrier function, also plays an important role in preventing the adhesion of various pathogenic bacteria (Parsons et al., 1990). Due to tight junction proteins, including claudins, and the GAG layer, the bladder epithelium is probably the tightest mammalian epithelium, with transepithelial resistance (TER) measured between 75 000 and 100 000 Ωcm^2. The method measures the resistance of a cell layer between two fluid compartments, which is maintained by the ionic balance

between the two spaces. When cells are damaged, the integrity of the cell layer is compromised, which means that their electrical resistance is reduced, hence their TER value is getting decreased (Lewis, 2000). Several previous studies have described that urothelial cells have not only a passive protective role, but also neuron-like, sensory properties, thus they themselves can be the target of many mechanical and chemical stimuli (Birder, 2004; Birder et al., 2001). Birder et al. described that certain receptor, such as alpha- and beta-adrenergic receptors – which are important components of the signaling function of noradrenergic neurons – are expressed not only in neurons of the bladder but also in the bladder epithelial cells themselves. The release of various chemicals, such as prostaglandins, nitric oxide (NO), adenosine triphosphate (ATP) and acetylcholine from the epithelial cells suggests that these cells have not only sensory, but also signal transduction properties, which allow mutual communication with neighboring urothelial cells and also with nerve cells (Birder et al., 2001; Chess-Williams, 2004).

2.2. Clinical implications of Feline Idiopathic Cystitis (FIC)

2.2.1. Occurrence, predisposing factors

FLUTD is most commonly of unknown origin, and in 54-64% of cases no specific diagnosis can be made when examining symptomatic cats, so the disease is considered as idiopathic (Gerber et al., 2005; Kruger et al., 1991). The first symptoms of FIC most often appear between 2 and 6 years of the age of the cat, and very rarely in cats younger than 1 year or older than 10 years. Of its two presentations (non-obstructive and obstructive), the non-obstructive form occurs in equal proportions in female and male cats, but in both sexes, it is more common in neutered individuals, regardless of the date of neutering (Hostutler et al., 2005). The obstructive form with urethral obstruction occurs in 20-55% of male cats (and only a minority of females). Although there is no difference in urethral diameter between neutered and intact males, urethral obstruction is more common in neutered male cats (Gerber et al., 2005; Hostutler et al., 2005). In addition to genetic and epigenetic causes, other predisposing factors, such as excess weight, exclusive housing, reduced activity, sedentary lifestyle, and environmental stress factors such as conflict between individuals in multi-cat households may also play an important role in the development of the disease (Buffington, 2002; Buffington et al., 2006).

2.2.2. Pathogenesis

The exact pathophysiology of FIC is still unknown, but based on numerous former studies it is suggested that the disease is rather a multi-organ syndrome than a single entity. As described in the introduction, abnormalities of the bladder, central nervous system and

hypothalamic-pituitary-adrenal axis are thought to be involved in the clinical manifestation of the disease.

2.2.2.1. Bladder abnormalities

In cats with FIC, the permeability of the bladder to water, urea and various ions is increased and the TER of the bladder wall is reduced *in vitro*. In addition, the bladder of cats with FIC shows a flattened image, covered with cells from the lower layers examined by electron microscopy, which is presumably related to altered cell maturation and differentiation processes (Gao et al., 1994; Lavelle et al., 2000). Further, the permeability of bladder epithelium is increased due to the diminished expression of tight junction proteins. This disruption of the bladder barrier may contribute to increased afferent nerve activity causing bladder symptoms such as pain and hypersensitivity (Hauser et al., 2015; Lavelle et al., 2000). Decreased urinary total GAG concentrations have been described in cats with FIC (Buffington et al., 1996; Pereira et al., 2004), as well as decreased levels of a specific GAG, GP-51 (Press et al., 1995). However, for years the role of GAGs in the pathogenesis of the disease remains unclear (Liebert, 2009) **(Fig.2.)**.

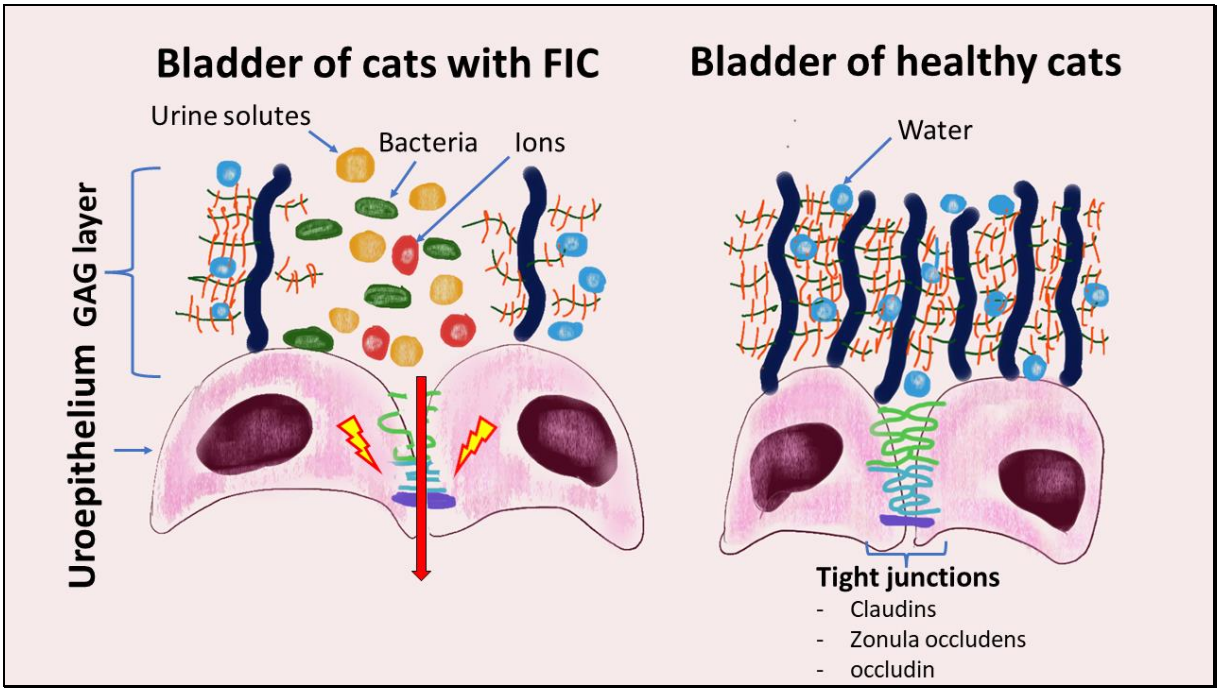

Fig.2.: Comparison of bladder wall in case of cats with FIC and healthy cats. FIC=Feline idiopathic cystitis, GAG=glucosaminoglycan (source: own editing)

2.2.2.2. Changes in the nervous system

Damage of the GAG layer covering the surface of the epithelium and increased epithelial permeability allow various urinary ions, such as hydrogen, calcium and potassium ions, or

chemical signal transporters released during epithelial cell damage, such as ATP, NO, acetylcholine, to come into contact with the pain nerve endings in the submucosa and irritate them. As a consequence, neurogenic inflammation of the bladder develops or the stimulation reaches the spinal cord, causing pelvic pain. The sensation of pain in the brain releases a neurotransmitter called substance P (SP), which is detectable at the site of inflammation. The local increase in SP concentration, together with the release of histamine from mast cells under the influence of SP, increases the permeability of the blood vessels around the inflammation, which is an important condition for the development of inflammation. Since SP receptors are also present in the smooth muscle of the urinary tract, contraction of the smooth muscle also occurs. This results in a condition similar to spasmodic urethral contraction, which makes urination difficult (Kruger et al., 2009).

According to previous studies the activity of tyrosine hydroxylase – which is the main regulatory enzyme of catecholamine production in the central nervous system – was increased in the area of *the locus coeruleus* and in the paraventricular nuclei of the hypothalamus of the cats with FIC. The *locus coeruleus* contains a large number of noradrenergic neurons and is also the most important site of norepinephrine (NE) synthesis. Consequently, increased NE level could be measured permanently in the blood (Forrester & Towell, 2015; Reche & Buffington, 1998; Sands et al., 2000). The described increased tyrosine hydroxylase activity can be observed in chronically stress-exposed, but otherwise healthy cats as well; however, the plasma NE concentration decreased over time. Complex changes in the brain due to prolonged, intermittent exposure to stressors resulted in a stimulated emotional response, leading to excitation of the *locus coeruleus*, rapid and elevated sympathetic nervous system activity, further, decreased effect of those inhibitory pathways maintaining the balance of stress response in healthy individuals (Correll et al., 2005; Jacobson et al., 2004; McEwen, 2000; McEwen, 2004; Shekhar et al., 2005).

2.2.2.3. Hormonal changes

Prolonged stress activates the hypothalamic-pituitary-adrenal axis (HPAA) in addition to the *locus coeruleus*/NE system: a neurotransmitter called corticotropin-releasing factor (CRF) is released in the hypothalamus, stimulating ACTH production in the anterior pituitary lobe, and activating the sympathetic nervous system in the nuclei of the brainstem, resulting in the release of adrenaline and NE. ACTH production leads to increased release of glucocorticoids, including cortisol, in the adrenal cortex. In healthy cats, the cortisol released inhibits further production of catecholamines (such as NE) and also has an inhibitory effect on the hypothalamus and pituitary gland. In cats with FIC, this inhibitory effect is lacking, and plasma NE concentrations are persistently elevated (**Fig.3**.) (Forrester & Towell, 2015;

Westropp & Buffington, 2004). It has been observed that cats with FIC have significantly lower ACTH-induced cortisol spikes in plasma and reduced adrenal gland size compared to healthy cats (Buffington & Pacak, 2001; Westropp et al., 2003, 2006). Histopathological examination of the adrenal glands reveals a reduction in the size of the *zona fasciculata* and *zona reticularis* layers of the adrenal cortex, suggesting that cats with FIC may also have mild primary adrenal insufficiency (Westropp et al., 2003).

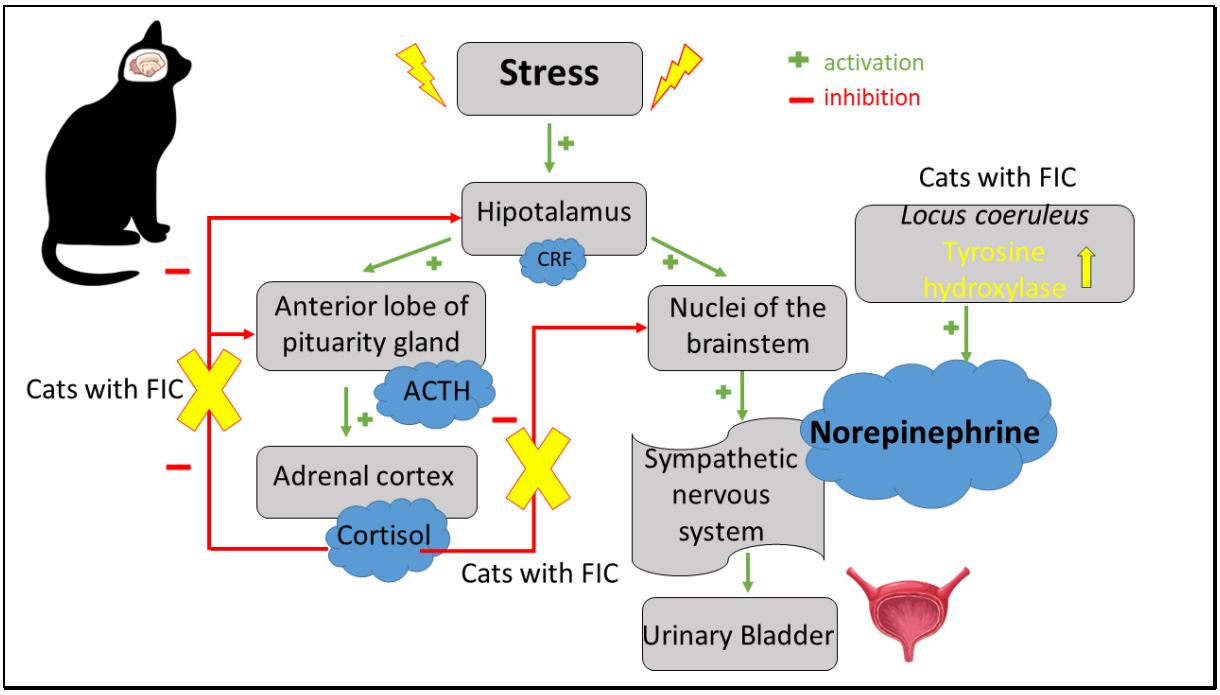

Fig.3.: Relationship between stress and the hypothalamic-pituitary-adrenal axis in cats with FIC . FIC= Feline idiopathic cystitis, CRF= corticotropin releasing factor, ACTH= adrenocorticotropic hormone (Source: own editing based on Forrester & Towell, 2015)

2.2.2.4. Epigenetic factors

The current state of science suggests that cats with FIC have an unusually high central stress response capacity. Some research suggested that the over-sensitivity of the stress response system is caused by altered gene expression due to epigenetic modulation (Jensen, 2013). This hypothesis is supported by a study describing that, when a pregnant cat is exposed to prolonged, high levels of stress, the hormonal mediators of the neuroendocrine stress response are transmitted across the placenta and affect fetal development (Gluckman & Hanson, 2006). If the stress effect occurs at a critical stage of fetal development, when the adrenal cortex is developing, the fetus may not develop the affected organ properly (Buffington, 2004).

2.2.3. Symptoms

The symptoms of idiopathic cystitis are very different. The manifestation of the symptoms depends on the form of the disease that develops. The obstructive form can lead to a complete cessation of urination and can quickly develop serious consequences. The non-obstructive form of the disease leads to the development of a non-life-threatening condition. Animals suffering from this form have difficulty passing urine with prolonged effort (stranguria). Symptoms may include blood in the urine (haematuria), urination in unusual places (periuria) and urination with a more frequent and persistent urge to urinate (pollakisuria). In terms of clinical presentation, in 80-90% of the non-obstructive cases, a long period of asymptomatic time is interrupted by an acute flare-up, which may be self-limited and resolve within a few days without treatment. In 2-15% of animals, these symptoms often occur in the form of recurrent episodes, between which the animal is asymptomatic, and in 2-15% of cases the disease is chronic, with persistent symptoms (Forrester & Towell, 2015; Osborne et al., 1996). Notwithstanding that the clinical sings of the disease can resolve within a few days without treatment, the recurrence of these symptoms is very common; based on some former studies up to 50% of cats with acute FIC will experience recurrence in one year, which can be related to the altered stress response of cats suffering from FIC (Defauw et al., 2011).

2.2.4. Diagnosis

Because of the complexity of the symptoms and the disease, it is not diagnosable based on these terms alone, and it is very difficult to recognize it at an early stage. Although lower urinary tract symptoms in young cats are most commonly caused by FIC, it is essential to exclude other diseases with similar symptoms, and to take a careful and detailed case history before making a diagnosis. Similar symptoms and lesions are most often caused by urolithiasis, less frequently by bacterial infection, tumors, anatomical abnormalities, lesions, narrowing of the urethra, or possibly infection with the parasite *Capillaria plica*. To exclude these illnesses, it is essential to perform an X-ray of the lower abdomen, a complete urinalysis and urine sediment analysis with bacteriological culture, and possibly an ultrasound examination. X-rays and ultrasound examinations can be used to detect the presence of urinary calculi or possible tumours, and by ultrasound the thickening of the bladder wall also can be detected, which is typically measured in cases of chronic inflammation of the bladder (Griffin, 2020; Buffington et al., 1997). Urinalysis shows an elevated urinary protein-to-creatinine ratio and higher pH in cats with FIC, probably related to increased permeability of the bladder wall, which results in leakage of plasma proteins into the urine (Panboon et al., 2017). Urine sediment from cats with FLUTD is characterized by

elevated numbers of red and/or white blood cells and bladder epithelial cells, but whereas bacterial cystitis is dominated by white blood cells, FIC is characterized by elevated numbers of red blood cells. In more complicated cases a cystoscopy examination may also be helpful, as well as the histopathological analysis of a biopsy in case of possible tumors. Blood biochemistry may also be performed to detect concomitant diseases (Buffington et al., 1997) **(Fig.4.)**.

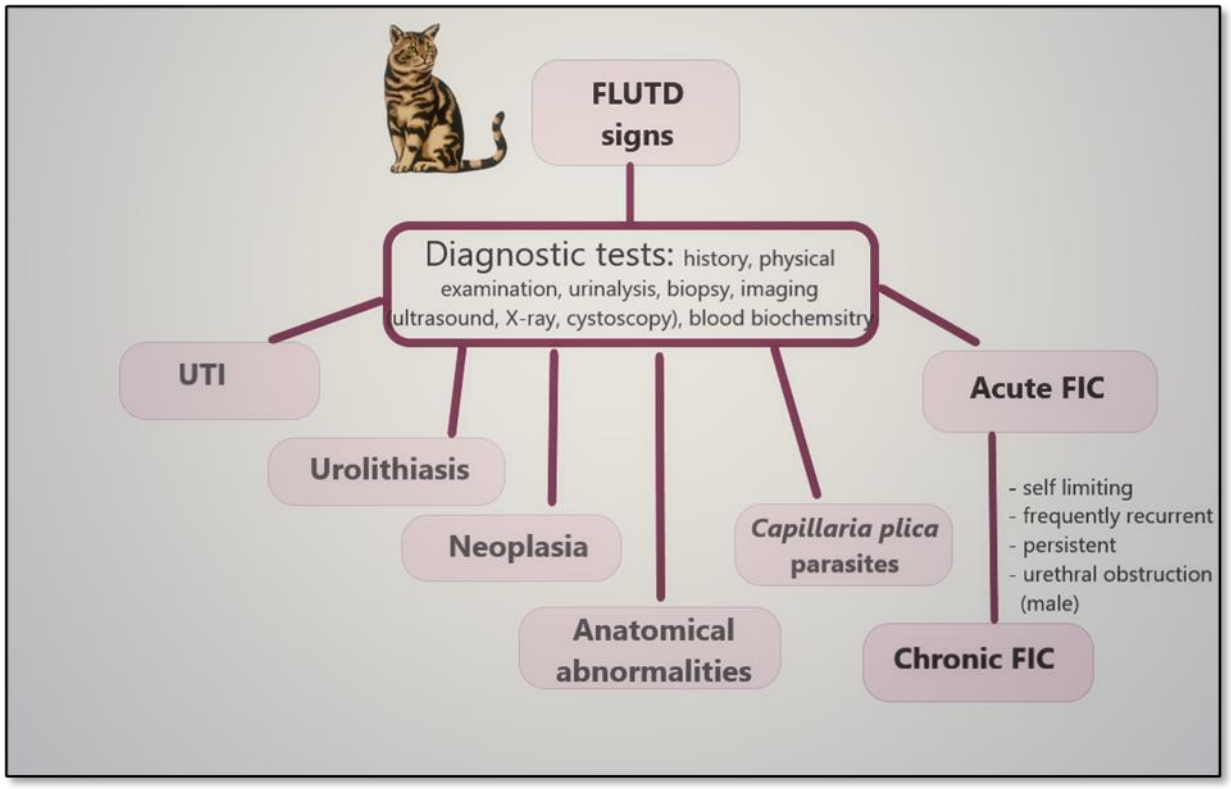

Fig.4.: Differential diagnosis of different FLUTD signs after appropriate diagnostic tests. FLUTD= feline urinary tract disorders, UTI=urinary tract infections, FIC=feline idiopathic cystitis (source: own editing)

A detailed medical history can help in the diagnosis. It is easy to identify the stress factors that are causing the symptoms by asking the owner specific questions about changes in the animal's environment. The age of the animal may also be helpful, as cats under 1 year and over 10 years of age are unlikely to develop the disease (Forrester et al., 2007).

At present, the number and availability of diagnostic tests for the disease is quite limited. In humans, several biomarkers such as antiproliferative factor or heparin-binding epidermal growth factor have been tested and shown to be specific in interstitial cystitis, but these tests are not yet available for feline clinical practice and their sensitivity has not yet been demonstrated in cats (Erickson et al., 2002). Elevated urinary fibronectin concentrations have been described in cats with FIC, however, fibronectin molecule plays an important role in cell migration, wound healing and blood coagulation, so it is assumed that the increase in its amount is rather secondary, which is always observed in obstructive processes due to

changes in the bladder wall, and therefore not specific for the disease (Lemberger et al., 2011). A group of researchers has shown differences in the profile of various intermediates and end products of tryptophan metabolism in the blood of affected cats compared with healthy individuals. These studies were performed using infrared spectroscopy on blood serum from cats, previously diagnosed with the disease but showing no symptoms at the time of sampling. The method was suitable to separate healthy individuals from cats with FIC, but further studies are needed to confirm the results (Rubio-Diaz et al., 2009).

2.2.5. Treatment

Based on our recent knowledge, there is no effective treatment that can completely eliminate the disease, so clinical practice should focus on managing symptoms, and prolonging symptom-free periods. Once the diagnosis has been made, based on the exact history and the animal's housing conditions, it may be necessary to introduce certain changes in the cat's environment (multimodal environmental modification, MEMO) to increase the cat's comfort and sense of security, in order to minimize the environmental stressors, present in the cat's home. These modifications may include, for example, optimizing the litter box: it should be placed in a location where other animals or children do not disturb the cat during use, the litter should be kept clean in the box, especially the strong odor as a possible stress factor should be prevented. An important consideration regarding the number of litter boxes is that if there are several cats in a household, the 'n + 1' rule should be applied, which means that there should always be one more litter box than the number of cats in the household (Overall, 1998). It is important to note that the feeding and watering habits of the animal may also play a role in symptomatic management. Fresh water should always be provided, and it is recommended to feed wet food to increase water intake. Changes in the cats' daily routine, perhaps moving house or renovating, or taking in another cat can be very important environmental stressors. In such cases, efforts should be made to re-establish the cats' daily routine according to their needs, or to place scratching posts in case of the arrival of another cat, thus helping to mark out the area (Hostutler et al., 2005).

Obesity is one of the main predisposing factors for the occurrence of FIC, therefore, if the affected cat is overweight, the use of reduced calorie feed is an important therapeutic tool (Michel & Scherk, 2012). In addition to environmental modifications, pheromone therapy can also be effective in cats with FIC. Pheromones are fatty acids that can transmit specific information between individuals of the same species. Synthetically produced feline facial pheromones are now commercially available and can be part of a FIC treatment strategy (Griffith et al., 2000; Gunn-Moore & Cameron, 2004; Michel & Scherk, 2012).

Reduction of the stress by medical treatment may be recommended if the environmental changes or the pheromone therapy have not led to an improvement in symptoms. In such cases, daily 12.5 mg/kg body mass oral addition of L-tryptophan – which is a precursor of serotonin synthesis – may be recommended. It plays an important role in the stress response, as elevated serotonin levels have an anti-anxiety effect in both humans and animals. The α-casozepine – a milk protein hydrolysate – administered daily at 15 mg/kg body mass orally can also be beneficial. In some clinical studies these substances have been shown to be effective in reducing stress-induced anxiety in cats (Beata et al., 2007; Landsberg et al., 2017). In addition, the beneficial effects of amitriptyline, a member of the tricyclic antidepressant family (2.5 to 7.5 mg/cat/24 h per os), have been investigated, and the treatment commonly starts to take effect after one week. However, if no improvement is observed or if the administration of the tablet causes distress to the animal, the treatment should be gradually discontinued over a week (Chew et al., 1998).

Analgesia is an inevitable part of the therapy of acute inflammatory episodes and the recommended agents are oral buprenorphine (0.01 mg/kg/8-12h) absorbed from the oral mucosa, subcutaneous butorphanol (0.2 mg/kg/8-12h) or, depending on the severity of the pain, possibly fentanyl patches.

2.3. Biochemical characterization of FIC

2.3.1. Inflammatory cytokines, with a special focus on IL-6 and SDF-1

Cytokine is a general name of small protein molecules that are produced by different cells and have important role in cell-cell interactions. Lymphokines are cytokines produced by lymphocytes, monokines are cytokines made by monocytes, interleukins (IL) are cytokines released by one leukocyte and acting on other leukocytes, and chemokines are cytokines that have chemotactic activity (Zhang & An, 2007). Cytokines can act on different cells like on the cells that secrete them, on nearby cells, or on distant cells, and it is also frequent that one single cytokine can act on different cell types at the same time (Papanicolaou & Vgontzas, 2000). Further, it is common that similar function of a cell can be stimulated by different cytokines, and they are often produced in a cascade process, which means that one cytokine stimulates its target cells to release additional cytokines. They can also act synergistically or antagonistically (Tanaka & Kishimoto, 2014; Zhang & An, 2007). Cytokines are mainly produced by macrophages and T-helper cells; however, they can be also synthesized by different epithelial cell type such as uroepithelial cells as well (Hedges et al., 1994; Zhang & An, 2007).

There are two major group of cytokines: pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, SDF-1 and TNF-α) and anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-11). Pro-inflammatory cytokines are involved in the up regulation of inflammatory reactions and anti-inflammatory cytokines are able to alleviate the pro-inflammatory cytokine response. Interleukins and chemokines are produced in high concentrations during the immune response, and IL-6 and SDF-1 have long been known as important pro-inflammatory cytokines whose expression is associated with several stressors (Arms et al., 2010; Tanaka & Kishimoto, 2014).

IL-6 is abundantly produced in almost all inflammatory processes, including cystitis as well, and is therefore an excellent tool for studying inflammation (Gonzalez et al., 2014; Malley & Vizzard, 2002; Scheller et al., 2011; Smaldone et al., 2009). In classical signaling, IL-6 directly stimulates target cells via the membrane-bound IL-6 receptor, which binds to a ligand associated with a signaling receptor protein called gp130. The gp130 dimerizes, leading to activation of Janus kinase (JAK) enzymes and phosphorylation of tyrosine residues, resulting in activation of the mitogen-activated protein kinase (MAPK) pathway. These processes result in activation of target genes and trigger distinct IL-6-specific effects. The membrane-bound IL-6 receptor is synthesized by a small number of cells, whereas all cells on the cell surface contain gp130. However, cells expressing gp130 can only respond to an IL-6 complex bound to a naturally occurring soluble form of the IL-6 receptor, not to IL-6 alone. This signaling pathway is called trans-signaling and is primarily responsible for the role of IL-6 in pro-inflammatory processes (Scheller et al., 2011).

CXCL-12 is a member of the CXC chemokine family and is also known as stromal cell derived factor-1 (SDF-1). It was found that CXCL-12 (henceforth SDF-1) was expressed constitutively by bone marrow stromal cells (Bleul et al., 1996), and has an important role also in pathological processes including inflammation, as it is able to activate the migration of most leukocytes (Janssens et al., 2018). During inflammatory processes, SDF-1 acts through the CXC chemokine receptor 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3) receptors and binds to GAGs in tissues like the endothelium to perform its functions, allowing the passage of leukocytes to display properly. The CXCR4 receptor only has SDF-1 as a ligand, and CXCR4 receptor can be found in many cell types including uroepithelial cells as well, therefore the CXCR4 and SDF-1 expression plays an important role in the regulation of inflammatory processes (Arms et al., 2010; Bleul et al., 1996; Janssens et al., 2018).

Based on earlier studies, the levels of the pro-inflammatory cytokine IL-6 and the pro-inflammatory chemokine SDF-1 were increased in the urine and uroepithelial cells in case of experimentally induced cystitis in rats, in the urine of patient with IC and in the plasma of cats with FIC (Arms et al., 2010; Corcoran et al., 2013; Erickson et al., 2007; Lamale et al., 2006;

Malley & Vizzard, 2002). Further, concentrations of IL-12, IL-18 and SDF-1 in blood plasma were elevated in cats with FIC (Parys et al., 2018).

The role of NE in inflammatory processes has been investigated in several previous studies, but the results are controversial in clarifying its pro- or anti-inflammatory role. Basically, the release of catecholamines during stress leads to the production of cAMP in microglial cells, which reduces the amount of pro-inflammatory cytokines, but this is also highly dependent on the environmental context (Elenkov et al., 2000; Tanaka et al., 2002). For example, NE is able to reduce the amount of pro-inflammatory cytokines such as IL-6 in LPS-treated astroglia- and microglia cells, but this anti-inflammatory effect is lacking in the absence of LPS, and rather, in this case, increases the amount of certain pro-inflammatory cytokines (Färber et al., 2005; Hinojosa et al., 2013). Further, the effect of NE was also examined on the production of various cytokines, such as IL-6 in human immortalized gastric epithelial cell culture, and it was found that NE triggered increased IL-6 production and up-regulated the IL-6 receptor, confirming the role of NE in inflammatory processes (Yang et al., 2014). These results are also in line with a previous study, in which the stimulatory effect of NE was detected on IL-6 production in rat cardiac fibroblast cells (Bürger et al., 2001). Taken together it can be concluded that current studies are often conflicting how NE influences cytokine production, and whether it has pro- or anti-inflammatory role. The outcome of such events might depend on several factors, such as the environmental context or the investigated cell types, further, to the best of our knowledge, there are no available literature data regarding the molecular effects of NE on uroepithelial cells in relation to inflammatory processes.

2.3.2. Free radicals and oxidative stress

Free radicals are molecules which contain unpaired electron(s) on their outermost shell(s), leading to their high reactivity. They are able to attack biomolecules such as cellular proteins, lipids, carbohydrates as well as nucleic acids. Free radicals can be derived from many elements, but the most important ones are maybe those involving oxygen, reactive oxygen species (ROS) and nitrogen, reactive nitrogen species (RNS) (Burton & Jauniaux, 2011). The most relevant ROS molecules are superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), peroxynitrite ($ONOO^-$), lipid hydroperoxide (LOOH), hypochlorous acid (HOCl), and ozone (O_3) (Glasauer & Chandel, 2013). ROS can be produced in the body by several enzymatic and non-enzymatic processes under physiological conditions and they play a key role in the functioning of a healthy body by keeping their production at low or moderate levels (Pizzino et al., 2017). The main sources of ROS production are the following physiological processes: respiratory chain, prostaglandin synthesis, phagocytosis and reactions of the

cytochrome P450 system (Halliwell, 2007). In these processes, superoxide radicals ($O_2^{\bullet -}$) are generated by certain enzymes such as NADPH oxidase and xanthine oxidase, leading to the production of additional oxygen radicals (OH•; $ONOO^-$; HOCl) and H_2O_2. However, H_2O_2 is not an oxygen radical, but it is able to generate hydroxyl radical (OH•) – which is the most reactive among all the free radical species – in the reaction with superoxide radical catalyzed by Fe^{2+} or Cu^+. H_2O_2 is produced in several biochemical processes by oxidase enzymes such as D-amino acid oxidases and xanthine oxidase (Genestra, 2007). Free radicals can be originated from both endogenous and exogenous sources. Immune cell activation, inflammation, ischemia, infection, cancer, excessive exercise, mental stress and aging are the main endogenous processes that generate free radicals (Frei, 1994; Genestra, 2007; Halliwell, 2007; Valko et al., 2007).

If there is an imbalance between the production of free radicals and the cells' ability to eliminate them, a condition known as oxidative stress occurs. It is a pathological process that can damage many cell structures such as membranes, lipids, proteins, lipoproteins, and deoxyribonucleic acid (DNA) (Dröge, 2002). For example, excess hydroxyl radicals can cause cell membrane and lipoprotein damage by lipid peroxidation, thereby releasing malondialdehyde (MDA), a cytotoxic and mutagenic molecule (Frei, 1994). The most common oxidative stress induced lesion in DNA is the formation of 8-hydroxy-2-deoxy-guanosine, which is – among other pathological factors – also responsible for mutagenesis (Nishida et al., 2013). Free radicals can also damage proteins, with direct oxidation of amino acid side chains leading to the formation of carbonyl groups (aldehydes and ketones). The resulting conformational changes can cause proteins to lose their original function, such as catalytic activity of enzymes. The most sensitive amino acids are proline, arginine, lysine and threonine (Dalle-Donne et al., 2003). The endoplasmic reticulum (ER) is a cellular organelle that contains the chaperone proteins and enzymes responsible for folding proteins. Under oxidative stress, the ER can accumulate unfolded or misfolded proteins, which is called ER stress. The cell then tries to restore homeostasis by increasing the expression of chaperone proteins, such as glucose-regulated protein 78 (GRP78), in an attempt to sequence or reformulate the misfolded proteins. If these restoration attempts fail, an apoptosis cascade is triggered to eliminate the defective cell (Bhandary et al., 2012) **(Fig.5.)**.

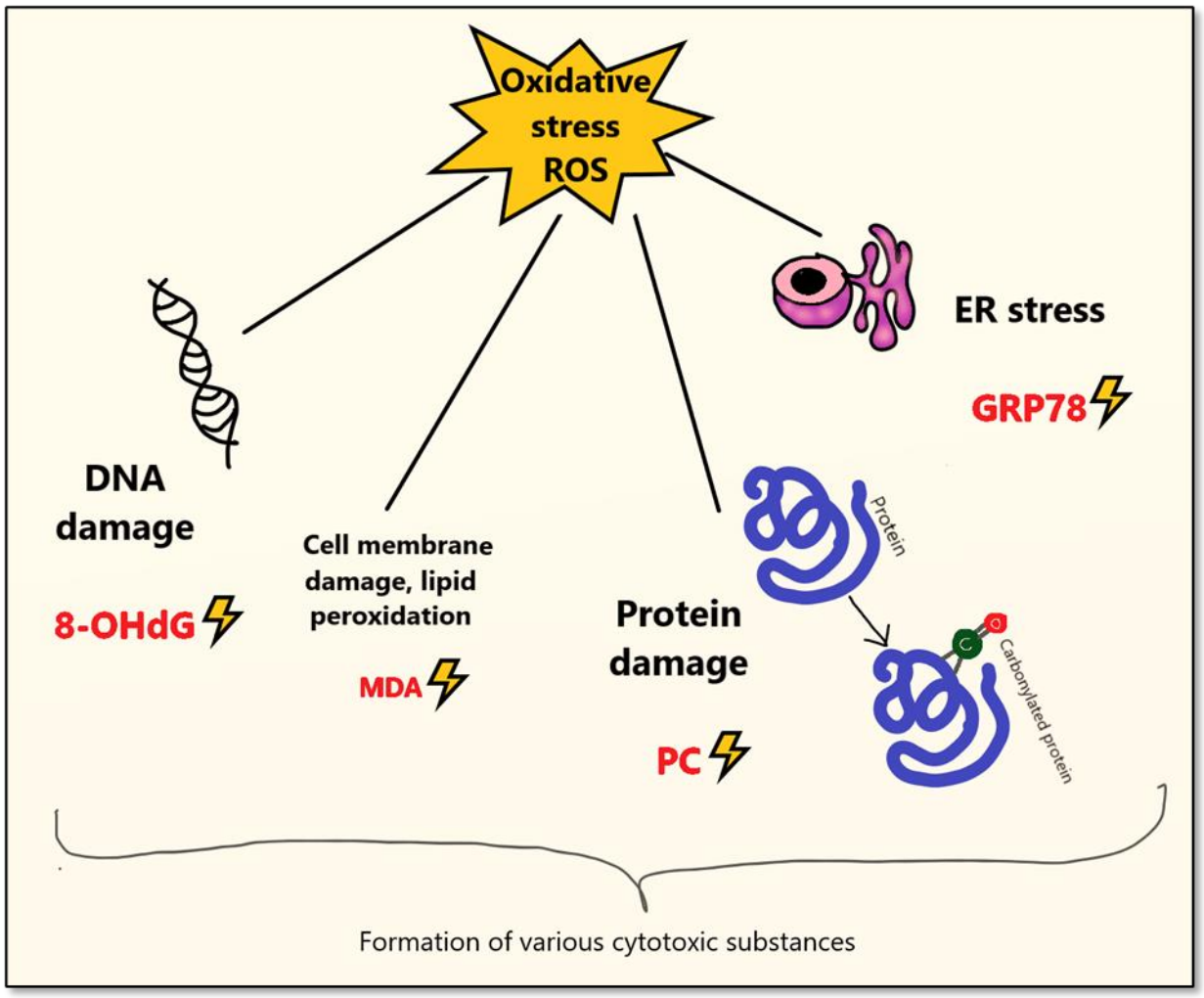

Fig.5.: Formation of various cytotoxic substances during oxidative stress. ROS=reactive oxygen species, ER=endoplasmic reticulum, 8-OHdG=8-hidroxy-2 deoxyguanosine, MDA=malondialdehyde, GRP78=glucose-regulated protein 78, PC=protein carbonyls (source: own editing)

Oxidative stress may have an important role in the bladder of individuals with interstitial cystitis due to hypoxia or the inflammatory character of the illness, as inflammatory stimuli are able to produce ROS. In addition, the oxidative stress can further increase the amount of inflammatory mediators, and also the permeability of the cells due to membrane damage (Forrester et al., 2018; Homan et al., 2013; Miyata et al., 2019; Treutlein et al., 2012). To the best of our knowledge, there is no research to suggest that elevated NE concentrations in cats with FIC can induce oxidative stress in the bladder, although Schralm and colleagues have described that peripherally elevated NE levels clearly led to increased oxidative stress in the blood of rats, therefore the investigation of this factor is essential for a deeper understanding of the biochemical background of the disease (Schraml et al., 2007).

2.4. Importance of *in vitro* cell culture models

Studies on living organisms are called *in vivo* studies, which have the great advantage of being able to monitor the effects of the experiment, and possibly unexpected side effects, by looking at the response of the whole organism in action. On the other hand, they have the disadvantage that the range of experiments is limited by animal welfare considerations, and that it is difficult to study certain mechanisms, since the organism is subject to the interplay of numerous processes. To overcome these problems, *in vitro* studies are used to examine an organ or tissue part, isolated or cultured cells, outside the body. By this method a mechanism can be studied in isolation from the rest of the body's metabolic processes, to determine whether a response to an external influence is the result of an independent, autonomous function of the organ or tissue (Kapałczyńska et al., 2018). It can therefore be said that *in vitro* experiments have many advantages over *in vivo* experiments, such as these testing methods are faster and cheaper, small amount of test material is needed and also small amount of toxic waste is produced. The variability between experiment is reduced as the chemical and physical environment are under tight control, the number of experimental animals is also reduced, and human cells and tissues also can be investigated by *in vitro* methods (Graudejus et al., 2018; Katt et al., 2016; Takhar & Mahant, 2011). However, it is not a complete replacement for *in vivo* studies, as the aim of *in vitro* experiments is usually to apply the results on living organism, although this requires that the extrapolability of the *in vitro* results to *in vivo* testing is demonstrated (Kapałczyńska et al., 2018).

Currently, primary cell cultures, which can be obtained directly from the tissue or organ to be investigated, or immortalized cell lines, which are commercially available, can be used for *in vitro* experiments (Burdall et al., 2003; Kaur & Dufour, 2012; Stacey, 2001). A major advantage of primary cell cultures is that they mimic the *in vivo* cells both phenotypically and genotypically better, allowing more accurate assays depending on the cell type. However, cells derived from living organisms typically contain multiple cell types depending on the source organ, making it important to use appropriate and accurate isolation protocols that allow the separation of the required cell types. In addition to isolation difficulties, another disadvantage of primary cell cultures compared to cell lines is that the lifespan and shelf life of primary cells is much shorter than those of cell lines (Dutton et al., 2019; Kapałczyńska et al., 2018; Kaur & Dufour, 2012; Stacey, 2001). However, cell lines are cost effective, easy to work with, contain pure population of cells, but there are some disadvantages compared to the primary cell cultures. Cell lines are usually genetically manipulated, which can alter their phenotype and response to stimuli, which can be further enhanced by continuous passaging, resulting in cell lines that do not adequately represent the primary cells and may give different results (Kaur & Dufour, 2012). Another major disadvantage associated with cell lines

is contamination with other cell lines and Mycoplasma (Hay et al., 1989). Cross-contamination of cell lines was first described by Walter Nelson-Rees in the early 1970s. He described that at that time most of the cell lines used worldwide and distributed by cell banks were contaminated with HeLa cells, which still causes serious problems today (Capes-Davis et al., 2010; Nelson-Rees et al., 1981). In addition, Mycoplasma contamination can persist undetected in cell cultures for long periods of time and cause extensive changes in gene expression and cell behavior (Hay et al., 1989; Kaur & Dufour, 2012). Taken together, great care should be taken when using cell lines and primary cell cultures should be included and preferred over cell lines in experiments where it is possible.

Cell cultures can be prepared and maintained in culture dishes, where cells are attached to a glass or plastic culture dish under adherent conditions, or in suspension, which – depending on the cell type, for example in the case of lymphocytes – can be more suitable for mimicking the natural environment of the cells (Ryan, 2008). The most commonly used cell culture type is the 2D model, but recently the 3D culture method has become increasingly popular due to the further benefits of 3D cultures to better mimicking the original *in vivo* conditions (Kapałczyńska et al., 2018). However, 3D cell cultures have some limitation also, for example the applied medium may not be able to deliver the appropriate substrates needed for survival to the cells in the middle of the 3D structure (Habanjar et al., 2021). Although there are some disadvantages regarding the 2D cell cultures, for example the cells may not behave exactly like in their tissue environment, they are still widely used, because it is relatively easy and cost effective to create and maintain them and most of the experiments can be easily carried out on 2D cultures (Kapałczyńska et al., 2018; Pampaloni et al., 2007). A cell culture can consist of one cell type, in which case it is called a monoculture, or different type of cells can be grown together in the same environment, called a co-culture. These co-cultures are excellent for studying intercellular relationships between cell or tissue types (Lawrence et al., 1978; Paschos et al., 2015). In summary, the appropriate cell culture model (2D, 3D; monoculture, co-culture) should be selected according to the objective of the experiments, and the results obtained with each model should be studied together and compared with each other.

The development of uroepithelial cell cultures started in the previous century. One of the first working groups was Eliot et al. who studied cell outgrowth from mammalian uroepithelial explants. Although, the cells were epithelial like, they did not have uroepithelial properties (Elliott et al., 1975). Chlapowski and Haynes were the first group to culture uroepithelial cells from rat origin that showed many morphological similarities with native tissues, including asymmetric unit membrane (AUM), tight junction formation and cytoplasmic vesicles (Chlapowski & Haynes, 1979), and a few years later Reznikoff and colleagues described a protocol for culturing human uroepithelium (Reznikoff et al., 1983). It was only in 1990 that

markers of uroepithelial differentiation became available. Surya and colleagues established a bovine uroepithelial cell culture and used a recently developed antibody (AE31) to show that the cells synthesize Uroplakin I., a component of AUM, but no data were available on TER values (Surya et al., 1990). Truschel et al. were the first to establish a rabbit-derived primary bladder epithelial cell culture containing differentiated umbrella cells, which were confirmed by uroplakin detection, and these cultured cells formed a tight monolayer with high TER values (Truschel et al., 1999). The first human uroepithelial cell line was developed by Perrone et al., who gained the cells from a patient with interstitial cystitis (Perrone et al., 1996). Birder et al. created and used primary uroepithelial cell culture from feline origin, however these cells were cultured only for 3 days, and uroepithelial cells need at least 4 days to differentiate *in vivo* (Birder et al., 2003; Truschel et al., 1999). Therefore, to create a useful tool for more accurate in vitro studies of bladder diseases, it seems necessary, culturing the cells with prolonged maintenance to establish a feline primary bladder epithelial cell culture containing differentiated uroepithelial cells. **(Table I.)**

Cell viability and cytotoxicity tests are essential for the reliability and accurate interpretation of results in *in vitro* experiments. These tests can be used in order to determine the cell death caused by different factors such as mechanical stimuli during cell isolation or after different treatment procedure, and they can be classified according to the type of measurement as follows: dye exclusion (such as Trypan blue, erythrosin B and eosin tests), colorimetric assays (such as CCK-8, LDH, MTT, MTS and neutral red uptake tests), fluorometric (alamarBlue assay) and luminometric (ATP assay) assays (Aslantürk, 2018). The proportion of viable cells in a cell suspension can be determined by Trypan blue staining. The principle of the trypan blue dye exclusion test is that the membrane of dead cells is disrupted, allowing the dye to enter the cytoplasm, while the intact membrane of healthy cells excludes the dye. During microscopic examination, the cytoplasm of the dead cells is blue, while the cytoplasm of living cells remains clear (Strober, 2015). CCK-8 test is a colorimetric assay which is suitable for measuring cell viability, cell proliferation and real time metabolic activity of the cultured cells. During the test a water-soluble tetrazolium salt reduced by nicotinamide adenine dinucleotide (phosphate) NAD(P)H to an orange-colored product formazan. The amount of the produced formazan dye is correlated with the concentration of the produced NAD(P)H and by this with the number of living cells (Aslantürk, 2018; Cai et al. 2019). NAD and NADP are important biological molecules serving as cofactors of dehydrogenase enzymes in different reactions essential for cellular metabolism, protection against oxidative stress, and cell death. CCK-8 test is able to measure the dehydrogenase enzymatic activity, therefore suitable to examine also the metabolic activity of the cells (Chamchoy et al., 2019). LDH assay is a colorimetric method to investigate cytotoxicity. In physiological conditions LDH is present only in the cytoplasm of the cells, but

in case of cell membrane damage it is released in high amount in the cell culture medium, therefore by measuring the extracellular LDH activity, information can be obtained on the cytotoxicity of the applied treatment procedure. (Legrand et al. 1992).

Table I.: Comparison of some uroepithelial cell culture based on their differentiation and permeability properties (ND=not investigated)

Work Group	Species	Uroepithelial cell differentiation marker (Uroplakin expression)	Tight junction protein expression	TER value (Ωcm^2)
Elliot et al. (Elliott et al., 1975)	Multiple	**No**	**No**	**ND**
Chlapowski et al. (Chlapowski & Haynes, 1979)	Rat	**No**	**Yes**	**ND**
Reznikoff et al. (Reznikoff et al., 1983)	Human	**No**	**Yes**	**ND**
Surya et al. (Surya et al., 1990)	Bovine	**Yes**	**Yes**	**ND**
Perrone et al. (Perrone et al., 1996)	Human	**ND**	**Yes**	**500-100**
Truschel et al. (Truschel et al., 1999)	Rabbit	**Yes**	**Yes**	**8000≤**
Birder et al. (L. A. Birder et al., 2003)	**Feline**	**ND**	**ND**	**ND**

3. Significance and aims of the study

As feline lower urinary tract disease caused by feline idiopathic cystitis (FIC) is one of the most common diseases in domestic cats, and the exact pathomechanism of the illness is not well-understood, investigation the biochemical background of the pathomechanism of the disease can be of special importance, possibly reflecting key points for the link between stress and inflammatory responses, redox homeostasis and barrier integrity in cultured feline uroepithelial cells. Since FIC is a complex multifactorial disease, a primary uroepithelial cell culture from feline origin would be a very useful tool to study the effect of different factors separately or in targeted combination.

Therefore, one of the main objectives of the current PhD thesis was to establish and characterize a novel primary uroepithelial cell culture of feline origin containing differentiated uroepithelial cells, which could serve as a useful tool for future studies not only regarding FIC but also concerning other urinary tract diseases. To demonstrate the uroepithelial origin and differentiation of the cultured cells, the cells needed to be characterized by immunofluorescence stainings, applying specific antibodies (pan-cytokeratin and uroplakin III.) **(Study I.).**

Furthermore, the direct molecular effect of a short term (1 h) NE exposure on the metabolic activity, oxidative status (MDA, PC, 8-OHdG, GRP78), pro-inflammatory cytokine production (IL-6 and SDF-1), and barrier function (GAG, Claudin-4, TER) of the cultured cells were aimed to be assessed on the newly established primary uroepithelial cell cultures mimicking acute sympathetic stress *in vitro* **(Study II.) (Fig.6.).**

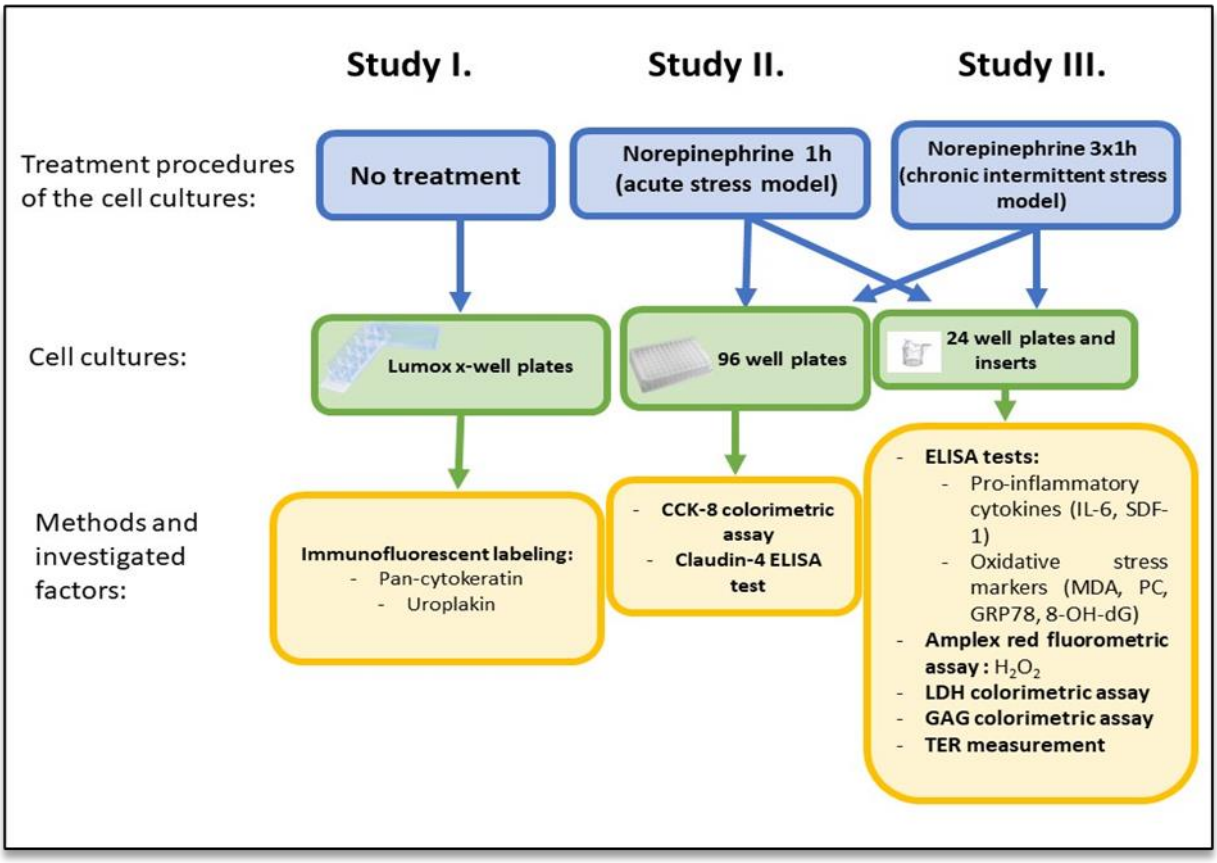

Fig.6.: Summary of the experimental design of studies I., II. and III.: treatments and investigated factors in the newly established primary uroepithelial cell culture of feline origin

Finally, a further aim of this present PhD study was to examine the pathological role of the chronic intermittent stress in the pathogenesis of FIC, by exposing the uroepithelial cell cultures to pulsative, 3x1 h NE treatment. Similarly, like in **Study II.** the oxidative status (MDA, H_2O_2), the pro inflammatory cytokine production (IL-6, SDF-1) and the barrier integrity of the cultured monolayer (GAG, Claudin-4, TER) were investigated **(Study III.) (Table II.)**.

Study II. can provide information on whether an acute stress has a direct molecular effect on bladder epithelial cells, and thus whether it has an effect on the characteristic symptoms that develop during FIC. As the recurrence of the disease is very common, **Study III**. can provide detailed information on the molecular effects of intermittent release of NE in cats with FIC on bladder uroepithelial cells, in particular with regard to inflammation, oxidative stress and permeability. The two experiments (**Study II. and Study III.)** can allow us to compare the effects of acute and chronic, intermittent stress, and their role in the pathogenesis of FIC.

Table II.: Overview of the performed studies

Study No.	Applied cell culture and treatment procedure	Aim of the study	Laboratory analyses (measured parameters)
Study I.	Feline primary uroepithelial cell culture	Development and characterization of novel feline primary uroepithelial cell culture model	Characterization with Giemsa staining and immunocytochemistry (pan-cytokeratin and uroplakin III)
Study II.	Feline primary uroepithelial cell culture, short term (1h) NE exposure	Investigation on the molecular effects of acute stress on uroepithelial cells	Metabolic activity (CCK-8), extracellular LDH release, redox parameters (MDA, PC, 8-OH-dG, GRP78), pro-inflammatory cytokine concentration (IL-6, SDF-1), barrier function parameters (GAG, claudin-4, TER)
Study III.	Feline primary uroepithelial cell culture, intermittent, pulsative (3x1 h) NE exposure	Investigation on the molecular effects of chronic, intermittent stress on uroepithelial cells, and comparing the effects of acute and chronic stress in the pathogenesis of FIC	Metabolic activity (CCK-8), extracellular LDH release, redox parameters (MDA, H_2O_2), pro-inflammatory cytokine concentration (IL-6, SDF-1), barrier function parameters (GAG, claudin-4, TER)

Summarized, the most important aims of this PhD study were:

Ad 1, to develop and characterize a novel primary uroepithelial cell culture from feline origin, which contains differentiated uroepithelial cells.

Ad 2, to examine the possible effects of acute sympathetic stress on metabolic status, oxidative stress, pro-inflammatory processes and barrier integrity of the uroepithelial cells *in vitro*, applying the established cell culture model.

Ad 3, to examine the pathological role of chronic intermittent stress mimicked by 3x1 NE treatment in the pathogenesis of FIC, and the comparison of the effects of acute and chronic stress in case of oxidative stress, pro-inflammatory processes and barrier integrity of the uroepithelial cells.

4. Material and methods

4.1. Ethic statement

In **Study I.**, in order to set up the cell isolation and separation procedure, some preliminary measurements were carried out using one cat in each trial (three totally). For the characterization of the cells gained by the finally established method (with Giemsa staining and immunocytochemistry) and to study the molecular effects of stress (**Study II. and Study III**.) the uroepithelial cells were gained from one cat in each trial. The cats were between 2-6 years old male European short haired breed, and they were euthanized at independent veterinary clinics, they did not suffer from urinary tract disease and the carcasses were donated by their owners for scientific purposes. The study was approved by the Local Animal Welfare Committee of the University of Veterinary Medicine, Budapest on 30 March 2018. and it was confirmed that no ethical approval is required for this study.

4.2. Chemicals and reagents

Unless specified otherwise, all chemicals were purchased from Merck KGaA (Darmstadt, Germany).

4.3. Study I.

4.3.1. Isolation and culturing of epithelial cells from cat bladder

A urinary bladder was obtained from a euthanized European short hair cat. The cat did not suffer from any urinary tract disease, and the cadaver was offered by the owner for scientific purposes. Immediately after the euthanasia, a median laparotomy was performed, the bladder was excised and rinsed with Krebs solution (VWR, Radnor, PA, USA) supplemented with 11.1 mM glucose, pH 7.4. Then the bladder was placed in the same Krebs solution at 4 °C and transported to the laboratory within 4 hours. After carefully removing excess adipose tissue and the smooth muscle layer, the stretched bladder was placed in sterile minimal essential medium (MEM) containing 2.5 mg/mL dispase, 1% penicillin/streptomycin/fungizone, and 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) at pH 7.4 and incubated overnight at 4 °C. Thereafter, the bladder was placed in a sterile Petri dish containing 20 mL trypsin-EDTA solution at 37 °C (0.25% trypsin and 1 mM EDTA). The epithelial layer was carefully separated from the connective

tissue **(Fig.7.)**, and the epithelial cells were dissociated by trypsinization at 37 °C for 30 min under continuous stirring. In order to remove non-uroepithelial cells from the cellsuspension, the freshly gained cell isolate was filtered through three layers of sterile gauze into a sterile centrifuge tube and brought up to 50 mL with MEM containing 5% fetal bovine serum (FBS), 1% penicillin/streptomycin/fungizone, and 20 mM HEPES at pH 7.4, followed by centrifugation at 120 g for 5 min. The supernatant was carefully discarded, and the cells were resuspended in 20 mL of the same FBS-containing MEM solution and centrifuged two additional times with the same parameters. For the third centrifugation, cells were resuspended and washed in 20 mL of defined keratinocyte medium (Thermo Fisher, Waltham, MA, USA) supplemented with 0.5% gentamicin and 1% fungizone. After the final centrifugation, the viability of the cells was examined by a trypan blue exclusion test and cell counting was performed using a Bürker's chamber. If the viability of the cells exceeded 80%, they were considered suitable for culturing, and the suspension was diluted with defined keratinocyte medium to yield the desired standard concentration (750.000 cells/mL).

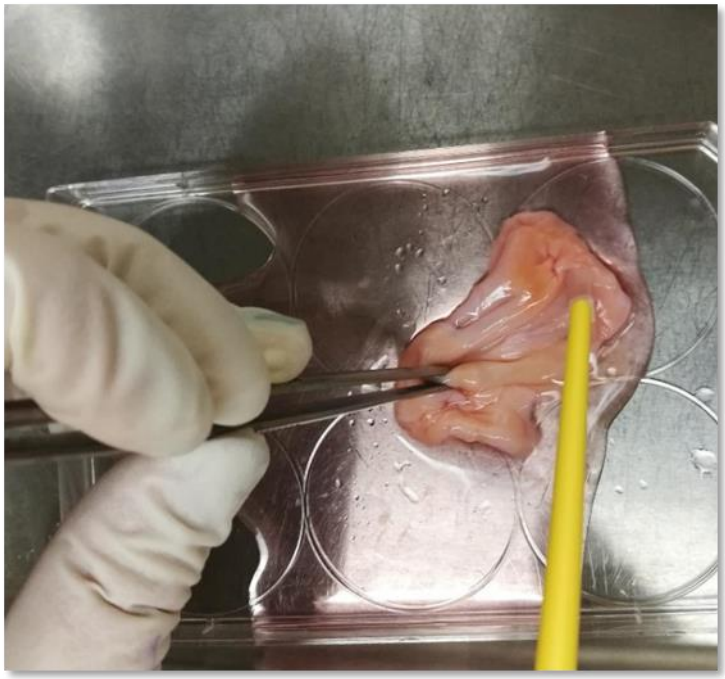

Fig.7.: Separating the uroepithelial layer from the connective tissue of a feline urinary bladder with a sterile cell-scraper

To perform Giemsa staining, cells were plated on 24-well plates (Greiner Bio-One, Frickenhausen, Germany), and the seeding volume was set as 0.6 mL cell suspension/well. Further, 8-well Lumox x-well plates (Sarstedt, Nümbrecht, Germany; seeding volume: 0.3 mL cell suspension/well) were used for the immunofluorescent staining. All cell culture plates were previously coated with collagen type IV according to the manufacturer's instructions.

The cells were cultured for 6 days in the presence of 5% CO_2 at 37 °C. The culture medium was changed 48 h after seeding and then every 24 h.

4.3.2. Characterization of the cell culture

Cell morphology and the confluency of the cell cultures were examined by Giemsa staining on day 1 and 6 of culturing, while immunofluorescent labeling was used to verify the uroepithelial origin of the cells also on day 1 and 6 of culturing. For the Giemsa staining, the cells were washed with phosphate buffered saline (PBS) and fixed in 10% PBS-formalin solution at room temperature for 10 min. Then the cells were covered with Giemsa dye at room temperature for 30 min and washed with distilled water. For the latter examinations, cultures were fixed in a mixture of 30% acetone and 70% methanol at −20 °C for 10 min. The cells were washed three times with PBS and then they were incubated in a blocking solution containing 3% bovine serum albumin (BSA) in PBS at room temperature for 30 min. To confirm the epithelial origin of the cells, eFluor labeled pan-cytokeratin antibody (Cat. No. 41-9003-82, Thermo Fisher, Waltham, Massachusetts, USA) was used. The cultures were incubated in PBS solution containing 1% BSA and pan-cytokeratin antibodies in a dilution of 1:200 for 1 h at room temperature. Then, to justify the uroepithelial origin of the cells by specific marker of uroepithelial differentiation, they were stained with fluorescein isothiocyanate (FITC) labeled feline-specific uroplakin III. antibody (Cat. No. Ac-12-00200-12, Abcore, Ramona, CA, USA). The antibody was used at a 1:500 dilution dissolved in PBS containing 1% BSA, the cultures were incubated for 1 h at room temperature. Thereafter, the cells were washed with PBS, and diamidino phenylindole (DAPI) containing mounting medium was used for staining the cell nuclei. The samples were examined by an Olympus CXK-41 type microscope (OLYMPUS, Tokyo, Japan), equipped with a Canon Eos 1100D camera (Canon, Tokyo, Japan). The ImageJ software (Center for Information Technology National Institutes of Health, Bethesda, MD, USA) was used to analyze the images.

4.4. Study II. and Study III.

4.4.1. Cell isolation and culturing conditions

In **Study II. and III.** cell isolation process and culturing conditions have been carried out in accordance with the developed methodology explained in **Study I.** For monitoring pro-inflammatory mediators (IL-6 and SDF-1), oxidative stress parameters (MDA, 8-OHdG, PC, GRP78), cell injury (lactate dehydrogenase, LDH) and GAG concentration, cells were plated on 24-well plates (Greiner Bio-One, Frickenhausen, Germany), and the seeding volume was set as 0.6 mL cell suspension/well. Further, 96-well plates (Greiner Bio-One, Frickenhausen, Germany; seeding volume: 0.2 mL cell suspension/well) were applied for assessing the

cellular metabolic activity (by the CCK-8 assay) and barrier function by claudin-4 abundance. For measuring the TER, cells were seeded on 24-well, high-density polyester membrane inserts (Greiner Bio-One, Frickenhausen, Germany; pore size: 0,4 µm, seeding volume: 0.2 mL cell suspension in the upper chamber and 0.4 mL defined keratinocyte medium in the bottom well). The cells were cultured in the presence of 5% CO_2 at 37°C for 6 days. The culture medium was changed 48 h after seeding and then every 24 h.

4.4.2. Treatments of the cell cultures

In **Study II.** after 6 day of culturing, cells on 24-well, 96-well plates and on 24-well membrane inserts were exposed to NE dissolved in defined keratinocyte medium at concentrations of 10, 100, and 1000 µM at 37 °C for 1 h (n=3/group on 24-well plates and n=6/group on 96-well plates), followed by a 24 h regeneration time (culturing without NE supplementation). Thereafter, cell culture supernatants were collected from 24-well plates, and these cells were lysed by using M-PER lysis buffer (Thermo Fisher, Waltham, MA, USA). For preparing the cell lysate, 300 µL M-PER reagent was added to each well of the cultures, and the cells were scraped after 5 min shaking with a sterile cell scraper and collected into Eppendorf tubes. The culture media and the lysates were stored at −80 °C until further examinations.

In **Study III.** the cell cultures were challenged to NE dissolved in defined keratinocyte medium at the same concentrations (10, 100, and 1000 µM) as in **Study II**. at 37 °C, but instead of 1 h treatment the NE solutions were applied 3x1 h to mimic *in vitro* chronic recurrent stress (n=3/group on 24-well plates and n=6/group on 96-well plates). Between the pulsative NE treatments simple defined keratinocyte medium without NE was added to the cultures for 1 h at 37°C. The last dose of NE treatment was followed by a 24 h regeneration time (culturing without NE supplementation). After all, similarly as in **Study II.**, the supernatants of the cultures from the 24-well plates were collected, and the cells were lysed with M-PER reagent and collected to Eppendorf tubes. The samples were also stored at -80°C until further examination.

4.4.3. Laboratory analyses

4.4.3.1. Assessment of cellular metabolic activity and cell injury

The evaluation of the metabolic activity of the cultures on 96-well plates was performed using the CCK-8 test according to the instructions of the manufacturer, adding 100 µL fresh culture medium and 10 µL CCK-8 reagent to each well. The reagent contained WST-8 (water soluble tetrazolium salt), which could be reduced by cellular dehydrogenase enzymes to orange-colored formazan, and the resulting color of the media was read at 450 nm by a Multiskan GO 3.2. reader (Thermo Fisher, Waltham, MA, USA).

To investigate the rate of plasma membrane damage caused by cell injury, the activity of lactate dehydrogenase (LDH) in cell culture media (the so called LDH release) was measured by a specific enzyme kinetic photometric assay (Diagnosticum Ltd., Budapest, Hungary) following the steps of the manufacturer's protocol. 10 µl cell culture medium and 200 µl working reagent (containing 56 mM phosphate buffer, pH=7.5; 1.6 mM pyruvate, and 240 µM $NADH+H^+$) were mixed and then the absorbance was measured at 340 nm six times in one-minute intervals during incubation at 37ºC using a Multiskan GO 3.2. reader. The enzyme activity was calculated based on the mean of the absorbance differences between consecutive time points.

4.4.3.2. Measurement of IL-6 and SDF-1 concentrations

IL-6 and SDF-1 concentrations were measured from both cell culture media and cell lysates. Concentrations were assayed using feline-specific IL-6 and SDF-1 ELISA kits (Cat. No. MBS085030 and MBS049100, MyBioSource, San Diego, CA, USA) according to the instructions of the manufacturer. The steps of the ELISA measurements were the same in case of both the IL-6 and SDF-1 detection. Briefly, 100 µL of horseradish peroxidase (HRP) conjugate reagent was added to 50 µL of sample solutions, followed by 60 min incubation at 37ºC. The wells were then washed four times, using 100 µL of washing solution per well, then 50 µL of Chromogen solution A and Chromogen solution B were added to each well and after 15 min incubation at 37ºC, the reaction was stopped with 50 µL of stop solution per well. After 5 min the resultant color was read at 450 nm using a Multiskan GO 3.2 reader.

4.4.3.3. Assessment of the redox state of the cells

As a marker of lipid peroxidation, MDA was measured from the cell culture media (Study II.) or cell lysate (Study III.) with a specific colorimetric test. According to the protocol, 300 µL freshly prepared thiobarbituric acid (TBA) stock solution was mixed with 100 µL cell culture media (Study II.) or cell lysate (Study III). Solutions were incubated at 95°C for 1 h, followed by 10 min cooling on ice. The absorbance of the samples was read at 532 nm with a Multiskan GO 3.2. reader.

Protein damage caused by oxidative stress was examined with a Protein Carbonyl ELISA Kit (Cat. No. MBS2600294, MyBioSource, San Diego, CA, USA) by measuring the protein carbonyl (PC) content of the cell lysate. To monitor the oxidative DNA damage, 8-OHdG concentration was assayed from the cell lysate with a specific 8-OHdG ELISA kit (Cat. No. MBS808265, MyBioSource, San Diego, CA, USA). The measurements were carried out according to instructions of the manufacturer's protocol. The absorbances of the samples were read at 450 nm by a Multiskan GO 3.2. reader.

As a marker of endoplasmic reticulum stress, a chaperone protein – GRP78 – was measured from cell lysate with a feline-specific ELISA kit (Cat. No. MBS072358, MyBioSource, San Diego, CA, USA), based on the instructions of the manufacturer's protocol. The absorbance was read at 450 nm via a Multiskan GO 3.2 reader.

The extracellular H_2O_2 concentration was monitored by Amplex Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. 50 µl Amplex Red working solution (composed of 100 µmol/l Amplex Red and 0.2 U/ml horseradish peroxidase) was measured into 50 µl cell culture media, and after 30 min incubation at room temperature the resultant fluorescence was measured by a Victor X2 2030 fluorometer (λex=560 nm; λem=590 nm).

4.4.3.4. Investigation of epithelial barrier function

The GAG concentration of the cell culture media was measured by a Blyscan sulfated glycosaminoglycan assay kit (Biocolor, Carrickfergus, UK) based on the instructions of the manufacturer. First, 50 µL of cell culture media was added to 1 mL of Blyscan dye reagent (containing 1, 9-dimethylmethylene blue) and incubated for 30 min during continuous mixing, followed by centrifugation (1300× g for 10 min). The supernatant was carefully discarded, and the bounded dye was released from the precipitate by a dissociation reagent. Thereafter 200 µL of the samples containing dissolved dye were transferred to a 96-well microplate and the absorbances were read by a Multiskan GO 3.2. reader at 656 nm.

In order to investigate the epithelial integrity, claudin-4 content of the cells was investigated by a feline-specific colorimetric cell-based ELISA kit (Cat. No. MBS070256, MyBioSource, San Diego, CA, USA). The relative amounts of claudin-4 were measured directly in cultured cells on a 96-well plate according to the instructions of the manufacturer's protocol. The absorbance was read at 450 nm by a Multiskan GO 3.2. reader.

For the examination of the permeability of the uroepithelial cell layer, the TER measurement of cultures on 24-well membrane inserts was carried out by a EVOM2 epithelial Volt/Ohm meter (World Precision Instruments, Sarasota, Florida, USA). The TER was measured directly after NE treatment, and also after 24 h of regeneration time.

4.4.4. Statistical analysis

For statistical analysis, R v. 4.2.2 (R Core Team, 2022) software was used. Differences between means were determined by one-way ANOVA, and Dunnett's post-hoc tests were used for pairwise comparisons. Data were checked for normal distribution by Shapiro-Wilk test before the statistical analysis was performed. Correlations between different variables were assessed using Pearson's correlation test. Differences were considered to be significant at $p<0.05$. All results are expressed as mean ± standard error of

the mean (details are shown in **Table S1** and **Table S2**). Principal component analysis (PCA) was performed with MetaboAnalyst 4.0 (https://www.metaboanalyst.ca).

5. Results

5.1. Study I.: Establishment and characterization of novel primary uroepithelial cell culture from feline origin

5.1.1. Characterization of the cell cultures by Giemsa staining

The cell morphology and the confluence of cell cultures were examined by Giemsa staining in the case of 1- and 6-day old cultures. On day 1, cells were adhered to the plates and started to multiply (**Fig.8.**), while, by day 6, the cell cultures grew to confluency (**Fig.9.**).

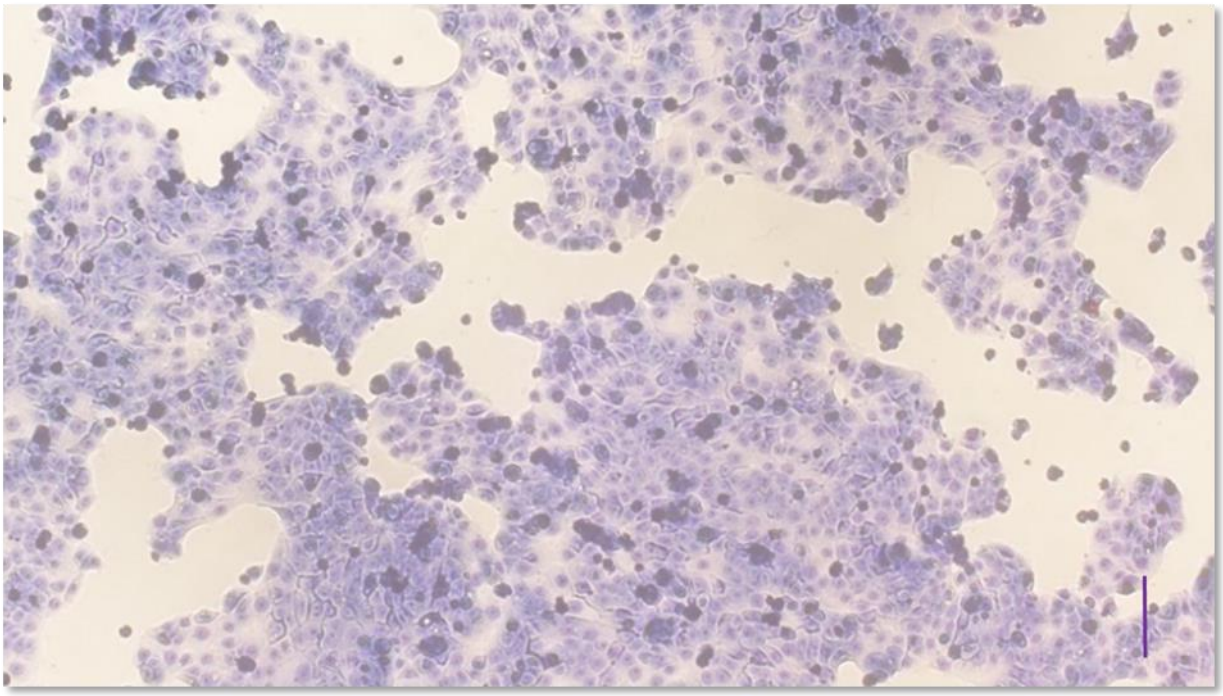

Fig.8.: Uroepithelial cell culture on day 1 after plating stained by Giemsa (bar=100 µm).

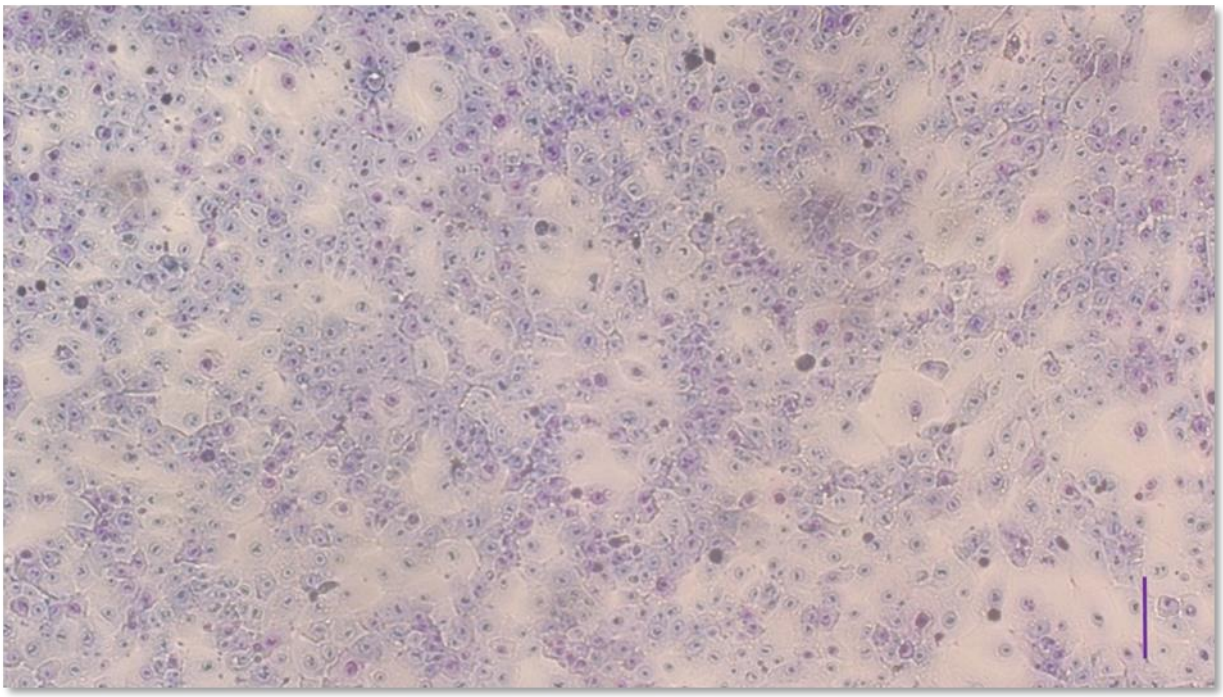

Fig.9.: Confluent uroepithelial cell culture on day 6 after plating stained by Giemsa (bar=100 µm).

5.1.2. Characterization of the cell cultures by immunocytochemistry

One day after plating, all the cells showed pan-cytokeratin positivity with eFluor labeled anti-pan-cytokeratin antibody, but no uroplakin III positivity was detected with FITC labeled antibody (**Fig.10.**). In the case of 6-day old cultures, all the cells showed pan-cytokeratin positivity, and the majority of the cells possessed uroplakin III positivity as well (**Fig.11.**).

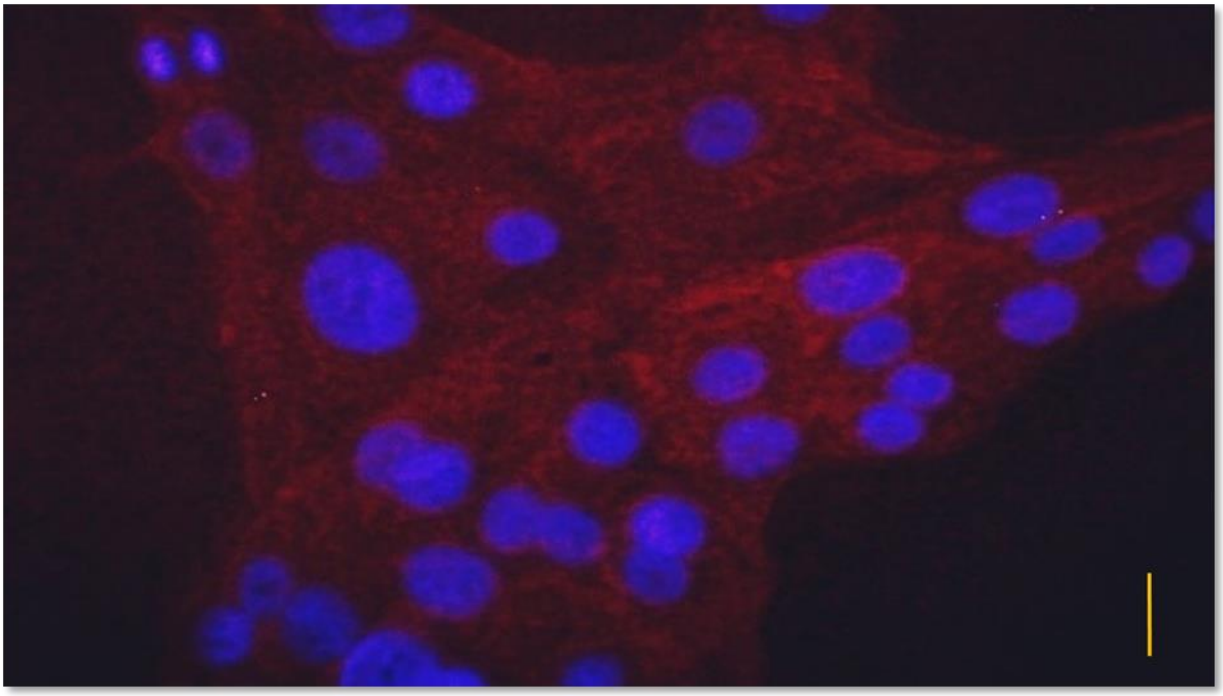

Fig.10.: Immunofluorescent staining of uroepithelial cell culture with eFluor labeled pan-cytokeratin antibody on day 1 after plating. Blue color indicates diamidino phenylindole (DAPI) labeled cell nuclei, while red color shows epithelial cells detected with eFluor labeled pan-cytokeratin antibody (bar=3.5 μm).

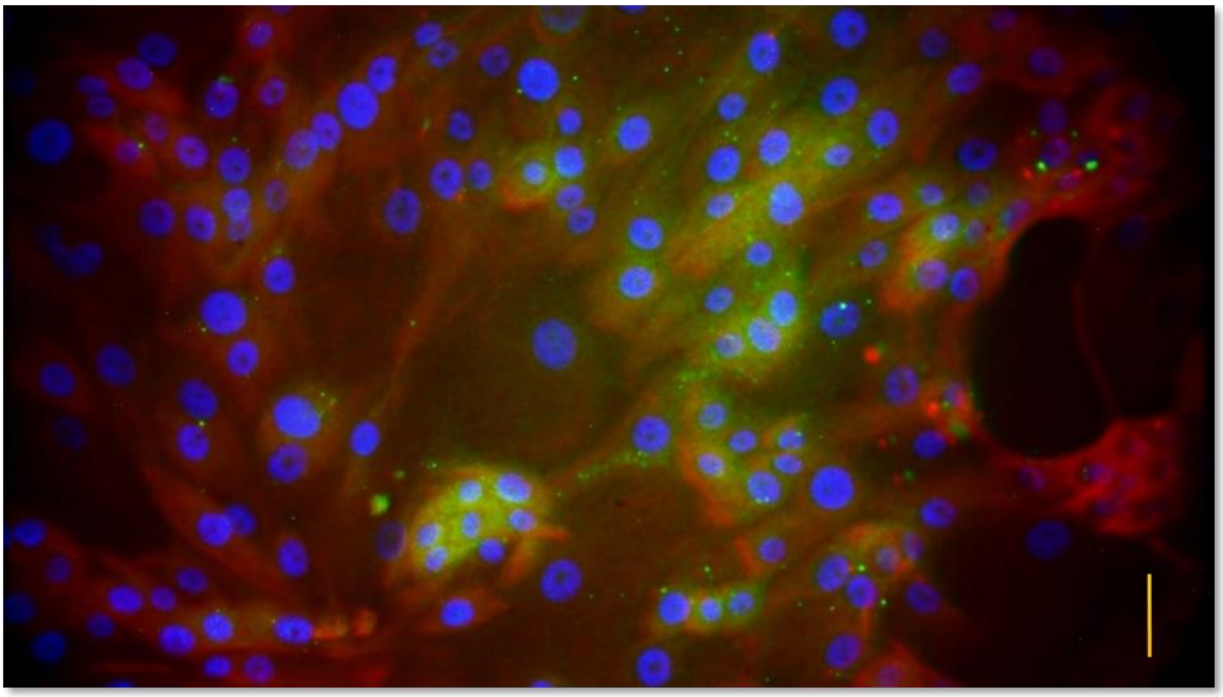

Fig.11.: Immunofluorescent staining of uroepithelial cell culture on day 6 after plating. Blue color indicates diamidino phenylindole (DAPI) labeled cell nuclei, red color shows epithelial cells detected with eFluor labeled pan-cytokeratin antibody, and green color refers to differentiated uroepithelial cells stained with fluorescein isothiocyanate (FITC) labeled uroplakin III antibody (bar=7 μm).

5.2. Study II.: Cellular effects of 1 h NE treatment modelling acute stress in feline uroepithelial cell cultures

5.2.1. Assessment of cellular metabolic activity and cell injury

The metabolic activity of the cells was significantly increased after 1 h 1000 µM NE treatment compared to the control group ($p<0.001$), while no significant changes were observed in groups with lower NE concentrations ($p=0.371$ and $p=0.056$ for 10 and 100 µM NE, respectively) (**Fig.12.**).

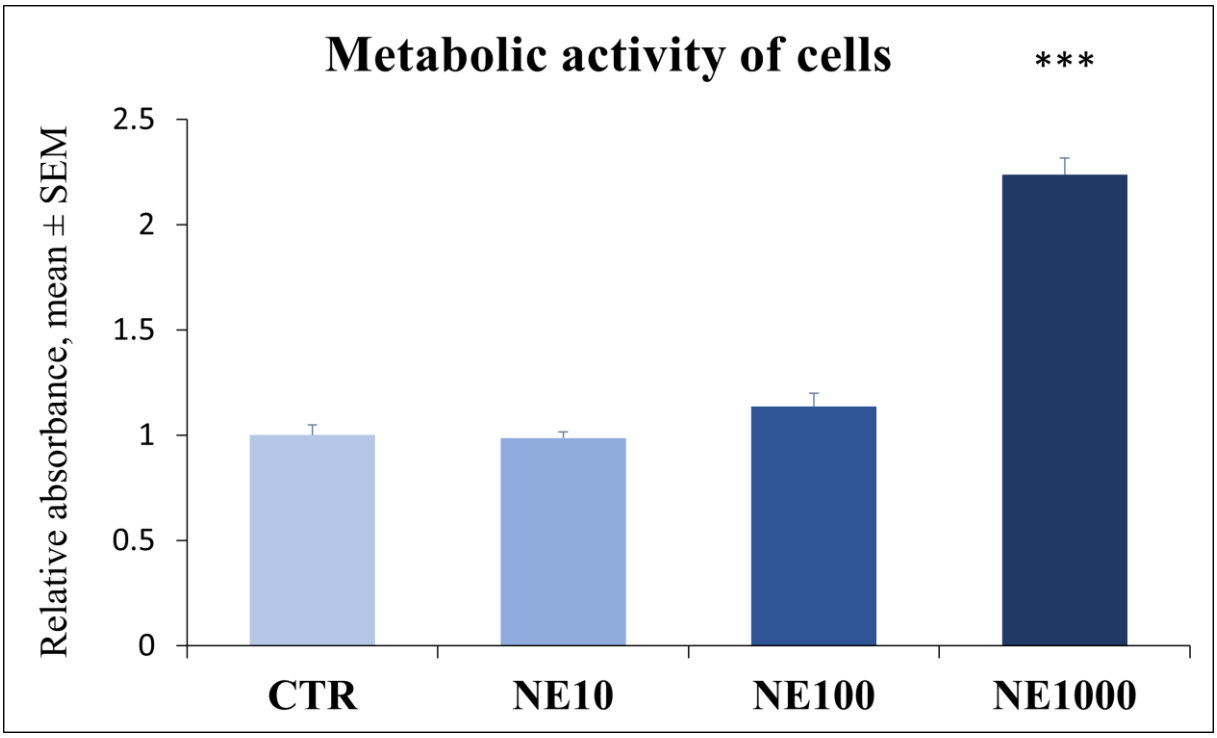

Fig.12.: Metabolic activity of cells after 1 h norepinephrine treatment. CTR=non-treated control cells, NE10=10 µM norepinephrine treated cells, NE100=100 µM norepinephrine treated cells, NE1000=1000 µM norepinephrine treated cells. n=6/group, results are expressed as mean±SEM, ***p<0.001.

There were no significant changes in extracellular LDH activity after NE exposures compared to the non-treated control cells (p=0.603, p=0.807, p=0.860 for 10, 100, and 100 µM NE, respectively) (**Fig.13.**).

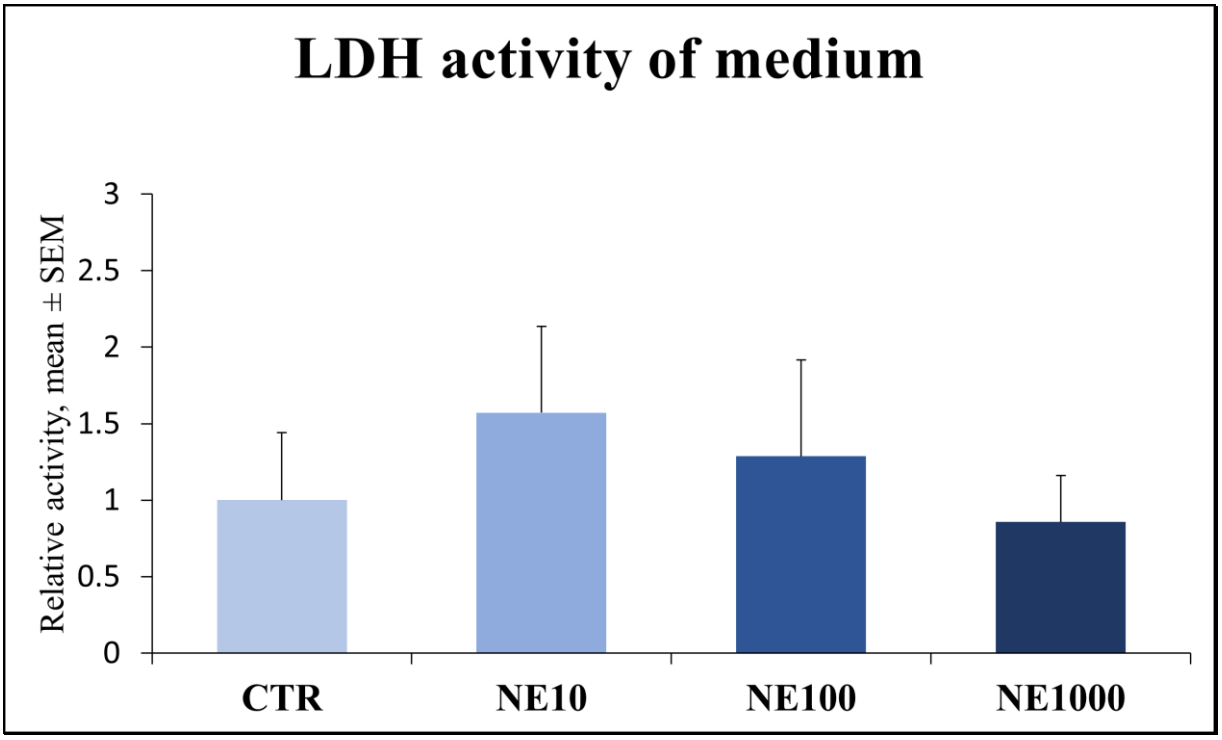

Fig.13.: Lactate dehydrogenase (LDH) activity in cell culture media after 1 h norepinephrine treatment. CTR=non-treated control cells, NE10=10 µM norepinephrine treated cells, NE100=100 µM norepinephrine treated cells, NE1000=1000 µM norepinephrine treated cells. n=3/group, results are expressed as mean±SEM.

5.2.2. Measurement of IL-6 and SDF-1 concentrations

In case of 1 h 1000 μM NE treatment, a significant increase in IL-6 level was detected in the cell culture medium (p=0.040), but no significant changes were observed in the extracellular IL-6 concentration concerning 10 and 100 μM NE treated cells compared to the control wells (p=0.670, p=0.665, respectively) **(Fig.14A).**

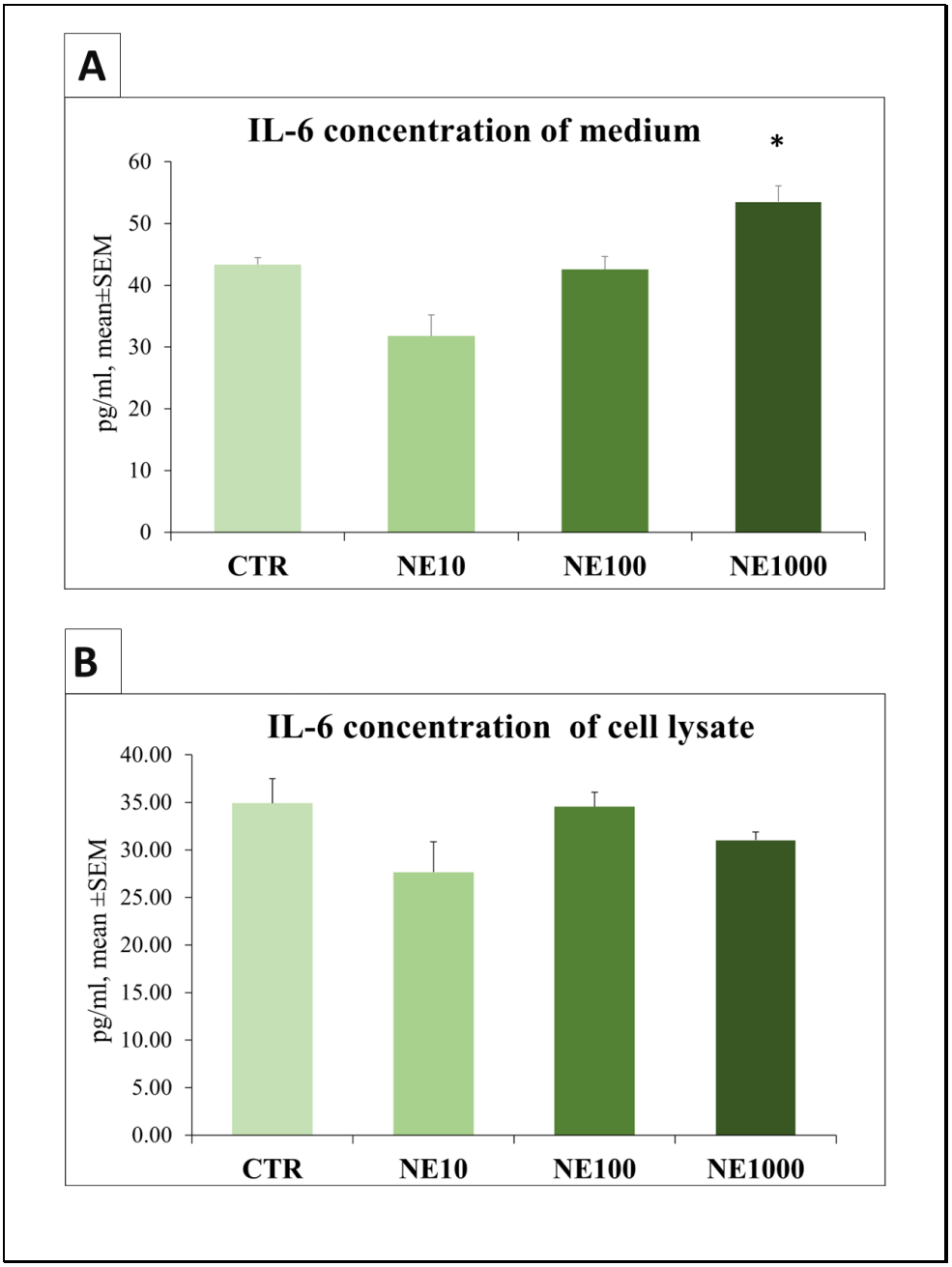

Fig.14.: Interleukin-6 (IL-6) concentrations in culture media (A) and cell lysate (B) after 1 h norepinephrine (NE) treatment. CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells. n=3/group, results are expressed as mean±SEM, * p<0.05.

The concentration of SDF-1 was significantly higher in the culture media of 10, 100, and 1000 μM NE exposed cells in comparison with the controls (p=0.012, p=0.009, p<0.001, respectively) (**Fig.15A**).

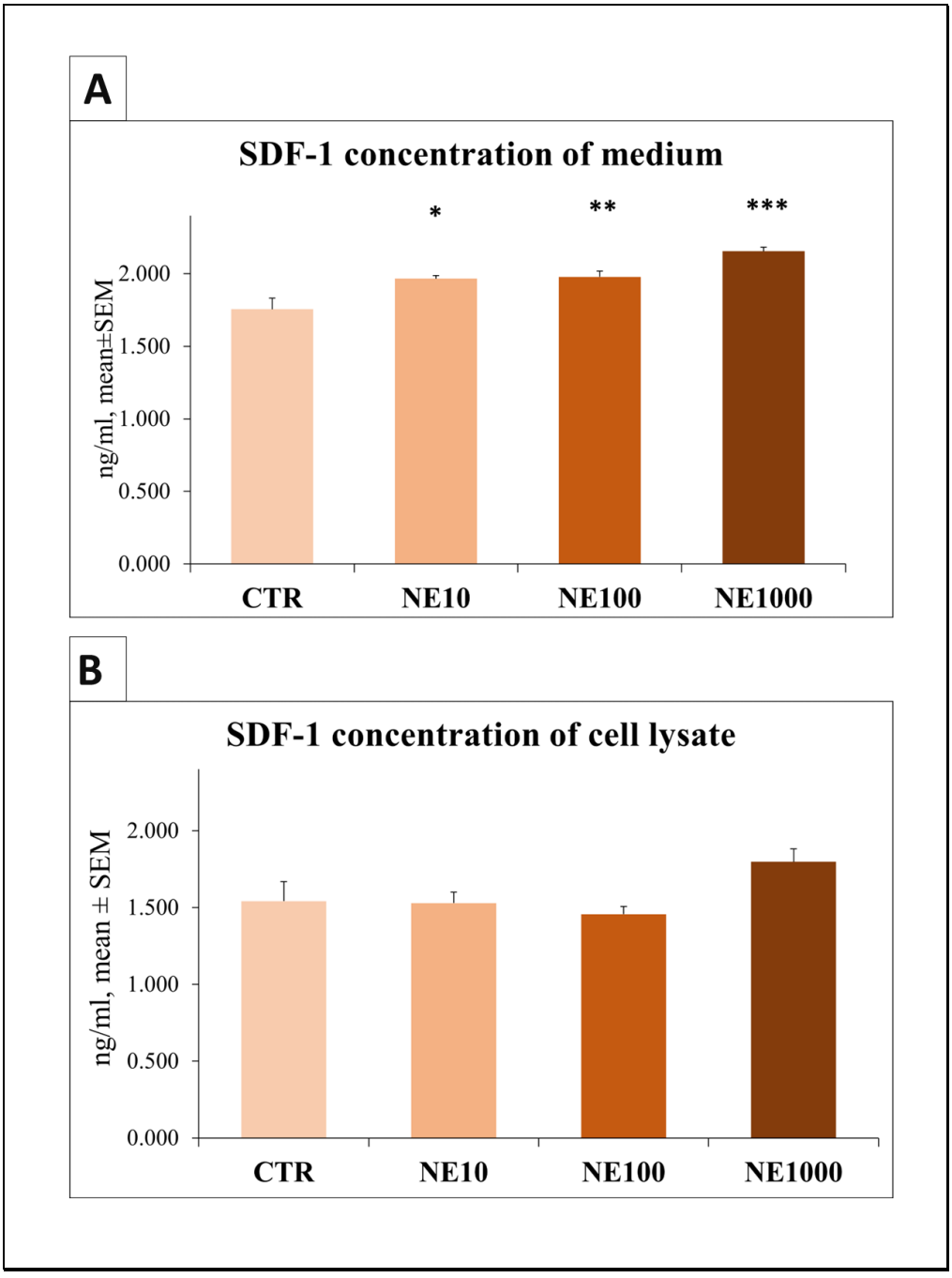

Fig.15.: Stromal-cell derived factor-1 (SDF-1) levels in culture media (A) and cell lysate (B) after 1 h norepinephrine (NE) treatment. CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells. n=3/group, results are expressed as mean±SEM, * p<0.05, ** p<0.01, *** p<0.001.

IL-6 and SDF-1 levels of the cell lysates showed no significant changes after NE treatments (p=0.432, p=0.919, p=0.273 for IL-6 and 10, 100, 1000 μM, respectively; p=0.941, p=0.624, p=0.243 for SDF-1 and 10, 100, 1000 μM, respectively) (**Fig.14B and Fig.15B**).

5.2.3. Assessment of the redox state of the cells

There were no significant changes in the GRP78 abundance of cell lysate (p=0.106, p=0.229, p=0.384, respectively) and the MDA levels of cell culture media (p=0.939, p=0.737, p=0.535, respectively) in the case of any NE supplementation compared with the non-treated control cells (**Fig.16A, C**). A significant increase in the PC level was measured in cell lysate in the case of 1000 µM NE exposure (p=0.049), but no significant alterations were detected in cells challenged with 10 and 100 µM NE in comparison with the control group (p=0.749, p=0.689, respectively) (**Fig.16B**). In case of 100 µM NE treatment, a significantly lower 8-OHdG concentration was observed in the cell lysate than in the non-treated control cells (p=0.019), but no significant changes were detected in the case of the other NE concentrations (p=0.977, p=0.828, respectively) (**Fig.16D**).

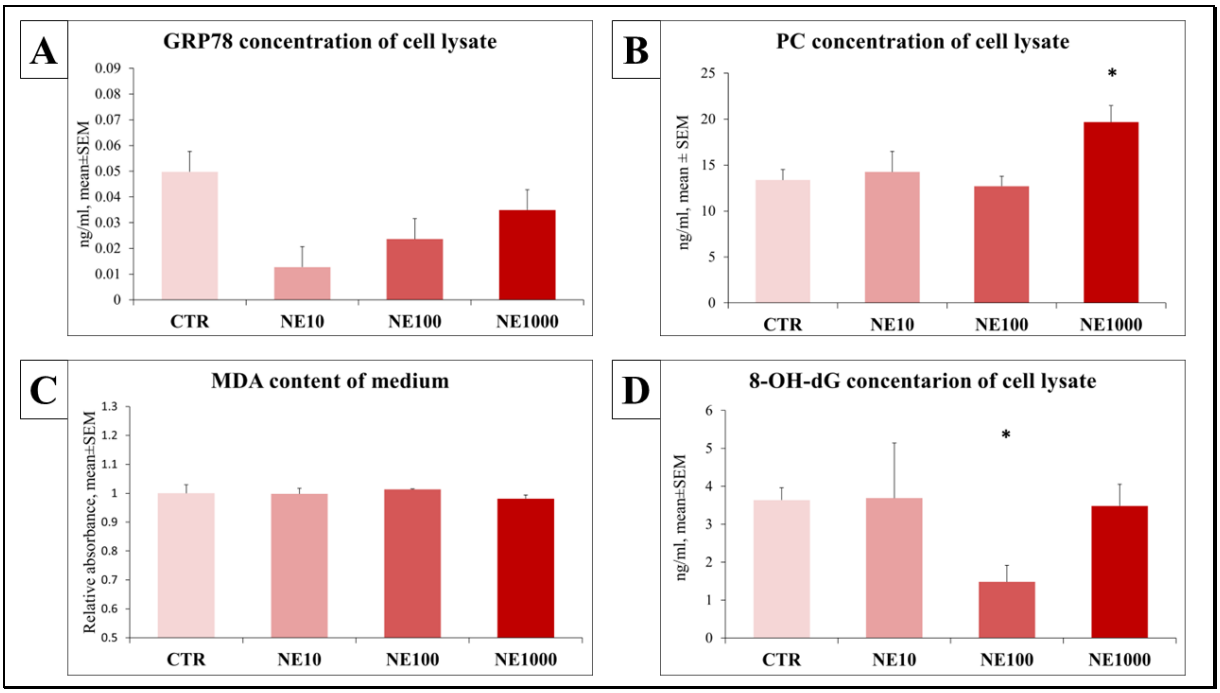

Fig.16.: Concentration of oxidative stress markers after 1 h norepinephrine (NE) treatment. Glucose-regulated protein 78 (GRP78) (A) and protein carbonyl (PC) (B) concentrations in cell lysate, malondialdehyde (MDA) concentration of cell culture media (C), and 8-hydroxy 2 deoxyguanosine (8-OHdG) level of cell lysate (D). CTR=non-treated control cells, NE10=10 µM norepinephrine treated cells, NE100=100 µM norepinephrine treated cells, NE1000=1000 µM norepinephrine treated cells. n=3/group, results are expressed as mean±SEM, * p<0.05.

5.2.4. Investigation of epithelial barrier function

Significantly lower GAG content was observed in the medium in the case of 100 μM NE addition (p=0.008), but there were no changes when cells were exposed to 10 and 1000 μM NE in comparison with the non-treated control wells (p=0.909, p=0.143, respectively) (**Fig.17**).

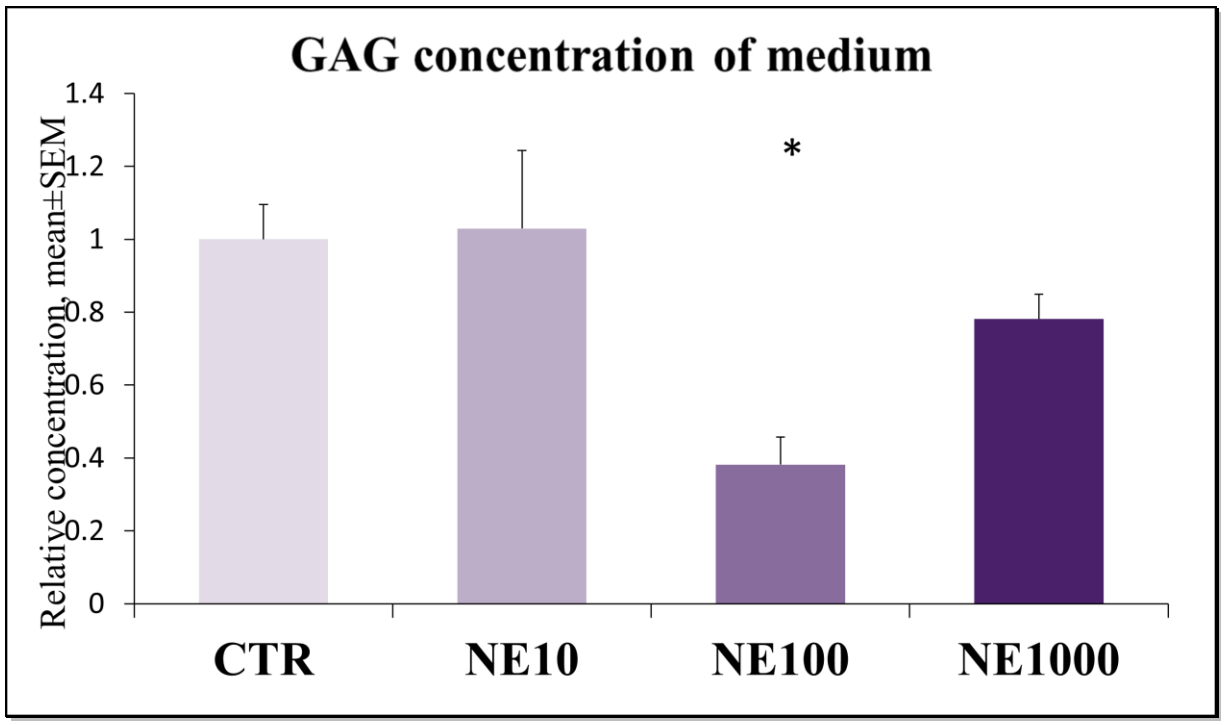

Fig.17.: Glycosaminoglycan (GAG) concentration of culture medium after 1 h norepinephrine (NE) treatment. CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells. n=3/group, results are expressed as mean±SEM, * p<0.05.

The claudin-4 content of the cultured cells was significantly decreased in the case of all of the applied NE treatments compared with the control cells (p=0.020, p=0.013, p=0.003, respectively) **(Fig.18)**.

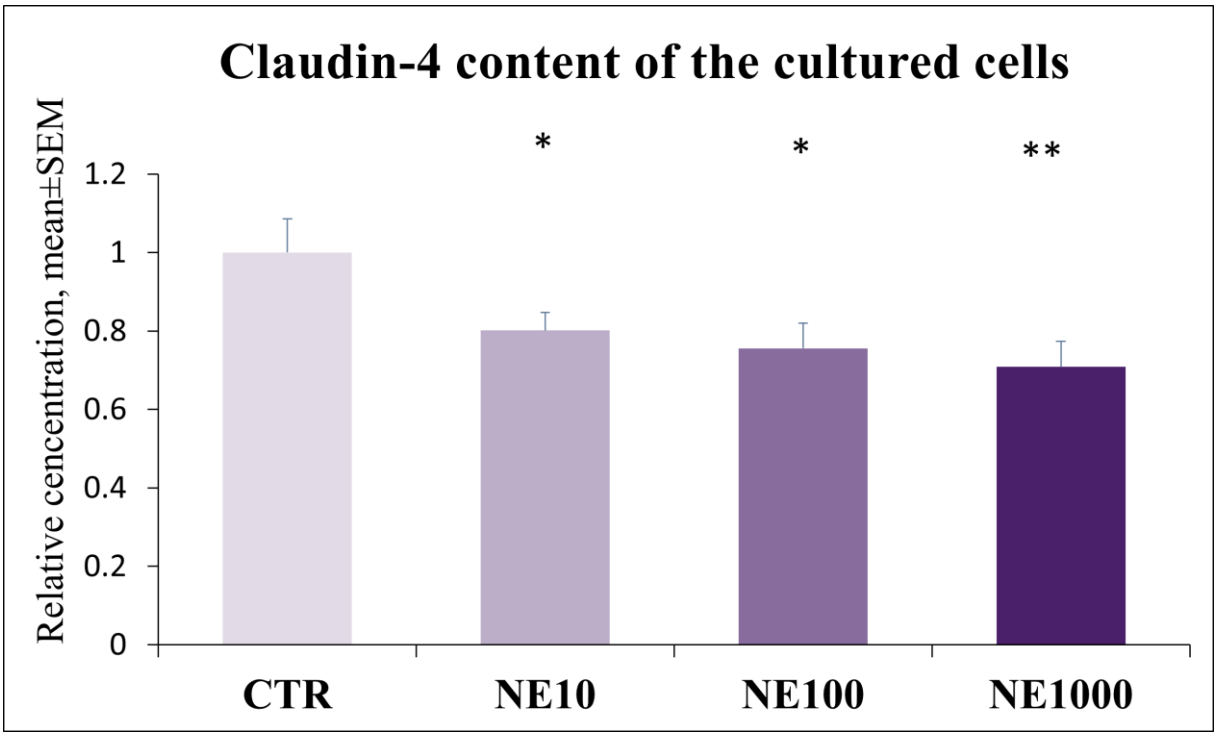

Fig.18.: Claudin-4 content of cultured uroepithelial cells after 1 h norepinephrine (NE) treatment. CTR=non-treated control cells, NE10=10 µM norepinephrine treated cells, NE100=100 µM norepinephrine treated cells, NE1000=1000 µM norepinephrine treated cells. n=6/group, results are expressed as mean±SEM, * p<0.05 ** p<0.01

Significant decline in TER values was measured in the case of 100 and 1000 μM NE treatment, both immediately after the treatment (p=0.040, p=0.030, respectively) and after 24 h regeneration time (p=0.038, p=0.037, respectively) compared with the controls, but there was no significant decrease after the 10 μM NE exposure (p=0.070, p=0.057 for immediate and 24 h measures, respectively) **(Fig.19)**.

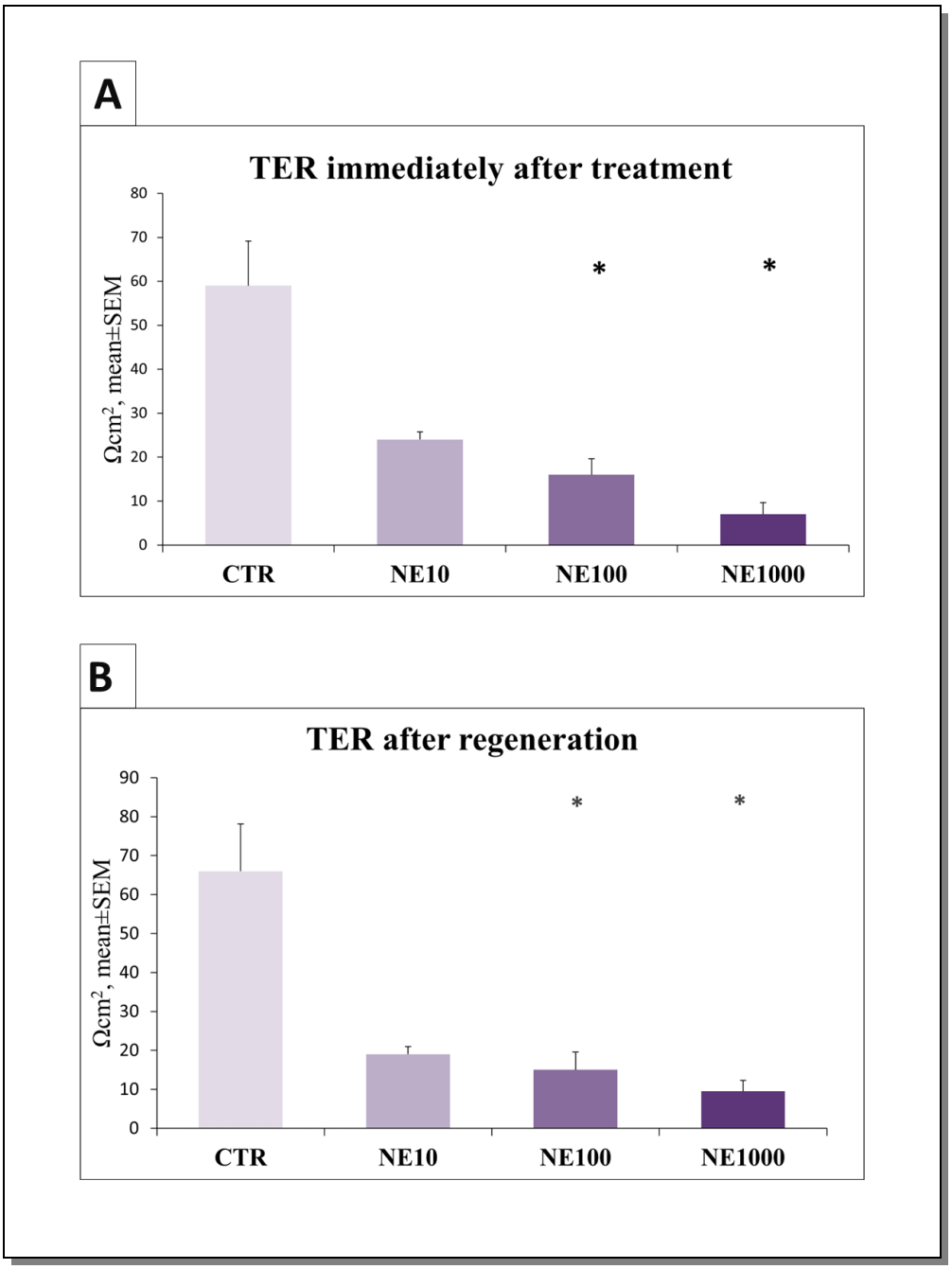

Fig.19.: Transepithelial electrical resistance (TER) of uroepithelial cell cultures measured immediately after norepinephrine treatment (A) and after 24 h regeneration time (B). CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells. n=3/group, results are expressed as mean±SEM, *p<0.05.

5.2.5. Pearson's correlation test

According to the Pearson's correlation test, a significant positive correlation was found between SDF-1 concentration of the medium, and PC and MDA levels (R^2=0.619, p=0.001 and R^2=0.99, p<0.001, respectively), between SDF-1 concentration of cell lysate and also PC and MDA levels (R^2=0.662 p<0.001, and R^2=0.911 and p<0.001, respectively). Further strong positive correlation was detected between the GAG content of the cultured cells and the TER values measured immediately after NE treatment and also after 24 h regeneration time (R^2=0.940, p<0.001 and R^2=0.931, p<0.001, respectively, **Table III.**)

Table III.: Correlation coefficients and p values as gained by Pearson's correlation test between various parameters assessed.

Analyzed factors	**Pearson correlation coefficient (R^2)**	**p value**
SDF-1 medium + PC	0.619	=0.001
SDF-1 medium + MDA	0.990	<0.001
SDF-1 lysate + PC	0.662	<0.001
SDF-1 lysate + MDA	0.911	<0.001
GAG + TER immediately	0.940	<0.001
GAG + TER after regeneration	0.931	<0.001

5.2.6. Principal component analysis (PCA)

For the better overall visualization of the molecular effects of acute NE treatment on cultured uroepithelial cells, principal component analysis (PCA) has been carried out (**Fig.20**). The PCA plot shows two distinct groups: one for control, 10, 100 μM NE treated cells and the other one for 1000 μM NE treated cultures (PC1: 77.3%; PC2: 7.1%).

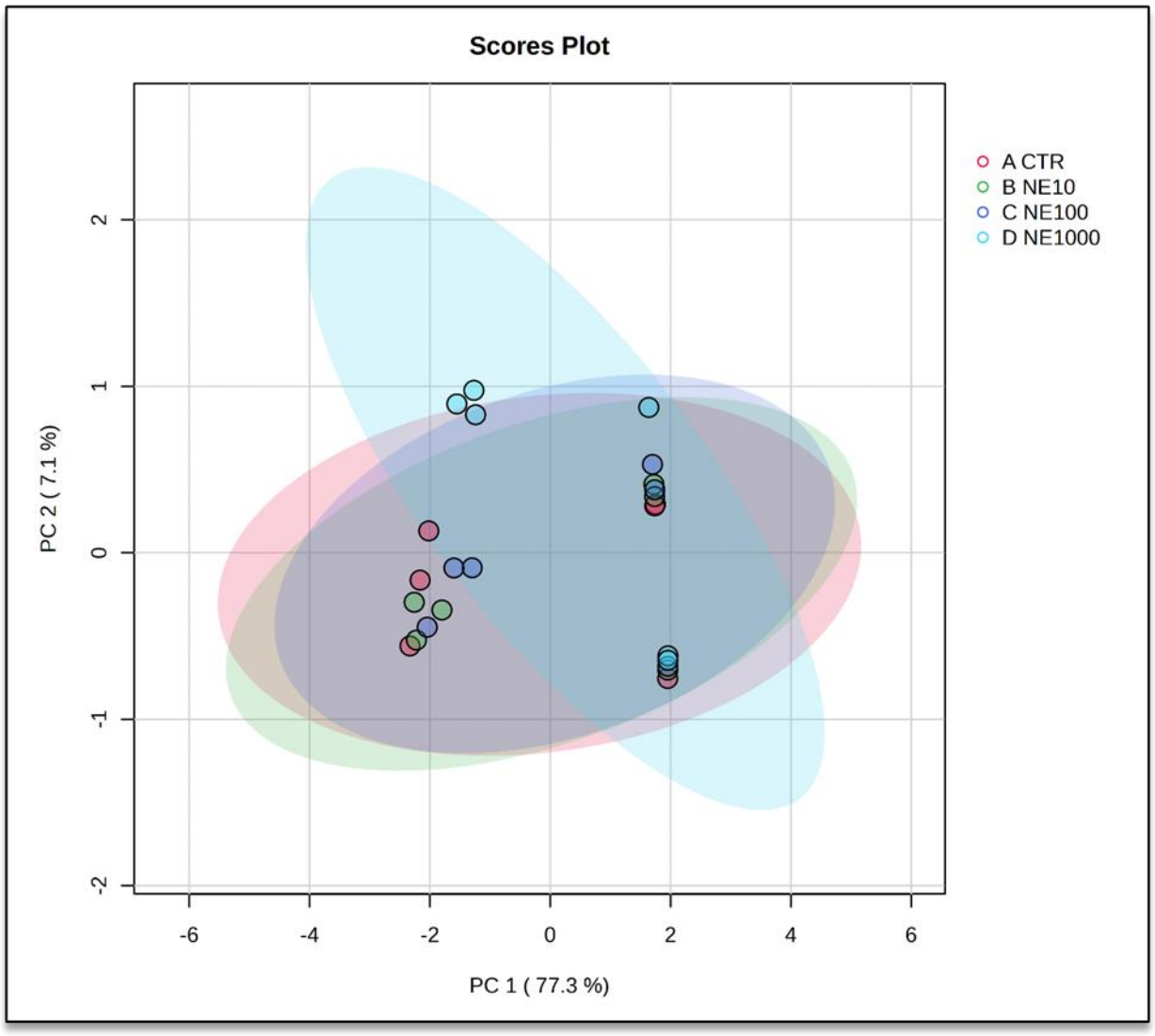

Fig.20.: Principal component analysis (PCA) after 1 h norepinephrine treatment shows two distinct groups for control, 10, 100 μM NE treated cells and for 1000 μM NE treated cultures. The two major components (PC 1 and PC 2) that accounted for the most variation of the metabolite abundance was used to plot. Each dot in the figure represents a single sample, and different colors indicate the different treatments. CTR=non-treated control cells (red), NE10=10 μM norepinephrine treated cells (green), NE100=100 μM norepinephrine treated cells (violet), NE1000=1000 μM norepinephrine treated cells (blue)

5.3. Study III.: Cellular effects of 3x1 h NE treatment modelling chronic, intermittent stress on feline uroepithelial cell cultures

5.3.1. Assessment of cellular metabolic activity and cell injury

No significant changes were observed in cellular metabolic activity (p=0.764, p=0.634, p=0.737 for 10, 100 and 1000 µM NE, respectively) (**Fig.21A**) and the LDH activities of cell culture media after NE treatments compared to the control group (p=0.563, p=0.755, p=0.766 for 10, 100 and 1000 µM NE, respectively) (**Fig.21B**).

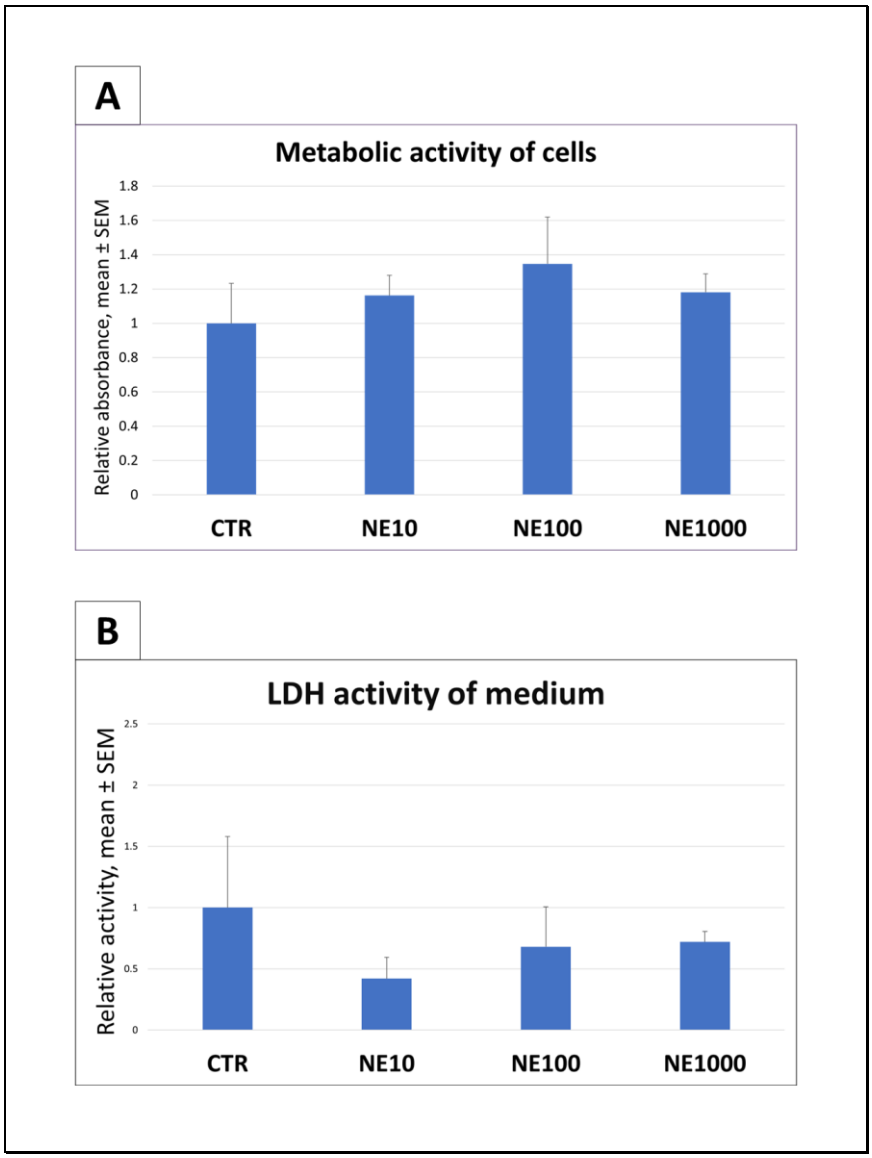

Fig.21.: The cellular metabolic activity measured by CCK-8 colorimetric assay (A) and lactate dehydrogenase (LDH) activity (B) of the cell culture media after 3x1 h norepinephrine (NE) treatment. CTR=non-treated control cells, NE10=10 µM norepinephrine treated cells, NE100=100 µM norepinephrine treated cells, NE1000=1000 µM norepinephrine treated cells. n=3/group (A) and n=3/group (B), results are expressed as mean ± SEM.

5.3.2. Measurement of IL-6 and SDF-1 concentrations

There were no significant changes in the level of IL-6, either in cell culture media or in cell lysate after any applied NE exposure (**Fig.22A, B**). However, elevated SDF-1 concentrations of the media were measured in case of 3x1 h, 10, 100 and 1000 μM NE supplementation (p=0.003, p=0.018, p=0.002, respectively) (**Fig.22C**), and decreased SDF-1 concentrations of the cell lysates were detected after 100 and 1000 μM NE exposure (p=0.022, p=0.005, respectively) (**Fig.22D**), compared to the non-treated control wells, while no significant difference was found in case of the 10 μM NE addition (p=0.197) (**Fig.22D**).

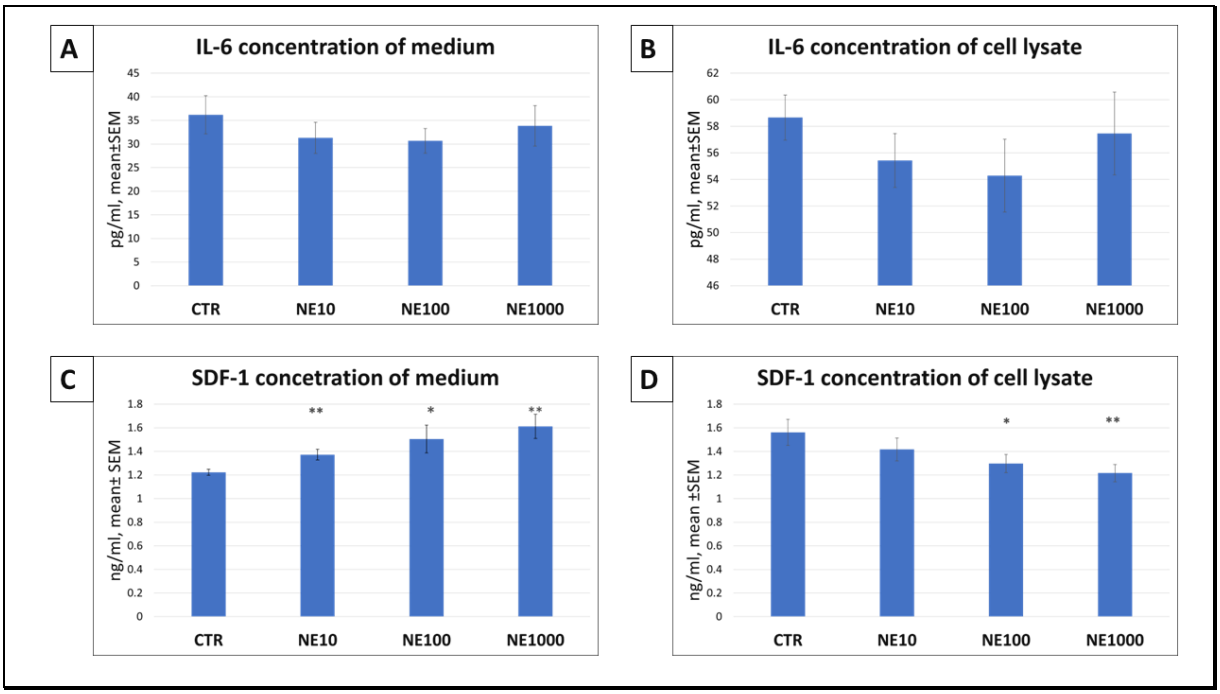

Fig. 22.: Interleukin-6 (IL-6) concentration in cell culture media (A), cell lysate (B) and stromal cell derived factor-1 (SDF-1) concentration in cell culture media (C) and cell lysate (D) after 3x1 h norepinephrine (NE) treatment. CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells. n=3/group, results are expressed as mean±SEM. * p<0.05, ** p<0.01

5.3.3. Assessment of the redox state of the cells

There were no significant changes in MDA levels of the cell lysates in case of any applied NE treatment (p=0.347, p=0.235, p=0.118 for 10, 100 and 1000 µM NE, respectively) (**Fig.23A**); however, the H_2O_2 production of the cultured cells, measured in the cell culture media was higher in case of 100 and 1000 µM NE exposure compared to the non-treated controls. (p=0.016, p=0.018, respectively) (**Fig.23B**).

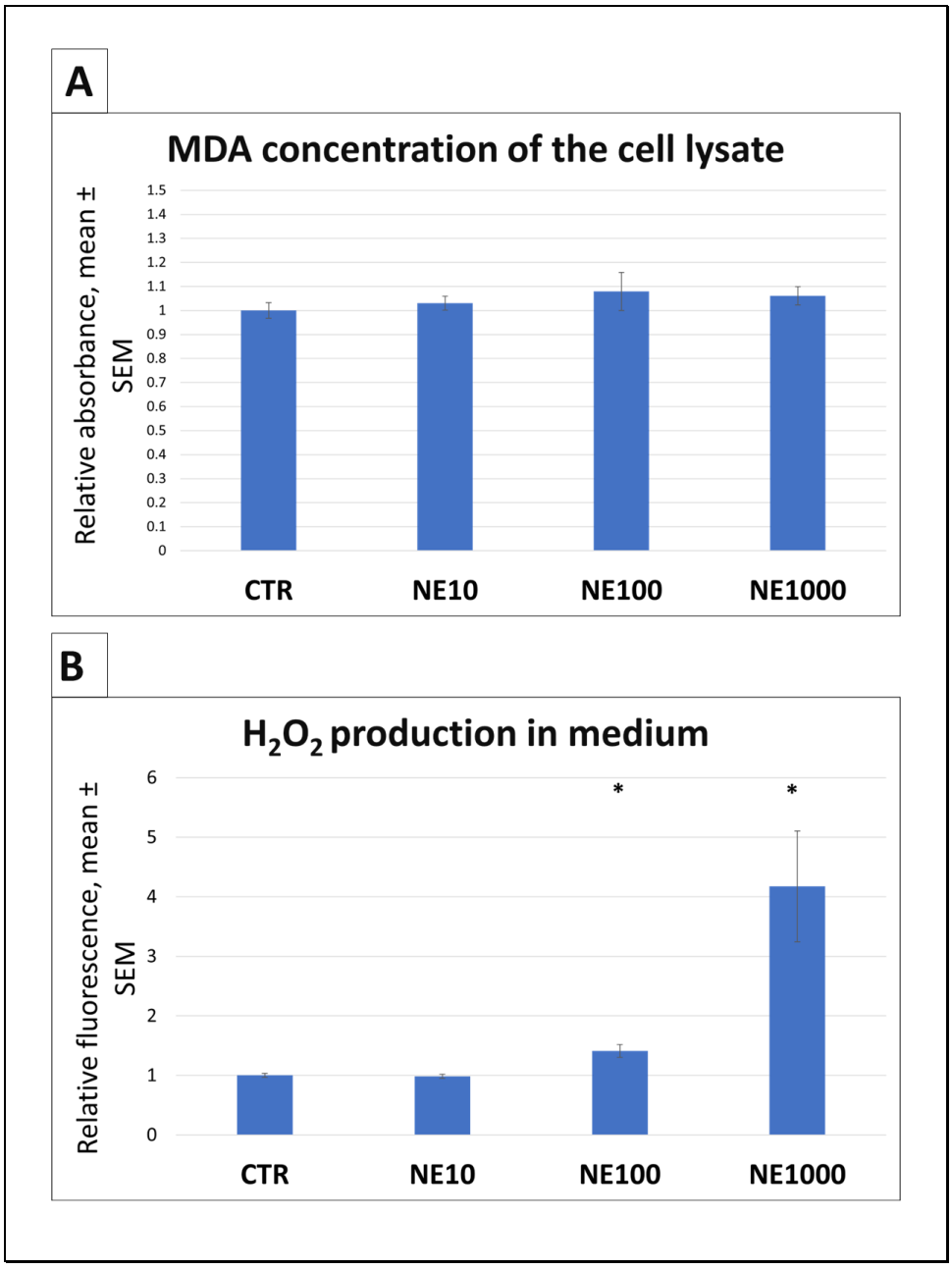

Fig.23.: Malondialdehyde (MDA) (A) and H_2O_2 (B) concentrations after 3x1 h norepinephrine (NE) treatment. CTR=non-treated control cells, NE10=10 µM norepinephrine treated cells, NE100=100 µM norepinephrine treated cells, NE1000=1000 µM norepinephrine treated cells. n=3/group, results are expressed as mean ± SEM. * p<0.05

5.3.4. Investigation of epithelial barrier function

Significantly lower GAG concentrations were observed in the cell culture media after all the applied NE treatments (p=0.024, p=0.021, p=0.025 for 10, 100 and 1000 μM NE, respectively) (**Fig.24A**). Further, significant decrease of claudin-4 content of the cultured cells was detected after each concentration of NE exposure, compared to the non-treated control cells (p=0.018, p=0.003, p=0.001 for 10, 100 and 1000 μM NE, respectively) (**Fig.24B**). Similarly, TERs were also significantly lower in case of all the applied NE treatments both immediately after the treatment (p=0.009, p=0.009, p=0.007 for 10, 100 and 1000 μM NE, respectively) (**Fig.24C**) and following 24 h regeneration time (p=0.012, p=0.016, p=0.014 for 10, 100 and 1000 μM NE, respectively) compared to the controls (**Fig.24D**).

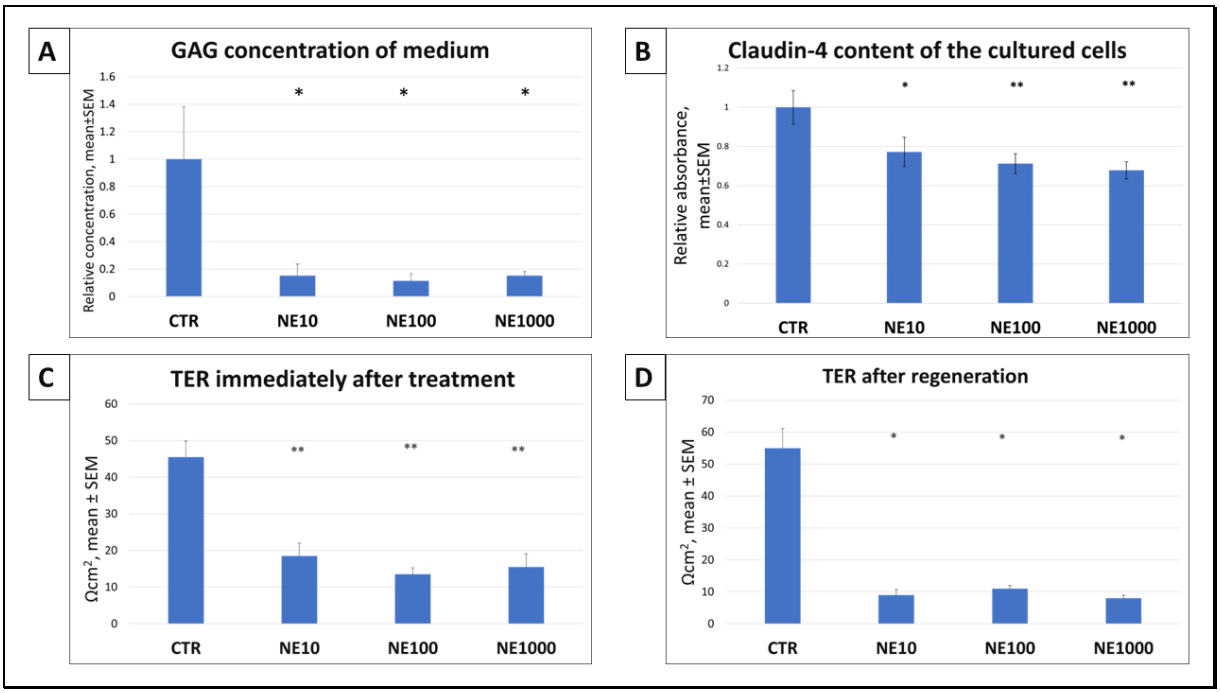

Fig.24.: Glycosaminoglycan (GAG) concentration of the cell culture media (A), claudin-4 content of the cells (B) and transepithelial electrical resistance (TER) immediately after 3x1 h norepinephrine (NE) treatment (C) and after 24 h regeneration time (D). CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells. n=6/group (B) and n=3/group (A,C,D), results are expressed as mean±SEM. * p<0.05, ** p<0.01.

5.3.5. Pearson's correlation test

According to the Pearson's correlation test, a significant positive correlation was found between SDF-1 and H_2O_2 content of cell culture media (R^2=0.51, p=0.009) and a significant negative correlation between SDF-1 in cell culture media and claudin-4 content of the cultured cells (R^2=-0.63, p<0.001), further significant negative correlation was observed between H_2O_2 and claudin-4 concentrations (R^2=-0.48, p=0.017). **(Table IV.)**

Table IV.: Correlation coefficients and p values as gained by Pearson's correlation test between various parameters assessed.

Analyzed factors	**Pearson correlation coefficient (R^2)**	**p value**
SDF-1 medium + Claudin-4	-0.63	<0.001
SDF-1 medium + H_2O_2	0.51	0.009
H_2O_2 + Claudin-4	-0.48	0.017

5.3.6. Principal component analysis (PCA)

For the better overall visualization of the molecular effects of 3x1h NE treatment on cultured uroepithelial cells, principal component analysis (PCA) has been carried out (**Fig.25.**). The PCA plot shows 2 distinct groups for control and treated (10, 100 and 1000 µM NE) cultures (PC1: 48,3%; PC2: 27,8%).

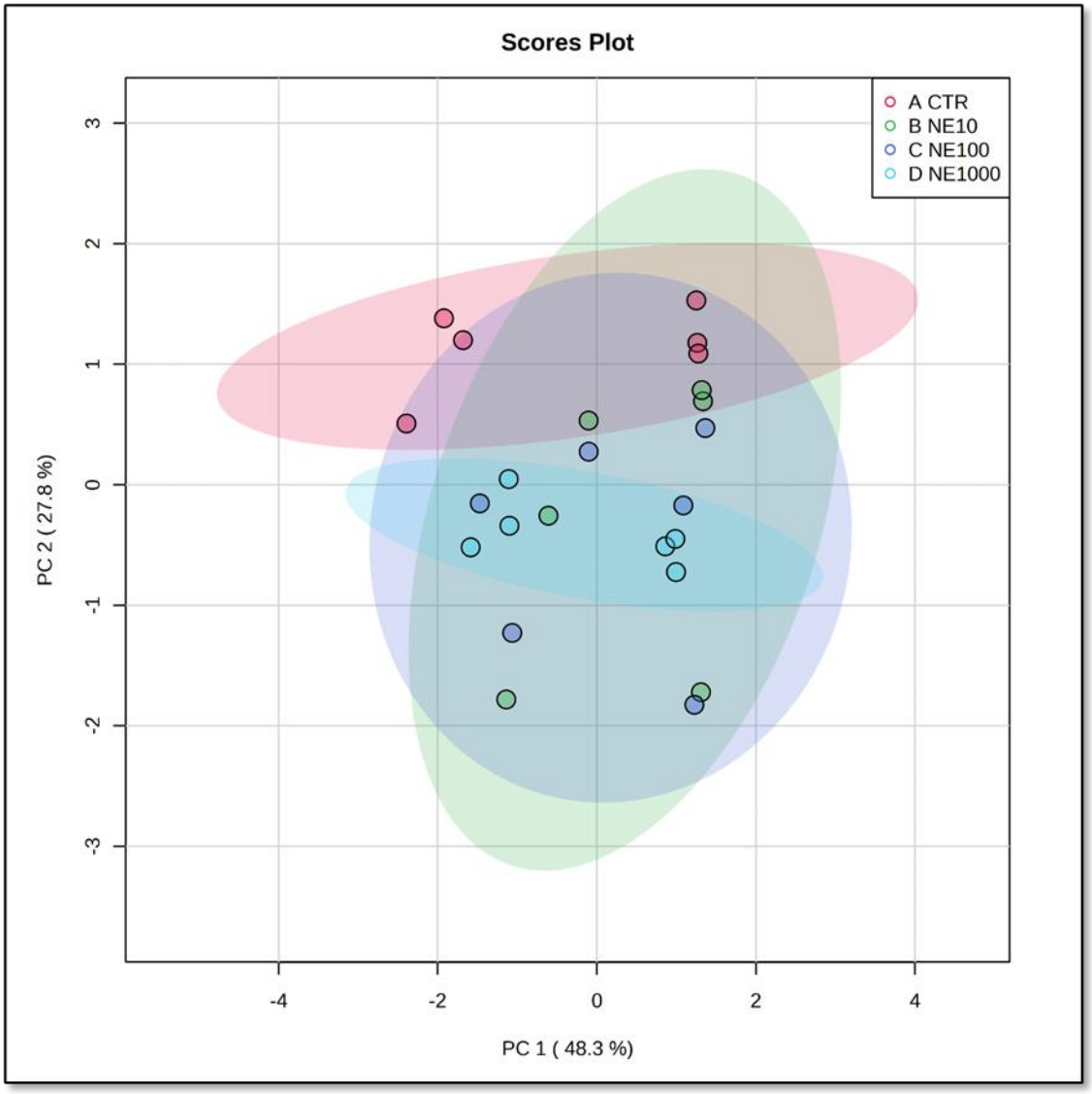

Fig.25.: Principal component analysis (PCA) after 3x1 h norepinephrine treatment shows two distinct groups for control and treated cultures. The two major components (PC 1 and PC 2) that accounted for the most variation of the metabolite abundance was used to plot. Each dot in the figure represents a single sample, and different colors indicate the different treatments. CTR=non-treated control cells (red), NE10=10 µM norepinephrine treated cells (green), NE100=100 µM norepinephrine treated cells (violet), NE1000=1000 µM norepinephrine treated cells (blue)

6. Discussion

6.1. Study I.: establishment and characterization of novel primary uroepithelial cell culture from feline origin

In **Study I.** of the recent thesis, a novel primary uroepithelial cell culture of feline origin was successfully established as an *in vitro* model suitable for investigating the pathomechanism of FIC. To the best of our knowledge, this model is the first cell culture from a cat which has been proven to contain differentiated uroepithelial cells, verified by the immunofluorescent detection of uroplakin III. As uroplakins, such as uroplakin III are specific urothelial differentiation products, uroplakin III is suitable to characterize differentiated uroepithelial cells in cultures (Kaufmann et al., 2000). Cytokeratins are characteristic of epithelial cells, therefore, the positivity confirms the epithelial origin of the cultured cells (Truschel et al., 1999; Wu & Sun, 1993; Yu et al., 1990). The cytokeratin positivity of the cells could be detected from the first day after plating, but the uroplakin III positivity could be observed only on the sixth day after seeding, which may suggest that several days are required to obtain differentiated uroepithelial cells in cell cultures.

Based on earlier studies it can be stated that FIC is a complex, multifactorial disease, developed on the basis of the interactions between the urinary bladder, adrenal glands, and nervous system, also affected by the environment where the cats live (Chew et al., 2013). Therefore, to understand the pathomechanism of the disease, our cell culture model could be beneficial as an *in vitro* system, in which the above-mentioned interactions and factors can be studied separately as well as in targeted combinations. Further, it should be highlighted that it is a non-tumorigenic primary cell culture, hence the results can be better extrapolated to the *in vivo* conditions of the feline urinary bladder than those obtained on cell lines.

6.2. Study II.: investigation of the molecular effects of acute stress mimicked by short term (1h) NE treatment on the newly established primary feline uroepithelial cell culture model

Under stress, the activity of tyrosine hydroxylase, the main regulatory enzyme of NE synthesis, is increased in the *locus coeruleus* of the brain of cats (Forrester & Towell, 2015; Reche & Buffington, 1998). Further, the bladder permeability of cats with FIC to water, urine and various ions is increased, and also inflammation and oxidative stress are reported in the bladder of affected cats (Kruger et al., 2009). Therefore, in **Study II**, it was investigated if there is a direct molecular link between the presence of NE and the appearance of the

mentioned lesions. Hence, the molecular effects of an acute NE treatment on cultured feline uroepithelial cells were examined, whereby the applied treatment induced a pro-inflammatory response, oxidative stress and barrier function impairment in cell cultures, confirming a direct role of NE in the pathogenesis of the disease **(Fig.26.)**.

NE acts on the alfa-1 adrenergic receptors in the smooth muscle layer and uroepithelial cells of the bladder wall, hence this hormone can have a significant effect on stress responses of the organ (Buffington et al., 2002). In our *in vitro* studies the applied, relatively high NE concentrations were set to cover a wide range and based on the limited available data from previous studies. Considering that, due to the release of NE at the noradrenergic nerve endings in the immediate vicinity of the bladder epithelial cells, it is assumed that the hormone concentration measured locally, near the bladder epithelial cells, may be much higher than in the blood plasma. Further, it should be also taking into account the fact that *in vitro* preparations often require higher agonist concentrations than those considered relevant *in vivo* (Buffington et al., 2002; Mansley et al., 2015; Yang et al., 2014; Reche & Buffington, 1998; Westropp et al., 2006; Flavahan et al., 1998). The treatment procedure (1 h NE exposure followed by 24 h regeneration) was set to mimic acute stress with appropriate time for triggering pro-inflammatory humoral response. Under *in vivo* conditions, a similar acute stressor can exacerbate the clinical signs of FIC in cats or those of interstitial cystitis in women by increasing the permeability of the bladder wall (Westropp et al., 2006; Lutgendorf et al., 2000;).

Metabolic activity of the cultures was monitored by CCK-8 test. This assay is based on a colorimetric method and is suitable for measuring the real-time aerobic catabolic activity of cell cultures. After 1 h of 1000 µM NE treatment, the metabolic activity was significantly higher than in the non-treated control group. In case of 10 and 100 µM NE treatments the metabolic activity of the cells has not elevated in a significant manner. Increased metabolic activity may suggest increased catabolic adaptation of cells exposed to NE induced stress. These results are consistent with those of previous studies, where the metabolic activity of a human osteoblast cell line was elevated after NE treatment (Kodama & Togari, 2013). Further, increased metabolic activity of chicken hepatocytes grown in primary cultures was measured by CCK-8 test after heat stress referring to the rapid metabolic stress adaptation of the cells (Mackei et al., 2020). Cell injury was examined by measuring the extracellular LDH activity, and according to our results, that was not affected by NE, which indicates that the applied treatments were not cytotoxic and did not induce necrosis of the cultured cells.

In the present study the inflammatory effect of NE was examined by measuring the IL-6 and SDF-1 concentrations of cell culture media and cell lysate. In the cell culture media, the IL-6 concentration was elevated after 1 h of 1000 µM NE treatment, but there were no significant changes in case of 10 and 100 µM NE application. However, in cell lysate no

significant changes could be measured after any treatments, which reflects that the cells secrete this cytokine into their environment. In a previous study the effect of NE was also examined on the production of various cytokines such as IL-6 on human immortalized gastric epithelial cell culture, and it was found that NE triggered increased IL-6 production and up-regulated the IL-6 receptor, confirming the role of NE in inflammatory processes (Yang et al., 2014). These results are also in line with a previous study, in which the stimulatory effect of NE was detected on IL-6 production in rat cardiac fibroblast cells (Bürger et al., 2001). The enhanced pro-inflammatory cytokine release in urinary bladder disorders was also reported by previous studies, in which significant increase of IL-6 levels was measured in the urine of women with interstitial cystitis, in rats with experimentally induced cystitis and in cats with FIC (Lamale et al., 2006; Malley & Vizzard, 2002; Mohamaden et al., 2020).

Significant increase of SDF-1 concentration in cell culture media was measured at all NE concentrations used, but there were no changes in case of cell lysate, similarly to the IL-6 measurements. As a pro-inflammatory chemokine, the role of SDF-1 and its receptor has been studied in rats with experimentally induced cystitis, where increased blood SDF-1 concentration as well as elevated uroepithelial SDF-1 receptor expression were observed (Arms et al., 2010). In addition to the above-mentioned experiment, other studies have addressed the role of this chemokine in the pathomechanism of FIC: elevated SDF-1 concentration was measured in the blood of cats with FIC (Parys et al., 2018), and in the urine of women with interstitial cystitis (Corcoran et al., 2013). Therefore, taking every detail into consideration it can be suggested that this chemokine, similarly to IL-6, may also have a role in the development of the disease. Based on our results, it should be underlined that the pro-inflammatory chemokine SDF-1 proved to be more sensitive to the presence of NE than IL-6. This finding corresponds to those of Parys et al. (2018), reporting a remarkable increase in plasma SDF-1 concentrations of FIC-affected cats, while plasma IL-6 levels remained unchanged.

To investigate if NE is able to induce oxidative stress in uroepithelial cells, certain oxidative stress markers (MDA, 8-OHdG, PC, GRP78) were monitored. According to our results, it could be concluded that the NE had significant effect only on the concentration of 8-OHdG and PC among the assessed stress markers. 8-OHdG is a common and sensitive biomarker of oxidative DNA damage (Dizdaroglu, 1998). Interestingly, in case of 100 μM NE treatment, the concentration of 8-OHdG was significantly decreased, however some previous papers highlighted that hormonal stress is able to induce oxidative DNA damage (reflected by elevated 8-OHdG level) in human oral keratinocyte cells, and also in hepatocytes of rats (Adachi et al., 1993; Valente et al., 2021). Further, increased 8-OHdG level was detected in the urine of patients with interstitial cystitis (Dokumacioglu et al., 2018; Lin et al., 2021). Based on these previous data, the above-mentioned paradoxical effect may be explained

rather by over-compensating processes of uroepithelial cells, than protective role of NE against oxidative stress.

PC measurement is a useful method to investigate the protein damage caused by oxidative stress (Dalle-Donne et al., 2003). In this present study the concentration of PC was elevated in case of the highest concentration (1000 µM) of NE treatment, in accordance with a previous study, in which increased stress hormone release induced slightly elevated PC level in cultured rat urothelial cells (Kullmann et al., 2019).

MDA is an oxidative stress marker resulting from lipid peroxidation (Da Costa et al., 2012), further, GRP78 is a stress related endoplasmic reticulum (ER) chaperon protein, which could also serve as an oxidative stress marker (Rao et al., 2002). Some earlier papers showed increased urinary MDA level in human interstitial cystitis (Dokumacioglu et al., 2018), elevated GRP78 expression in uroepithelial cells of rats with cystitis (Vera et al., 2009) and also increased GRP78 level in different cell types after NE treatment (Mao et al., 2006; Yang et al., 2011). However, in the present study both the MDA and GRP78 levels remained unchanged, further, the concentration of 8-OHdG was not increased, it seems that the applied short term NE treatment was not able to cause significant lipid peroxidation, ER distress or DNA damage in cultured feline uroepithelial cells.

In addition, significant positive correlation was found between SDF-1 concentrations and PC and MDA levels, suggesting a strong link between oxidative stress and inflammatory processes. These results are in line with results of several previous studies where oxidative stress was able to induce increased pro-inflammatory marker production (Biswas, 2016; Collins, 1999; Flohé et al., 1997; Homan et al., 2013; Sezginer et al., 2019)

Another goal of the present study was to investigate the effect of stress on barrier function of the cultured uroepithelial cells, hence GAG concentration of the cell culture media, claudin-4 content of cultured cells and TER of the monolayer of cultured epithelial cells were investigated, after NE exposure. In healthy cats, the apical surface of bladder urothelium is covered by GAG layer which may have an important role in maintaining barrier function (Parsons et al., 1990). Certain previous studies highlighted some changes in bladder GAG layer, such as declined total urinary GAG concentration and decreased abundance of a specific GAG, GP-51 in the bladder of cats suffering from FIC, although it is still remained unclear that this decrease is due to the failure of GAG synthesis or metabolism (Buffington, Blaisdell, et al., 1996; Liebert, 2009; Parsons et al., 1990). These findings are in line with our results showing that the 100 µM NE treatment has significantly decreased the concentration of GAGs in cell culture media, indicating that NE may impair physiological GAG metabolism. However, there was no decrease in GAG concentrations following treatment with 1000 µmol/l NE, which may be explained by the overcompensation and defense mechanisms of the cultured cells triggered by a single, acute, high dose of NE.

To investigate the barrier function of the uroepithelium, it is inevitable to examine the tight junction proteins as well, since the barrier function is supported by the structural integrity of tight junctions. (Acharya et al., 2004) Claudins form a multigene protein family with 24 members, being able to form ion channels and to regulate the membrane permeability depending on the size, charge and electrical resistance of the ions (Tsukita et al., 2001). Claudin-4, -8, and -12 can be found in tight junctions of the umbrella cells. (Acharya et al., 2004) Previous studies have already indicated alterations in the abundance and distribution of certain tight junction proteins, such as zonula-occludens 1, occludin, E-cadherin, and uroplakins in cats with FIC and human patients with interstitial cystitis (Kullmann et al., 2018; Lavelle et al., 2000; Lee & Lee, 2014). In this present study, all the applied NE treatments (10-, 100-, and 1000 µM) had significant decreasing effect on the claudin-4 concentration of the cultured cells, indicating that the NE-triggered stress can diminish the barrier function of urothelial cells. Similarly, 100 and 1000 µM NE exposures had decreasing effect on the TER of the uroepithelial monolayer both immediately after treatment and following 24 h regeneration time compared to the non-treated control cells. These findings are in line with an earlier study, where reduced TER was measured in bladder of cats suffering from FIC (Lavelle et al., 2000). Taken together, these results demonstrate that NE has a direct influence on uroepithelial barrier functions and this modified barrier functions may be in association with changes in the expression of tight junction protein claudin-4 and GAG content of urothelial cells. The results described above may also explain the strong positive correlation between GAG and TER values found in the present study, since, the GAG layer is a very important component of the bladder barrier function, as confirmed by the previous study of Hurst et al. (2016), where reduced TER values were measured after digestion of the bladder GAG layer (Hurst et al., 2016).

According to our recent knowledge this was the first study, which investigated the direct molecular effect of NE on cultured feline uroepithelial cells to gain deeper knowledge of the pathomechanism of FIC; however, for a more accurate understanding of the disease, further research is necessary.

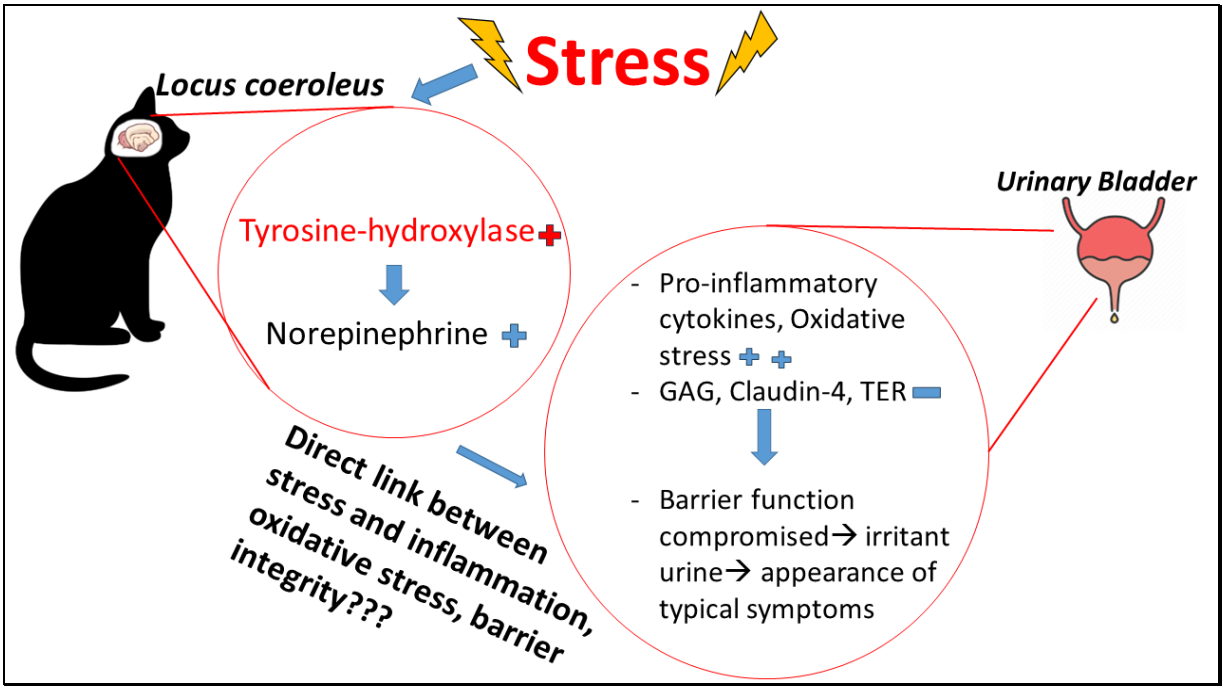

Fig.26.: Summarizing the main questions and results of **Study II**.

6.3. Study III.: investigation of the molecular effects of chronic, intermittent stress mimicked by pulsative (3x1h) NE exposure on primary feline uroepithelial cell culture model and comparison of the molecular effects of acute and chronic stress in the pathomechanism of FIC

In **Study III.** the molecular effects of intermittent NE exposure on cultured feline uroepithelial cells were investigated to examine the role of recurrent stress in the development of FIC. In case of increased exposure to environmental stress, the synthesis of catecholamines (such as NE) is getting stimulated together with the activation of the sympathetic nervous system (SNS). Hence, the pain fibers in the bladder wall are stimulated and Substance P is released, causing vasodilatation, increased bladder wall permeability, submucosal edema, smooth muscle contraction, and mast cell degranulation, Based on these results it was supposed by Kruger et al. (2009), that NE has an indirect role in the development of FIC symptoms (Kruger et al., 2009). However, Birder et al. described that alfa- and beta-adrenergic receptors – as important elements of noradrenergic signaling pathways – can be found not only in the nerve cells of the bladder, but in the uroepithelial cells as well. Hence NE may possess a significant, direct effect on the stress response of the organ by stimulating the alfa-1 adrenergic receptors (Birder, 2004; Birder et al., 2001). While the symptoms of acute FIC cases are usually resolved in a few days, the recurrence of them is very common, possibly in association with the altered stress response of the individual

(Buffington et al., 2002). Therefore, to investigate whether the molecular changes in the urinary bladder cells that may lead to the symptoms of the disease are associated with repeatedly elevated presence of NE, the applied NE concentrations were set to cover a wide range, and the treatment procedure (3x1 h NE treatment followed by 24 h regeneration time) was designed to mimic an *in vitro* chronic intermittent SNS stress, taking into consideration the properties of primary cell cultures and the chronic stress conditions of some previous in vitro experiments (Bassil et al., 2022; Vernazza et al., 2019; R. Yang et al., 2014).

For greater clarity, a comparison of the results of **Study II**. and **Study III**. is presented in **Table V**. The real-time metabolic activity of the cell cultures was monitored by CCK-8 colorimetric assay. According to the results of **Study III**. no significant changes were observed in the metabolic activity of the cultured cells after 3x1 h NE treatment, although in **Study II** the acute single high-dose treatment increased the metabolic activity of the cells. This result may indicate an efficient metabolic adaptation of the cultured cells to chronic presence of NE, further it shows that the applied treatment procedure had no effect on cell viability. Cell injury was examined by measuring the extracellular LDH activity, and similarly as in **Study II.,** no significant changes were detected in case of any applied NE treatment. Based on these results it could be concluded that the neither the acute nor the chronic intermittent NE exposures were cytotoxic for the cultured feline uroepithelial cells.

Table V: Comparison the molecular effects of acute and chronic intermittent norepinephrine (NE) treatment (10, 100, 1000 µM) on cultured feline uroepithelial cells

	Examined factors	Study II (1h NE treatment)	Study III (3x1h NE treatment)
Cell injury and metabolic activity	Metabolic activity of cells (CCK-8)	**Increased in case of 1000 µM NE treatment**	No significant cahnges
	LDH activity of medium	No significant changes	No significant changes
Pro-inflammatory mediators	IL-6 concentration of medium	**Increased in case of 1000 µM NE treatment**	No significant changes
	IL-6 concentration of cell lysate	No significant changes	No significant changes
	SDF-1 concentration of medium	**Increased in case of all applied NE treatment**	**Increased in case of all applied NE treatment**
	SDF-1 concentration of cell lysate	No significant changes	**Decreased in case of 100 and 1000 µM NE treatment**
Redox parameters	PC concentration of cell lysate	**Increased in case of 1000 µM NE treatment**	-
	MDA content of cell lysate	No significant changes	No significant changes
	H_2O_2 production of medium	-	**Increased in case of 100 and 1000 µM NE treatment**
Barrier function of the cultured cells	GAG concentration of medium	**Decreased in case of 100 µM NE treatment**	**Decreased in case of all applied NE treatment**
	Claudin-4 content of cultured cells	**Decreased in case of all applied NE treatment**	**Decreased in case of all applied NE treatment**
	TER immediately after treatment	**Decreased in case of 100 and 1000 µM NE treatment**	**Decreased in case of all applied NE treatment**
	TER after 24h regeneration time	**Decreased in case of 100 and 1000 µM NE treatment**	**Decreased in case of all applied NE treatment**

To investigate the inflammatory role of chronic stress on the uroepithelium, the concentrations of the pro-inflammatory cytokine IL-6 and the chemokine SDF-1 were measured in cell culture media and also in cell lysates. There was no significant effect of NE treatment on the IL-6 concentration either in the cell culture media, or in the cell lysates compared to the control group. However, in **Study II.** the concentration of IL-6 was elevated in the cell culture media in case of the highest applied NE treatment. These findings are in line with the results of a previous paper, where chronic cystitis caused more moderate IL-6 production in rat urinary bladder than acute inflammation, and also with the finding of a study where acute (1h) NE treatment induced greater IL-6 mRNA synthesis than chronic (3h and 6h) NE exposure on human gastric epithelial cell culture (Malley & Vizzard, 2002; Yang et al., 2014). Based on these data it can be concluded that the changes in the bladder in the chronic FIC cases may be influenced to a greater degree by other cytokines and further mediators (for example SDF-1). The concentration of SDF-1 was elevated by all applied NE treatments in the cell culture media similarly as in **Study II.**, while parallelly decreased in the cell lysate. These results suggest that the NE-exposed cells intensely secreted this chemokine into their environment. The role of SDF-1 in the pathomechanism of the disease has been already examined, and elevated SDF-1 level was detected in the blood of cats suffering from FIC, and in the urine of human patients with IC (Corcoran et al., 2013; Parys et al., 2018). Based on the present results it can be suggested that NE had a direct stimulatory effect on the pro-inflammatory processes of uroepithelial cells by increasing the level of the pro-inflammatory chemokine SDF-1, which may contribute to the development of inflammatory symptoms in cats with FIC. Furthermore, the fact that, similar to what was observed in **Study II.**, both acute and chronic treatment induced an elevated pro-inflammatory cytokine response reflects the inability of bladder epithelial cells to adapt to the repeated elevated presence of NE demonstrating the prominent role of NE in the pathogenesis of the disease.

Reactive oxygen and nitrogen species can be also involved in the inflammatory processes by inducing oxidative stress in cells and increasing the levels of various pro-inflammatory molecules. In addition, they also play an important role in altering cell permeability by damaging cell membranes (Forrester et al., 2018; Homan et al., 2013; Masuda et al., 2009; Miyata et al., 2019; Treutlein et al., 2012). Notwithstanding that the results of **Study II.** suggest that acute NE treatment can induce oxidative stress without lipid peroxidation, DNA damage or endoplasmic reticulum distress, it was aimed to investigate whether the intermittent presence of NE affects MDA production in cultured uroepithelial cells as previous studies have shown that the appearance of elevated MDA concentrations can be detected only in the chronic cases of bladder inflammation (D'Amico et al., 2021; Tanik et al., 2017; Vysakh et al., 2017). Furthermore, we investigated the amount of hydrogen peroxide

as a general oxidative stress marker, since this molecule also serves as a potential diagnostic marker in the chronic cases of human interstitial cystitis (Masuda et al., 2009). In **Study III.** the level of MDA as an oxidative stress marker from lipid peroxidation did not show significant changes after 3x1h NE treatments similarly as in **Study II**., suggesting that NE was unable to induce lipid peroxidation on the applied uroepithelial cell culture. These findings are in line with the results of a new study, where MDA level remained unchanged in the plasma of cats with FIC compared with healthy ones (Nishi et al., 2023). In contrast, the concentration of H_2O_2 in cell culture media was increased in case of 3x1 h 100 and 1000 µM NE exposure and the H_2O_2 level showed positive correlation with the extracellular SDF-1 concentration similarly as in **Study II.,** where a positive correlation was also observed between SDF-1 concentration and redox parameters. These data are in line with the results of previous studies indicating that urinary H_2O_2 level was increased in patients with IC (Masuda et al., 2009) and intravesical H_2O_2 injection was able to provoke inflammation in rat urinary bladder by increasing the amount of IL-6, IL-1 and TNF-α of the bladder wall (Homan et al., 2013). These correlations may also be caused by the oxidative stress caused by the production of numerous reactive oxygen and nitrogen species during inflammation, and the fact that these can initiate intracellular signaling cascades contributing to increased pro-inflammatory gene expression, reflecting the strong link of the inflammatory and oxidative stress response (Anderson et al., 1994; Biswas, 2016; Collins, 1999; Flohé et al., 1997).

To investigate the effect of intermittent stress on barrier function of uroepithelial cells and to comparison the effect of acute and chronic stress, similarly as in **Study II.**, GAG concentration of cell culture media, claudin-4 content of the cultured cells, and TER values were measured after NE supplementation. According numerous former studies, GAG layer of the bladder wall is damaged in bladder of human patients with IC and in cats with FIC; however, it is not clear if it is a cause or consequence of FIC (Buffington, Blaisdell, et al., 1996; Buffingtonet al., 1996; Kruger et al., 2009; Nordling & Ophoven, 2008; Pereira et al., 2004). Based on our recent results, it can be concluded that NE treatment significantly reduced the GAG content of cultured bladder epithelial cells, confirming the key role of NE in altered permeability of bladder epithelia in FIC affected cats. Moreover, while in **Study II**., only the 100 µM NE treatment was able to reduce GAG concentrations, in the present **Study III.**, all the applied treatments induced a significant reduction in GAG levels, demonstrating that the uroepithelial cells are more sensitive to repeated elevated NE levels than to a single acute stressor. Furthermore, not only the GAG content, but also the abundance of claudin-4 as an important tight junction protein showed significant decrease after all the applied NE treatments similarly as in **Study II**. Investigating the level of tight junction proteins is a useful tool to gain information concerning the barrier function of the uroepithelium as it is strongly maintained by the structural integrity of tight junctions (Acharya et al., 2004). In addition to

the above-mentioned facts, in our research, the TER values of the uroepithelial monolayer cell cultures were significantly decreased following all used NE treatments both directly after exposure and after 24 h regeneration time, similarly to a former study, where declined TER was measured in the bladder of cats with FIC (Lavelle et al., 2000). Further, similarly to the GAG measurements in **Study III.** even lower NE concentrations were able to induce decline of uroepithelial barrier function, compared to the results of **Study II.**, where only 100 and 1000 µM NE exposures induced TER reductions. These findings also confirm that cultured bladder cells are more sensitive to repeated NE signals compared to a single acute addition. Further, the results confirm that intermittent NE exposure can directly disturb the barrier function of uroepithelial cells, which may be in association with the altered GAG metabolism and diminished function of the *tight junctions*. However, in **Study II.** positive correlations were observed between GAG levels and TER values, and in **Study III.** negative correlations were found between claudin-4 abundance and H_2O_2 concentration, and between claudin-4 and SDF-1 levels, suggesting the complex interplay of the inflammatory and oxidative stress response with the impaired barrier integrity of the uroepithelium. These correlations can be explained by the results of different previous studies, where reactive oxygen species such as H_2O_2 was able to increase paracellular permeability by disruption of *tight junction* proteins. Further, the production of pro-inflammatory mediators also enhanced the uroepithelial barrier permeability (Han et al., 2004; Hasegawa et al., 2021; Liu et al., 2019; Rao et al., 2000). The PCA shows that there were some differences between the molecular effects of acute and chronic NE exposure, hence on the PCA plot of the acute NE treatment two distinct group for a control, 10 and 100 µM NE treated cells against the 1000 µM NE treated cells could be detected, compared to the chronic stress model. There two distinct groups could be observed for the control against all treated cells suggesting that chronic intermittent NE treatment may have a stronger molecular effect on uroepithelial cells than a single acute exposure. Furthermore, the small differences between the correlation analysis and PCA of the two models can be explained by the different response of the cells in case of diverse dynamics of the stressor.

In the present PhD study, a primary bladder epithelial cell culture of feline origin was established, which can serve as a useful tool for *in vitro* studies of the pathogenesis of various urinary tract disorder such as FIC. Since previous literature has shown that stress plays an important role in the development of the disease and NE is the most important stress hormone in FIC affected cats, the biochemical role of this hormone was investigated, with special emphasis on the direct link between the changes in the bladder epithelium – manifested by FIC symptoms – and stress. As a limitation of the model used in present PhD dissertation, it is important to note that since FIC is a complex, multi-organ disease, in addition to the studied NE release, other factors may also have a major impact on the

changes in the bladder epithelial cells, and further studies are needed to the better understanding of the pathogenesis and biochemical background of the disease. Further, as a limitation of the work, it is also important to mention the relatively low number of samples in certain measurements of Study II., and Study III., resulting from the limited number of uroepithelial cells could be isolated from one urinary bladder according to the applied primary feline uroepithelial cell culture protocol.

6.4. Conclusions

On the basis of our results, the conclusions of the present PhD thesis can be summarized as follows. Short term NE exposure was able to increase the metabolic activity of the cultured uroepithelial cells, suggesting that the enhanced cellular metabolism might have an important role in the stress response of the organ. Further, both acute and intermittent NE treatment could increase the SDF-1 production of uroepithelial cells, even at lower concentration; however, NE-triggered IL-6 secretion was observed only after applying short term NE exposure at higher concentrations. Acute NE treatment could induce the oxidative protein damage of cultured urothelial cells, but no DNA damage, lipid peroxidation, or ER stress was detected in the case of the applied short term NE treatments, however the pulsative NE treatment was able to induce oxidative stress by increasing the H_2O_2 concentration of the cell cultures. As a result of the applied acute and chronic intermittent stress, the barrier function of feline uroepithelial cells was damaged, as reflected by the reduced TER values, which may be in association with the reduced expression of claudin-4 tight junction protein and the decreased GAG concentration.

As previously described, in cats with FIC, the inhibitory effect of cortisol on catecholamine synthesis is lacking during chronic stress, therefore it is essential to examine the pathological role of NE, as a main regulatory stress hormone in the affected cats. Further, recurrence of symptoms in response to repeated stress is very common, therefore, we investigated whether the onset of symptoms might be related to the presence of repeatedly elevated NE, or whether uroepithelial cells might be able to adapt to chronic stress. There are numerous studies in the literature describing the ability of certain cell types to adapt to the persistent presence of different stressors, for example hepatocytes can successfully adapt to heat stress, or endothelial cells to mechanical stress (Mackei et al., 2020; Matthews et al., 2006). In other studies, it was described that acute and also chronic NE exposure could induce oxidative stress and elevated pro-inflammatory cytokine expression in rat myocardial cells (Neri et al., 2007), however, until now no information could be found regarding the effect of chronic presence of NE on uroepithelial cell adaptation.

The results of the present PhD thesis suggest that NE as a stress hormone may have pro-inflammatory action, can contribute to oxidative stress, and may impair the barrier function of the urothelium in case of acute and also chronic intermittent cases, hence it may play an important role in the pathogenesis of the FIC as well. Further, the established feline primary uroepithelial cell culture can be considered as a proper *in vitro* model for studying the molecular mechanisms of urinary disorders, such as FIC in cats.

7. New scientific results

Ad 1,

A novel primary uroepithelial cell culture, which contains differentiated uroepithelial cells from feline origin has been successfully established and characterized by Giemsa staining and immunocytochemistry. This newly established cell culture can serve as proper tool for studying the biochemical background of FIC and other urinary tract diseases.

Ad 2,

Acute stress mimicked by short term (1h) NE treatment was able to increase the metabolic activity of the cultured uroepithelial cells and elevated the extracellular concentrations of the pro-inflammatory mediators IL-6 and SDF-1, confirming that NE can trigger an inflammatory response in the uroepithelium. Oxidative stress markers such as cellular PC levels were elevated by NE exposure, while the concentration of MDA, 8-OHdG and GRP78 were not increased, indicating that NE may provoke the oxidative damage of proteins without inducing lipid peroxidation, DNA damage or endoplasmic reticulum stress. Further, the acute NE challenge might diminish the barrier function of uroepithelial cells, as reflected by the decreased GAG concentration, claudin-4 protein expression, and reduced TER values of the NE-treated cell cultures. Hence, it is highly expected that stress-associated NE release may play an important mediatory role in the pathogenesis of FIC.

Ad 3,

Chronic intermittent stress mimicked by pulsative (3x1 h) NE treatment was also able to trigger pro-inflammatory response and oxidative-stress in the uroepithelial cells by increasing the level of SDF-1 and H_2O_2 in cell culture media. In addition, similarly to the acute stress exposure, NE increased the permeability of the uroepithelium, since decreased GAG concentration, claudin-4 abundance, and TER values were measured after the NE treatments. The obtained results suggest that bladder epithelial cells are unable to adapt to recurrently elevated NE levels, further supporting the important role of NE in the pathogenesis of FIC.

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9. Own scientific publications

9.1. Publications related to the topic of the present PhD thesis

9.1.1. Full text papers in peer-reviewed journals

Hatala P, Lajos A, Orbán K, Kulcsár A, Mátis G, Neogrády Zs: **Neurohumoral and biochemical background of feline idiopathic cystitis.** *Magyar Állatorvosok Lapja* 142:367–376, 2020.

Hatala, P, Lajos A, Mackei M, Sebők C, Tráj P, Vörösházi J, Neogrády Zs, Mátis G: **Feline Uroepithelial Cell Culture as a Novel Model of Idiopathic Cystitis: Investigations on the Effects of Norepinephrine on Inflammatory Response, Oxidative Stress, and Barrier Function.** *Veterinary Sciences*, 10, 132. 2023.

Hatala P, Sebők C, Mackei M, Kárpáti K, Gálfi P, Neogrády Z, Mátis G: **Molecular effects of intermittent stress on primary feline uroepithelial cell culture as an *in vitro* model of feline idiopathic cystitis.** *Frontiers in Veterinary Science*, 6;10:1258375, 2023.

9.1.2. Oral and poster presentations on international conferences

Hatala P, Lajos A, Orbán K, Mátis G and Neogrády Zs: **Investigating the inflammatory effect of noradrenaline in a feline urinary bladder cell culture.** Clinical/Research Abstracts Poster Presentation at ISFM European Feline Congress, Cavtat, Croatia 2019.

9.1.3. Oral presentations on Hungarian national conferences

Hatala Patrícia, Lajos Andrea, Mackei Máté, Orbán Kata, Kulcsár Anna, Neogrády Zsuzsanna, Mátis Gábor: ***Macskák ismeretlen oktanú cisztitiszének biokémiai háttere*****,** MTA Akadémiai beszámolók, 2019. január, Budapest

Hatala Patrícia, Kárpáti Karina, Sebők Csilla, Mackei Máté, Mátis Gábor és Neogrády Zsuzsanna: ***Az intermittáló stressz molekuláris hatásainak vizsgálata macska eredetű primer húgyhólyaghámsejt-tenyészeten*****,** MTA Akadémiai beszámolók, 2024. január, Budapest

9.2. Publications not related to the topic of the present PhD thesis

9.2.1. Full text papers in peer-reviewed journals

Mátis Gábor, Hatala Patrícia, Kulcsár Anna, Kulcsárné Petrilla Janka, Neogrády Zsuzsanna **A Kupffer-sejtek szerepe a máj gyulladásos és metabolikus folyamatainak szabályozásában: Irodalmi áttekintés: Role of Kupffer-cells in the regulation of hepatic inflammatory and metabolic processes** *Magyar Állatorvosok Lapja* 137:(9) pp. 569-575, 2015.

9.2.2. Oral and poster presentations on international conferences

Anna Kulcsár, Dénes Dudás, Gábor Mátis, Patrícia Hatala, Hedvig Fébel, Zsuzsanna Neogrády **The effect of age and diet type on the hepatic and intestinal CYP activity in broiler chicken** XVth European Poultry Conference, Dubrovnik, Horvátország, 2018.

Gábor Mátis, Anna Kulcsár, Patrícia Hatala, Máté Mackei, Zsuzsanna Neogrády **Investigations on the effects of heat stress on hepatic cell culture models of chicken origin** XVth European Poultry Conference, Dubrovnik, Horvátország, 2018.

9.2.3. Oral and poster presentations on Hungarian national conferences

Kulcsár Anna, Sebők Csilla, Mátis Gábor, Talapka Petra, Hatala Patrícia, Petrilla Janka, Fébel Hedvig, Neogrády Zsuzsanna **Az inzulin és a glukagon jelpálya különböző takarmányozási faktorok segítségével történő szabályozása brojlercsirkében** MTA Akadémiai Beszámolók, Budapest, Hungary, 2018.

Mátis Gábor, Kulcsár Anna, Hatala Patrícia, Tóth Adrienn, Mackei Máté, Neogrády Zsuzsanna **A T-2 toxin sejtkárosító hatásainak összehasonlító vizsgálata csirke primer bélhámsejt- és májsejttenyészeten** MTA Akadémiai Beszámolók, Budapest, Hungary, 2018.

Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Hatala Patrícia, Kővágó Csaba, Neogrády Zsuzsanna **Bakteriális lipopoliszacharidok által kiváltott gyulladás vizsgálata sertés hepatocyta – Kupffer-sejt ko-kultúra modellen** MTA Akadémiai Beszámolók, Budapest, Hungary, 2015.

Mátis Gábor, Kulcsár Anna, Kulcsárné Petrilla Janka, Hatala Patrícia, K_vágó Csaba, Csikó György, Neogrády Zsuzsanna **A Kupffer-sejtek arányának meghatározása sertés primer májsejttenyészeten** MTA Akadémiai Beszámolók, Budapest, Hungary, 2014.

9.3. Supervision of DVM theses

Sebők Csilla: **Egyes takarmányozási faktorok inzulin jelpályára gyakorolt hatásának vizsgálata brojlercsirkében** TDK thesis, supervisors: Kulcsár Anna, Hatala Patrícia, Budapest, 2017.

Lajos Andrea: **A noradrenalin gyulladáskeltő hatásának vizsgálata macska eredetű primer húgyhólyaghámsejt-tenyészeten** TDK thesis, supervisors: Hatala Patrícia, Mátis Gábor, Budapest, 2018

Kárpáti Karina: **A krónikus stressz molekuláris hatásainak vizsgálata macska eredetű primer húgyhólyaghámsejt-tenyészeten** TDK thesis, supervisors: Hatala Patrícia, Mátis Gábor, Budapest, 2023.

10. Supplementary materials

Table S1.: Means and standard errors of the means (SEM) of the results of the Study II. measurements (CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells rel.abs.=relative absorbance, rel.conc.=relative concentration).

Examined parameters	CTR		10NE		100NE		1000NE	
	mean	**SEM**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**
CCK-8 (rel.abs.)	1.000	0.047	0.986	0.028	1.135	0.063	2.236	0.079
LDH (rel. act.)	1.000	0.440	1.571	0.562	1.285	0.630	0.857	0.303
IL-6 medium (ng/ml)	43.350	1.145	31.773	3.437	42.554	2.109	53.527	2.555
IL-6 lysate (ng/ml)	34.910	2.633	27.776	4.466	34.550	1.535	31.070	0.862
SDF-1 medium (pg/ml)	1.755	0.076	1.966	0.02	1.978	0.040	2.154	0.028
SDF-1 lysate (pg/ml)	1.542	0.127	1.529	0.072	1.456	0.049	1.797	0.084
PC (ng/ml)	13.370	1.136	14.250	2.233	12.700	1.090	19.660	1.805
MDA (rel. abs.)	1.000	0.029	0.997	0.019	1.010	0.002	0.981	0.012
GRP78 (ng/ml)	0.049	0.013	0.013	0.001	0.023	0.012	0.035	0.005
8-OHdG (ng/ml)	3.639	0.324	3.688	1.455	1.481	0.436	3.484	0.573
GAG (rel. conc.)	1.000	0.095	1.029	0.214	0.382	0.074	0.781	0.068
claudin-4 (rel. abs.)	1.000	0.085	0.801	0.045	0.755	0.065	0.708	0.065
TER immediately (Ωcm^2)	59.000	10.140	28.000	1.730	16.000	3.605	7.000	2.640
TER regeneration (Ωcm^2)	66.000	12.120	19.000	2.000	15.000	4.582	9.500	2.780

Table S2.: Means and standard errors of the means (SEM) of the results of the Study III. measurements (CTR=non-treated control cells, NE10=10 μM norepinephrine treated cells, NE100=100 μM norepinephrine treated cells, NE1000=1000 μM norepinephrine treated cells rel.abs.=relative absorbance, rel.conc.=relative concentration, rel. fluor.=relative fluorescence).

	CTR		10NE		100NE		1000NE	
Examined parameters (mean)	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**
CCK-8 (rel. abs.)	1.000	0.233	1.162	0.117	1.347	0.272	1.181	0.106
LDH (rel. act.)	1.000	0.580	0.420	0.173	0.680	0.326	0.720	0.085
IL-6 medium (ng/ml)	36.170	4.030	31.302	3.288	30.663	2.597	33.859	4.278
IL-6 lysate (ng/ml)	58.658	1.693	55.427	2.019	54.287	2.739	57.454	3.119
SDF-1 medium (pg/ml)	1.222	0.025	1.372	0.045	1.503	0.117	1.611	0.102
SDF-1 lysate (pg/ml)	1.560	0.110	1.465	0.097	1.297	0.077	1.216	0.073
MDA (rel. abs.)	1.000	0.032	1.030	0.028	1.079	0.079	1.060	0.038
H_2O_2 (rel. fluor.)	1.000	0.032	0.982	0.035	1.409	0.107	4.170	0.930
GAG (rel. conc.)	1.000	0.381	0.152	0.084	0.115	0.050	0.152	0.030
claudin-4 (rel. abs.)	1.000	0.085	0.772	0.075	0.712	0.050	0.667	0.044

TER immediately (Ωcm^2)	45.500	4.359	18.500	3.500	13.500	1.732	15.500	3.605
TER regeneration (Ωcm^2)	55.000	6.080	9.000	1.732	11.000	1.000	8.000	1.000

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