Genotoxic potential of *Fasciola gigantica* infection in experimentally infected rabbits

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Abstract A number of parasitic platyhelminthes are known to cause genotoxicity in humans and animals. However no such information is available on tropical liver fluke, *Fasciola gigantica*, which incurs huge economic losses worldwide. In the present study the genotoxic potential of *F. gigantica* infection in rabbits, experimentally infected with the metacercarial cysts of this parasite, has been investigated using the standard comet assay and micronucleus (MNi) test on the isolated hepatocytes and the whole blood from the infected rabbits. The tail length of the comet in both hepatocytes and reticulocytes from the infected animals was significantly prominent ($p < 0.05$) as compared to the controls. About 61.17 % of the hepatocytes from the infected rabbits were positive for MNi formation. A number of blood cells also showed cellular deformities, which were recognised as spicule type, schistocytes, tear drop type, acanthocytes and dumbbell type. It is possible that during the establishment of host-parasite relationship the worms might have released some products which could have contributed to the induction of cellular and DNA damage. However, long term studies are required to understand the serious implications of such an effect caused by *F. gigantica*, though hepatic carcinoma has not been reported so far due to fasciolosis, however, considering the present results the possibility may not be rule out for the disease progression in this direction.

Keywords *Fasciola gigantica* · Metacercariae · Genotoxicity · Micronucleus · Comet assay · Excretory/secretory (E/S) products

Introduction

Fasciolosis caused by the liver fluke species in livestock incurs huge revenue losses, approximately reaching to US \$3.2 billion worldwide (Spithill 1999). In Asia, distribution of the causative agents, *Fasciola gigantica* and *F. hepatica* overlaps and hence they are often referred to simply as *Fasciola spp.* The incidence of fasciolosis in Indian livestock due to *F. gigantica* has been estimated to be around 31.14–43.28 % (Yadav et al. 2009; Garg et al. 2009; Khan et al. 2014). But this disease has remained neglected with regard to human infection where it has been steadily increasing since 1970s, and this increasing trend started attracting the global medical concern due to the zoonotic potential of the parasite (Mas-Coma et al. 2009). Beside infecting the animals around 27.7 million people were considered at risk of contracting the fasciolosis (Mas-Coma et al. 1999). Some rural areas in Egypt were found endemic with the prevalence of 7–17 % infection of this trematode (Mas-Coma et al. 1999). Fasciolosis affects more than 51 countries worldwide (Marcos et al. 2008) and about 17 million people are affected by it while millions of people are at risk of contracting the infection (Tolan 2011).

The parasitic flatworms are also known to cause genotoxicity in humans and animals (Salazar et al. 2013). The biological agents including parasites contributed to 16.1 % of total cancer cases diagnosed and 2 million new cancer cases were attributed to infections. The percentage appeared to be higher in less developed countries as compared to the developed nations (de Martel et al. 2012).

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Although the parasitic helminths were relatively attributed to a small portion of infection but they are considered as an important group since the number of people exposed to infection with this group is very high (WHO 2002; Sripa et al. 2007).

Chronic infection by other trematode parasites such as *Schistosoma haematobium*, *Opisthorchis viverrini*, *Clonorchis sinensis* promote malignancies but evidences for carcinogenesis due to *Schistosoma japonicum* and *Opisthorchis felinus* was considered insufficient although increased incidence and related risk of cholangiocarcinoma has been considered by International Agency for Research on Cancer (Parkin 2006). However, no such information is available on tropical liver fluke, *F. gigantica*. Helminthic infection causes cancer due to the exposure of the host for many years resulting in chronic inflammation of infected tissues. Some of the excretory secretory metabolic products of these parasites are highly immunogenic (Sripa et al. 2007) and can lead to genetic instability and malignant transformation (Salazar et al. 2013). Excretory secretory (E/S) products released by the parasite during its establishment within the host could be developmentally regulated and correlate with the migration of parasite through the host tissues. The E/S products may also include proteases which not only contribute to the virulence potential but can also act as tissue damaging factor (Robinson et al. 2009). It has been pointed out that parasites can damage host's DNA either by directly releasing its products during the infection or by inducing inflammatory responses that can cause DNA damage (Salazar et al. 2013) that is mediated by the production of free radicals such as reactive oxygen and nitrogen species, which may damage the host's DNA, leading to genetic and epigenetic processes that regulate cell proliferation, thereby influencing carcinogenesis (Herrera and Ostrosky-Wegman 2001). However, the information on the genotoxic effect of fasciolosis, if any, due to *F. gigantica* is almost non-existent. Therefore, in the present study rabbits were experimentally infected with *F. gigantica* metacercariae and a possible genotoxic effect due to host parasite interactions during the acute phase of infection has been investigated.

Materials and methods

Materials

EDTA, ethidium bromide (EtBr), Triton-X 100, low melting point agarose (LMPA) and Tris-HCl were purchased from Sigma-Aldrich (MO, USA). Normal melting agarose (NMA) was purchased from Bio-Rad laboratories (CA, USA). RPMI-1640 and fetal bovine serum (FBS) were bought from HIMEDIA, India. Common salts namely

NaH₂PO₄, Na₂HPO₄, NaOH, NaCl were purchased from Qualigens Fine Chemical Company, India.

Establishment of infection

Male New Zealand white rabbits, about 6 weeks old, were purchased from the local vendor and maintained in the departmental animal house as per the guidelines of the institutional ethical committee of Department of Biochemistry, Aligarh Muslim University. The animals were acclimatized for 4 weeks before the experiments were started and allowed to feed on commercially available sterilized diet and drink water ad libitum. Thereafter, three rabbits were infected with 50 *F. gigantica* metacercariae through oral gavage while one rabbit was kept uninfected and used as a control. All the rabbits were observed daily for 28 days before their sacrifice on the 29th day post-infection to collect livers and blood from each of the animal and processed them separately for various studies.

Genotoxicity assays

Micronucleus test

Liver: Micronucleus (MNi) test was carried out according to the method of Schmid (1975). Briefly, 200 mg liver tissue was homogenized in 5.0 ml of 100 mM phosphate buffered saline (PBS) pH 7.4 and then centrifuged at 3000×g for 10 min at 4 °C in a refrigerated centrifuge (Sigma SVI, Germany). The supernatant was discarded and fresh 0.4 ml PBS, pH 7.4, was added to re-suspend hepatocytes and a small drop of suspension was poured onto a pre-cleaned glass slides to prepare a thin smear, which was air dried and fixed in methanol for 10 min and stained in undiluted May-Grunwald solution for 90 s followed by washing with distilled water and then slides were stained with Giemsa (1:10 diluted) for 15 min, rinsed twice with distilled water, dehydrated in ascending grades of ethanol, cleared in xylene and mounted in DPX (Bharrhan et al. 2011). A total of 1000 cells were counted per sample to get the frequency of micronuclei on Motic BA210 Trinocular Microscope (Hong Kong) and photographs were taken with a 3.0 megapixel Nikon digital camera.

Blood: Micronucleus (MNi) test for reticulocytes was done according to Schmid (1975) with some minor modifications. A drop of blood was taken on a pre-cleaned air dried glass slide and a uniform smear was prepared. Slides were first air dried and then fixed in methanol for 10 min followed by staining as described earlier. The MNi and cellular deformities observed in the cells were photographed as mentioned above.

Comet assay

Liver: The comet assay was performed accordingly Singh et al. (1988) protocol with few modifications (Singh et al. 1988; Oshida et al. 2008). The liver tissue was submerged in cold buffer (NaCl; 75 mM, EDTA-2Na; 24 mM, pH 7.5) and then cut into 2–3 mm thick pieces in separate petri dishes. The cell suspension was collected and centrifuged at 4 °C at 700×*g* for 10 min. The supernatant was removed and the cells were re-suspended in cold buffer (Oshida et al. 2008). The frosted slides were coated with 1 % (w/v) agarose prepared in phosphate buffered saline (PBS), pH 7.4. The suspension containing about 10,000 cells was mixed with 100 µl of 1 % low melting point agarose (LMPA) and spread over the slide and cover slip was placed immediately over it and allowed to solidify. Then coverslip was removed and another thin layer of 0.5 % LMPA (100 µl) was laid. The slides were removed and immersed in cold lysis solution, pH 10 (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1 % Triton X-100) for 1 h at 4 °C. Thereafter slides were dipped in electrophoretic running buffer (300 mM NaOH, 1 mM EDTA), pH 13, for 20 min in the dark. Electrophoresis was performed at 4 °C for about 30 min at 300 mA current. The slides were washed thrice with cold saline and placed in neutralising buffer, pH 7.5 (0.4 M Tris base). Washing was repeated thrice and then staining was done with aqueous ethidium bromide (20 µg/ml) for 15 min followed by washing with chilled saline and then slides were kept at 4 °C in a humidified chamber before analysis. Scoring of slides was carried out on CX41 fluorescence microscope (Olympus, Japan), equipped with 510–560 nm excitation and 590 nm barrier filters and attached with integrated CC camera, COHU 4910. Images were analysed with Komet 5.5 software, (Kinetic Imaging, Liverpool, UK). The size of the comet and its tail length was measured at a magnification of 1000× using Komet 5.5 image analyzing system to assess the nuclear DNA damage. The comets were scored and images of 50 cells (25 cells from each replicate slide) for each sample were taken.

Blood: Comet assay was performed under alkaline condition as described earlier by Singh et al. (1988) with some modification (Singh et al. 1988; Chuang and Hu 2004). Whole blood was used for the comet assay. A total volume of 20 µl of whole blood was mixed with 150 µl of 0.5 % low melting point agarose (LMPA) and out of which 100 µl was spread on the fully frosted slides which were pre-coated with 100 µl of 0.6 % normal melting agarose (NMA) prepared in phosphate buffered saline, pH 7.4. Afterwards another agarose gel layer of 100 µl of 0.6 % NMA was laid. Following polymerization slides were immersed in cold lysis solution, pH 10.0 (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1 % Triton X-100) at

4 °C for 1 h. Then slides were placed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA), pH 13, for 30 min. The remaining procedure was same as described above. The results were analysed as described earlier.

Cellular deformity

A small drop of blood was poured onto a pre-cleaned air dried glass slide. A thin film was prepared and air dried before fixation in methanol for 10 min and staining in Giemsa (1:10 diluted) for 15 min. Then the slides were rinsed twice with distilled water, again air dried, mounted in DPX and observed at 400× magnification on Motic BA210 Trinocular Microscope (Hong Kong). The photographs were taken with a 3.0 megapixel Nikon digital camera.

Statistical analysis

All the data were analyzed for mean values and standard error (mean ± SE). Statistical comparisons were made using Students *t* test on software GraphPad Prism version 5.01, and *p* < 0.05 were considered significant.

Results

The tropical liver fluke, *F. gigantica*, infection in vertebrate laboratory host, i.e. rabbits, produced genotoxicity as revealed by the MNi test and the comet assay, showing DNA damage predominantly in hepatocytes and reticulocytes. The results are summarised in Figs. 1, 2, 3, 4 and Table 1.

It was observed that the rabbits infected with *F. gigantica* exhibited about 61.17 % micronuclei formation in hepatocytes during the acute phase of infection (Figs. 1, 2). The comet assay in which single cell electrophoresis of liver cells was performed which also confirmed the DNA damage, an indicator of genomic instability. The mean tail length of the comet was 34.8 ± 0.08 µm in the liver of the infected rabbits and it was significantly (*p* < 0.05) longer as compared to the control (4.6 ± 0.12 µm) (Fig. 3; Table 1). A similar trend was observed for comet assay (*p* < 0.05) of whole blood when compared to the control (Table 1). The cellular deformities were also evident in the form of schistocytes, acanthocytes, spicule and tear drop like cells etc. and the MNi were also observed in the blood cells (Fig. 4) of the infected rabbits.

Discussion

The micronucleus test and comet assay are the meaningful tools to investigate the qualitative and quantitative DNA damage using single cell analysis and also they can be used

Fig. 1 Hepatocytes showing normal cell type in control rabbit (a) and micronucleus (MNi) formation in *F. gigantica* infected rabbit (b)

Fig. 2 Micronucleated analysis in liver cells of metacercariae infected rabbits with compare to control rabbit (uninfected). Values are expressed as percentage of micronucleated cells. * $p < 0.05$ versus control

to detect in vivo DNA damage (Schmid 1975; Singh et al. 1988). The molecular epidemiological studies in human population have shown that the increased MNi frequency in peripheral lymphocytes can be used to predict cancer risk (Salazar et al. 2013). It has been estimated that the viruses, bacteria and parasites contribute 16.1 % of the global burden of cancer. If these infections were prevented or treated in developing countries, there would be about 26.3 % decline in cancer cases (de Martel et al. 2012). Chronic infections and inflammations are also responsible for about 25 % of cancers cases worldwide (Hussain and Harris 2007; Vennervald and Polman 2009). DNA damage due to helminthic infections may certainly involve inflammatory responses which may act as DNA damaging factor and may also act as initiation factor for genotoxicity, leading to malignancy (Balkwill and Mantovani 2001; Hussain and Harris 2007). Evidences show that the chronic inflammation caused by parasite or parasite released products deposited in the host tissues could be a key factor in helminth induced carcinogenesis (Vennervald and

Polman 2009) either by direct DNA damage or by inducing inflammatory responses (Salazar et al. 2013). However, no such information is available on the tropical liver fluke, *F. gigantica*, an important veterinary parasite which is responsible for huge economic losses to livestock owners. The parasite causes enormous damage to liver parenchyma during its migration before reaching to their final destination, the bile duct, where they lodge and mature to produce eggs and also cause bile duct hypertrophy and hyperplasia (Slutzky 1981).

The enlargement of lymph nodes and macrophages infiltration in intestine occurs during the infection of a closely related species, *F. hepatica* (Spithill 1999). Human fasciolosis has always been considered as secondary or neglected disease which has steadily increased since 1970s and attracted the global medical concern due to its zoonotic potential (Mas-Coma et al. 2009). According to International Agency for Research on Cancer (IARC) many parasitic infections can lead to human cancer (Parkin 2006). Studies on the excretory secretory (E/S) products of *C. sinensis* revealed that they can increase the level of free radicals and the expression of proinflammatory cytokines (Nam et al. 2012). In hamsters, inflammation induced nitric oxide production in gallbladder epithelial cells can also produce genotoxicity (Kitasato et al. 2007). Therefore, inflammation can produce free radicals, which are genotoxic and may lead to genetic instability.

A considerable increase in MNi frequency (61.17 %) was found in hepatic cells of rabbits infected with *F. gigantica* as compared to controls and the same pattern was also observed in blood. An increased frequency of MNi formation probably reflects the accumulation of damaged genetic material throughout the infection (Salazar et al. 2013). In blood cells beside MNi formation the cell membrane deformities were also observed. The occurrence of spicule like cells as well as schistocytes, acanthocytes and tear drop like cells further reflect cellular instability

Fig. 3 A representation image of the comet assay of normal hepatocytes (a) and the hepatocytes from infected rabbits (b)

Fig. 4 Blood smear slide showing distribution of normal blood cells in control rabbit and the different types of cellular deformities and micronucleus in *Fasciola gigantica* infected rabbit

Table 1 Showing the mean comet tail length in liver and whole blood of metacercariae infected (experimental) and uninfected (control) rabbits

Rabbits	Mean comet tail length in liver (μm)	Mean comet tail length in whole blood (μm)
Control (normal)	4.6 ± 0.12	5.3 ± 0.10
Infected with <i>F. gigantica</i> metacercariae	34.8 ± 0.08	25.27 ± 0.10

All the values have been expressed in mean $\pm$ SE

$p < 0.05$ is considered significant as compared to control

possibly due to the E/S products released by *F. gigantica*. After penetrating the intestinal mucosa, the parasites reach liver where they migrate towards their final destination, the bile ducts, leaving behind fibrosed tracts which appear like tunnels. The exposure of liver tissue to parasitic E/S products is supposed to be highest during this stage. The E/S products of the migrating worms could induce the

production of free radicals (Herrera and Ostrosky-Wegman 2001) and may be the possible reason for the high level of MNi frequency in hepatic cells. The results of comet assay, which reflect qualitative DNA damage, were significant ($p < 0.05$) in both whole blood samples as well as the hepatic cells of infected rabbits. So far any molecule from *F. gigantica* has not been identified as human carcinogen

but the possibilities of existence of such a component cannot be ruled out considering the present results, however, further studies are necessary to rule out this assumption. It has been established for *T. Solium* cysticerci that they secrete a factor which is responsible for the formation of micronucleus in cultured human lymphocytes (Herrera et al. 2003). Many helminth parasites have been recognised as the source of human carcinogens by IARC (Parkin 2006), though their contributing percentage to worldwide cancer is very low but the emerging trend of zoonosis with helminth parasite makes it an important aspect to be under taken for further genotoxic studies.

Taken together it is concluded that *F. gigantica* infection contribute to the genotoxicity of the experimental host in the present study. Therefore, the possibility for the disease progression towards malignant transformation in future may not be ruled out.

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References

- Balkwill F, Mantovani A (2001) Inflammation and cancer: back to Virchow? *Lancet* 357:539–545
- Bharrhan S, Koul A, Chopra K, Rishi P (2011) Catechin suppresses an array of signalling molecules and modulates alcohol-induced endotoxin mediated liver injury in a rat model. *PLoS One* 6:e20635. doi:10.1371/journal.pone.0020635
- Chuang CH, Hu ML (2004) Use of whole blood directly for single-cell gel electrophoresis (comet) assay in vivo and white blood cells for in vitro assay. *Mutat Res Genet Toxicol Environ Mutagen* 564:75–82. doi:10.1016/j.mrgentox.2004.07.013
- de Martel C, Ferlay J, Franceschi S et al (2012) Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol* 13:607–615. doi:10.1016/S1470-2045(12)70137-7
- Garg R, Yadav CL, Kumar RR et al (2009) The epidemiology of fasciolosis in ruminants in different geo-climatic regions of north India. *Trop Anim Health Prod* 41:1695–1700. doi:10.1007/s11250-009-9367-y
- Herrera LA, Ostrosky-Wegman P (2001) Do helminths play a role in carcinogenesis? *Trends Parasitol* 17:172–175
- Herrera LA, Tato P, Molinari JL et al (2003) Induction of DNA damage in human lymphocytes treated with a soluble factor secreted by *Taenia solium* metacystodes. *Teratog Carcinog Mutagen* 23(S1):79–83. doi:10.1002/tcm.10063
- Hussain SP, Harris CC (2007) Inflammation and cancer: an ancient link with novel potentials. *Int J Cancer* 121:2373–2380
- Khan YA, Khan MAH, Abidi SMA (2014) 2D-PAGE analysis of the soluble proteins of the tropical liver fluke, *Fasciola gigantica* and biliary amphistome, *Gigantocotyle explanatum*, concurrently infecting *Bubalus bubalis*. *J Parasit Dis*. doi:10.1007/s12639-014-0603-7
- Kitasato A, Tajima Y, Kuroki T et al (2007) Inflammatory cytokines promote inducible nitric oxide synthase-mediated DNA damage in hamster gallbladder epithelial cells. *World J Gastroenterol* 13:6379–6384. doi:10.3748/wjg.13.6379
- Marcos LA, Tagle M, Terashima A et al (2008) Natural history, clinicoradiologic correlates, and response to triclabendazole in acute massive fascioliasis. *Am J Trop Med Hyg* 78:222–227
- Mas-Coma MS, Esteban JG, Bargues MD (1999) Epidemiology of human fascioliasis: a review and proposed new classification. *Bull World Health Organ* 77:340–346. doi:10.1371/journal.pone.0024567
- Mas-Coma S, Valero MA, Bargues MD (2009) Chapter 2 *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv Parasitol* 69:41–146. doi:10.1016/S0065-308X(09)69002-3
- Nam JH, Moon JH, Kim IK et al (2012) Free radicals enzymatically triggered by *Clonorchis sinensis* excretory-secretory products cause NF- κ B-mediated inflammation in human cholangiocarcinoma cells. *Int J Parasitol* 42:103–113. doi:10.1016/j.ijpara.2011.11.001
- Oshida K, Iwanaga E, Miyamoto-Kuramitsu K, Miyamoto Y (2008) An in vivo comet assay of multiple organs (liver, kidney and bone marrow) in mice treated with methyl methanesulfonate and acetaminophen accompanied by hematology and/or blood chemistry. *J Toxicol Sci* 33:515–524. doi:10.2131/jts.33.515
- Parkin DM (2006) The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 118:3030–3044. doi:10.1002/ijc.21731
- Robinson MW, Menon R, Donnelly SM et al (2009) An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. *Mol Cell Proteomics* 8:1891–1907. doi:10.1074/mcp.M900045-MCP200
- Salazar AM, Mendlovic F, Cruz-Rivera M et al (2013) Genotoxicity induced by *Taenia solium* and its reduction by immunization with calreticulin in a hamster model of taeniosis. *Environ Mol Mutagen* 54:347–353
- Schmid W (1975) The micronucleus test. *Mutat Res* 31:9–15. doi:10.1016/0165-1161(75)90058-8
- Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191. doi:10.1016/0014-4827(88)90265-0
- Slutzky GM (1981) *The Biochemistry of Parasites*. Pergamon Press, Oxford
- Spithill TW (1999) *Fasciola gigantica*: epidemiology, control, immunology and molecular biology. In: Dalton JP (ed) *Fasciolosis*. CAB International Publishing, Wallingford, pp 465–525
- Sripa B, Kaewkes S, Sithithaworn P et al (2007) Liver fluke induces cholangiocarcinoma. *PLoS Med* 4:1148–1155
- Tolan RW (2011) Fascioliasis due to *Fasciola hepatica* and *Fasciola gigantica* infection: an update on this “neglected” neglected tropical disease. *Lab Med* 42:107–116. doi:10.1309/LMLFBB8PW4SA0YJI
- Vennervald BJ, Polman K (2009) Helminths and malignancy. *Parasite Immunol* 31:686–696
- WHO (2002) Evaluation of certain mycotoxins in food. Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization Technical Report Series 906
- Yadav CL, Garg R, Banerjee PS et al (2009) Epizootiology of *Fasciola gigantica* infection in cattle and buffaloes in Western Uttar Pradesh, India. *J Vet Parasitol* 23:135–138