

## Original Article

# Obesity influence on bladder inflammation and cancer: a cystitis model

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**Abstract:** Background: Recently, the role of subclinical inflammation in obesity has gained prominence. An association between obesity and chronic inflammation has been observed in several studies that show a relationship between increased morbidity and high Body Mass Index (BMI). This study aims to compare inflammatory pathways in obese (by high-fat diet) and non-obese mice after exposure to an intravesical carcinogen in a cystitis model. Methods: We divided 16 female, 7 week old mice into two groups: 1) CONTROL: standard diet, and 2) OBESE: high fat diet for 8 weeks. Both groups underwent a protocol for *N*-Nitroso-*N*-methylurea (MNU) pro-inflammatory bladder instillation. Bladder was analyzed by histopathology and western blotting for proteins of the inflammatory pathway (JNK, NFκB, c-JUN, IKK), and immunohistochemistry (proliferation and apoptosis). Results: While mice eating standard diet showed minimal histologic alteration in 4 of 5 (80%) bladder tissues, those eating a high fat diet showed moderate (60%) and intense (40%) chronic active inflammation with dysplasia foci, increased proliferation, apoptosis and inflammatory pathway activation with increased NFκB, and also IKKβ, JNK, and c-JUN phosphorylation in the urothelium. Conclusion: A high-fat diet causes increased urothelial proliferation, apoptosis, and NFκB expression with cystitis exacerbation and dysplasia. Together, these results suggest that obesity induced by a high-fat diet increases the inflammatory pathway in the bladder with possible pre-malignant alterations.

**Keywords:** Inflammation, obesity, cystitis, MNU, mice, cancer

### Introduction

Numerous studies have linked obesity with cancer. About 20 percent of all cancer cases are related to weight, weight increase, and obesity, and there is evidence that obese people have worse cancer prognoses and survival rates [31]. The complicated biological mechanisms that underlie the link between obesity and cancer comprise a number of diverse elements, including increased steroid hormone release, persistent inflammation, chronically elevated insulin levels, and insulin resistance [7, 8].

According to multicenter retrospective research with 892 patients, obesity is also linked to worse bladder cancer outcomes. Older age and higher BMI were strongly linked to an elevated

risk of recurrence, progression, and cancer-specific death [20]. After adjusting for the traditional clinicopathologic characteristics, another large trial with 1155 patients revealed that overweight and obesity were strongly related to an elevated risk of recurrence and progression [8].

Evidence suggests that chronic inflammation brought on by insulin, insulin-like growth factor-1, proinflammatory cytokines, oxidative stress, and growth factors [28] is characteristic of cancer evolution in diabetes mellitus (DM) patients. In particular, type 2 DM typical low-grade systemic inflammation may interfere in bladder cancer biology. The contribution of immune cells to the development of bladder cancer is now being investigated. Particularly, it

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has been suggested that innate immune system deregulation might have a protumor effect and that the adaptive immune system has an anticancer effect [29].

More research is being done on the role of inflammation in cancer to better understand mechanisms like carcinogenesis, resistance, progression, and metastasis. Microenvironment activation of the immune system occurs in the tumor [9] with reported increases in host components, expanding blood arteries, and inflammatory infiltrates. Additionally, the growth and spread of bladder cancer are significantly influenced by cytokines that the tumor microenvironment releases. Proinflammatory cytokines might play a role in the tumor microenvironment and cancer stem cells, which is important for the initiation, growth, and spread of cancer [14].

This study aims to compare inflammatory pathways in obese and non-obese mice after exposure to a pro-inflammatory carcinogen as a cystitis model.

### Material and methods

#### *Animals*

All animal handling and experiments were performed following the National Institute of Health guidelines for the use of experimental animals and the approval of the Care of Animals and Ethical Committee for Animal Research of the State University of Campinas (CEUA Protocol 6001-1). To carry out the study, 7 week old female C57BL/6J mice were provided by the University of Campinas Central Breeding Center (Campinas, Brazil). Mice were first numbered and then randomly selected for standard chow or high-fat diet using a random number generator. Mice were kept in the animal facility with constant light/dark cycle (12 h/12 h), room temperature (22°C), humidity, and receiving water ad libitum.

#### *Protocol for the induction of bladder inflammation*

The animals were kept on a control diet (20% protein, 70% carbohydrate, 10% lipid) or high fat diet (20% protein, 35% carbohydrate, 45% lipid) from week 0 (zero), thereby forming two

distinct groups containing 8 animals each. Both groups were anesthetized with halothane and received 4 doses of 1.5 mg of intravesical *N*-Nitroso-*N*-methylurea (MNU, Sigma, St. Louis, MO), with an interval of two weeks between each application [26]. In the 15th week of treatment, the weight of all animals was measured, animals were euthanized and the extraction of the bladders performed. Three animals from each group were randomly selected for western blotting analysis.

#### *Extraction of tissues*

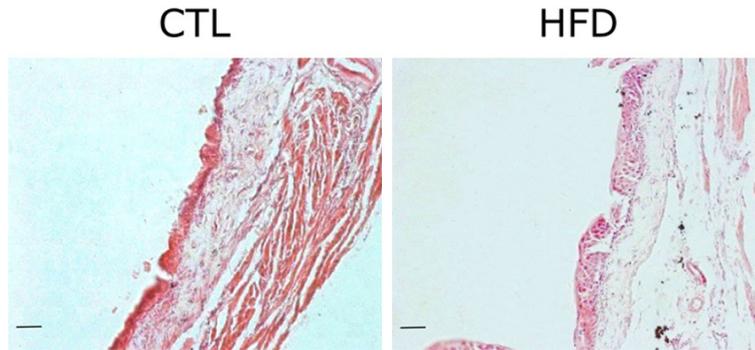
Animals were anesthetized intraperitoneally with thiopental (15 mg/kg) until loss of the corneal reflex and paw withdrawal to pain. Incision was made in the ventral region of the animal for total removal of the bladder. The extracted material was immediately placed in an extraction buffer (1% Triton X-100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethyl-sulphonyl fluoride and 0.1 mg/ml aprotinin) and homogenized with Polytron PTA 20S Generator Brinkmann Instruments Model E 10/35 set at full speed.

#### *Protein analysis by immunoblotting*

The insoluble material was removed by centrifugation for 30 minutes at 11,000 rpm in an Eppendorf 5804 centrifuge at 4°C. Protein was measured by the method of Bradford using the Bio-Rad reagent and BSA as standard. The supernatants were resuspended in Laemmli buffer containing 100 mM DTT, heated in boiling water for 5 minutes, and subjected to polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrically transferred to a nitrocellulose membrane. The membranes with transferred proteins were incubated in blocking solution (skimmed milk 5% 10 mM Tris, 150 mM NaCl and 0.02% Tween) for two hours at room temperature and incubated with specific antibodies (JNK, NFκB, c-JUN, IKK), at 4°C overnight under continuous stirring. The bands of autoradiographs were analyzed by optical densitometry.

#### *Histopathology*

The mice were euthanized and the bladder was infused with formalin through an intravesi-



**Figure 1.** Histopathology of bladders from mice in a standard diet group (CTL) and high fat diet (HFD) group (Scale bar represents 20  $\mu$ m, magnification 40 $\times$ ).

cal catheter. The bladders were extracted and immersed in formalin for 12 hours for fixation. Then, the material was dehydrated with different concentrations of ethanol and processed for paraffin embedding. The sections were cut, the paraffin was removed and the sections were stained with H&E (hematoxylin and eosin) for determination of tissue morphology.

Histopathology was classified according to the proposed WHO/International Society of Urological Pathology [24] nomenclature.

#### *Immunohistochemistry*

The same animal material used in tumor histopathology was subjected to immunohistochemistry also comprising 5 animals per group.

#### *Cell proliferation*

Antigen retrieval was performed in a microwave oven (Panasonic Junior) at 700 W. The slides were immersed in citrate buffer (0.01 mol/L, pH 6.0) for 10 minutes and cooled to room temperature. Histological sections were blocked with serum blocking solution and afterwards, incubated overnight at 4°C with primary antibody to Ki-67 (Dako Cytomation) (diluted 1:150). Next, the sections were incubated with the avidin-biotin LSAB + Kit (Dako Cytomation) for 30 minutes followed by staining with the chromogen solution diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich). The experiments were performed in triplicate for each animal.

#### *Apoptosis assay (TUNEL)*

Apoptosis was determined using the TUNEL assay (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling), using a cell death Detection Kit (Roche) according to the recommendations of the manufacturer (Promega, Madison, WI, USA).

#### *Statistics*

Variables were expressed as mean  $\pm$  standard deviation. The statistical analysis was

performed by Student's t-test, with  $P \leq 0.05$  considered significant.

## **Results**

#### *Histopathologic analysis*

No urothelial carcinoma was identified. Mice eating a standard diet showed mild histologic alteration in 4 of 5 (80%) bladder tissues and 20% had inflammation with submucosal layer hyalinization. On the other hand, mice eating a high-fat diet showed urothelial moderate chronic active inflammation in 3 of 5 (60%) and intense urothelial chronic active inflammation with dysplastic foci in 40% (**Figure 1**).

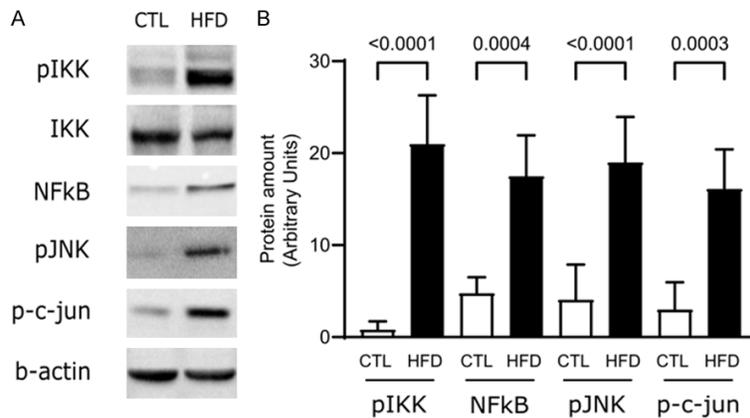
#### *Western blotting*

When we analyzed the bladders from mice undergoing standard (CTL) or a high-fat diet (HFD), we observed an increase in markers of inflammation, with increase in IKK $\beta$  phosphorylation accompanied by an increase in total NF $\kappa$ B in HFD compared to CTL. Furthermore, JNK and c-JUN phosphorylation also showed an increase in HFD compared to CTL, indicating that the inflammatory pathway was activated in the HFD tissues (**Figure 2**).

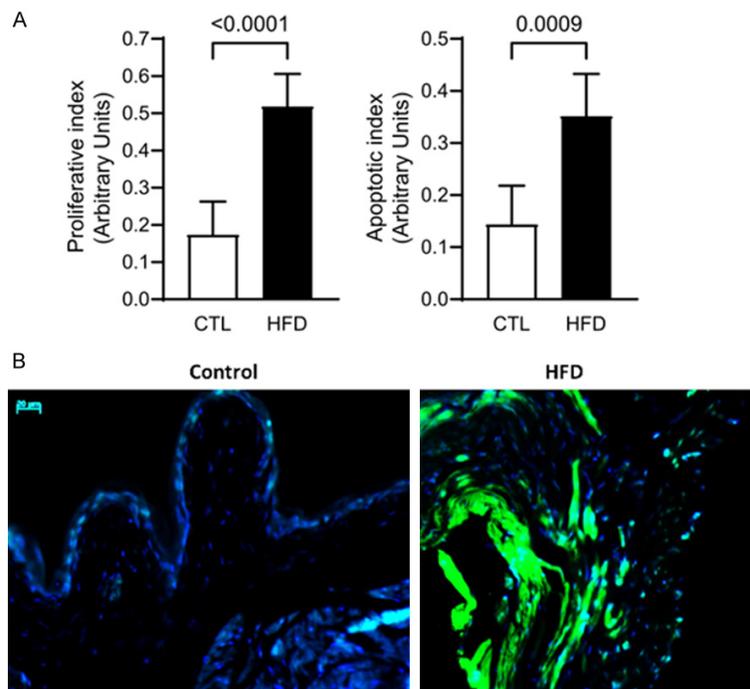
#### *Immunohistochemistry*

Cell proliferation rates were higher in mice eating a high-fat diet. The mean value of the proliferative index in the obese group was 0.52, while standard diet mice had a lower mean proliferative index of 0.17 ( $P < 0.05$ ) (**Figure 3**).

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**Figure 2.** Western blot (A) and quantification (B) of the inflammatory signaling pathway in bladders from mice in a standard diet group (CTL) and high fat diet (HFD) group.



**Figure 3.** Quantification of proliferative index and apoptotic index of bladders from mice in a standard diet group (CTL) and high fat diet (HFD) group (A) and (B) representative images of proliferation (Ki-67, Green) and apoptosis (TUNEL, Blue).

The apoptotic index was also higher in mice eating a high fat diet. The mean value of apoptotic index in the obese group was 0.35, while standard diet mice had a mean apoptotic index of 0.14 ( $P < 0.05$ ) (Figure 3). These results showed an increased cellular turnover in the obese group.

## Discussion

The current study showed a higher potential for bladder inflammation and premalignant alterations (dysplasia) in mice under a high fat diet. Mice eating a standard diet showed minimal histological alteration in 4 of 5 (80%) bladder tissues, those eating a high-fat diet showed moderate (60%) and intense (40%) chronic active inflammation with dysplasia foci, increased proliferation, apoptosis and inflammatory pathway activation with increased NFκB, and also IKKβ, JNK, and c-JUN expression in the urothelium. The tumor microenvironment has an important role in the development, growth, and progression of cancer. There is evidence that obesity is associated with inflammation, and the presence of chronic inflammation has been associated with fibrosis and cancer [12, 16, 19].

Studies on the impact of BMI on bladder cancer are scarce and controversial. It was [1, 2, 21] observed through the analysis of a population of 471,760 people with 1,719 positive cases of bladder cancer, that obesity was associated with a 28% increased risk for this cancer compared to patients of normal weight, following the same trend observed by others [5]. In contrast a different study [13], using a similar sample of U.S.

adults with 866 positive cases of bladder cancer did not observe an association between BMI and the risk for this cancer.

Although a relationship between the natural course of bladder cancer and obesity is present [11, 23], firm conclusions have not been

made until the present time. Future research is needed to delineate the biological mechanisms for such a relationship and, thus, clarify the influence of obesity in bladder cancer.

Inflammation is a key promoter of the pathogenesis of diseases such as rheumatoid arthritis, Crohn's disease, and atherosclerosis, as well as cancer of the liver, stomach, and colon [3, 10, 17, 18, 22]. It is well established that obesity is associated with a state of chronic subclinical inflammation (metabolic inflammation) and has an important role in the pathogenesis of several metabolic disorders, including type 2 diabetes and metabolic syndrome [15].

The stress-activated protein kinases, c-Jun N-terminal kinase 1 (JNK1) and Inhibitor of NF $\kappa$ B kinase  $\beta$  (IKK $\beta$ ) are central to signaling in innate immunity and the stress response that control the expression of many pro-inflammatory genes [6]. In general, JNK and IKK complex are situated on different central signaling pathways involved in inflammation and innate immunity stress positions, whose main function is to activate the host defense (inducing TNF, IL-6, IL-1, and other pro-inflammatory cytokines) and maintain homeostasis [22].

Additionally, prolonged use of non-steroidal anti-inflammatory drugs such as aspirin reduces the incidence of colon, lung, stomach, esophagus, ovary, and Hodgkin's lymphoma [30]. However, there are controversies as to this relationship, and their mechanisms are still not well understood.

Commonly, inflammation and innate immunity exert pro-tumorigenic effects, whereas adaptive immunity may have an anti-tumorigenic action [4, 22]. These effects are mediated by various types of leukocytes, including macrophages, tumor associated macrophages, dendritic cells, neutrophils, mast cells, and T cells recruited to the tumor microenvironment through interactions with the local stromal cells and malignant cells. These leukocytes produce cytokines, angiogenic factors, and growth, as well as metalloproteinases and their inhibitors, which allow proliferation of malignant cells, invasion, and distant metastases.

The expression of several proinflammatory cytokines such as TNF, IL-1, and IL-8 is regulated by means of the target-dependent activa-

tion of NF $\kappa$ B and of the IKK $\beta$  gene. Many oncogenes and carcinogens cause activation of NF $\kappa$ B, whereas substances with known chemopreventive properties may interfere with its activation. Recent studies in animal models have given strong and direct genetic evidence that the activation pathway of NF $\kappa$ B-dependent IKK $\beta$  is a crucial mediator in tumor promotion [10, 17, 25].

There are two pathways for activation of NF $\kappa$ B. The classical pathway is activated by pro-inflammatory stimuli, among which cytokines (TNF, IL-1), proteins of the bacterial cell membrane (lipopolysaccharide) or virus. These substances promote phosphorylation of IKK $\beta$ , resulting in proteasomal degradation of I $\kappa$ B and consequent release of the dimers of NF $\kappa$ B (usually a p50-REL-A) to nuclear migration and ubiquitination and transcription of target genes. The alternative pathway is activated by members of the TNF family, independently of IKK $\beta$ , and promotes phosphorylation of p100, resulting in nuclear translocation of the dimer p52 REL-B [3].

The contribution of the classical pathway of activation of NF $\kappa$ B in inflammation and cell proliferation is well accepted, and sustained activation of NF $\kappa$ B has been described in various malignancies. Due to the variety of the classical pathway target genes, including those that express inflammatory mediators, it was proposed that the activation of the classical pathway of NF $\kappa$ B determines the relationship between inflammation and tumor promotion and progression [3, 10, 17, 18, 22].

The use of mice in studies of liver and colon cancer associated with inflammation supports this hypothesis and explains how inflammation drives tumor promotion and progression [10, 17, 25]. Our results so far have shown that obese mice express more inflammatory proteins and have a faster urothelial turnover than non-obese mice. Even though cancer was not present in neither of the groups, the obese group presented inflammatory lesions suggesting a more disease-prone microenvironment. We have previously shown that the intravesical MNU model has wide potential, from bladder cancer animal model when using Fisher 344 strain, to Lewis strain as a carcinogen resistance model [27]. According to current results, C57BL/6J mice are also less suscepti-

ble to carcinogenesis compared to the Fisher 344 rat strain.

Our study is not without limitations. When using MNU, a previous validated complete intravesical carcinogen, the protocol is limited to relatively short-term evaluation (4 MNU doses and 15 weeks) in mice, which showed low sensitivity to carcinogenesis. Future studies should explore the potential for malignant transformation in the mid and long term.

### Conclusion

A high-fat diet can increase urothelial proliferation, apoptosis, and NF $\kappa$ B expression with cystitis exacerbation and dysplasia. Together these results suggest that obesity induced by a high-fat diet increases the inflammatory pathway of the bladder with possible pre-malignant alterations.

### Disclosure of conflict of interest

None.

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