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Development of urinary bladder preneoplasia by injection of *Schistosoma haematobium* eggs and administration of chemical carcinogen in mice

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Development of urinary bladder preneoplasia by injection of *Schistosoma haematobium* eggs and administration of chemical carcinogen in mice

by

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ABSTRACT

Development of urinary bladder preneoplasia by injection of *Schistosoma haematobium* eggs and administration of chemical carcinogen in mice

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Background: Human schistosomiasis is a chronic disease caused by the blood flukes belonging to the genus *Schistosoma* including *Schistosoma haematobium* (Sh). According to International Agency for Research on Cancer (IARC), *S. haematobium* is categorized as group 1 biocarcinogen of human urinary bladder (UB). The present study investigated developing UB cancer mouse model by injecting Sh eggs into the bladder wall and introduction of chemical carcinogens. The association of experimental Sh eggs injection into the mouse bladder wall and co-administration of N-nitrosodimethylamine (NDMA) for bladder cancer induction has not been studied yet.

Methods: A total of 72 female ICR mice were divided into 6 group with different combinations of Sh eggs, NDMA, and N-butyl n-(4-hydroxybutyl) nitrosamine (BBN): control group (Group 1); Sh eggs (Group 2); NDMA (Group 3); BBN (Group 4); Sh eggs + NDMA (Group 5); Sh eggs + BBN (Group 6). Group 2, 5 and 6 animals underwent anaesthesia by isoflurane and after midline lower abdominal incision was made 1000 Sh eggs in 50 μL saline were injected submucosally into the anterior aspect of the bladder dome. The control group was injected with 50 μL saline. NDMA and BBN, prepared in drinking water with a concentration of 12.5 part per million (ppm) and 0.05%, respectively were provided to mice *ad*

libitum throughout the experimental process. Two mice from each group were sacrificed after 4, 12, 20, 28 and 36 weeks post injection of Sh eggs and NDMA or BBN treatment. Histopathological and immunohistochemical (IHC) analyses of mouse bladder tissue, relative expression of tumor suppressor and epithelial mesenchymal transition (EMT) marker genes by qRT-PCR were studied. Mouse cytokines and IgG subtypes analyses were also investigated.

Results: Significant reduction (20.5%) of body weight was observed in Sh eggs + BBN group (36.26 ± 7.2 g; $P < 0.01$) and Sh eggs + NDMA group with 11.6% weight loss (40.33 ± 8.5 g; $P < 0.01$), compared to the control group (45.61 ± 8.3 g). The histopathological findings of the bladder wall showed variable degrees of abnormalities ranging from mild hyperplasia to epithelial vacuolar change, squamous metaplasia and dysplasia. In particular, squamous metaplasia and dysplasia, urinary schistosomiasis related pre-neoplastic changes were observed in Sh eggs + NDMA group at week 12 but not in the Sh eggs group. Moreover, few *S. haematobium* eggs were observed from the mouse bladder tissue sections but there were no inflammation or granuloma surrounding the eggs. IHC revealed that *Ki-67* expression intensity for urothelial epithelial cells was significantly high for the Sh eggs + BBN group at week 20 while weak or no staining for the remaining groups. With the exception for IgG2b, the mean serum levels of IgG1, IgG2a and IgG3 of the Sh eggs and Sh eggs + NDMA group showed more or less strong significant difference compared to those of control in week 4, 12 and 20. IgG1 also showed significant increase for the Sh eggs + BBN group at week 4 and 12 while IgG2b only for the Sh eggs + NDMA group at week 12. Results from qRT-PCR of Sh eggs group showed strong relative mRNA expression of *p53* gene at week 4 compared to the rest group. Expression level of *p53* gene in the week 20 showed a slight significant variation for the treated group but the remaining group did not show significant variation over the remaining weeks. Relative mRNA expression of *E-cadherin* among Sh eggs + BBN group at week 12, 20 and 28 and all treated groups was downregulated whereas *vimentin* level was upregulated for Sh eggs + BBN group at week 12 and 20.

Conclusions: Sh eggs and nitrosamines may transform UB cells into squamous metaplasia and dysplasia in correlation with increased expression of *Ki-67*, *p53*, and *vimentin* with decreased *E-cadherin.* Such

inverse expressions of *E-cadherin* and *vimentin* mainly in Sh eggs + BBN group may be an indication for EMT. The present study provides a possibility of the mouse model for bladder cancer study.

Key words: bladder cancer, mouse, *Schistosoma haematobium* eggs, N-nitrosodimethylamine, N-butyl n-(4-hydroxybutyl) nitrosamine

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LIST OF ABBREVIATIONS

ABL-2	Animal biosafety level 2
BBN	N-butyl n-(4-hydroxybutyl) nitrosamine
CCA	Cholangiocarcinoma
CD-1	Caesarean Derived-1
EMT	Epithelial mesenchymal transition
H&E	Hematoxylin and eosin
IACUC	Institutional animal care and use committee
IARC	International agency for cancer research
IBC	Institutional biosafety committee
ICR	Institute for cancer research
IHC	Immunohistochemistry
NDMA	N-nitrosodimethylamine
ppm	parts per million
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Sh	*Schistosoma haematobium*
SNU	Seoul National University
WHO	World Health Organization

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INTRODUCTION

Human schistosomiasis is a chronic disease caused by the blood flukes belonging to the genus *Schistosoma* such as *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. intecalatum* and *S. mekongi*. *Schistosoma haematobium* accounts for about 112 and 436 million individuals' infection and risk of infection, respectively. More people are infected with *S. haematobium* than with the other schistosomes (King, 2010). It has been reported that the estimated annual mortality rates of 150,000 due to urogenital schistosomiasis-induced non-functioning kidney makes *S. haematobium* one of the most lethal worms worldwide (Van Der Werf et al., 2003).

The adult female worms lay eggs that are deposited in the wall of the urinary bladder. The individual ovum is home to the miracidium that produces proteolytic enzymes which aid the eggs to move either towards the lumen of the bladder or towards the host intestine (Gryseels et al., 2006). The eggs act as a mechanical irritant to the bladder epithelium, inducing chronic inflammatory lesions which frequently lead to conversion of the normal transitional epithelium of the bladder to a metaplastic squamous cell epithelium (Hodder et al., 2000; Parkin, 2008). As a result of inflammation, a range of urothelial abnormalities and related signs such as hematuria, dysuria, lesions of the bladder, kidney failure and bladder cancer are linked (Poggensee and Feldmeier, 2001; Fenwick et al., 2003; Parkin, 2006).

Bladder cancer is any of several types of cancers arising from the epithelial lining (urothelium) of the urinary bladder. An estimated 429,800 new cases of bladder cancer and 165,100 deaths occurred in 2012 worldwide. The majority of bladder cancer occurs in men, and there is about a 10-fold variation in incidence rates internationally (Siegel et al., 2012). It is more common in developed countries especially in Western world where it stands fourth and ninth most common cancer in men and women, respectively and the 7th most common cancer in men and the 17th most common in women worldwide (Ferlay et al., 2010). The highest incidence rates are found in the countries of Europe, North America, and Northern Africa, causing an estimated 150,000 deaths per year (Jemal et al., 2011).

There are four types of bladder cancer which include transitional cell carcinoma (TCC) also recently called urothelial carcinoma (UC), squamous cell carcinoma (SCC), adenocarcinoma and small cell carcinoma. UC is the most common type in Western countries (Ploeg et al., 2009); whereas SCC also called bilharzial bladder cancer predominates in some countries in Asia and Africa owing to endemic schistosomiasis (Amr et al., 2014). In those urinary schistosomiasis endemic nations, there have been reports indicating the presence of *S. haematobium* in the bladder cancer of human formalin-fixed paraffin embedded tissues (Gatta et al., 2012).

SCC is one of the most severe complications of chronic schistosomiasis. SCC differs from UC in its clinicopathological and molecular characteristics. For instance, most SCC cases present with stage T3 and T4, with a lower incidence of nodal metastasis. Furthermore, the age ranges of squamous cell carcinoma schistosomiasis dependent bladder cancer patients are lower than non-schistosomiasis bladder cancers i.e. third or fourth decade in SCC while seventh decade in UC (Shaw et al., 1999).

Schistosoma haematobium has been classified as a class 1 carcinogen by IARC of the World Health Organization (IARC, 2012). The epidemiologic association is based on both case control studies and the close correlation of bladder cancer incidence with prevalence of *S. haematobium* infection within different geographic areas. It has been estimated that schistosoma associated bladder cancer incidence ranges 3–4 cases per 100,000 (Shiff et al., 2006). Available data suggest that SCC of bladder arises from transformation of squamous metaplastic lesions, which occurs secondary to inflammation initiated by urinary schistosomiasis (Rausch et al., 2012). Previous studies reported that SCC accounts for up to 75% (Mostafa et al., 1999) and 83% (Shirahama and Sakakura, 2001) of bladder cancers in certain schistosomiasis endemic regions of the world. In schistosomal endemic areas of Africa, the prevalence of SCC has been reported to be high compared to conventional UC, especially in rural areas (Heyns and van der Merwe, 2008). Felix et al. (2008) reported that the occurrence of UC of the bladder has supplanted SCC in Egypt following a major decline in the prevalence of urinary schistosomiasis, which suggests the importance of schistosome-associated bladder cancer (Shiff et al., 2010). Recently, a retrospective review of 185 patients in north-western Tanzania reported 55.1% of SCC whereas 40.5% UC and 44.9% of all

cancer cases were found to have schistosomal eggs (Rambau et al., 2013). Similarly, study from Angola reported a prevalence of 71.7 % *S. haematobium* infection with 3.4% vesical tumor, classified as SCC (Botelho et al., 2015).

Although risk factors, such as parasite, host immune, genetic and molecular abnormalities, chemical or environmental exposures, and chronic irritation, may contribute to the development of bladder cancer (Kaufman et al., 2009; Honeycutt et al., 2014), it has been reviewed recently that several studies overwhelmingly document the epidemiologic and experimental association between the parasite and the development of SCC of the UB (Nweke et al., 2015). The studies showed that the chronic inflammatory processes induced by the eggs on the bladder wall induced macrophages to produce toxic free radicals, with the ensuing generation of a variety of nitrogenous compounds, which are eventually activated by the p-450 of the liver to genotoxic N-nitroso compounds.

Chronic inflammation is a well-established risk factor for the development of bladder cancer, as seen in *S. haematobium* infection (shown by hypothetical model in Fig. 1). Evidences showed that the eggs not only release highly inflammatory antigens but also have proliferative and anti-apoptotic effect (Botelho et al., 2009 & 2013; Gouveia et al., 2015). These eggs-induced chronic inflammation and irritation in the urinary bladder are associated with increased cancer initiation at the site of inflammation (Rosin et al., 1994). Inflammatory cells such as macrophages and neutrophils are important sources of endogenous oxygen radicals, which are also implicated in the formation of carcinogenic N- nitrosamines (Marletta, 1988) and hydroxyl radicals from the inflammatory cells (Dizdaroglu et al., 1993).

Cell proliferation is a hallmark of cancer including bladder cancer. *Ki-67* is a nucleolar protein expressed by proliferating cells in all active phases of the cell cycle but it is not present in non-cycling cells (Gerdes et al., 1984; Zheng et al., 2006). The relative number of *Ki-67*-positive cells is used to determine the proliferation index or the relative number of cells actively involved in the cell cycle (Scholzen and Gerdes, 2000) and can be observed immunohistochemically. Nuclear *Ki-67* antigen expression is a measure of cell growth fraction and hence biological aggressiveness of a malignancy (Margulis et al., 2006). It has been reported that immunohistochemical assessments of female C57BL/6/c

mice revealed significantly higher percentages of *Ki-67*–positive cells in each of the BBN-treated group, ranging from 0.66% to 4.88% in comparison to the BBN naïve group (0.14 ± 0.05%) (George et al., 2013). There has been increasing evidence indicating that *Ki-67* may be a promising molecular and effective target in cancer diagnosis and therapy including bladder cancer (Li et al., 2015). Furthermore, overexpression and aberration of cell-cycle proteins including *p53* and *Ki-67* was associated with high-stage/grade tumors of urothelial cells (Lenz et al., 2012).

N-nitrosamines are carcinogenic compounds that occur widely in the environment and can also be formed endogenously from the interaction of ingested nitrate or nitrite with secondary amines (Lijinsky, 1972). Exogenous nitrosamines are produced as a by-product of industrial processes that use nitrates and/or nitrites and amines under a range of pH conditions (WHO, 2002). NDMA is the simplest dialkylnitrosamine (Fig. 2A) and its precursors in particular, are widely present in wastewater treatment plants (Wang et al., 2014). It has been suggested that nitrosamines and the nitrosamides are carcinogenic, inducing tumorigenic alkylation of specific bases and DNA sequences (Hecht, 1999).

Interestingly, endogenous volatile N-nitroso compounds appear to be of particular importance since they were found at high levels in the urine of patients in schistosomiasis-associated bladder cancer (Tricker et al., 1989). Various strains of bacteria that can mediate nitrosation reaction leading to the formation of N-nitrosamines have been identified in the urine of subjects with schistosomiasis at higher intensities of infection than in normal subjects (Mostafa et al., 1999). It has also been reported that these nitrosamines are carcinogenic and act on metaplastic epithelium, with a subsequent progression to squamous cell carcinoma (Sheweita et al., 2004).

There is conclusive evidence that NDMA is a potent carcinogen in experimental animals by several routes of exposure, including through ingestion of drinking-water. Some studies report that current bladder cancer models in rodents rely on the administration of nitrosamines in drinking water, leading to onset of invasive carcinoma after 3–4 months of exposure. NDMA has been classified by IARC as Group 2A which means probably carcinogenic to humans. The mechanism by which NDMA produces cancer

has been suggested through biotransformation by liver microsomal enzymes, generating the methyldiazonium ion where the reactive metabolites form DNA adducts (Hecht, 1999).

Some earlier and recent experimental studies reported that urothelial tumors in primates and rodents have been induced by urinary bladder carcinogens like BBN (Fig. 2B) combined with *S. haematobium* infection. Such infection of *S. haematobium* in conjunction with BBN in baboons (*Papio* sp.) showed that four of ten baboons but none of the control group developed neoplastic disease of the urothelium (Hicks et al., 1980). Similarly, earlier animal experiments reported noninvasive papillary and nodular UC of the UB in a talapoin monkey (*Cercopithecus talapoin*), a capuchin monkey (*Cebus appella*), and opossums (*Didelphys marsupialis*) when infected with *S. haematobium* (Kuntz et al., 1971& 1972).

Rodents are commonly used for *in vivo* urothelial cell carcinoma models since they have a lower urinary tract comparable to humans and neoplasms in the bladder are morphologically very alike (Oyasu, 1995). In mice, the pathogenesis can follow a sequence of marked dysplasia with or without hyperplasia, leading to carcinoma *in situ* and ultimately to high-grade invasive carcinoma (Cohen, 1998). Reports showed the induction of urothelial tumors in rodents by chemical carcinogens alone like BBN and N-nitroso-N-methylurea (NMU) in *in vivo* experimental model. Recent studies revealed that mice exposed to different doses of BBN exhibited tumors or preneoplastic lesions (Chuang et al., 2014; Henriques et al., 2014). Similarly, 0.05% BBN carcinogen treatment in drinking water *ad libitum* resulted in a 76% tumor incidence and increased mean bladder weights in comparison to controls in mice (George et al., 2013). However, depending on the dosage, time and animal strains used, some differences in bladder histopathology between different rodents exposed to BBN have been identified. All, except for transgenic and knockout animals, require 8-12 months experimental periods in order to generate a high yield of neoplasias (Oliveira et al., 2006).

One of the major problems in relation to developing tractable animal model for *S. haematobium* has been the inability of its cercariae to efficiently mature and migrate to the bladder venous plexus in the mouse (Loker, 1983; Rheinberg et al., 1998). Rheinberg et al. (1998) reported that in mice experimental infection *S. haematobium* as opposed to other schistosomes such as *S. intercalatum, S. japonicum, S.*

mansoni and *S. rodhaini* showed the lowest yield of adult recovery and established later as well as persisting in lung for at least two weeks. In the experiment *S. japonicum* showed early establishment and rapid departure from lungs. Non-murine rodent models like hamsters develop strange clinical outcomes which can differ from the human disease while primates showed promising recapitulation but seemingly expensive and difficult to manipulate. Therefore, the need for developing a modified animal model is unquestionable.

In recent studies, new mouse model was designed to study immunological phenomena of the UB by artificial injection of *S. haematobium* eggs into bladder wall of experimental mice (Fu et al., 2011). By doing so it has been shown that egg deposition alone was sufficient to reproduce several important aspects of urogenital schistosomiasis, even in the absence of the other life stages of this important human pathogen. The results showed that egg-injected mice developed urinary tract fibrosis, bladder dysfunction, chronic granulomatous inflammation, upregulation of Th2 associated cytokines like IL-4 and various urothelial changes morphologically reminiscent of human urogenital schistosomiasis (Fu et al., 2012).

So far, although there is sufficient evidence for the initiation of bladder neoplastic cells by administering chemical carcinogens like BBN alone or BBN followed by NMU in primates as well as rodents under experiments, there were no recent studies reporting the scientific association of artificial injection of *S. haematobium* eggs into mouse UB wall accompanied with exogenous administration of NDMA for possible urothelial neoplastic development. In fact, the NDMA administration in conjunction with liver flukes (*Opisthorchis viverrini* and *Clonorchis sinensis*) infection in hamster model has been reported to have successfully induced cholangiocarcinoma (CCA) (Prakobwong et al., 2010). The present study attempted to investigate the development of urothelial tumor mainly by histopathological and immunohistochemical observation of mouse bladder tissue. Tumor suppressor and epithelial mesenchymal transition (EMT) marker genes were also analyzed to better understand the mechanism of bladder neoplasia. Furthermore, immune response study was conducted using mouse IgG subtypes and selected cytokines analysis post egg injection and NDMA or BBN treatment.

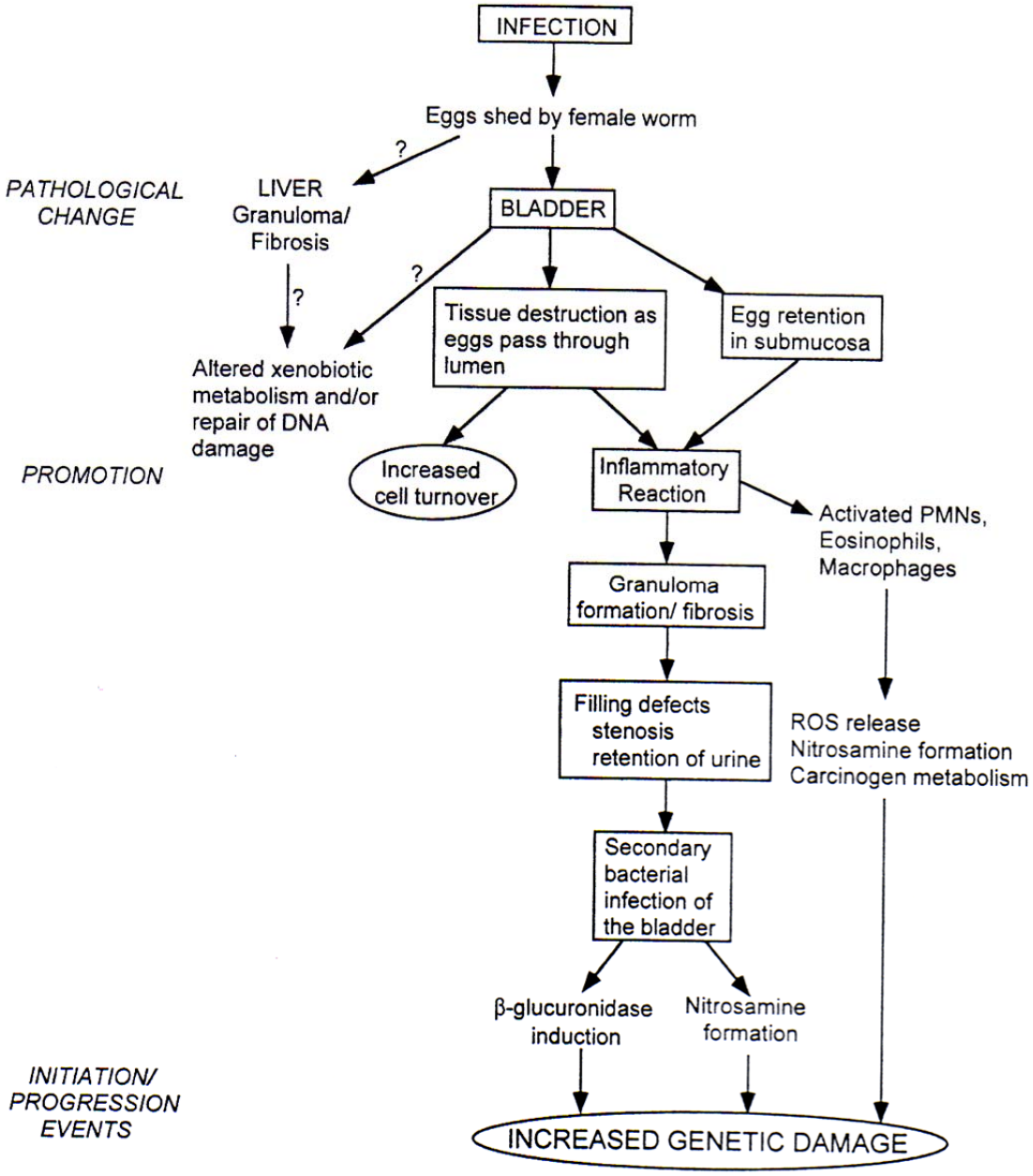

Fig. 1. Interrelationship between bladder pathology and cellular changes associated with schistosomal bladder carcinogenesis (Source: http://slideplayer.com/slide/5277662/, slide 70).

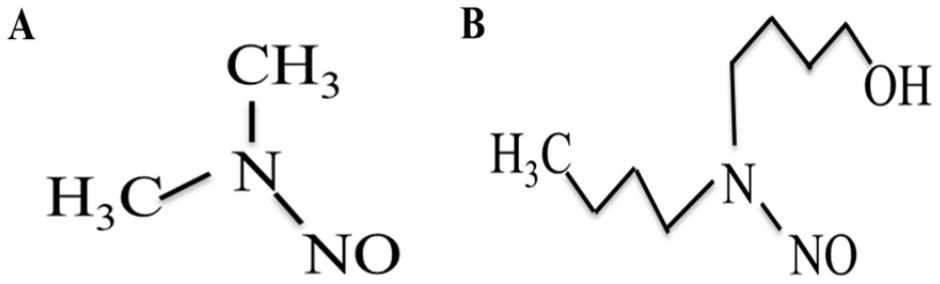

Fig. 2. Chemical structures of NDMA (A) and BBN (B).

MATERIALS AND METHODS

1. Ethical statement

This animal experiment was conducted after approval of the protocol by the institution of animal care and use committee (IACUC) of Seoul National University (SNU), Seoul, Korea (SNU-141126-2-2). The institution of biosafety committee (IBC) approval was also obtained before starting the research.

2. Animals, parasites and reagents

Female ICR (CD-1) mice, 5 weeks old (22 to 38 g; average weight of 30 g) on arrival were purchased from the Orient Bio Inc. (Seongnam, Korea). The mice were grouped and allowed to acclimatize for two weeks under animal biosafety level-2 (ABL-2) laboratory conditions before starting the experiment. The animals were maintained under standard and sterile laboratory conditions in ventilated cages starting from their arrival up to the end of the experiment.

Eggs of *S. haematobium* were collected from patients in Sudan. The eggs were isolated from urine samples in the Laboratory of the Department of Parasitology and Tropical Medicine, SNU, Korea, in September 2014. The eggs were counted and kept in sterile saline solution under frozen conditions until required for experimentation (Fig. 3).

NDMA and BBN reagents were purchased from Sigma Aldrich and stored undiluted in the dark at -20°C. Both NDMA and BBN were diluted in distilled water under chemical biohazard hood. The final concentration of solution was kept in a dark container, and transferred to brown bottles used only for the respective mice group with NDMA and BBN administration.

3. Experimental design

A total of 72 female ICR mice were randomly divided into 6 group (twelve per group): Group 1 (control) mice injected with 50 μL sterile saline solution into the bladder wall, Group 2 (Sh eggs) injected

with Sh eggs into the bladder wall, Group 3 (NDMA) supplied with NDMA, Group 4 (BBN alone) supplied with BBN, Group 5 (Sh eggs + NDMA) injected with Sh eggs + supplied with NDMA, Group 6 (Sh eggs + BBN) injected with Sh eggs and supplied with BBN (Table 1). Animals in Group 2, 5 and 6 animals were injected with 1000 *S. haematobium* eggs in 50 µL saline into the bladder wall. The mouse UB wall egg injection was performed in accordance with the protocol designed by research group of Stanford University (Fu et al., 2011). After putting the mouse under isoflurane anaesthesia, which was administered in a fume hood with a standard vaporizer, a midline lower abdominal incision was made, and the bladder exteriorized. The eggs were injected in sterile saline submucosally into the anterior part of the bladder dome using Hamilton Syringe. Abdominal incisions were then closed with 4-0 Vicryl suture, and the surgical site was treated once with topical antibiotic ointment (Fig. 4).

Concerning the dose of chemical treatment, 12.5 ppm concentration of NDMA was used in the present experiment on the basis of earlier reports on the induction of cholangiocarcinoma in hamster model (Prakobwong et al., 2010). On the other hand, a 0.05% concentration of BBN dissolved in drinking water was administered to mice *ad libitum* throughout the experimental process (Arantes-Rodrigues et al., 2013; George et al., 2013). The prepared solutions of NDMA and BBN were changed every week. Two mice from each group were sacrificed after 4, 12, 20, 28 and 36 weeks starting from injection of *S. haematobium* eggs and administration of NDMA as well as BBN post experiment (Fig. 5).

4. Body weight measurement

Body weight of all mice group was measured every week using electronic balance (Sartorius, Germany). The body weight of all the six mice groups was recorded until 29th week. Five mice from all of the six groups were included in the analyses of mean mice body weight and standard deviation.

5. Histopathological analysis

After mice sacrifice at serial time points of 4, 12, 20, 28 and 36 weeks post experiment, the whole urinary bladder organ was harvested and divided into two-third and one-third section for histopathology

and mRNA expression, respectively. The two-third sized urinary bladder was kept in neutral buffered formalin 10% (v/v) for few days until paraffin embedding and blocking for routine histology. Then, the bladder tissue sections (4–5 μm) were processed and followed by the routine hematoxylin and eosin (H & E) staining. Histological lesions and abnormalities of tissue sections found in different group were classified and staged according to standard histopathological examination (Eble et al., 2004). All morphological readings were double-checked by pathologist blinded to the slide information. Images were captured with a light microscope (Olympus CKX41 Microscope, Tokyo, Japan) linked to a computer.

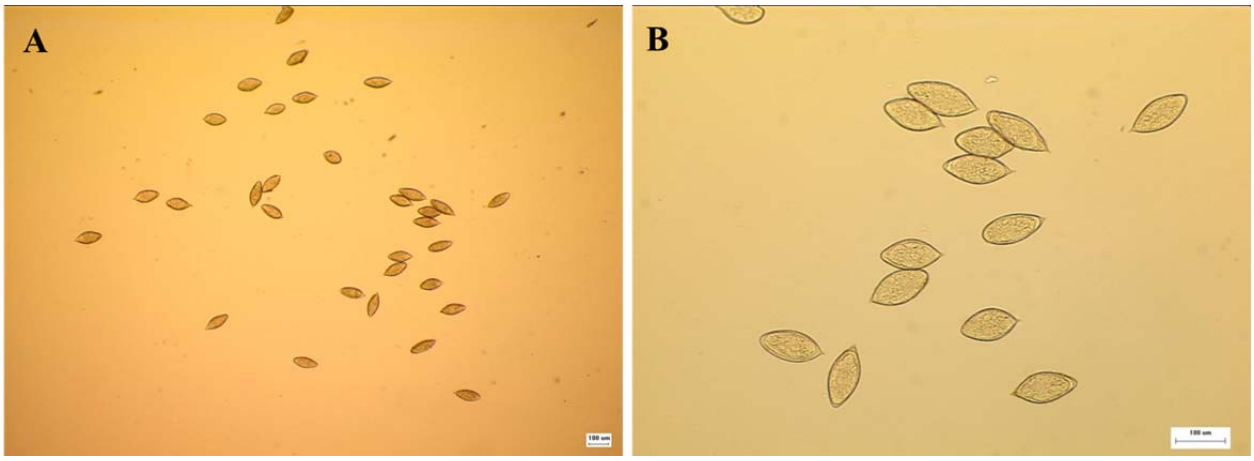

Fig. 3. *S. haematobium* eggs isolated from urine samples of patients collected from Sudan in September 2014. (A) 40x, (B) 100x, scale 100 µm.

Table 1. Experimental design and treatment profile of mice group

Treatment	Mice group (n=72; 12/group)					
	Control (G-1)	Sh eggs (G-2)	NDMA (G-3)	BBN (G-4)	Sh eggs + NDMA (G-5)	Sh eggs + BBN (G-6)
Sterile saline	✓					
S.haematobium eggs		✓			✓	✓
NDMA			✓		✓	
BBN				✓		✓

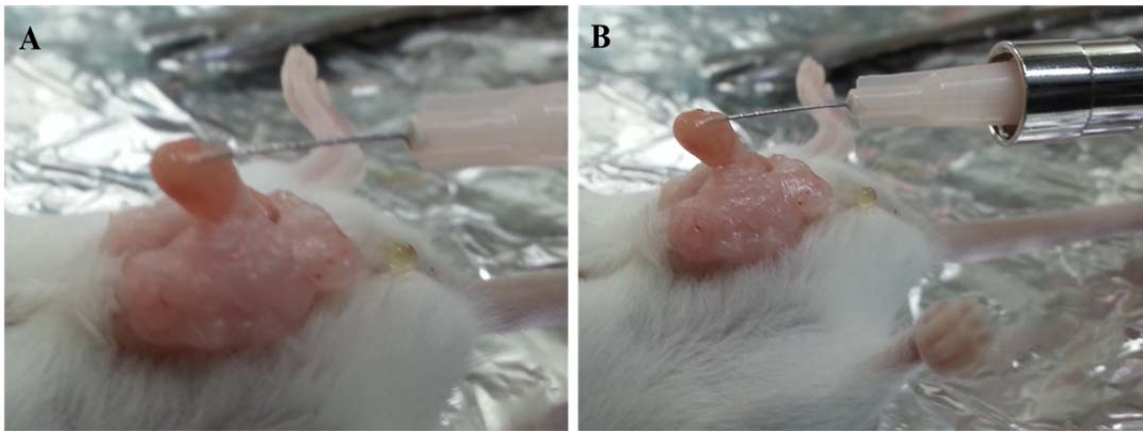

Fig. 4. Picture showing mouse urinary bladder wall injection with *S. haematobium* eggs.

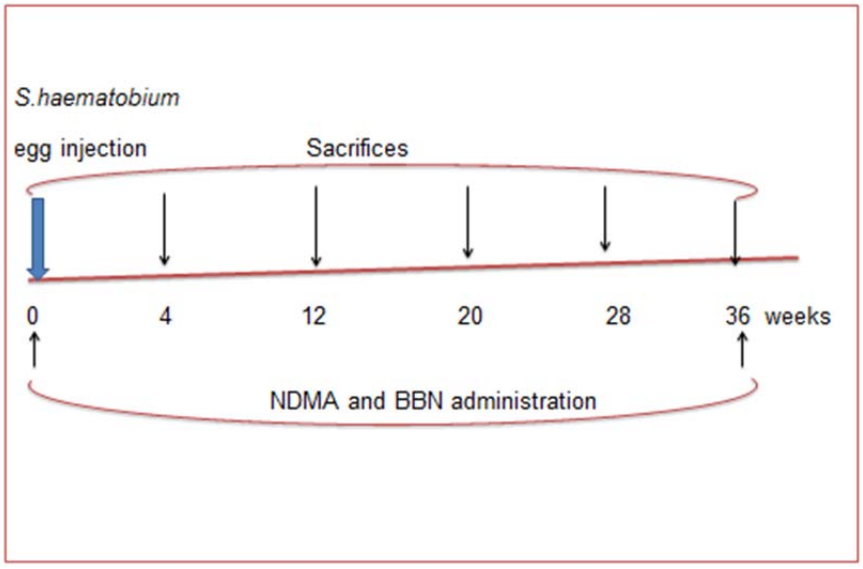

Fig. 5. *S. haematobium* eggs injection, NDMA and BBN administration and sacrifice schedule of experimental mice.

6. Immunohistochemical analysis

Paraffin embedded sections were processed for immunohistochemical analysis of nuclear cell proliferation marker using monoclonal rat anti-mouse *Ki-67* antigen according to the manufacturer's instructions (Dako, Carpinteria, CA, USA). Secondary biotinylated goat anti-mouse antibodies were used. Antibodies were diluted in the recommended antibody diluting buffer. The working dilutions and the final concentrations of the primary antibody for anti-*Ki-67* was 1:200 and 0.005 mg/mL. The used dilution and concentration of the biotinylated goat anti-mouse antibody was 1:200 at final concentration 0.0025 mg/mL. The expression was scored using a semiquantitative composite scoring system as follows: (1) staining intensity, defined as 0 for negative, 1+ for weak, 2+ for moderate, and 3+ for strong. The positive immunostained samples for *Ki-67* proteins were graded based on the ratio (%) of positively immunostained cells. The immunostained samples were also compared among the group as well as across the weeks of sacrifice. The mean of proliferation of the nuclear antigens was used for comparison.

7. Mouse serum immunoglobulin and cytokine analysis

Blood sample was directly collected from the heart after anesthesia and kept at 4^{o}C over night. Serum samples were collected by centrifugation at 3000 rpm at 4°C for 10 min and stored at -70°C until use. The serum immunoglobulins levels of IgG1, IgG2a, IgG2b and IgG3 subtypes of the first three sacrifice phases i.e. week 4, 12 and 20 mice group were determined by Luminex bead-based multiplex kit according to the instructions of the manufacturer (R&D Systems biotechne, San Diego, CA, USA). Similarly, Th-1 associated pro-inflammatory cytokines like IFN-γ, IL-1β and TNF-α and Th-2 associated cytokines such as IL-4, IL-5 and IL-13 as well as regulatory cytokine IL-10 were also evaluated among week 4, 12 and 20 group of mice using Luminex bead-based multiplex kit from the R&D Systems. The values of the cytokine assay were expressed in bar graph.

8. RNA extraction and cDNA synthesis

About one-third proportions of urinary bladder tissues of the respective group of the sacrificed mice were preserved in liquid nitrogen for the expression of mRNA. Total RNA was isolated using PureLink^{R} RNA Mini kit (Cat. #12183555, Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. After quantified by a NanoDrop 280 nm spectrophotometer, up to 2 µg RNA sample was reversely transcribed into cDNA according to the instructions provided by High-Capacity cDNA Reverse Transcription Kits (Cat. #4368814, Applied Biosystems, USA). The cDNA samples were kept at -70°C until use.

9. Analysis of gene expression by real-time PCR assay

The resulting cDNA was amplified by TOPreal qPCR 2X PreMix (SYBR Green with high ROX) (Cat. #RT501M, Enzynomics, Daejeon, Korea) using a primer set specific for *E-cadherin*, *p53*, *vimentin* and *GAPDH* (reference gene) (Bionics, Korea). The expression of the target genes was performed using mRNA transcripts quantified by real-time PCR. The primer design for the genes was obtained from NCBI database and published journal articles as shown in Table 2 (Madka et al., 2013; Liang et al., 2015). All comparative expression data were analyzed using Microsoft Excel 2010 version with a cycle threshold (Ct) in the linear range of amplification.

10. Data analyses and statistics

Data generated from the experiments were analyzed using Microsoft Excel 2010 version and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data were presented as mean ± standard deviation (SD). Comparisons of results were performed using a Student's t-test. P value < 0.05 was considered as significant. P values < 0.05 and < 0.01 were indicated as asterisk and dagger, respectively in the figures.

Table 2. Tumor suppressor and EMT marker genes targeted primer pairs for real-time PCR

Genes	Sequence Upper line: forward primer 5'-3' Bottom line: reverse primer 5'-3'	GenBank accession
E-cadherin	GATCCTGCTGCTCCTACTGT GCTCAAATCAAAGTCCTGGTCT TGAAACGCCGACCTATCCTTA	NM_009864.3
p53	GGCACAAACACGAACCTCAAA CCTTGACATTGAGATTGCCA	NM_001127233.1
vimentin	GTATCAACCAGAGGGAGTGA GGTGAAGGTCGGTGTGAACG	NM_011701.4
GAPDH	GCGCTCCTGGAAGATGGTG	NM_001289726.1

RESULTS

1. Body weight measurement of mice

The mean body weights of the mice recorded during the course of this study were compared. According to mice body weight measurement follow up there was no significant difference between the control and the treated group prior to 14 weeks post experiment. However, mean body weight showed significant decrease among the treated group but Sh eggs group after week 14. From week 15 to week 29, a highly significant reduction (20.5%) of body weight was observed in Sh eggs + BBN (36.26 ± 7.2; $P < 0.01$) compared to the control group (45.61 ± 8.3). On the other hand, from week 24 onwards a slightly sharp decline in body weight curve of Sh eggs + BBN was observed in comparison to the rest group. Similarly, between week 18 and 29, the average body weight curve for Sh eggs + NDMA group fell below all of the groups except Sh eggs + BBN, with minor decline between weeks 27 and 28. The Sh eggs + NDMA group also showed significant (11.6%) weight loss (40.33 ± 8.5; $P < 0.01$). Similarly, BBN group (42.2 ± 7.29; $P < 0.01$) and NDMA group (42.69 ± 9.11; $P < 0.01$) also showed (7.5%) and (6.4%) weight loss compared to control group respectively, but Sh eggs group didn't show significant reduction (2.2%) with respect to weight loss (43.35 ± 9.7; $P = 0.12$) (Fig. 6). A total of eight mice, two from each of BBN and Sh eggs + NDMA group and four from Sh eggs + BBN group but no deaths were recorded from control, Sh eggs and NDMA group. The death of two mice, one from each of Sh eggs + NDMA and Sh eggs + BBN group happened few days after egg injection process, which may be due to injury during surgical operation. The reason for death of the rest six mice which happened during the experimental process was unknown.

2. Histopathological findings

Based on H&E staining of mouse UB tissue from the existing mice group the results were evaluated and compared by professional pathologist. The histopathology of the control group showed normal appearance with clear epithelial cell arrangement. No sign of inflammation was also seen in the tissues of the control. Unfortunately, a few Sh eggs were identified from the tissue sections of Sh eggs and Sh eggs + BBN group. On the other hand, there was no inflammation or granuloma formation around the eggs. BBN and Sh eggs + BBN group showed marked focal tissue reaction and inflammatory aggregates almost at all weeks of observation whereas Sh eggs + NDMA group showed focal inflammation at week 12 and 20 (Fig. 7). Further microscopic evaluation of the H&E stained mouse UB tissue was categorized as normal urothelium, hyperplasia, dysplasia, squamous metaplasia and epithelial vacuolar change. In control and NDMA group, urothelial epithelial cells with 2 to 3 layers look normal with clear basal, intermediate and umbrella layers at week 4. Mouse UB tissues from BBN, Sh eggs + NDMA and Sh eggs + BBN group revealed abnormal histopathological manifestations such as prominent nucleoli, enlarged and pleomorphic cells (red arrow), hyperplasia and dysplasia. Moreover, the urothelial cell layers when compared to the control exhibited more than 6 layers. Other than the commonly aforementioned histopathological abnormalities, epithelial vacuolar change was observed in Sh eggs + BBN group, at week 20 post experiment. The epithelial vacuolar changes were whitish spot and scale like structures sparsely distributed in the tissue sections (Fig. 8). Comparison for major histopathological abnormalities in BBN, Sh eggs + NDMA and Sh eggs + BBN group was summarized in Fig. 9. In addition to hyperplasia and dysplasia, a premalignant indicator of urinary bladder called squamous metaplasia of UB was exclusively observed in Sh eggs + NDMA group at 12 week post eggs injection and NDMA treatment as shown in Fig. 10.

3. Immunohistochemical results of Ki-67 staining

The IHC analysis showed positive *Ki-67* staining for urothelial epithelial cells only in Sh eggs + BBN group at week 20. The remaining treated group showed insignificant or no expression of *Ki-67* proteins. Likewise, there was strong staining of *Ki-67* for lymphocytes but not for urothelial epithelial cells in Sh eggs + NDMA and Sh eggs + BBN group at week 4 and 12 (Fig. 11). The intensity of lymphocyte staining slowly declined to moderate at week 20 and later. On the other hand, the control and Sh eggs group showed no expression at all weeks of observation.

4. Serum immunoglobulin and cytokine results

4.1 Serum IgG subtypes

The mean serum immunoglobulin levels of IgG1 showed strong significant increase for Sh eggs group ($P < 0.05$), Sh eggs + NDMA group ($P < 0.01$) and Sh eggs + NDMA group ($P < 0.05$) at week 4. On the other hand, Sh eggs + NDMA and Sh eggs + BBN group showed a marked increase at week 12 and only Sh eggs + BBN group at week 20 ($P < 0.05$) (Fig. 12A). The mean serum levels of IgG2a of Sh eggs and Sh eggs + NDMA group presented a significant increase compared to control at week 4 and 12 ($P < 0.01$). At week 20, similar situation was observed for Sh eggs group ($P < 0.01$) but significance level decreased for Sh eggs + NDMA ($P < 0.05$). On the contrary, Sh eggs + BBN group didn't show any difference compared to the control across the weeks (Fig. 12B). For IgG2b levels, only Sh eggs + NDMA group at week 12 exhibited a sharp increase ($P < 0.01$) while there was a decrease or a slight increase in the remaining group and the other weeks (Fig. 12C). The Sh eggs group showed a consistently higher IgG3 levels at all the three weeks ($P < 0.01$) for week 4 and 20, and Sh eggs + NDMA group also showed relatively comparable levels at the week 4 and 12 ($P < 0.05$) except for week 20. However, Sh eggs + BBN group didn't show difference compared to the control through the observation time (Fig. 12D).

4.2 Cytokine analysis

The serum levels for cytokines such as IFN-g, IL-1b, IL-13 and TNF-a showed low values below standard range. Because of the low detectability of these cytokines, the data was not included in the result. The serum levels of IL-4, IL-5 and IL-10 showed relatively good detectable status. The IL-4 level showed a slight increase only for Sh eggs + BBN at weeks 12 and 20 though not statistical significance (Fig. 13A). IL-5 levels showed more or less increased values except for week 12 in all group (Fig. 13B). The IL-10 levels increased for Sh eggs and Sh eggs + NDMA group except at week 20 in the later and for Sh eggs + BBN group only at week 4 (Fig. 13C).

5. Tumor suppressor and EMT marker genes expression

Relative expression of *p53* by qRT-PCR significantly increased in Sh eggs group at week 4 ($P < 0.01$) and Sh eggs + BBN group at week 12 and 20 ($P < 0.05$) compared to the control (Fig. 14). Relative expression of *E-cadherin* showed significant decrease compared to control in BBN and Sh eggs + BBN group at week 12, 20, 28 and 36. Remarkably, all treated group presented significant reduction in the expression of *E-cadherin* at week 12 and 36 (Fig. 15). On the contrary, relative expression of *vimentin* showed a marked increase at week 20 with peak among BBN, Sh eggs + NDMA and Sh eggs + BBN group. At week 12, only Sh eggs + BBN showed significant increase relative to control group. At week 28 and 36, there was no significant variation among the group (Fig. 16). Amplification curve and melting peak chart of PCR product were shown in Fig. 17 A, B. The target genes were resolved in 1.5% agarose gel electrophoresis (Fig. 17 C, D).

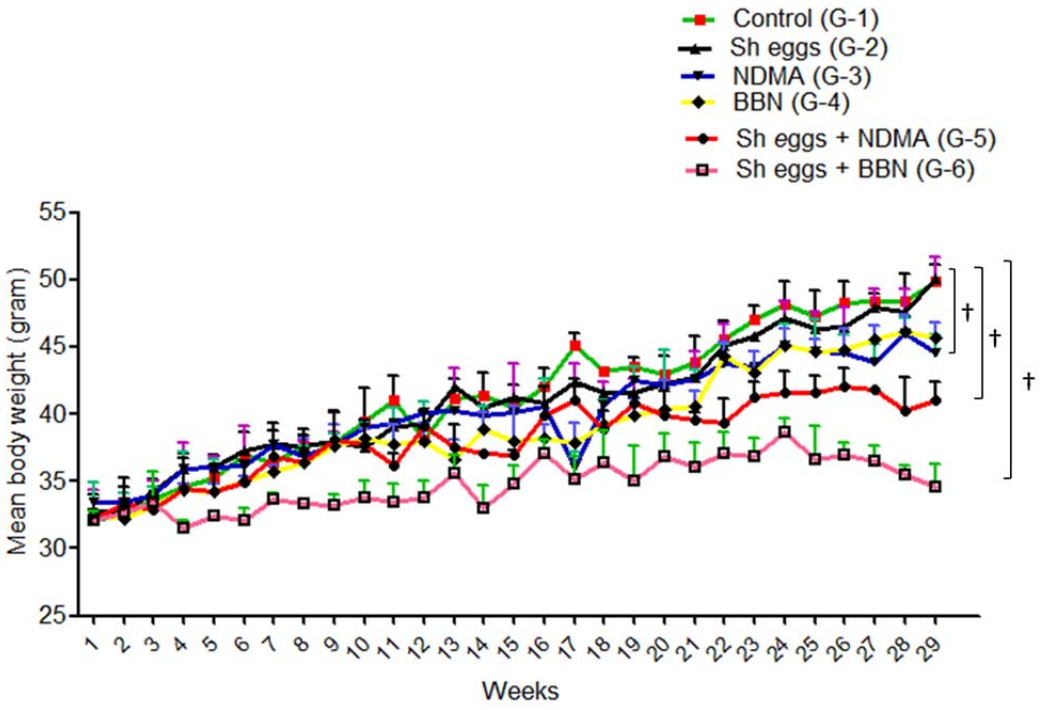

Fig. 6. Body weight curve of selected mice per their respective group over 29 weeks.

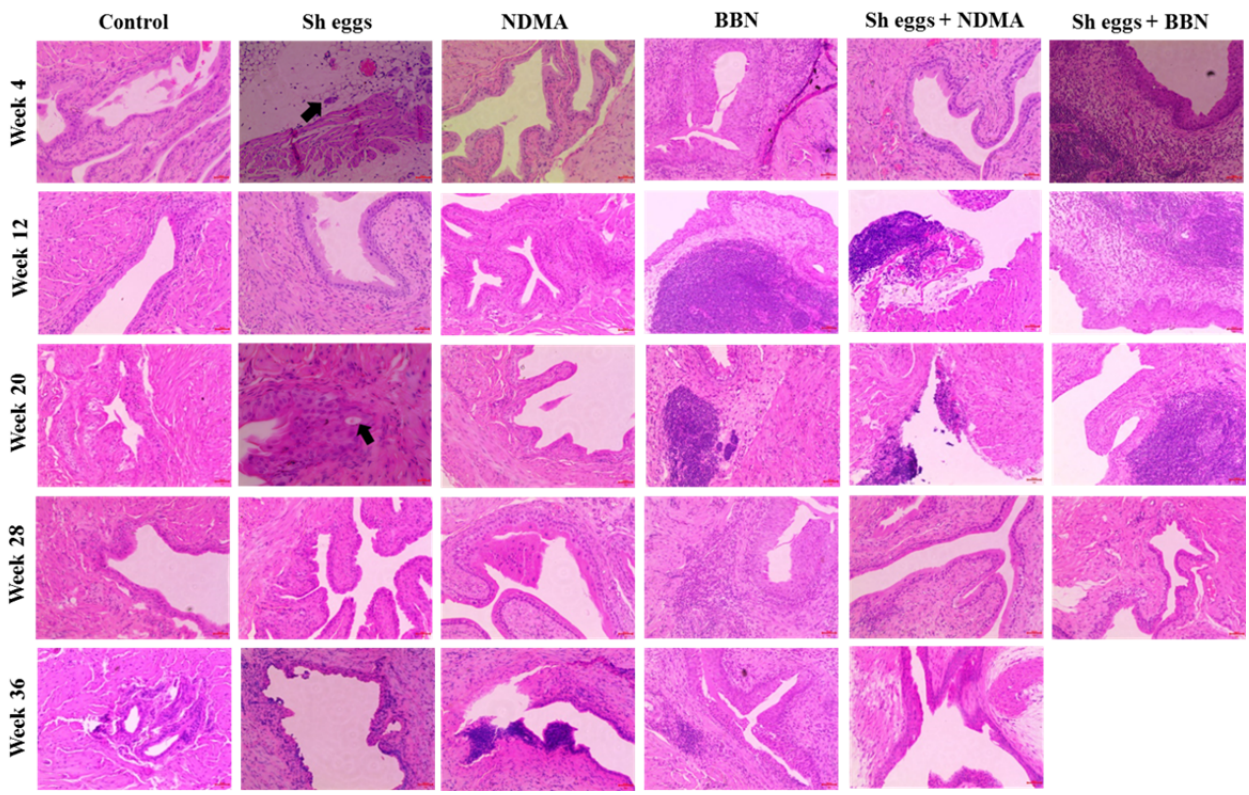

Fig. 7. H&E stained tissue sections of mouse UB showing Sh eggs and inflammation. Tissue structures in control group look normal from week 4 to week 36, arrows indicating Sh eggs in tissue section (x200), NDMA group showing fairly normal tissue structure across the weeks except mild focal inflammation at week 36, BBN and Sh eggs + BBN group showed marked inflammation at all weeks of observation, Sh eggs + NDMA group showed focal inflammation at week 12 and 20, (magnification = x100).

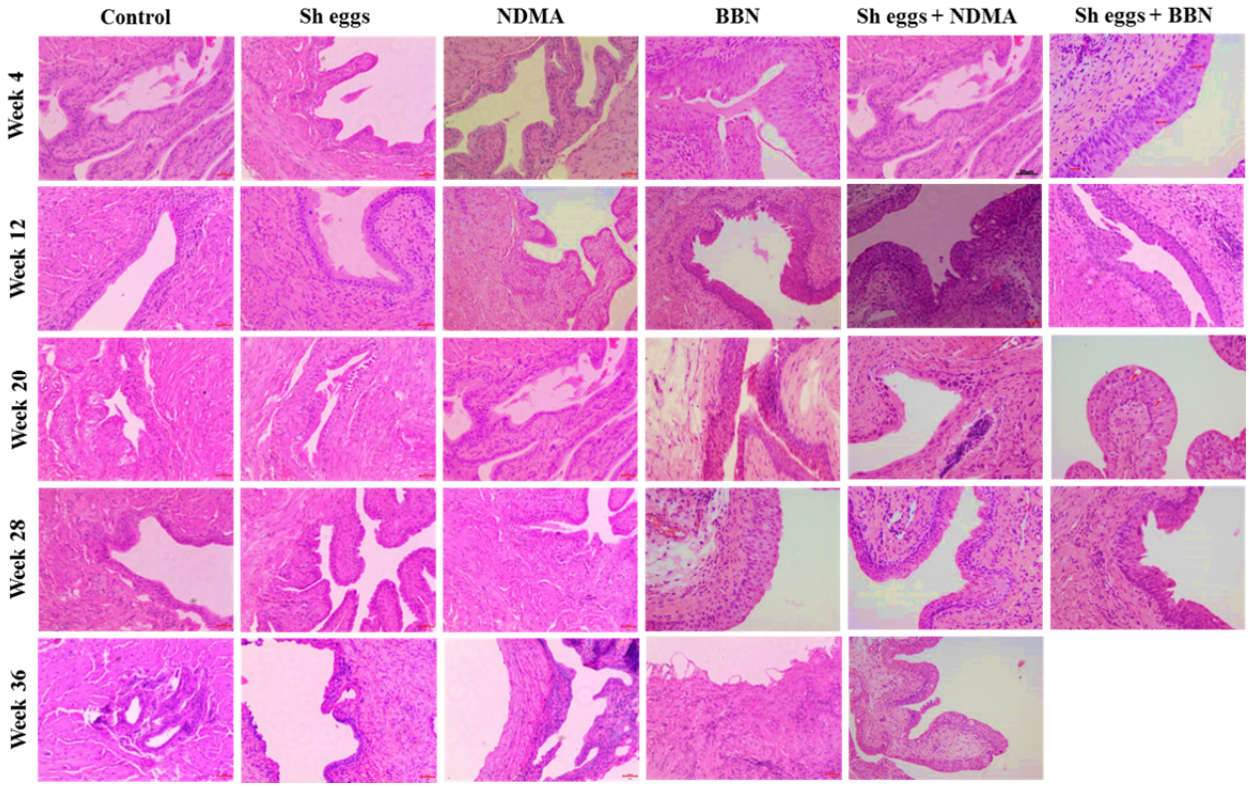

Fig. 8. H&E stained tissue sections of mouse UB showing various histopathological abnormalities of mouse UB tissue sections. Tissue sections look normal (control and Sh eggs group), mild hyperplasia at week 12 and inflammation at week 36 (NDMA group), hyperplasia at week 4 and hyperplasia and dysplasia at week 12 and 20 and mild hyperplasia at week 28 (BBN group), dysplasia at week 12 and hyperplasia and dysplasia at week 20, 28 and 36 (Sh eggs + NDMA group), hyperplasia and/or dysplasia, enlarged and pleomorphic urothelial cells (red arrow), epithelial vacuolar change at week 4, 12, 20 and 28 (Sh eggs + BBN group), (Original magnification = x400).

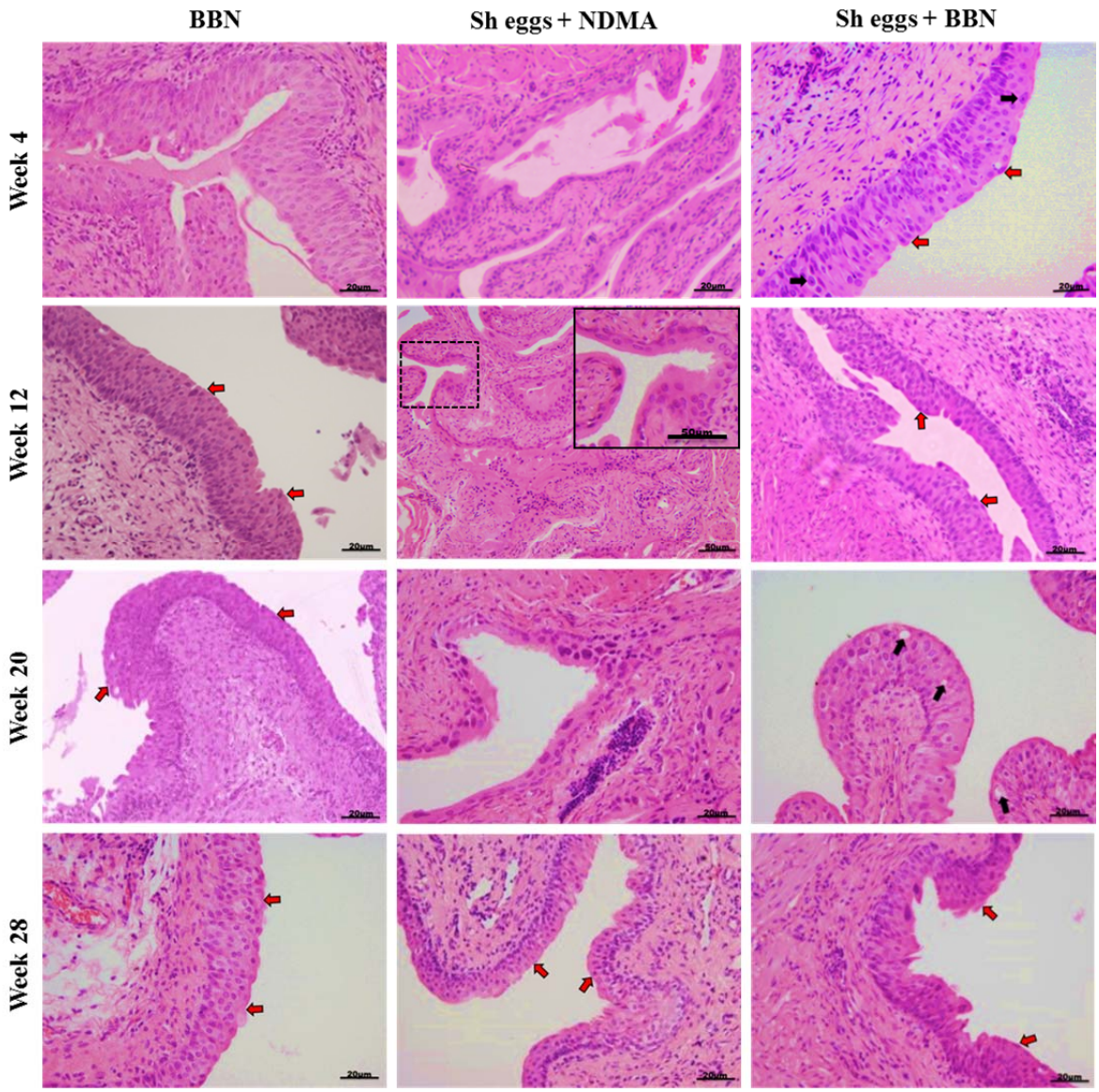

Fig. 9. Selected H&E stained tissue sections of mouse urinary bladder showing major histopathological abnormalities. BBN group showed hyperplasia at week 4, hyperplasia and dysplasia at week 12, 20 and 28 (red arrows), Sh eggs + NDMA showed normal at week 4 and squamous metaplasia at week 12, dysplasia and hyperplasia (red arrows) at week 20 and 28, in Sh eggs + BBN group hyperplasia and dysplasia (red arrows) were observed at week 4 and 12 with enlarged and pleomorphic cells (black arrows) and epithelial vacuolar change and hyperplasia and dysplasia (red arrows) at week 20 and 28, respectively, (Original magnification = x400).

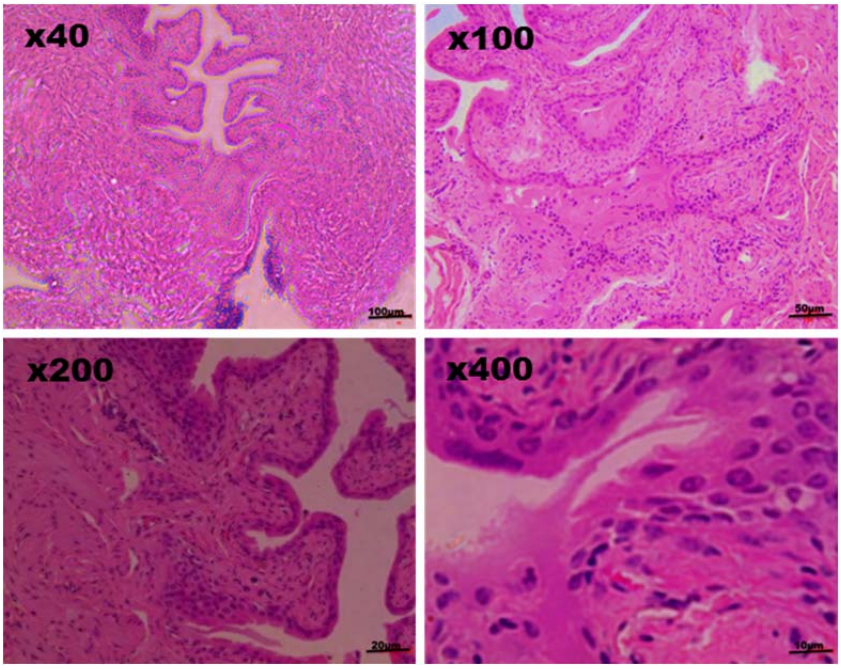

Fig. 10. Histopathology of mouse urinary bladder wall showing squamous metaplasia and dysplasia in the Sh eggs + NDMA group at week 12, H&E stained, magnification from x40 to x400.

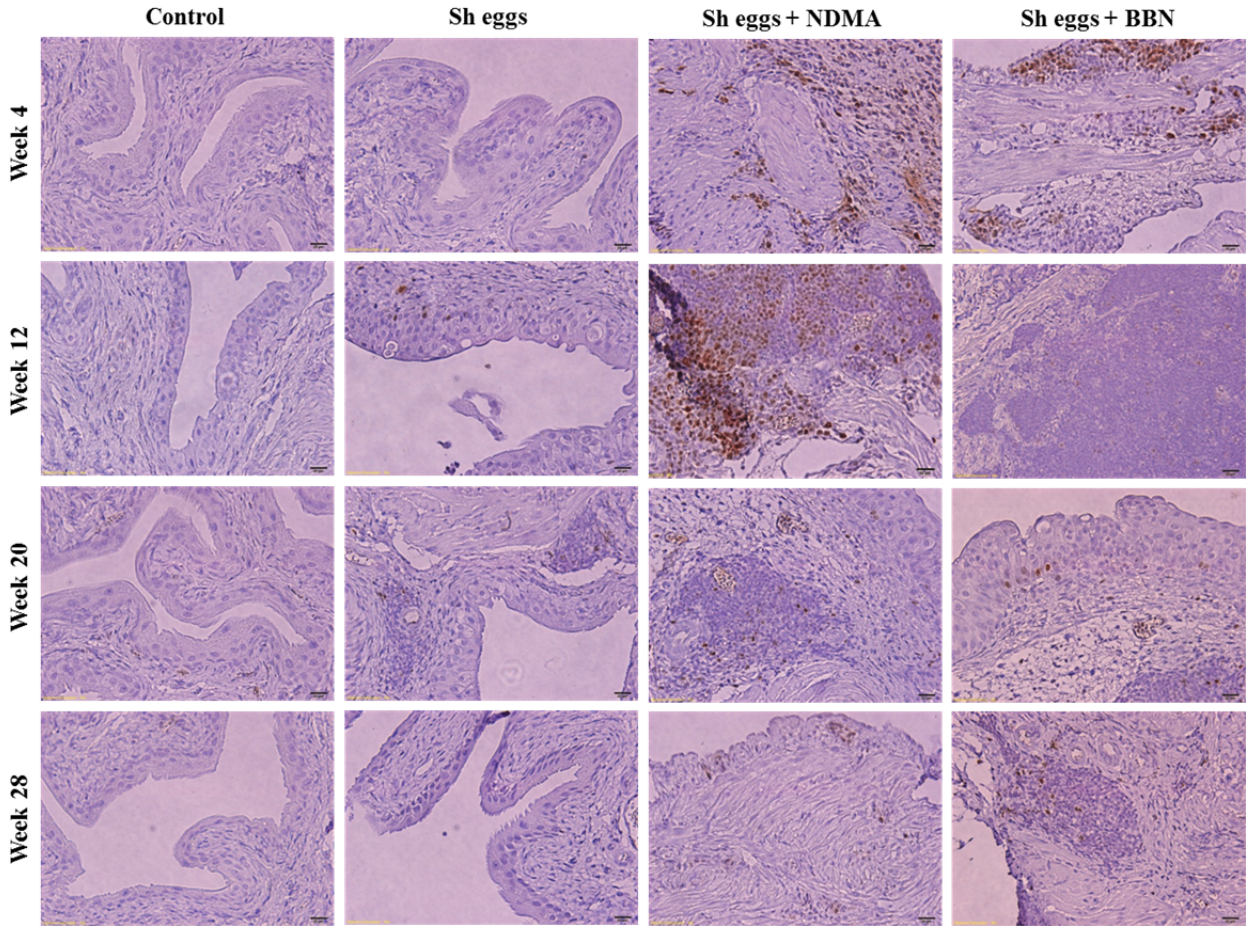

Fig. 11. Immunohistochemistry for staining of *Ki-67* expression (brown color for positive cells) in the mouse urinary bladder wall by group and the experiment week. Negative for Control and Sh eggs group, positive in the groups of Sh eggs + NDMA and Sh eggs + BBN, (magnification = x400).

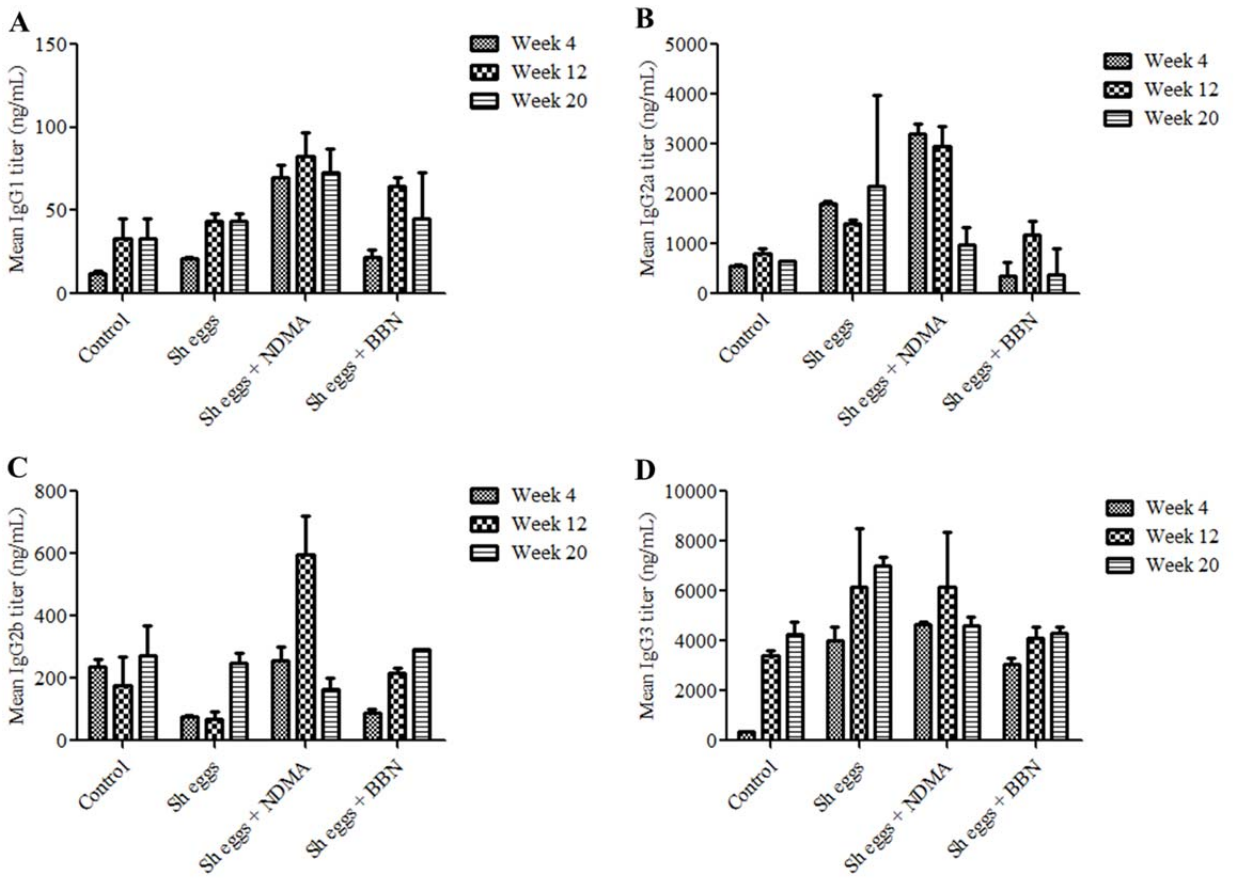

Fig. 12 Serum levels of mouse IgG subtypes at weeks of experiment by group, Luminex assay. The graphs show mean titers in ng/ml for control, Sh eggs, Sh eggs + NDMA and Sh eggs + BBN groups (A) IgG1, (B) IgG2a, (C) IgG2b and (D) IgG3 at week 4, 12 and 20, post experiment.

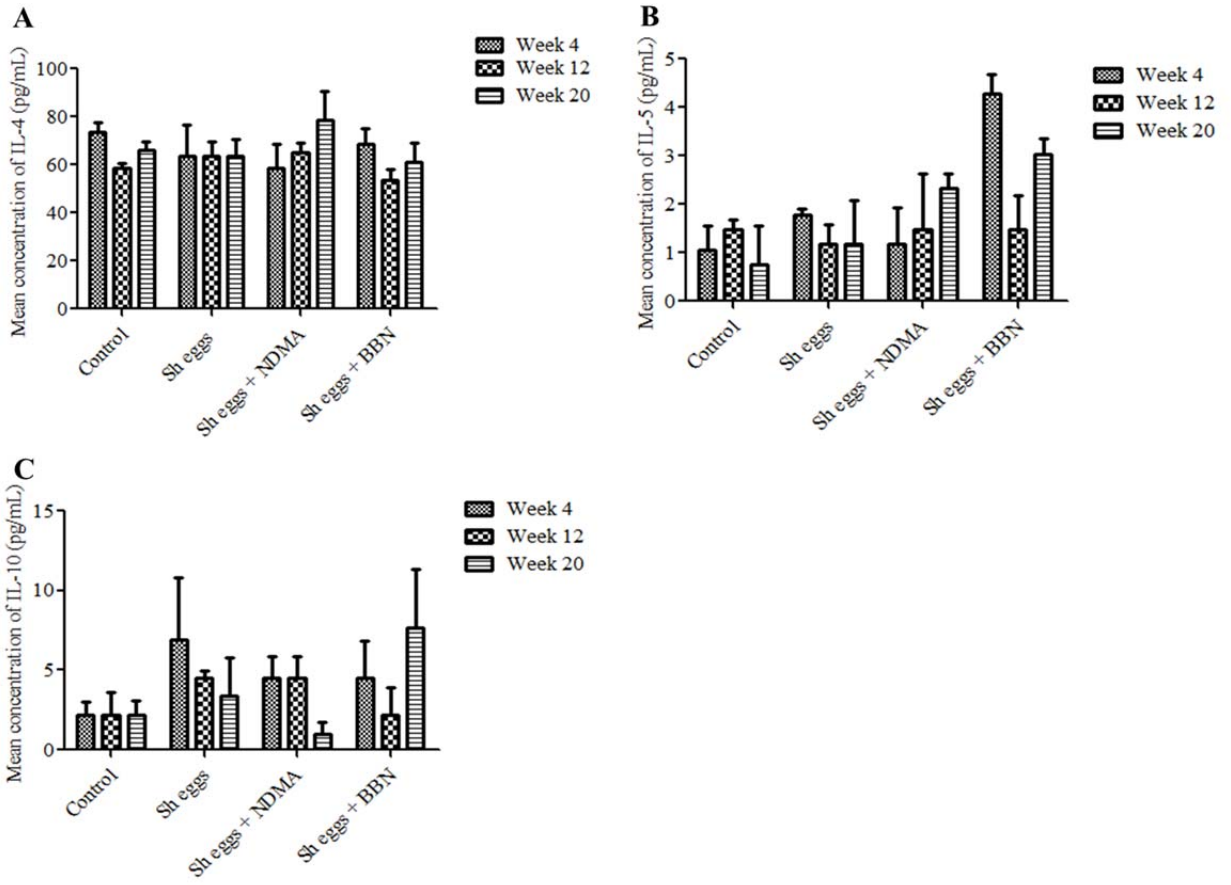

Fig. 13 Serum levels of mouse interleukins at weeks of experiment by group, Luminex assay. The graphs show mean concentrations in pg/ml for control, Sh eggs, Sh eggs + NDMA and Sh eggs + BBN groups (A) IL-4, (B) IL-5 and (C) IL-10 at week 4, 12 and 20, post experiment.

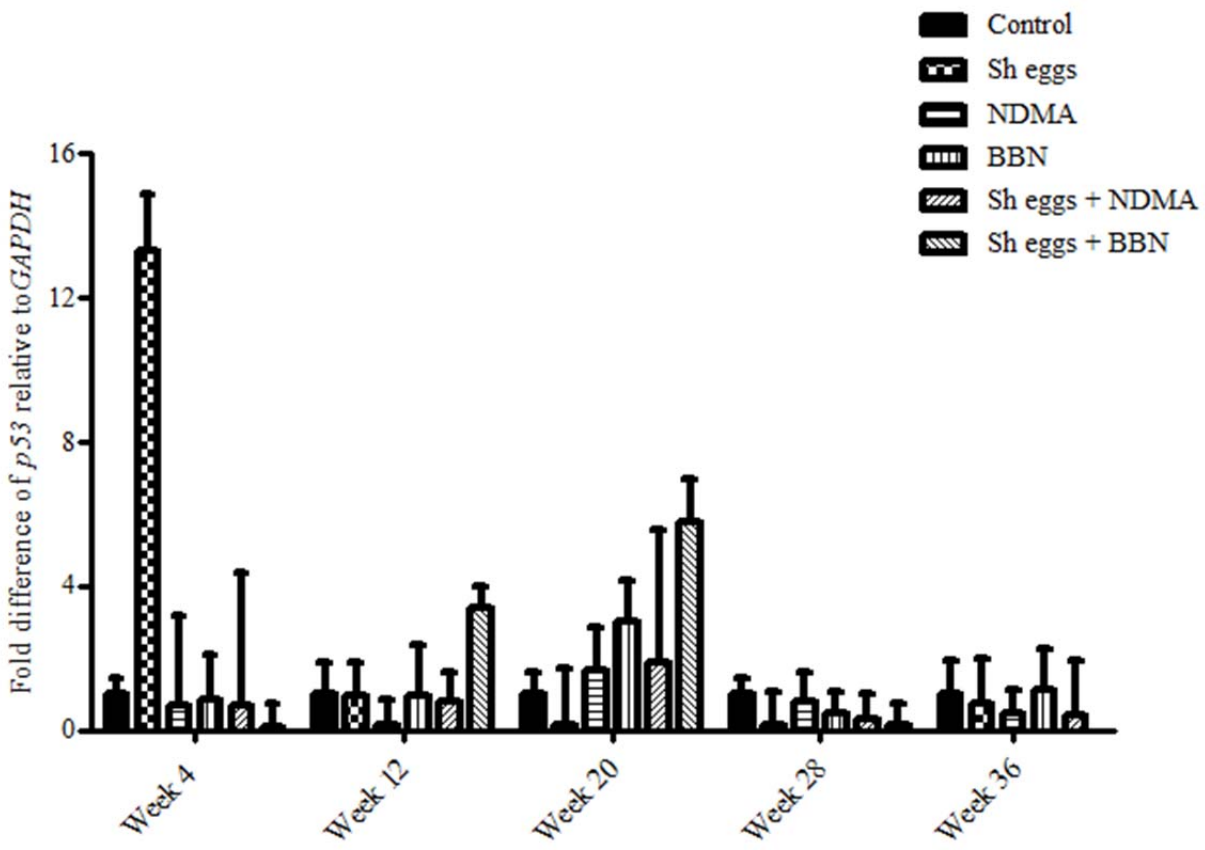

Fig. 14. Comparison of transcriptional expression of *p53* gene according to the treatments of Sh eggs, NDMA and BBN in different combination by qRT-PCR analysis. Saline injection was used as negative control. Relative mRNA expression is normalized to *GAPDH* expression levels, and shown by fold changes as mean ± S.E.M (n = 2).

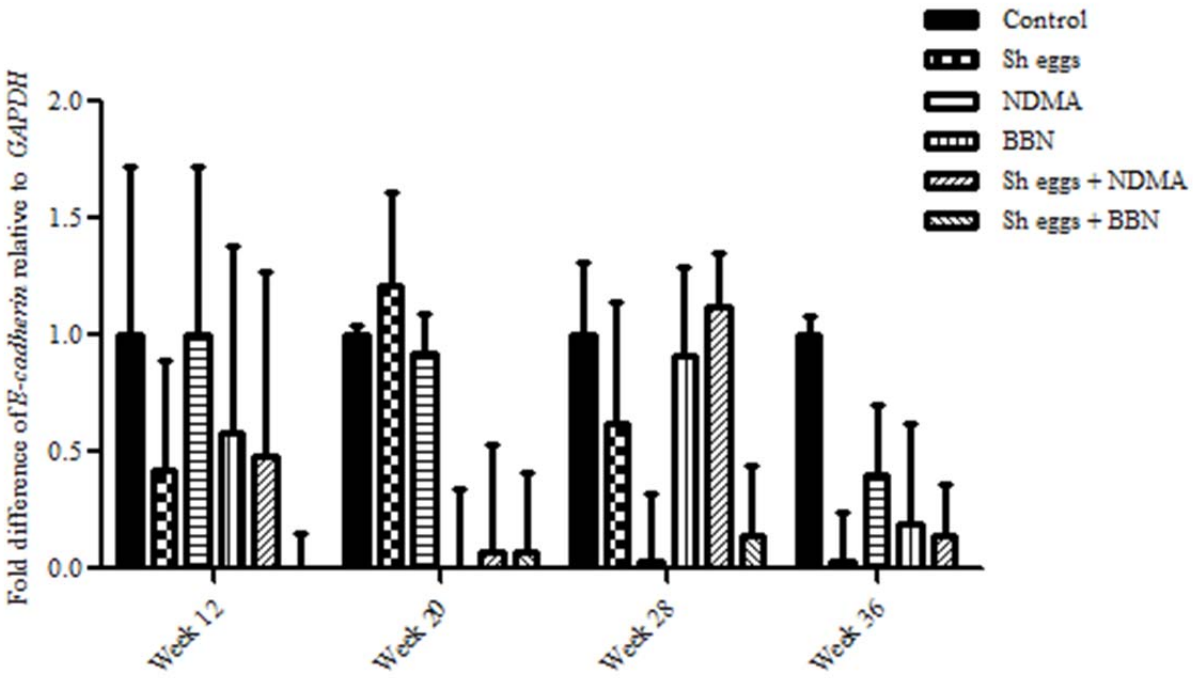

Fig. 15. Comparison of transcriptional expression of *E-cadherin* gene according to the treatments of Sh eggs, NDMA and BBN in different combination by qRT-PCR analysis. Saline injection was used as negative control. Relative mRNA expression is normalized to *GAPDH* expression levels, and shown by fold changes as mean ± S.E.M (n = 2).

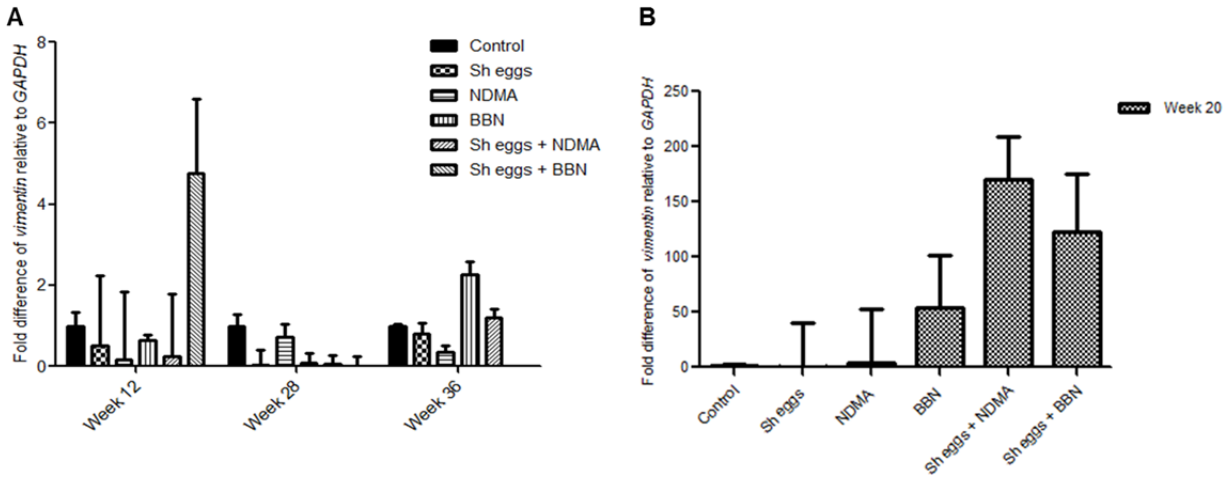

Fig. 16. Comparison of transcriptional expression of *vimentin* gene according to the treatments of Sh eggs, NDMA and BBN in different combination by qRT-PCR analysis. Saline injection was used as negative control. Relative mRNA expression is normalized to *GAPDH* expression levels, and shown by fold changes as mean ± S.E.M (n = 2) (A) fold differences at week 12, 28 and 36, (B) week 20.

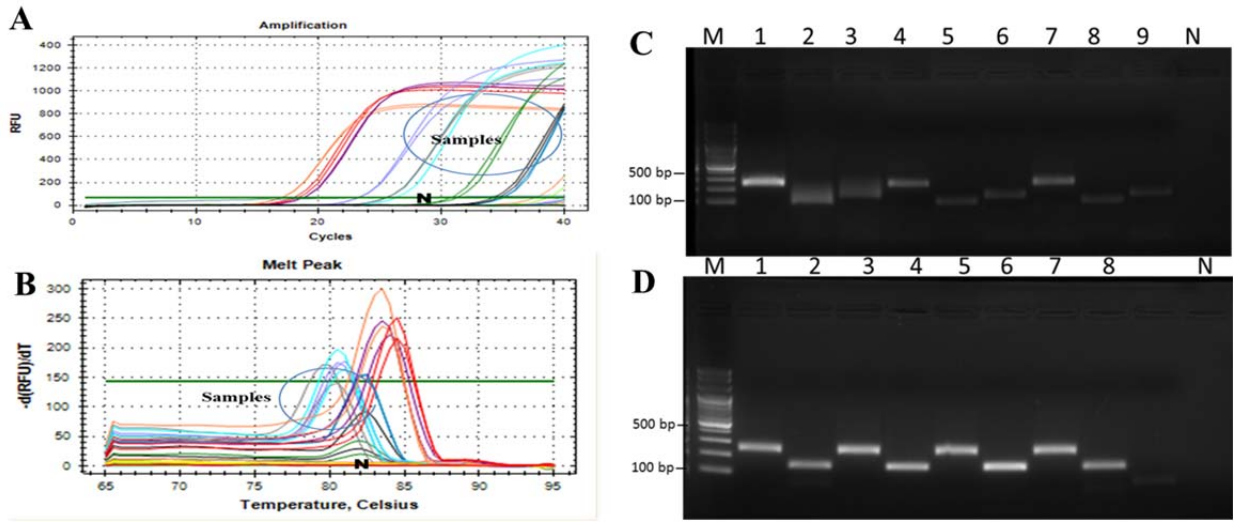

Fig. 17. Real-time PCR using SYBR Green I DNA binding dye. Amplification curve with negative RT product (A); melting peak chart of same amplified sample (B); agarose gel electrophoresis of PCR product of *p53* and *vimentin* for confirmation of single band (M = 100 bp molecular DNA size marker, 1,4,7 = *GAPDH*, 2,5,8 = *p53*, 3,6,9 = *vimentin*, N = RT negative control (C); and *E-cadherin* PCR product (1,3,5,7 = *GAPDH*, 2,4,6,8 = *E-cadherin*, N = RT negative control (D).

Table 3. Summarized histopathological, IHC and qRT-PCR findings at weeks of experiments by group

Measured parameter	Variable observed	Mice group									
		NDMA Vs Sh eggs + NDMA					BBN Vs Sh eggs + BBN				
		Results in time frame (week)					Results in time frame (week)				
		4	12	20	28	36	4	12	20	28	36
Histopathological results	Hyperplasia	-/+	-/+	+/+	-/+	-/-	+/+	+/+	+/+	+/+	-/ϕ
	Dysplasia	-/-	-/+	-/+	-/+	-/+	+/+	+/+	+/+	-/+	-/ϕ
	Squamous metaplasia	-/-	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/ϕ
	Inflammation	-/+	-/+	-/+	-/+	+/+	+/+	+/+	+/+	+/+	+/ϕ
	Epithelial vacuolar change	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/+	-/ϕ
	Nuclear abnormalities	-/-	-/+	-/+	-/-	-/-	+/+	+/+	+/+	+/+	+/ϕ
IHC results	*Ki-67* staining	-/*	-/*	-/*	-/*	-/-	*/*	*/*	*/+	*/*	-/ϕ
Gene expression results	*p53*	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/+	-/-	-/ϕ
	E-cadherin	-/-	+/-	-/-	-/+	-/-	-/-	ϕ/-	-/-	-/-	-/ϕ
	vimentin	-/-	-/-	-/+	-/-	-/+	-/-	-/+	+/+	-/-	+/ϕ

Key: '+'=positive (histopathological results, *Ki-67* for urothelial cells and increase for gene expression), '*'=positive (*Ki-67* staining for lymphocytes), '-'=negative (histopathological results, *Ki-67* & decrease for gene expression), 'ϕ'=missing data

DISCUSSION

The present study investigated a mouse model of UB carcinogenesis by injecting Sh eggs and introducing nitrosamines. The mice were monitored for their body weight change, histopathological changes, IgG and cytokine levels in serum and oncogene expressions in the urothelium. The findings could confirm that Sh eggs and nitrosamines induced preneoplastic findings that suggested neoplastic transformation in the UB wall of the mice.

Significant weight loss was observed among all treated groups except the Sh eggs group compared to the control after 15 weeks post experiment in the present study. Comparatively, the body weight measurement data showed more significant reduction among Sh eggs + BBN and Sh eggs and NDMA implying synergistic effect of the Sh eggs and nitrosamines. The mice revealed delayed response to stimuli and general lethargy among the Sh eggs and NDMA or BBN treated groups. The weight loss data may provide a clue for the general impact of administration of Sh eggs and nitrosamines on physical conditions.

Histopathological findings presented ranges of urothelial cell abnormalities from mild hyperplasia to high grade dysplasia which were interpreted as preneoplastic changes. These preneoplastic lesions were manifested by anomalous transformations such as several urothelial layers, abnormal nuclear structure, pleomorphic and prominent nucleoli, and marked local inflammation observed mainly in the BBN and Sh eggs + BBN group. The Sh eggs and BBN may have stimulated the urothelial cells to be transformed.

Squamous metaplasia and dysplasia were also observed in the Sh eggs + NDMA group at 12 week post eggs injection and NDMA treatment. On the contrary, tissues from the Sh eggs or NDMA group showed no comparable histopathological changes. The coexistence of squamous metaplasia and squamous cell carcinoma of the UB has been well established since the description of the condition (Khan et al., 2002; Rausch et al., 2012). Squamous metaplasia represents replacement of the urothelium by stratified squamous epithelium occurring commonly in response to local stimuli like injury or infection. It may be

of non-keratinizing squamous metaplasia or keratinizing squamous metaplasia, the latter is clinically associated with the development of bladder cancer. Vitamin A deficiency has been reported to induce keratinizing squamous metaplasia of urothelium in mice (Liang et al., 2005). The present finding of squamous metaplasia and dysplasia supports preneoplastic transition of the UB urothelial cells in the Sh egg + NDMA group as well as in the Sh eggs + BBN group.

In addition to hyperplasia and dysplasia, unusual epithelial vacuolar change was observed in the Sh eggs + BBN at week 20. The epithelial vacuolar change was scattered in the tissue with whitish keratin like structure. The squamous metaplasia and dysplasia in the UB wall can suggest cellular transformation possibly progressing to develop squamous cell neoplasia although there is no definite evidence of neoplasia in the present study. The present histopathological changes from the mice group treated with Sh eggs and nitrosamines may indicate progress of transformation to preneoplastic cells in the UB wall of the animals.

The carcinogenic effects of *S. haematobium* infection have been studied in experimental animals mostly through the evaluation of the pathological changes that occur in the bladder mucosa. According to previous reports of animal experiment, urothelial neoplastic induction attempts were reported in gibbons (*Hylobates lar*) and non-human primates like baboon (*Papio* sp.) infected with *S. haematobium* and supplied with or without N-nitroso chemicals (Kuntz et al., 1975; Hicks et al., 1980). Heavy egg deposits in the bladder mucosa and submucosa were observed during the acute phase of *S. haematobium* infection in mice though eggs were not discharged in urine (Agnew et al., 1988). Recently it has been reviewed that the initiation and progression of schistosomal bladder cancer, being a complex and multistep process, involves the actions of several risk factors including the parasite, the host immune and tissue regenerative response, and environmental exposures to carcinogens (Kaufman et al., 2009; Honeycutt et al., 2014; Nweke et al., 2015).

Immunohistochemical results of the present study revealed that *Ki-67* expression intensity in the UB epithelial cells was significantly stronger in Sh eggs + BBN group at week 20 than in the remaining groups. The lymphocytes were strongly stained for *Ki-67* in Sh eggs + NDMA and Sh eggs + BBN

group at week 4 and 12. The strong intensity of the nuclear marker staining for lymphocytes slowly declined to moderate at week 20 and 28. The control group showed *Ki-67* expression neither in urothelial cells nor in lymphocytes at all weeks of experiment. It has been reported that the expression of *Ki-67* is strongly associated with tumor cell proliferation and growth, and is widely used in routine pathological investigation as a proliferation marker (Ding et al., 2014). A number of diagnostic applications for *pKi-67* have been described, where it is significantly more expressed in malignant than in normal tissues (Hu et al., 2011). Initiation and promotion of UB carcinogenesis by BBN was accompanied by increased cell proliferation in rodents owing to changes in the expression of genes involved in cell cycle control (Tyagi et al., 2007; Williams et al., 2008). Previous studies reported no difference of *Ki-67* expression between schistosomal and non-schistosomal bladder tumors (Abdulamiret al., 2009). Nevertheless, an increase in *Ki-67*–positive cells induced by BBN has recently been reported in female C57BL/6/c mice (George et al., 2013). More recently, it has been reported that *Ki-67* elimination might have minor effects on cell cycle exit and mitosis, mammalian cells can nevertheless proliferate efficiently in the absence of detectable *Ki-67* (Sobecki et al., 2016). The present study finding on strong *Ki-67* expression supports its role in the prevailing urothelial transformation to dysplasia and epithelial vacuolar change in the Sh eggs + BBN group.

A tumor suppressor gene, *p53*, is normally kept at very low levels and its level and activity increase dramatically in response to DNA damage, oncogenic stress, and other stimuli that are viewed by a cell as anomalous (Lozano, 2010). It has been reported that roughly 50% of chemically induced tumors in mice develop *p53* mutations (Yamamoto et al., 1997), which are similar to those found in humans. UB cancers in schistosomiasis have also been reported to show peculiar profiles of *p53* and *bcl-2* mutations compared to non-schistosome related bladder cancers (Helal Tel et al., 2006). A recent study reported that urothelial *p53* signaling indeed seemed to affect urothelial homeostasis during *S. haematobium* infection, albeit in a sex-specific manner (Honeycutt et al., 2015). In the present study of relative gene expression by qRT-PCR, *p53* showed a sharp increase in the Sh eggs group at week 4 compared to the control. Similarly, the relative high expression level in the Sh eggs + BBN group at week 12 and 20 was recognized. Such a

variation in expression level of *p53* in the Sh eggs and Sh eggs + BBN group might also indicate that the neoplastic change was under progress.

Relative expression of *E-cadherin* in Sh eggs + BBN group at weeks 12, 20 and 28 and all experiment group except the Sh eggs + BBN group at week 36 was highly downregulated compared to that of the control. On the contrary, *vimentin* level was upregulated in the Sh eggs + NDMA group at week 20 and Sh eggs + BBN group at week 12 and 20. *Vimentin* has been reported to play important roles in cell adhesion, migration, and signaling (Ivaska et al., 2007). Recent studies showed that the expression of *vimentin* was observed in 43% of bladder cancers, whereas it was not expressed or found negative in all normal urothelia (Ding et al., 2014). It has been implicated that loss of *E-cadherin* and acquisition of *vimentin* are two critical steps in EMT, where epithelial cells undergo a transformation into spindle-shaped mesenchymal cells, lose cell polarity and cell-cell adhesion and acquire malignant properties, and become more migratory and invasive (Kalluri et al., 2009; Thiery et al., 2009). In addition, cadherin switching has been observed to be an extremely crucial process in bladder cancer development (Bryan and Tselepis, 2010). It has been suggested that loss of *E-cadherin* and acquisition of *vimentin* are two critical steps in EMT of the present study.

Mouse IgG has 4 subclasses namely IgG1, IgG2a, IgG2b and IgG3 as opposed to the equivalent IgG1, IgG2, IgG3 and IgG4 subtypes in human. In the present study, the luminex bead-based multiplex analysis of the three IgG subtypes levels showed high levels during the early weeks than late weeks with statistical significance. The mean serum immunoglobulin levels of IgG1, IgG2a and IgG3 showed strong increase for the Sh eggs and Sh eggs + NDMA groups at week 4. For IgG2b levels, only Sh eggs + NDMA group at week 12 showed statistically significant increase while there was a decrease or a slight increase in the remaining group. On the other hand, IgG1 level among Sh eggs + NDMA and Sh eggs + BBN showed a moderate increase at week 12 and only Sh eggs + NDMA at week 20. The mean serum levels of IgG2a in the Sh eggs and Sh eggs + NDMA groups showed strong significance compared to that of the control at week 12. Thus, the present data on the major IgG subtypes were in agreement with previous reports and may indicate the role of adaptive immune response towards Sh eggs.

Previous studies reported a significant increase in anti-schistosome IgG1, IgG2a and IgG2b levels but a decreased IgG3 in *S. mansoni* infected mice was reported at 8 weeks post infection (El Ridi et al., 2001). On the other hand, human infection with *S. haematobium* and *S. mansoni* has consistently found that levels of specific IgG1 against worm antigen and IgG4 against both worm and egg antigens correlate with infection intensity (Satti et al., 1996; Ndhlovu et al., 1996; Naus et al., 1998).

Th2 associated cytokine analysis of the present study revealed significant increase among the treated groups compared to the control. IL-5 showed a dramatic increase at week 4 and week 20 among all treated groups but more in the Sh eggs + BBN group. IL-4 level also showed an increase for the Sh eggs + NDMA group at week 12 and 20. Previous studies reported that schistosome eggs can induce Th2 responses independently of schistosome worms (Vella and Pearce, 1992; Fu et al., 2012). On the other hand, there was no difference in cytokine levels of control and mouse injected with Sh eggs in female genital schistosomiasis model (Richardson et al., 2014).

From human infection in *S. haematobium* endemic areas, egg-positive people showed significantly higher levels of specific antibodies, IL-2, IFN-γ and IL-23 while egg-negative individuals had significantly higher circulating IL-10, IL-4 and IL-13 (Milner et al., 2010). Other recent study reported that urinary IL-10 was negatively correlated to infection intensity and urinary tract inflammation in *S. haematobium*-infected children (Njaanake et al., 2014). In this study, IL-10 level was similar at week 20 compared to week 4 and week 12. At week 4, the entire treated group showed relatively higher levels but Sh eggs group showed the highest peak of all. At week 12 both Sh eggs + NDMA and Sh eggs + BBN group showed higher level compared to that of the control group and only Sh eggs group showed higher level at week 20. Previous studies reported that IL-10, part of the immunomodulation mechanism and regulatory feedback of Th2-type response (Fairfax et al., 2012), limits the initial granulomatous inflammation that peaks in size and intensity at 8 weeks of *S. mansoni* infection (Hoffmann et al., 2000; Pesce et al., 2009).

The present study had some technical and egg sample size related limitations. Sufficient number of eggs was not injected into the mouse UB wall since the eggs were collected from infected humans. The

number of Sh eggs might have been over 2000 per mouse for better outcomes. Perhaps following lack of experience in mouse UB eggs injection and less number of injected eggs, only a few eggs were identified from tissue section. Appropriate bladder wall injection skill with more eggs may improve the result. Since it has been reported that UB oncogenesis in rodents usually takes as long as 8-12 months (Oliveira et al., 2006), the duration of experiment was not enough to detect neoplastic change.

In conclusion, Sh eggs and nitrosamines synergistically transform UB epithelial cells into squamous metaplasia and dysplasia. The present histopathological findings were also further supported by increased expression of *Ki-67*, *p53*, and *vimentin* with decreased *E-cadherin*. Such inverse expressions of *E-cadherin* and *vimentin* mainly among Sh eggs + BBN group may be an indication for EMT. The present study provides a possibility of the mouse model for bladder cancer study by *S. haematobium*.

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국문초록

방광주혈흡충은 사람에서 방광암을 일으킬 수 있는 생물학적 발암물질이다. 본 연구에서는 마우스의 방광벽에 방광주혈흡충의 충란을 주입하고 화학적 발암물질을 마우스에게 섭취시켜 방광암 마우스모델 확립하였다. 총 72마리의 암컷 ICR 마우스를 6개 군; 대조군 (군 1), 방광주혈흡충 충란만을 주입한 군 (군 2), N-nitrosodimethylamine (NDMA)만을 섭취시킨 군 (군 3), N-butyl n-(4-hydroxybutyl) nitrosamine (BBN)만을 섭취시킨 군 (군 4), 방광주혈흡충 충란을 주입하고 NDMA를 섭취시킨 군 (군 5), 방광주혈흡충 충란을 주입하고 BBN을 섭취시킨 군 (군 6)으로 나누어 실험을 진행하였다. 군 2와 군 5 그리고 군 6의 동물들에겐 isoflurane으로 마취를 진행한 뒤 하복부 정중선을 절개하여 방광 원개 (bladder dome)의 앞쪽 측면에 점막 하 주사를 통해 1000개의 방광주혈흡충 충란을 50 μL 생리 식염수를 통해 주입하였다. 대조군에겐 방광주혈흡충 충란을 제외한 50 μL의 생리식염수만을 주입하였다. NDMA와 BBN은 각각 12.5 part per million (ppm)과 0.05%의 농도로 음수에 혼합되어 준비되었고, 실험 일정 전체에 걸쳐 마우스에게 자율적으로 제공되었다. 마우스들은 각각 4주, 12주, 20주, 28주 그리고 36주 간의 실험 후에 희생되었다. 마우스의 방광조직을 통해 암억제유전자 (tumor suppressor gene)와 상피간엽이행 epithelial-mesenchymal transition (EMT)과 관련된 유전자 마커들의 발현을 분석하기 위해 면역조직화학염색 (immunohistochemical analysis)과 실시간 중합효소연쇄반응법 (qRT-PCR)을 실시하였다. 마우스의 사이토카인과 IgG subtypes그리고 소변을 통해 분비된 NDMA을 분석하기 위한 실험도 실시하였다. 대조군 (45.61 ± 8.3)에 비교해서 군 6 (36.26 ± 7.2; $P < 0.01$) 에서 체중의 유의적인 감소 (20.5%)가 나타났고 군 5 (40.33 ± 8.5; $P < 0.01$)에서도 유의적인 체중손실 (11.6%)이 일어났지만 군 2 (43.35 ± 9.7; $P = 0.12$)에서는 나타나지 않았다 (2.2%). 방광의 조직병리학적인

소견에서는 방광상피세포의 경미한 과형성부터 상피조직의 공포성 변화, 그리고 높은 수준의 이형성이 나타났다. 면역조직화학염색을 통해서 20주 경의 군 6 마우스의 요로상피세포에서 *Ki-67* 유전자의 발현이 유의적으로 높게 나타나는 것을 알 수 있었다. 실시간 중합효소연쇄반응 결과에서는 4주 및 20주 경의 군 2 에서*p53* 유전자가 유의적으로 높게 발현되었다. 군 6에서는 *E-cadherin*과 *vimentin*의 대조되는 발현이 결과로 관찰되었다. 이러한 *E-cadherin*과 *vimentin*의 대조되는 발현은 방광조직에서의 상피간엽이행 (EMT)이 일어났다는 것을 짐작할 수 있게 해준다. IgG2b를 제외하고, 혈청에서 IgG1와 IgG2a 그리고 IgG3가 4주, 12주, 20주에서 대조군에 비해 군 2와 군 5에서 유의적으로 큰 차이를 보였다. 종합해보면, 방광주혈흡충과 nitrosamines는 방광 세포에서 편평상피화생과 이형성을 일으킬 수 있고, 이는 곧 증가된 *Ki-67*과 *p53* 그리고 *vimentin*의 발현과 감소된 *E-cadherin*의 발현과 관련지어 생각해볼 수 있다. 본 연구는 방광주혈흡충 충란과 nitrosamines을 이용한 방광암의 재현 가능한 동물모델 가능성을 제시하였다.

주제어: 방광암, 마우스, 방광주혈흡충 충란, N-nitrosodimethylamine, N-butyl n-(4-hydroxybutyl) nitrosamine

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