**RESEARCH ARTICLE**

**BLASTOCYSTIS SP. ENHANCES OXIDATIVE STRESS-INDUCED CARCINOGENESIS IN COLORECTAL CANCER: IN VIVO EXPERIMENTAL STUDY.**

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**Abstract**

**Background:** Blastocystis sp. is a common intestinal parasites that known to have a crucial role in the pathogenesis of cancer via promoting the oxidative stress pathway. Colorectal cancer (CRC) is the second leading cause of cancer deaths worldwide. Objectives: we aimed to prove the carcinogenic effect of Blastocystis sp on oxidative stress status in vivo mice model. Methods: three groups of mice were infected with Blastocystis spp. isolated from CRC patients, symptomatic and asymptomatic in fected patients. Nitric oxide (NO) and Glutathione peroxidase (GSH) levels were measured in plasma two weeks post-infection using biochemical assays.

**Results:** High levels of NO and GSH were observed in infected mice that were infected with Blastocystis sp. Isolated from CRC patients in comparison to those that infected with Blastocystis sp. Isolated from patients with intestinal Blastocystis.

**Conclusion:** our study supports the evidence that Blastocystis sp. enhances carcinogenesis by promoting oxidative stress damage in Blastocystis sp. infected mice.

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**Introduction:**

Blastocystis sp. is one of the most widespread parasites found in the gastrointestinal tract of humans and animals [1]. The pathogenic potential of Blastocystis sp. was widely debated in the literature during the last two decades because the parasite has been found in both symptomatic and asymptomatic patients [2]. Various reports have shown that Blastocystis sp. may be associated with some intestinal disorders such as IBS [3] and acquired immune deficiency syndrome (AIDS) [3]. The opportunistic nature of Blastocystis sp. was revealed when the parasites were confirmed in CRC patients during chemotherapy treatment [3].

Evidence has been detected that patients with both CRC and parasitic infections secrete significant amounts of serum and plasma metabolites derived from oxidative stress damage [3]. Oxidative stress, that plays an important role in the molecular mechanisms of CRC development, has been shown to occur in Blastocystis infection [4]. In vitro experimental studies had demonstrated that solubilized antigen from Blastocystis has the ability to promote human colorectal cells proliferation [5]. In vivo experimental study in rats showed that Blastocystis have a significant role in enhancing carcinogenesis by inducing intestinal epithelium damage and promoting oxidative damage [5 and 6].

The study of oxidative stress and antioxidants among humans with parasitic infections may help to clarify the effect of asymptomatic intestinal parasitic infections. Oxidative stress can be of benefit to host inflammatory cells,

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stimulating the production of reactive oxygen species (ROS) to suppress invading parasites [6-9]. However, they can also damage the body, leading to diseases like cancer, diabetes and ischemic heart disease [10-13].

Oxidative enzymes are elevated in individuals with intestinal parasitic infections, even in those with less pathogenic protozoa such as Blastocystis [14, 15 and 16] Antioxidants, which play a role in suppressing oxidation, have been studied in tissue and blood parasites [17 and 18]. The production of NO in intestinal epithelial cells occurs by inducible nitric oxide synthase (iNOS) that catalyzes the conversion of L-arginine to reactive oxygen species of nitrogen. Consumption of L-Arginine by microbial arginase has been considered a survival mechanism of pathogens against host macrophage NO response [18-20].

The present study aimed to assess oxidative / nitrosative stress biomarkers level in blood of experimental mice induced by Blastocystis sp. isolated from CRC patients in comparison to Blastocystis sp. isolated from symptomatic and asymptomatic patients without CRC.

**Results:-**
In order to investigate the carcinogenic effect of blastocytosis.sp on oxidative stress status, mice were infected with blastocytosis.sp that was isolated from CRC patients (CRC isolates), moreover, the comparative group was inoculated with blastocytosis.sp that was isolated from asymptomatic and symptomatic intestinal blastocytosis (IB isolates) infected patients. Our results revealed that plasma NO and GSH levels were higher in mice infected with CRC isolates compared to the mice infected with IB isolates. The activity of NO and GSH isolated from mice different Blastocystis isolates is presented in Table 1. The value of NO and GSH activities of the control group were 12 µmol/l and 39 mg/dl; respectively. The mean levels of NO among CRC isolates was 48±9.0 µmol/l and 98±9.0 mg/dl compared to 30±15 µmol/l and 71±23mg/dl, in the intestinal Blastocytosis isolates, a statistical significant differences were obtained between both groups for each both oxidative biomarker ($\chi^2 : 12$, $p<0.001$, $\chi^2 : 11$, $p<0.01$); respectively, Fig 1A and 1B. In the IB isolates (symptomatic isolates), the NO activity was 24±4 µmol/l while in asymptomatic isolates it was 19 ± 3µmol/l, no significant difference was achieved between asymptomatic and symptomatic isolates ($\chi^2 : 2.5$, $p>0.05$), Fig 1C. For GSH activity, symptomatic IB group showed higher values than asymptomatic group and a statistical significance was reached (($\chi^2 : 6.8$, $p=0.009$), Fig 1F. The mean level of NO and GSH activities were very low in control (un-infected) group, the difference was statistical significant (NO: $\chi^2 : 10.5$, $p=0.001$ and GSH: $\chi^2 : 11$, $p=0.01$), Fig 1A and 1D.

**Table 1:**-Plasma levels of NO and GSH levels in studied groups

| Groups                     | NO (µmol/l) | Statistics | GSH (mg/dl) | Statistics |
|----------------------------|-------------|------------|-------------|------------|
| Blastocystis sp. infected groups | 12±2 (10 – 15) | $\chi^2 : 10.2$ | 39±4 (33 – 42) | $\chi^2 : 11.0$ |
| Non-infected               | 30±15 (15–59) | P=0.001    | 71±23 (42–110) | P=0.001    |
| Blastocystis sp. isolates | 48±9 (37 – 59) | $\chi^2 : 9.4$ | 98±9 (89 – 110) | $\chi^2 : 8.6$ |
| CRC patients               | 21±4 (15–30) | P=0.002    | 57±12 (43–76) | P=0.004    |
| Intestinal Blastocystis patients | 19±3 (15 – 23) | $\chi^2 : 2.5$ | 47±3 (43 – 50) | $\chi^2 : 6.8$ |
| Asymptomatic               | 24±4 (20–30) | P=0.12     | 67±7 (59–6) | P=0.009    |

$\chi^2$: Chi-Square value, CRC: Colorectal cancer
Figure 1: Boxplot graphs illustrating the plasma level of Nitric oxide and GSH in mice [(A and D: infected with Blastocystis sp. versus negative uninfected group), (B and E: mice transfected with Blastocystis sp. from CRC patients versus intestinal blastocystosis) and (C and F: mice infected with Blastocystis sp. from asymptomatic intestinal Blastocystosis infected patients versus symptomatic patients)]; respectively. Higher levels of NO and GSH in Blastocystis sp infected mice isolated from CRC patients (p<0.01). No significant difference was detected for NO level between asymptomatic and symptomatic intestinal Blastocystosis (p>0.05), although, a significant difference was reached for the GSH level (p=0.009).

Discussion:-
The incidence of parasitic infections in CRC patients is high. Parasitic infection and CRC may contribute to oxidative stress independently, but when present together, the oxidative stress burden may be attenuated [21 and 23]. The present study aimed to assess oxidative / nitrosative stress biomarkers level in blood of experimental mice induced by Blastocystis sp. isolated from CRC patients in comparison to that induced by Blastocystis sp. isolated from symptomatic and asymptomatic patients without CRC [24 and 25].

The biological injury caused by free-radical-mediated mechanisms can be prohibited by particular chemical scavengers which entrap the specific radicals, as well as by protective antioxidant enzymes including SOD, GPx and catalase, which remove hydrogen peroxide or superoxide radicals. GPx is a part of the resistance mechanisms...
against free radicals [26 and 27]. GPx protects the cell from damage from many oxidizing species, organic hydroperoxides, and hydrogen peroxide [28-31].

Our study showed maximum increase of GPx activity of up to 2,310.1 U/ml in GI (CRC isolates). A significant increase was noticed in GII (Symptomatic isolates) while no significant increase was found in GIII (asymptomatic isolates), as compared to control group [32-35]. This indicates that this isolate GI (from CRC patients) induced a higher degree of oxidative protein stress than that of GII (symptomatic isolate) and GIII (asymptomatic isolate) as compared to the control group [35-37]. This may be explained by one possibility that, in order to fight parasites, monocytes produce O2 by stimulating xanthine oxidase activity (O2 is formed when xanthine oxidase converts hypoxanthine to xanthine). This excess O2 is then converted to H2O2 by glutathione peroxidase [38 & 39].

Our results are supported by a previous study that provided evidence that patients with both CRC and parasitic infections secrete significant amounts of serum and plasma metabolites derived from oxidative stress damage. Parasitic infection and CRC may contribute to oxidative stress independently, but when present together, the oxidative stress load imposed by the parasites may be attenuated [32-34].

Another study detected higher levels of H2O2 in the CRC patients and the parasite-infected subject (without CRC) than the healthy controls. Interestingly, the H2O2 levels in parasite-infected subjects were almost two-fold higher than in CRC patients, but were attenuated in parasite-infected subjects with CRC [35, 37 and 39]. Elevated level of GPx in the blood of mice in the course of trichinellosis induced by T. spiralis was reported. It was suggested that such antioxidant enzyme GPx, is a part of a defense mechanism against oxygen-derived free radicals, and may help in the host’s biochemical defense mechanisms during trichinellosis [40]. These results have been supported by other studies in which the significance of antioxidant processes in host defense mechanisms during parasitic infections (Plasmodium spp., Schistosoma mansoni, Dicrocoelium dendriticum, Wuchereria bancrofti, Trypanosoma cruzi) has been confirmed [31, 33, 35, 37, 39, 40 and 41].

Moreover, a recent in vivo study recorded that the GSH level and GPX activity were substantially increased on the 7th day of infection with Toxoplasma gondii in experimental rats when compared to noninfected one [41 and 42].

The cytotoxicity and therapeutic potential of NO against a range of microbial agents are well recognized [41 and 42]. NO prevents growth as well as encystation of Giardia [42], while it induces apoptosis-like features in Entamoeba [41].

NO production requires tight control to limit cytotoxic damage to the host's own cells. Unregulated production may lead to a variety of damaging effects including alterations to normal neurological functions during cerebral malaria and intestinal pathology during trichinosis [42 and 43].

In the present study, NO level in serum of mice infected with Blastocystis isolates derived from CRC patients (GI) showed significant decrease, while in GII (symptomatic isolates) significant increase occurred, and in GIII (asymptomatic) no significant increase was detected [44, 46 and 47].

This may be explained by a possible ability of this isolate derived from CRC patients (GI) to cope with host defense mechanisms such as enterocyte-generated nitrosative stress and hence might provide it with a survival advantage over other isolates from GII and GIII in colonizing the human gut [48-51], by inhibiting epithelial iNOS production as a defense mechanism that might help it to colonize and exacerbate host pathology and help it to defeat the disadvantage of increased vulnerability to nitrosative stress [51 and 52].

This finding is in agreement with a previous study done by [53 and 54] in which a metronidazole resistant strain, Blastocystis ST-7 (B) inhibited epithelial NO production by down regulating epithelial iNOS expression. The author postulated possible association of NO susceptibility with Mz resistance in which this metronidazole resistant strain Blastocystis ST-7 (B), although more susceptible to nitrosative stress [48, 49 and 52], exhibited significantly higher arginase activity than another metronidazole sensitive strain Blastocystis ST-4 (WR-1) and inhibited epithelial NO production.
Our findings also agree with another in vivo study that detected high levels of NO in mice infected with Blastocystis sp. Isolated from symptomatic patients compared to low changes in control group, moreover; a study done on Trichomonas vaginalis showed that the mean level of NO was significantly low in severe symptomatic than moderate and mild symptomatic subgroups [53].

Noninvasive, lumen-dwelling, extracellular pathogens like Giardia and Blastocystis rarely come in contact with macrophages. Since these organisms are in close immediacy to NO producing enterocytes, thus in order to survive this hostile environment, it is reasonable to suggest that they suppress intestinal epithelial NO production [54-56].

All this previous data suggested the presence of diverse pathogenic roles of Blastocystis sp. isolates derived from different clinical presentations. Significant difference in surface ultrastructure and protein profiles between Blastocystis sp. isolates derived from CRC and Non-CRC patients was demonstrated [57]. Moreover, variation in growth kinetics and MTZ sensitivity of Blastocystis sp. isolated from different clinical groups including asymptomatic, symptomatic and CRC patients was also established [57 and 58].

In conclusion, Blastocystis sp. isolates associating colorectal carcinoma differ in their pathogenic capabilities than symptomatic and asymptomatic isolates. There was a higher degree of oxidative protein damage and intensification of the antioxidant processes associating this infection. Further understanding of mechanisms developed by Blastocystis sp. in immune evasion would help in the improvement of host immune-modulating strategies.

Materials and methods:
Experimental animals and treatments
Three-weeks-old BALB/c mice (n = 20) with a mean weight of 25 g were categorized into 4 groups consisting of 5 rats per group and treated as follows for the entire duration of two weeks of the study: control group (untreated) was administered with 0.5ml Jones' medium by the oral route followed by PBS injection intraperitoneally once a week for two weeks, second group was inoculated with Blastocystis sp. cyst isolated from CRC patients (ten thousand cysts/ ml sterile saline) orally followed by PBS injection intraperitoneally once a week for two weeks, third group was inoculated with Blastocystis sp. cyst isolated from patients with asymptomatic intestinal Blastocystis sp parasitic infestation ((ten thousand cysts/ ml sterile saline) orally followed by PBS injection intraperitoneally once a week for two weeks and the last group was inoculated with Blastocystis sp. cyst isolated from patients with symptomatic intestinal Blastocystis sp parasitic infestation (ten thousand cysts / ml sterile saline) orally followed by 15mg/kg AOM injection intraperitoneally once a week for two weeks. The concentration of Blastocystis sp. cyst and in this study was referred to on a previous study by and Gosse et al. (2005) [34]. All the rats were sacrificed on the 3rd week. A week prior to the experiment they were pre-screened and found to be negative for intestinal parasitic infections. Animals were caged individually under standardized conditions (22°C, 60% relative humidity, 12 h light/12 h dark cycle, 20 air changes/h). They were fed with standard diets and water was given ad libitum .The cages and paper bedding was changed at weekly intervals. One additional mouse for each group was inoculated with one ml parasite-free culture medium to be considered as negative control and was kept under the same conditions. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, Ain Shams University, Cairo, Egypt under registration number FWA 00006644

Measurement of Nitric Oxide (NO) and Glutathione Peroxidase (GSH)
Three weeks post-inoculation, peripheral blood samples were collected from all mice early morning prior sacrifice by placing them in a closed cylindrical jar with diethyl ether to ensure the absence of righting reflex. Any possible anesthetic influence on the results was reduced by using a standardized euthanizing technique to all the animals including the control group. Blood was drained via cardiac puncture whilst mice were under general anesthesia. Blood samples were collected in plain and EDTA coated tubes, centrifuged at 4000 rpm for 20 minutes, then plasma is separated and analyzed within 2 hours of collection to avoid enzymatic degradation. Nitric Oxide was measured spectrophotometrically using Nitric Oxide Assay colorimetric kit (ThermoFisher Scientific, USA), and Glutathione Colorimetric Detection kit (ThermoFisher Scientific, USA) was used for determination of GSH level.
Statistical analysis:-
All data were analyzed using SPSS version 23. Values are expressed as mean ± SD. Correlations between the parameters for both control and parasite infected subjects were identified by Kruskal-Wallis test and differences were considered significant when P < 0.05.

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Conflict of Interest
All authors declare no conflict of interest.

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