Beneficial effect of folic acid on kidney and testes of adult albino rats after exposure to methomyl

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Abstract

The aim of the study was to evaluate the protective effect of folate against methomyl induced toxicity on kidney and testes of male rats. Adult male albino rats were divided into four groups; Group I served as control (vehicle), Group II received folic acid (1.1 mg/kg b.wt.), Group III methomyl (1 mg/kg b.wt.) and Group IV folic acid and methomyl. Treatments were administered via oral gavage on a daily basis for 14 weeks. Thereafter blood samples were collected and serum creatinine, testosterone and total antioxidant capacity (TAC) determined. Animals were sacrificed and semen analysis conducted. The kidneys and testes were excised and malondialdehyde (MDA) levels were determined. Histopathological and immunohistochemical analysis for caspase-3 was also undertaken. Methomyl treatment resulted in a significant ($p < 0.001$) elevation of creatinine and MDA levels and significant ($p < 0.001$) reduction in testosterone and TAC levels. Furthermore, methomyl caused a significant ($p < 0.001$) reduction in sperm quality. Histopathological examination indicated testicular and renal damage with strong immunoreactivity for caspase-3. Functional and tissue damage was prevented in rats treated with a combination of methomyl and folic acid. This is ascribed to the ability of folate to directly scavenge reactive oxygen species and indirectly enhance cellular redox homeostasis. This study identified that folic acid supplementation may have a beneficial effect in preventing or reducing the deleterious effects of methomyl exposure on kidney as well as testis structure and function. Future studies should focus on the fertility outcome/pregnancy index in rats.

Key words: Antioxidant capacity, folic acid, caspase -3, kidney, methomyl, testes

1. Introduction

Methomyl, a carbamate insecticide, is a broad spectrum insecticide used for foliar treatment of fruits, vegetables, field crops, cotton, and commercial ornamentals as well as to repel flies in and around poultry houses and dairies.[1] In humans, methomyl may enter the body either orally, via inhalation or dermal absorption.[2]. Exposure is mainly due to occupational handling, fly spraying and ingestion of food where methomyl was used during spraying.[3]

Methomyl is categorized as a restricted-use insecticide due to its toxicity to non-target species.[2] In rats, methomyl exerts oral toxicity at an LD_{50} of 20 mg/kg body weight.[4] Although the primary target organ of methomyl is the brain, other organs such as the kidney and testes are known to be sensitive to pesticides. The high blood volume supplied to the kidney and the role of this organ in concentrating solutes increases susceptibility to pesticide toxicity.[5] Deleterious effects to the liver and kidney due to the overproduction of free radicals is known.[6] Inhibition of male fertility by pesticides involves endocrine disruption and reactive oxygen species (ROS) mediated disruption of testicular function and sperm development.[7]

Pesticides cause ROS overproduction resulting in oxidative stress with subsequent damage to lipids, membranes, proteins and nucleic acids.[6] Lipid peroxidation has been implicated as a main mechanism involved in carbamate-induced toxicity.[8] Under normal conditions, cells are equipped with non-enzymatic and enzymatic pathways that ensures cellular redox homeostasis.[9] This includes glutathione (GSH) and the antioxidant enzymes; superoxide dismutase (SOD), glutathione peroxidases (GPx) and catalase

(CAT). Disruption of this homeostasis by either the depletion of antioxidant molecules such as GSH or the inhibition of the antioxidant enzymes, enhances ROS formation.[10] This results in cellular dysfunction and associated apoptosis, an essential homeostatic process, which allows for the elimination of damaged or redundant cells in an ordered process of cellular disintegration.[11] Apoptosis is initiated by activation of caspase-3, which leads to DNA fragmentation, nuclear protein degradation, chromatin condensation, fragmentation, formation of apoptotic bodies and cellular death.[12]

Non-enzymatic antioxidants, usually dietary in origin, assist in maintaining cellular redox homeostasis. These include fat-soluble antioxidants associated with the cellular membrane such as vitamin E, coenzyme Q and β-carotene. The most potent is vitamin E, which protects the cell membrane against lipid radicals, and removes reactive intermediates. In contrast, vitamin C is a water-soluble antioxidant scavenger and protects hydrophilic environments such as the cytoplasm and the extracellular environment against oxidative damage.[9] Another water-soluble vitamin is folate, which has antioxidant activity comparable to vitamin C and E. Also, folate in conjunction with vitamin B12 and B6, is involved in nucleotide synthesis, the regeneration of methionine from homocysteine, and oxidation and reduction of one-carbon units required for normal cellular function.[13] In liver cells, homocysteine enters the transsulfuration pathway and in conjunction with vitamin B6 forms cysteine required for the synthesis of GSH, which is essential for the maintenance of cellular redox homeostasis.

Folic acid supplementation is widely used to reduce the risk of neural tube development (NTD), improve male fertility and diminish the risk of development of non-communicable diseases such as diabetes and cancer.[13] Therefore folate, may have several

additional beneficial effects besides an antioxidant effect, which includes normal one-carbon metabolism that is essential for nucleotide synthesis, DNA and histone methylation, and maintenance of genomic integrity especially during processes such as spermatogenesis.

Therefore, the aim of this study was to determine if folic acid co-administration can prevent methomyl induced kidney and testes damage in a chronic rat model of methomyl toxicity.

2. Material and methods

2.1 Animals

All rat experiments were performed in compliance with the relevant laws and guidelines of Zagazig University of Medicine, Egypt, which are in accordance with the National Institute of Health Guidelines for Animal Care. The Ethics Committee of Zagazig University of Medicine approved the study (Ethics approval number ZU-IRB# 1015-258-2013). A total of 40 male albino rats (aged 16-18 weeks; weighing 150-200 g) were bred and reared at the animal care center. Animals were acclimated for two weeks under standard laboratory conditions prior to commencement of treatment. Food and water was made available *ad libitum*. The room temperature was maintained at 23 ± 2°C, 12 h light-dark cycles, with a relative humidity of 40-60%.

Animals were assigned at random to one of four groups consisting of 10 animals per group. Group I: control, received 1 mL distilled water, Group II: received folic acid at 1.1 mg/kg b.wt. (El Gomhoryia, Egypt), Group III: received methomyl at a dose of 1 mg/kg

b.wt. (Kafr El-Zayat Pesticides & Chemicals, Egypt) and Group IV: received co-administered folic acid and methomyl. All treatments were given once daily for 14 weeks via oral gavage.

The dose of methomyl, 1 mg/kg b.wt., was selected on the basis that it would mimic chronic exposure. This dose represents 1/20 of oral LD50 in rats[4] and relates to a human equivalent dosage of 0.1675 mg/kg b.wt.[14] The folic acid dose of 1.1 mg/kg b.wt. was selected based on the average daily intake for humans and experimental animals.[4,15]

2.2 Specimen collection

Blood: Twenty four hours after the last dose, animals were anaethetized using ether and venous blood (5-7 mL) was collected from the retro-orbital plexus using a capillary glass tube. Samples were then ejected into non heparinized glass tubes and allowed to clot for 30 min at 25°C after which serum was separated by centrifugation (600*g*, 15 min, 4°C) and stored at -20°C until analysis.

Organs and tissue: For organ and tissue collection, rats were anaesthetized using ether and sacrificed by cervical dislocation. Epididymal content (spermatozoa) of each rat was collected by cutting the tail of the epididymis and squeezing it into a petri dish. Thereafter the semen was incubated at 37°C for 30 min for liquefaction and examination.

The left kidney and left testes of all test animals were excised, washed with ice cold saline and weighed. Half of the organ was used in malondialdehyde analysis and the other half was fixed in 10% formalin saline, embedded in paraffin blocks and processed for histopathological and immunohistochemical examination.

2.3 Serum analysis

Serum creatinine levels were determined using commercially purchased kits obtained from Spinreact (Spain, Cat. No. 1002011).

Serum testosterone concentrations were assessed using kits purchased from Cusabio (USA, Cat. No. CSB-E05100r1). The rat/mouse ELISA method as described by Chen and Zirkin[16] was employed. Briefly, into a 96-well plate was added 10 µL calibrator, sample or control then 100 µL of incubation buffer followed by 50 µL of the enzyme conjugate. The plates were incubated with shaking at room temperature for 60 min after which the plate was washed, before 200 µL of the substrate solution was added. After a 30 min incubation period, 50 µL stop solution was added to each well and the absorbance measured at 450 nm.

Total antioxidant capacity (TAC) was determined using kits obtained from Biodiagnostic (Egypt, Cat. No. TA 2512) which measures the ability of serum to scavenge free radicals generated from hydrogen peroxide (H_2O_2). TAC was assessed according to the spectrophotometric method of Koracevic *et al.*[17]

2.4 Tissue analysis

Malondialdehyde (MDA) levels were measured using kits obtained from Abnova (Cat. No. KA37362) and the method of Ohkawa *et al.*[18] The excised tissues were homogenized in 10% (w/v) ice cold phosphate buffer (0.12 M, pH 7.2) and centrifuged at 10,000 *g* for 30 min at 4°C. The tissue supernatant was mixed with 1 mL trichloroacetic acid (TCA, 20%) and 2 mL thiobarbituric acid (TBA, 0.67%), where after the mixture was heated for 1 h at 100°C. The mixture was allowed to cool and the supernatant was collected by

centrifugation at 3000 *g* for 10 min. The absorbance of the sample was measured spectrophotometrically at 532 nm, using a blank containing all the reagents excluding the sample.

2.5 Semen parameters

The amount of progressive sperm was determined according to the method of Bearden and Fuquay.[19] Sperm viability was assessed using eosin-nigrosin stain where 200 sperm cells were counted.[20] Sperm concentration was determined according to the method of Blazak *et al.*[21] and sperm morphology was assessed according to Mori's classification.[22]. All analysis was carried out using a light microscope (Olympus) at 40x magnification.

2.6 Histopathology

2.6.1 Haematoxylin and Eosin staining

Paraffin blocks were sectioned at 5 µm and stained with haematoxylin and eosin (H & E) according to Bancroft and Stevens.[23] Sections were de-waxed in xylene, rehydrated using descending grades of alcohol (100%, 96%, and 70%), then stained in hematoxylin for 20 min and eosin (1%) for 10 min and examined under light microscopy.

2.6.2 Immunohistochemical examination

Immunohistochemical examination was carried out according to the method of Ramos-Vara[24] using monoclonal antibody kits (ABclonal, USA; Cat no A10765). Paraffin sections (4 µm) were mounted, dewaxed with xylene, rehydrated with phosphate buffered

saline (PBS, pH 7.2). The endogenous peroxidase activity of the sections was blocked with 3% hydrogen peroxide and washed in PBS. The non-specific binding of the immunoglobulin was quenched by adding rabbit serum and incubating for 30 min in a humidified chamber. Thereafter, slides were incubated overnight with primary caspase-3 antibodies (rabbit anti-caspase antibody; 1:50 dilution in PBS) and then rinsed with PBS. Subsequently, slides were incubated with labelling antibody (streptavidin horse radish peroxidase; 1:50 dilution) in PBS for 30 min in a humid chamber at room temperature. Localization of caspase-3 was achieved by incubating the slides with the chromagen, 3-3diaminobenzidin tetrahydrochloride (DAB) until suitable staining had developed (5 min). Sections were counterstained with Mayer's hematoxylin, washed, dehydrated, mounted and then examined microscopically.

The height of the germinal epithelium was measured using photomicrographs (x200) and Digimizer 4.3.2. image analysis software (MedCalc, Belgium).

2.8 Statistical analysis

Data was captured and analyzed using SPSS version 19. Results are expressed as mean ± standard deviation (SD) of at least three repeats carried out in triplicate. One-way analysis of variance (ANOVA) was used to compare the means of the groups. Least significant difference (LSD) was used to compare the means between groups. A *p*-value <0.05 was considered statistically significant.

3. Results and Discussion

3.1 Serum parameters

Elevated serum creatinine is an indicator of impaired renal function.[25] Serum creatinine levels of the control group, and folic acid-treated group showed no statistically significant differences (Table 1). Methomyl treatment resulted in an increase (1.41 ± 2.9 mg/dL) in serum creatinine level when compared to the control group (0.54 ±0.08 mg/dL). This finding is corroborated by other studies in which rats were exposed to ~ 2 mg/kg of methomyl administered over a 5 day period[26] and 28 day period,[27] respectively. In addition, mice exposed to 1 mg/kg of methomyl for 10, 20 and 30 days were also found to have increased serum urea and creatinine levels,[5] similar to what has been noted in rats given a dose of 8 mg/kg b.wt. methomyl.[6] Co-administration of folic acid with methomyl resulted in a lower (0.76 ± 0.04 mg/dL) serum creatinine level compared to methomyl (1.41 ± 0.29 mg/dL) treatment alone (Table 1).

Table 1. Serum biochemistry and antioxidant capacity of kidney and testes.

Parameter	**Treatment group (n = 10)**			
	Group I Control	**Group II Folic acid**	**Group III Methomyl**	**Group IV Methomyl-Folic acid**
Serum creatinine (mg/dL)	0.54 ± 0.08	0.55 ± 0.09	1.41 ± 0.29[b]	0.76 ± 0.04[a,b,c]
Serum testosterone (ng/dL)	5.17 ± 0.09	4.99 ± 0.48	2.32 ± 0.82[b]	4.54 ± 0.41[a,b,c]
Serum TAC (mmol/L)	1.26 ± 0.02	1.25 ± 0.17	0.78 ± 0.07[b]	1.14 ± 0.14[a,b,c]
MDA kidney (mmol/g)	49.44 ± 8.05	47.77 ± 18.19	103.21 ± 14.71[b]	62.81 ± 14.05[a,b,c]
MDA testes (mmol/g)	49.22 ± 12.62	48.39 ± 15.93	155.60 ± 37.31[b]	52.68 ± 7.06[a,b,c]

[a] *$p<0.05$ when values are compared to control group.*

[b] *$p<0.001$ when values are compared to control group.*

[c] *$p<0.001$ when values of Group IV are compared to Group III.*

Serum testosterone levels of the control and folic acid-treated groups showed no significant differences, however, treatment with methomyl caused a significant ($p < 0.001$) decrease (2.32 ± 0.82 ng/dL) in the serum testosterone level when compared to the control group (5.17 ± 0.09 ng/dL) (Table 1). Similar findings have been reported in male albino rats treated with 0,5 and 1 mg/kg methomyl for 65 days.[4] A decrease in testosterone levels has also been noted after treatment with other pesticides.[28,29] Treatment of rats with a methomyl-folic acid combination resulted in an increase (4.54 ± 0.41 ng/dL) in serum testosterone levels, compared to animals treated with methomyl only (Table 1). However, levels were still significantly ($p < 0.05$) lower than the control group, indicating only partial protection (Table 1). In the present study, only a single dosage was used and a future dosage based study, will identify the optimal folate concentration required for the normalization of testosterone levels. Interestingly, in the present study 2.49 µmol/kg folic acid effectively reduced the effect of 6.16 µmol/kg methomyl. Improvement of the testosterone level in rats treated with a combination of folic acid and methomyl compared to methomyl treatment only, has previously been reported.[4] Also, a combination of vanadylfolate with carbofuran has been found to alleviate reproductive toxicity and increase testosterone levels.[29] These effects are ascribed to the ability of folic acid to scavenge ROS, thereby protecting the Leydig cells and consequently resulting in an increase in plasma testosterone levels.[30]

Serum TAC levels of the control and folic acid-treated groups showed no statistically significant differences (Table 1). A significant ($p < 0.001$) decrease (0.78 ± 0.07 mmol/L) in serum TAC level was noted in the methomyl treated group when compared to the control group (1.26 ± 0.02 mmol/L; Table 1). A similar decrease in TAC has been

reported after exposure of rats to methomyl (0.03 mg/kg/day) for 14[26] and 28[31] consecutive days. Similarly, the pesticide, diazinon, has been found to result in a decrease in TAC in rats.[32] Combination treatment with methomyl and folic acid resulted in an increase (1.14 ± 0.14 mmol/L) in serum TAC compared to the methomyl-treated group alone (Table 1).

3.2 Tissue levels

Malondialdehyde (MDA), the end product of polyunsaturated fatty acid (PUFA) degradation, is used as an indicator of lipid peroxidation.[33] No statistical differences in the MDA levels in the renal and testicular tissues between the control and folic acid-treated groups were noted (Table 1). Kidneys and testes of the methomyl-treated group showed a significant (p <0.001) increase in tissue MDA compared to the control (Table 1). The MDA level in kidneys of the East Asian bullfrog treated with methomyl have been found to be higher than that of the control group.[34] This is supported by the finding that methomyl induces lipid peroxidation and causes perturbations in various antioxidant enzymes in kidneys[26] and testes of rats.[35] The elevated MDA levels noted after methomyl treatment is thought to be caused by direct conjugation of methomyl (or its metabolites) to PUFA or liberation of ROS which react with PUFA or accumulation of conjugated methomyl liphophilic components with fatty acids.[36] The increased MDA levels in rats after methomyl treatment was found to alter the antioxidant defense system, specifically GPx and CAT levels.[6] Methomyl has been reported to increase lipid peroxidation in mice kidneys, when compared to control animals.[5] GSH, total sulfhydryl group, SOD, CAT and GPx were found to be decreased in the methomyl treated animals.[5] Chronic exposure of Nile Tilapia to methomyl resulted in significant increases in renal antioxidant enzymes

and GSSG and a reduction in GSH levels.[37] Low levels of GSH and higher GSSG levels translates into a poor GSH:GSSG ratio which is associated with increased oxidative stress.

Co-administration of methomyl and folic acid caused a significant ($p < 0.001$) decrease in both the renal and testicular tissue MDA levels when compared to the methomyl-treated group (Table 1). In a previous study the combinational treatment of folic acid and methomyl was found to decrease MDA levels in testes of rats.[38] Similarly, co-administration of methomyl with the antioxidants, vitamin C and selenium, in rats was found to partially lower MDA levels which resulted in the normalization of the renal antioxidant status.[6]

Folate has a wide range of biochemical effects and these include the synthesis of DNA, transfer of RNA as well as the synthesis of cysteine and methionine[39] which are important precursors for the synthesis of GSH. In the rat, the predominant metabolic pathway of methomyl involves the displacement of the S-methyl moiety by GSH, and with further metabolism the mercapturic acid derivative is formed.[40] This results in GSH depletion and down regulation of the antioxidant enzyme pathway, which will cause bioaccumulation of ROS. Excessive accumulation of ROS can then cause cellular dysfunction due to radical induced changes to membranes, protein and DNA.[39]

Supplementation with folate can increase cysteine and methionine levels and consequently GSH levels.[39] The direct antioxidant effect and the ability to inhibit lipid peroxidation indicates that folate is a multifunctional molecule that restores the antioxidant status of cells and tissue via the normalization of GSH levels, the direct

scavenging of ROS and preventing the conjugation of methomyl (or its metabolites) to PUFA.

3.3 Semen parameters

Sperm count correlates with fertility[41] where changes in sperm motility and morphology indicates testicular damage often caused by toxins.[42] Semen analysis with regard to sperm count, sperm motility, viability and abnormal forms, of the control and folic acid-treated groups was found not to be statistically different (Table 2). Methomyl resulted in a significant ($p < 0.001$) decrease in sperm count, sperm motility, sperm viability and an increase in abnormal sperm forms when compared to the control group (Table 2). The latter includes the presence of double headed sperm, detached heads and coiling of the sperm tail. Previously it has been shown that a daily oral dose of 1 mg/kg methomyl administered for 65 consecutive days caused a progressive decline in sperm motility and concentration, as well as an increase in the percentage of sperm abnormalities, which included double heads, detached heads, and coiled tails.[4] In addition, a decrease in the diameter and the number of spermatogenic and Leydig cells in the testes has also been observed.[36] The toxic effects of methomyl on the male reproductive tract may be ascribed to either direct oxidative effects or indirect effects which lower the serum testosterone levels.[4] Low testosterone level is known to inhibit spermatogenesis and fertility.[43]

Table 2. Semen parameters and organ weights of experimental groups.

Parameter	Treatment group (n = 10)			
	Group I Control	**Group II Folic acid**	**Group III Methomyl**	**Group IV Methomyl-Folic acid**
Sperm count (10^6/mm^3)	75.32 ± 2.95	75.89 ± 3.89	50.85 ± 2.25[b]	69.79 ± 5.99[a,b,c]
Sperm motility (%)	90.03 ± 3.80	89.36 ± 3.02	57.45 ± 3.50[b]	84.09 ± 3.42[a,b,c]
Abnormal sperm forms (%)	2.79 ± 0.38	2.78 ± 0.31	4.51 ± 0.64[b]	3.16 ± 0.59[c]
Sperm viability (%)	87.67 ± 3.33	88.15 ± 1.68	46.91 ± 4.19[b]	81.65 ± 5.88[a,b,c]
Germinal epithelium height (µm)	120.39 ± 20.15	113.56 ± 16.19	72.51 ± 15.25[b]	100.08 ± 15.25[a,b,c]
Weight of kidney (g)	1.76 ± 0.03	1.73 ± 0.02	0.94 ± 0.01[b]	1.30 ± 0.01[b,c]
Weight of testis (g)	2.82 ± 0.02	2.80 ± 0.08	1.95 ± 0.03[b]	2.36 ± 0.04[b,c]

[a]*p<0.05 when values are compared to control group.*

[b]*p<0.001 when values are compared to control group.*

[c]*p<0.001 when values of Group IV are compared to Group III.*

Lipids are the target cellular components for free radicals.[44] Consequently, a high concentration of poly unsaturated fatty acids renders the spermatozoa more liable for lipid peroxidation.[30] The decreased sperm count and density may be attributed to the direct detrimental effect of methomyl on the Sertoli cells which results in disruption of the blood testes barrier, disorganization of epithelium, poor spermatogenesis and tubular atrophy.[44] A reduced number of germinal cells, spermatocytes and spermatids following methomyl treatment can also be attributed to the reduced nucleic acid levels secondary to ROS induced disruption of cell regulatory pathways, meiosis and cell division.[36]

Methomyl has been reported to alter the level of sialic acid, which is responsible for sperm plasma membrane stabilization, ionic balance maintenance and antigen interaction between epididymal epithelium and sperm.[36] The altered sialic acid level results in sperm abnormality, decreased viability and motility.[36]

In testes and epididymis, methomyl has been found to decrease the activity of Na^{+}-K^{+} adenosine triphosphatase (ATPase), Mg^{++} ATPase, and Ca^{++} ATPase.[44] The inhibition of ATPases alters cellular energy of sperms. Thus, a deficiency of ATP, even slight, will reduce motility and lead to infertility.[44].

Treatment with a combination of methomyl and folic acid caused a significant ($p < 0.001$) increase in sperm motility and viability and a decrease in the amount of abnormal sperms when compared to the methomyl-treated group (Table 2). Folic acid has been found to ameliorate the detrimental effects of methomyl on semen parameters, by resulting in an increase in sperm count, motility, viability and a decrease in abnormal forms.[4] A marked reduction in sperm motility and count has been noted in rats fed a folic acid-deficient diet.[45] Folic acid deficiency leads to the accumulation of homocysteine, secondary to the disruption of methionine synthesis, with an associated decrease in GSH levels, which causes a disruption in cellular antioxidant pathways and increased ROS mediated cellular and tissue damage.[39]

Although ROS is physiologically important, excess ROS can have detrimental effects. Increased ROS has been implicated in the seminal fluids of males with subfertility.[46] A positive correlation between ROS and both decreased sperm motility and abnormal

morphology has been identified while a negative correlation between ROS and the folic acid levels in semen has been reported.[46] This implies that folic acid has antioxidant properties that protects sperm and semen against ROS. Collectively, folic acid can improve male fertility through its antioxidant properties, sperm production enhancement and its anti-apoptotic activity against oxidative damage of DNA in spermatozoa.[47]

3.4 Histopathology

The weight of kidneys and testes in control and folic acid groups showed no statistical significant differences (Table 2). In methomyl treated rats there was a ignificant ($p < 0.001$) decrease in the weights of the kidneys and testes. Co-administration of methomyl and folic acid resulted in the organ weights being higher than the methomyl treated group (Table 2). The lower organ weights has also been reported for the livers of rats treated with methomyl compared to control rats.[6]

3.4.1 Haematoxylin and Eosin staining

H & E stained sections of the kidneys of the control group and folic acid-treated group revealed normal cell and tissue structure. The structure of the renal corpuscles and convoluted tubules of the cortex was normal (Figures 1A and 1B). The renal corpuscle presented as a glomerular tuft of capillaries surrounded by Bowman's capsule. The convoluted tubules included more abundant proximal convoluted tubules (PCT), lined by large pyramidal cells and less abundant distal convoluted tubules (DCT), lined by

cuboidal epithelium. The tubular epithelium showed acidophilic cytoplasm and vesicular nuclei.

Exposure of rats to methomyl resulted in several histological alterations in the glomeruli, tubules and the interstitium. The glomeruli were distorted, shrunken and even emaciated with enlarged Bowman's spaces (Figures 1C and 1D). The renal tubules were distorted and had marked alterations in structure (Figure 1C). Dilation and abundant intracytoplasmic vacuoles were also evident in the convoluted tubules (Figure 1E). The latter is an indication of fluid retention which in turn is indicative of kidney damage.[48] The interstitium showed extensive congestion, homogenous acidophilic material (Figure 1D), fibrosis and inflammatory cell infiltration (Figure 1F). These findings are corroborated by El-Demerdash *et al*.[5] and Abu-Basounie,[26] where rodents treated with methomyl showed diffuse degenerative changes of the epithelial cells lining the tubules, severe congestion of cortical blood vessels and focal inflammatory cells infiltration in between the tubules. In the East Asian bullfrog, methomyl has been found to cause both renal tubule and corpuscle, damage.[34] Dilatation of the capsule as well as blood congestion and amorphous substances in the interstitial tissues were observed.[34]

The degenerative changes and nephritic damages associated with methomyl have been attributed to methomyl induced oxidative stress, lipid peroxidation and the resultant free radical accumulation.[5,10] Cellular vacuolation has been ascribed to increased cell membrane permeability secondary to the oxidative stress resulting in subsequent accumulation of water within the cells.[49] Vacuolation is considered to be an adaptive physiological response attempting to limit cell damage. When this limitation fails, rapid

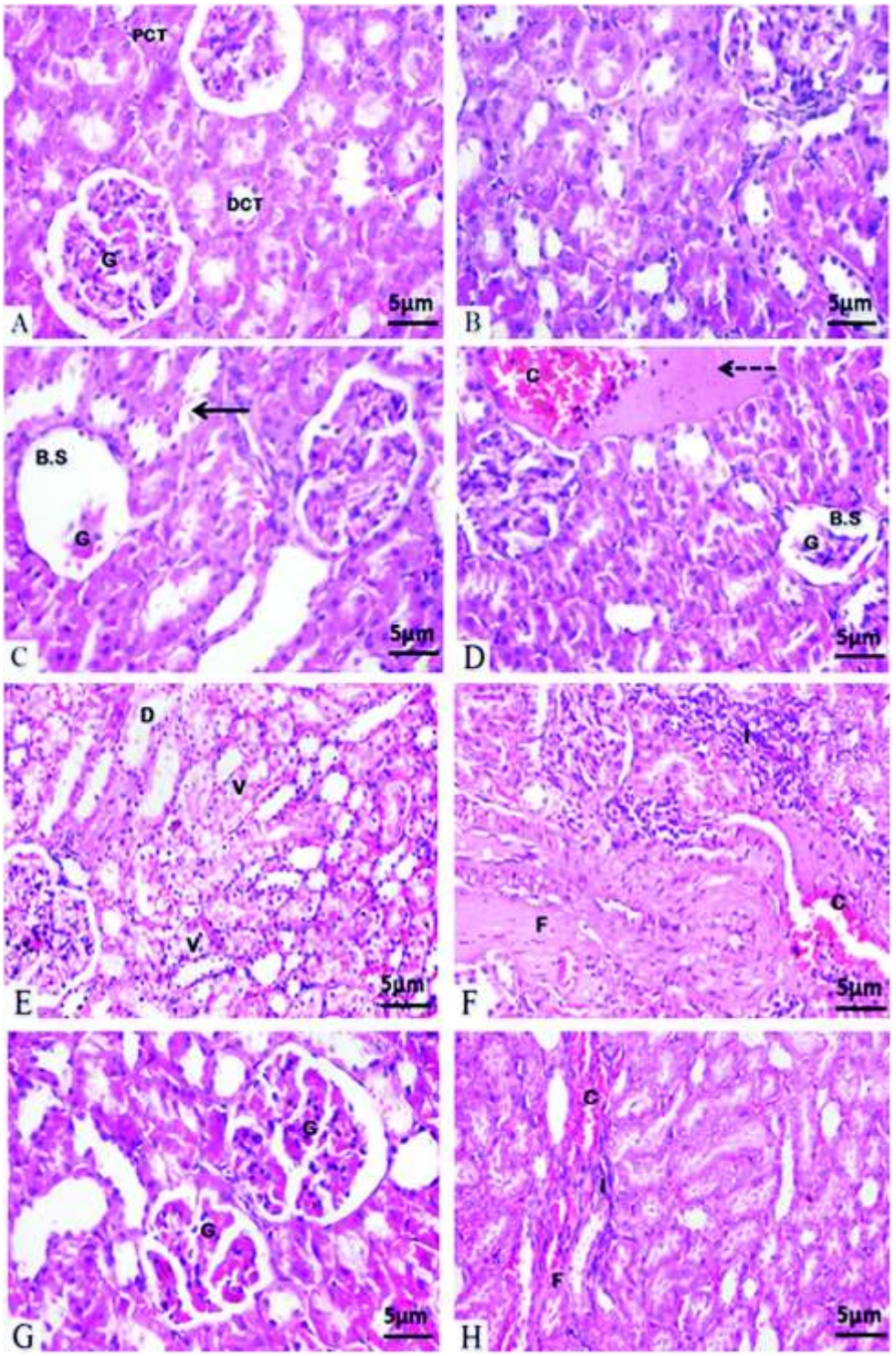

Figure 1. Photomicrographs of the kidneys of adult male albino rats. (A) Control, (B) folic acid, (C, D, E, F) methomyl and (G, H) methomyl and folic acid. G= glomerulus; PCT & DCT= proximal & distal convoluted tubules; B.S= Bowman's space; Arrow= distorted tubule; C= congestion; Dashed arrow= homogenous acidophilic material; D= dilation; V = vacuolation; f = fibrosis; I= infiltration. H & E staining. (Total magnification = x200; Scale bars = 5µm.)

cell death follows.[50] Furthermore, it is proposed that vacuolation precedes the mitochondrion-dependent caspase activation associated with nuclear apoptosis.[51] The inflammatory reaction is considered a defensive response of the tissues to the oxidative stress induced injury imposed by methomyl.[49] Additionally, the homogenous acidophilic material may be a result of the oxidative stress insult whereas ROS, cytokines and growth factors are generated at the site of injury by the inflammatory cells leading to exacerbated renal damage and poorly ordered matrix deposition.[52] ROS has been reported to cause renal toxicity.[53] The damage to tissue structure is hypothesized to be attributed to an increase in the MDA levels.[34]

Combination treatment with methomyl and folic acid reduced methomyl associated kidney damage. The structure of the renal corpuscles, proximal and distal convoluted tubules and collecting ducts (Figure 1G) were nearly similar to the control and folic acid treated groups. In addition, the levels of fibrosis, inflammatory cell infiltration and congestion were also less (Figure 1H).

Histological examination of H & E stained sections of the testes of the control group (Figure 2A) and folic acid-treated group (Figure 2B) revealed densely packed seminiferous tubules separated by narrow interstitial tissues, containing blood vessels and clusters of interstitial Leydig cells. The seminiferous tubules were lined with the stratified germinal epithelium with formed spermatogonia resting on the basement membrane, below the primary spermatocytes, spermatids and spermatozoa which filled the lumen. Secondary spermatocytes were not detected. Sertoli cells with a pale cytoplasm were observed between the spermatogenic cells.

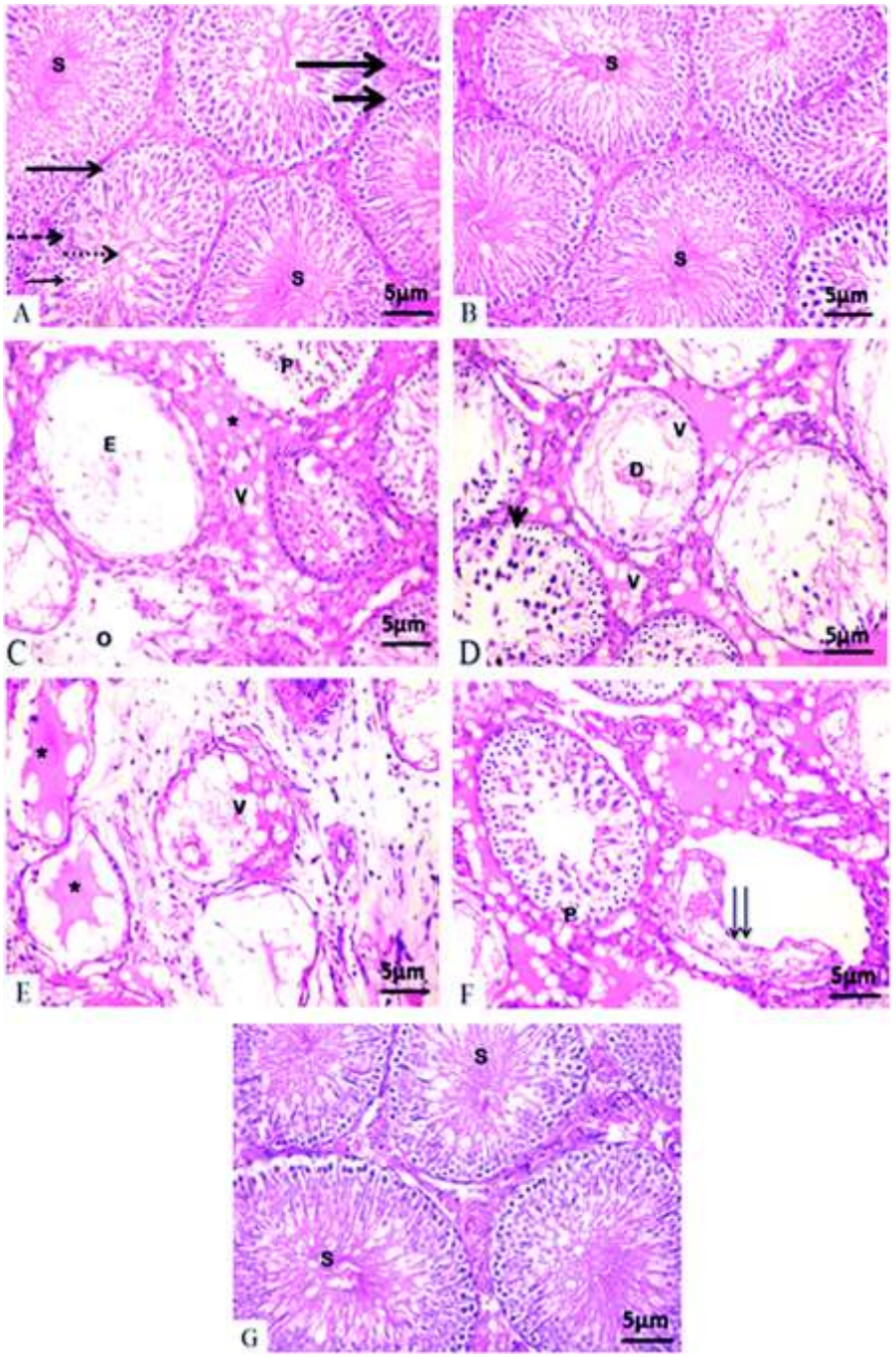

Figure 2. Photomicrographs of the testes of adult male albino rats. (A) Control, (B) folic acid, (C, D, E, F) methomyl and (G) methomyl and folic acid. S= seminiferous tubule; long thin arrow= spermatogonia; ===→ = spermatocytes; short thin arrow= spermatids; ·····→ = spermatozoa; thick long arrow= Leydig cell; thick short arrow= Sertoli cell; E= empty tubule; P= Pyknosis of nuclei; V= vaculation; *= homogenous acidophilic material; D= desquamation; arrow head= disorganization; Double arrow= separated germinal epithelium. H & E staining. (Total magnification = x200; Scale bars=5µm.)

The testes of rats treated with methomyl (Figures 2C, 2D, 2E) showed variable changes to the seminiferous tubular structure and these included; distortion of normal shape, disorganization with loss of germinal layers, decreased height of tubular epithelium, incomplete series of spermatogenic cells (spermatogonia, spermatocytes, and spermatids), total absence of spermatozoa in some tubules, empty spaces between spermatogenic cells, intra luminal desquamation of cells (Figure 2D), accumulation of homogenous acidophilic material (Figure 2E), degeneration with dark stained nuclei of pyknotic cells (Figures 2C, 2F) and the presence of diffuse intra cytoplasmic vacuoles (Figures 2D, 2E). Furthermore, separation of the germinal epithelium was evident in some seminiferous tubules (Figure 2F). The interstitium showed marked oedema (Figure 2C), homogenous acidophilic material accumulation and abundant vacuoles (Figure 2C).

Shalaby *et al.*[4] also found similar testicular degenerative changes in rats treated with methomyl. Additional degenerative changes to the seminiferous tubules of rats exposed to methomyl for 30 days has also been reported.[54] Methomyl treatment has been found to lead to an incomplete series of spermatogenic cells of the seminiferous tubules, degeneration and necrosis of spermatogenic cells with cellular desquamation in lumena.[35] Likewise other pesticides have been found to cause testicular degenerative changes and cellular damage.[44] The deleterious effects of methomyl on the testes could be attributed to the induction of oxidative damage due to increased lipid peroxidation and perturbations of antioxidant enzymes.[55] The presence of dark stained nuclei (pyknosis) in the germ cells indicates apoptosis secondary to the oxidative stress induced DNA damage. Desquamation of germinal epithelium could be explained by the direct toxic effect of methomyl on the cell-cell junctions between Sertoli and germ cells.[16] Contrary to these

findings, methomyl was not found to cause any tissue damage in the gonadal tissue of the bullfrog.[34]

Combination treatment with folic acid showed an overall alleviation of the histopathological findings, with nearly normal structure of seminiferous tubules and complete spermatogenic series (Figure 2G). This protective effect of folic acid on tissues is supported by Shalaby *et al.*[4]

3.4.2 Immunohistochemistry

Immunohistochemical analysis of the kidneys of the control (Figure 3A) and folic acid treated groups (Figure 3B) showed negative reactivity for caspase-3, which is in contrast to the strong immune reactivity noted in the cytoplasm of the tubular epithelium of the methomyl-treated group (Figure 3C). In the methomyl-folic acid treated group, weak immune reactivity for caspase-3 in the cytoplasm of the tubular epithelium was noted (Figure 3D).

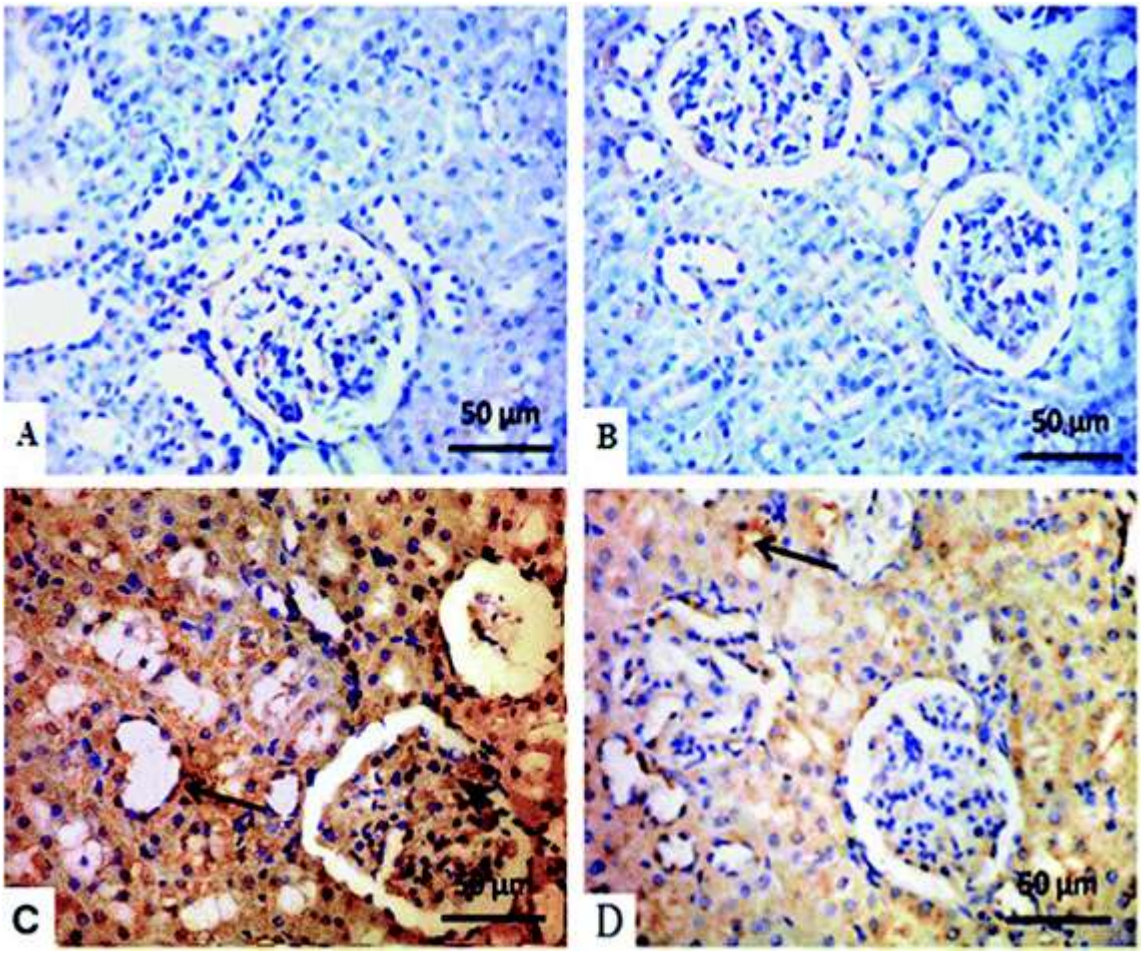

Figure 3. Photomicrographs of immunohistochemical staining of caspase-3 in the kidneys of adult male albino rats. (A) Control, (B) folic acid-treated, (C) methomyl-treated and (D) treatment of a combination of methomyl and folic acid. Arrow = brown stain indicating positive immunoreactivity for caspase-3 in cytoplasm of tubular epithelium; dashed arrow= brown stain indicating positive immunoreactivity for caspase-3 in cytoplasm of glomerular endothelium. (Total magnification = x400; Scale bars=50µm.)

A similar result was found for the testes of the control (Figure 4A) and folic acid-treated group (Figure 4B), where negative immunoreactivity for caspase-3 was noted, whereas for the methomyl-treated group strong reactivity for caspase-3 in the nuclei of germinal epithelium and interstitial cells of the seminiferous tubules was observed (Figure 4C). The testes of animals treated with methomyl and folic acid showed weak caspase-3

reactivity in the nuclei of the germinal epithelium and interstitial cells of the seminiferous tubules (Figure 4D).

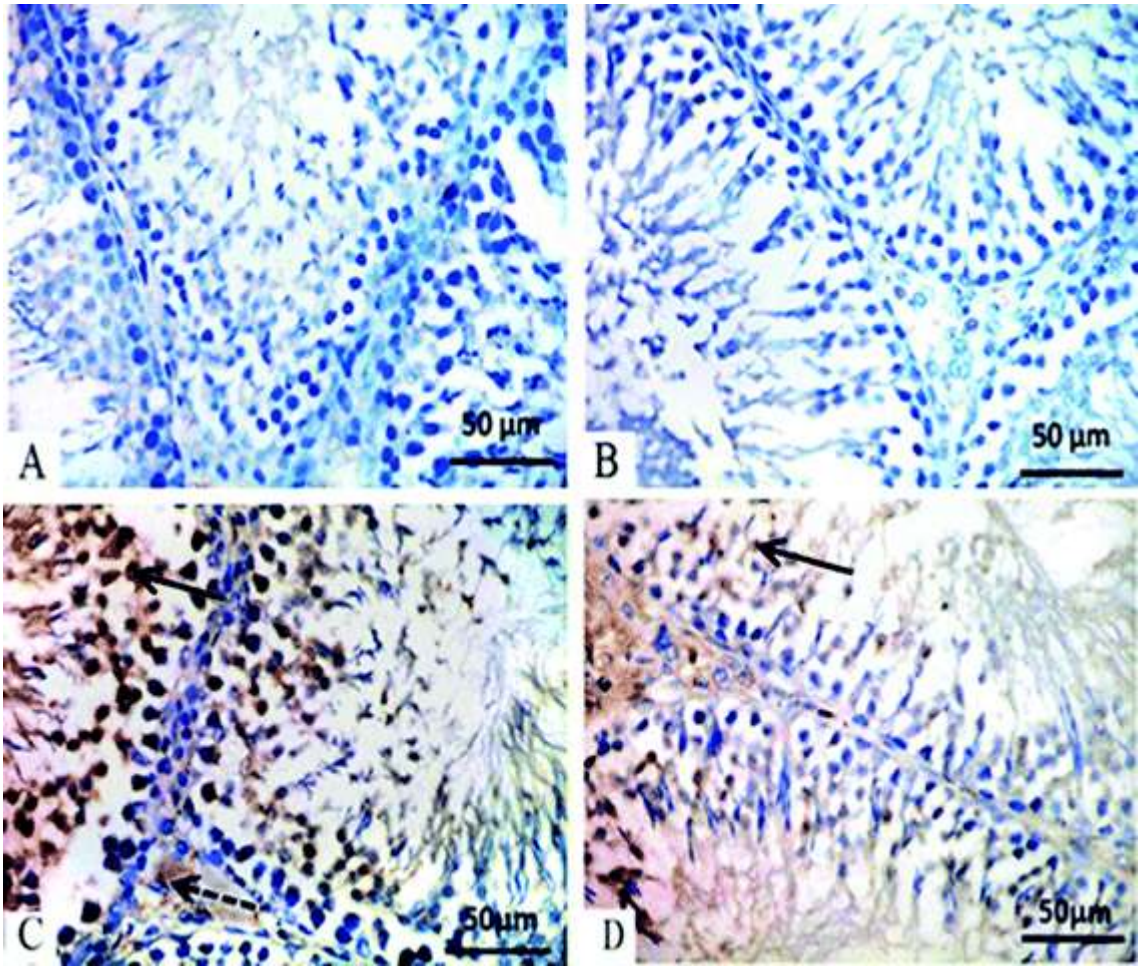

Figure 4. Photomicrographs of immunohistochemical staining of caspase-3 in the testes of adult male albino rats. (A) Control, (B) folic acid-treated, (C) methomyl-treated and (D) treatment with a combination of methomyl and folic acid. Arrow = brown stain indicating positive immunoreactivity for caspase-3 in the nuclei of germinal epithelium; dashed arrow= brown stain indicating positive immunoreactivity for caspase-3 in cytoplasm of interstitial cells. (Total magnification = x400; Scale bars=50µm.)

ROS has been reported to induce mitochondria-dependent apoptosis in different cell types.[56] Various apoptotic inhibitors have been found to exert antioxidant actions or improve antioxidant defenses,[57] as was the case for folate in the present study.

A significant ($p < 0.001$) decrease in the height of the testicular epithelium was noted when the animals were treated with methomyl, compared to the control group (Table 2). Combination treatment with methomyl and folic acid resulted in a significant ($p < 0.001$) increase in the thickness of the epithelium in comparison to the animals treated with methomyl only (Table 2). No studies could be found to expand on the current findings.

This study has shown that methomyl adversely affects several different organs such as the kidney and testes as well as spermatogenesis. Folate effectively prevented methomyl induced organ damage, maintained cellular organization of the testes required for spermatogenesis and improved semen parameters. It will be important in future studies to determine whether folate would prevent methomyl induced DNA damage, maintain genomic integrity as well as prevent infertility. Folate supplementation, of workers exposed to methomyl, may be an effective strategy to reduce the renal and the male reproductive toxicity of methomyl. However, this needs to be assessed.

4. Conclusions

Chronic exposure to methomyl exerts renal and reproductive toxicity. The significant reduction of renal and testicular MDA levels and increased serum TAC levels in the methomyl-folic acid treated group indicated strong antioxidant effects of folic acid either due to direct scavenging or the normalization of antioxidant pathways. By preventing oxidative damage, folic acid exerted partial protection against methomyl-induced nephrotoxicity as was proven by reduced creatinine levels and nearly normal kidney histology. Folic acid antagonized methomyl-induced reproductive toxicity as indicated by elevated testosterone levels, improved seminal parameters and nearly normal testes histology. Additionally, folic acid alleviated the oxidative stress mediated apoptosis in

both kidneys and testes as was indicated by weak immunoreactivity for caspase-3 in rats treated with a combination of methomyl and folic acid. This study identifies folate as a vitamin, with strong antioxidant effects which can counteract the toxicity of methomyl. Future studies should focus on the fertility outcome/pregnancy index in rats.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

The work was supported by the departments involved in this study.

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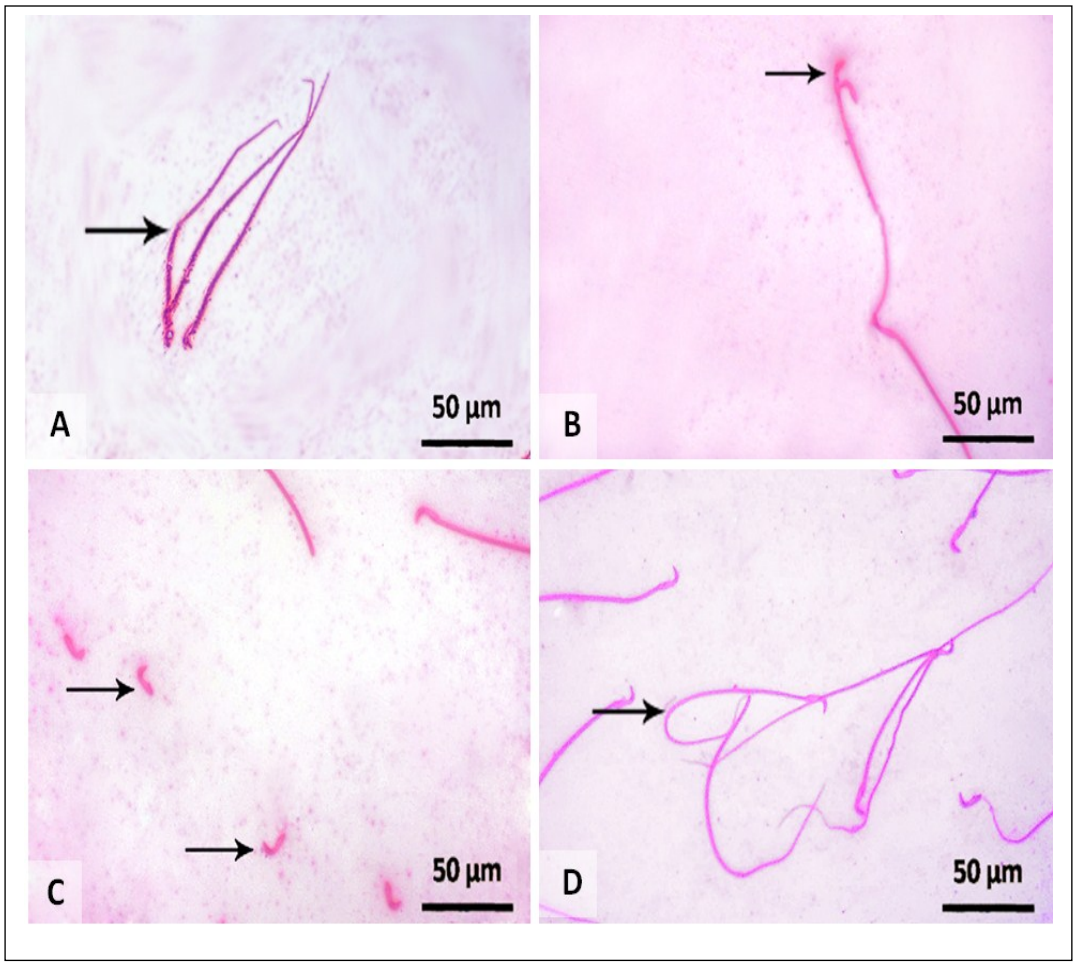
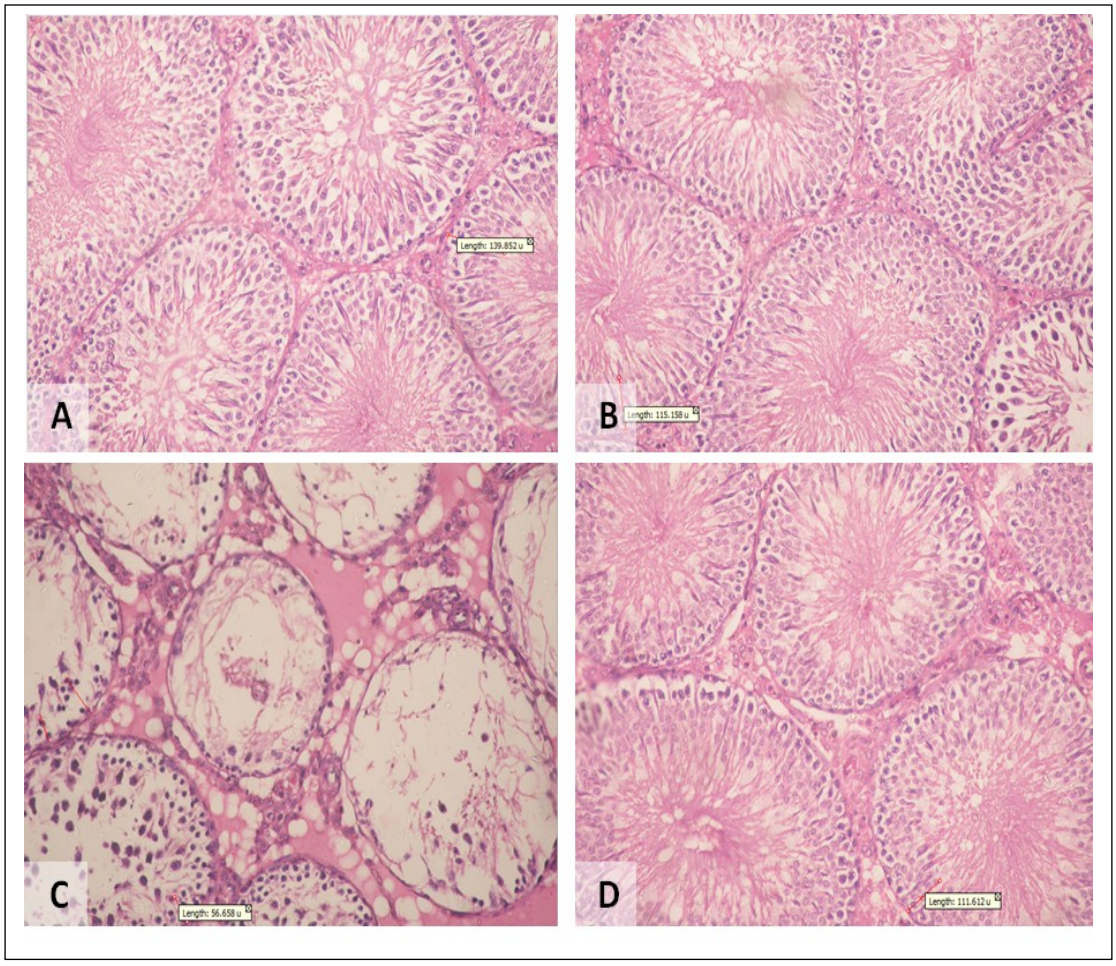
Supplementary Material



Supplementary Figure 1. Photomicrographs of sperms of adult male albino rats. (A) Normal sperm, (B) double headed sperm, (C) multiple detached heads and (D) coiled sperm tail. Nigrosin & Eosin. (Total magnification = x400; Scale bars=50µm.)



Supplementary Figure 2. Print screen of Digimizer to measure the height of germinal epithelium. (A) Control, (B) folic acid, (C) methomyl and (D) methomyl and folic acid treated.