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Impact of parotoid gland secretions of the Egyptian toad (*Bufo relgularis*) on kidney functions of Ehrlich ascites carcinoma bearing mice

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ABSTRACT

Cancer is the second killer disease of the world, and its management is a major socio-medical problem. Although, several chemotherapeutic approaches have been taken to tackle the complexity of different cancer, toxicity and resistance may represent the potential obstacles to successful clinical development. Search for novel and safe drug from natural plant products are ventured worldwide. This study aims to show the impact of Parotoid gland secretions (PGS) of the Egyptian toad (*Bufo regularis*) on the kidney functions of Ehrlich ascites carcinoma bearing mice (EAC). PGS were collected to evaluate their *in vivo* anti-tumor effect, the median lethal dose (LD_{50}) of PGS was determined. Forty female Albino mice were separated into four groups. Group 1 considered the control. Groups 2 to 4 were injected 1×10^6 EAC intraperitoneally (ip). Group 2 considered the positive control. Groups 3 received ip injection of cisplatin (Cis) (2 mg/kg), while Group 4 injected with PGS 1/10 (78 mg/kg). PGS and Cis were ip injected daily for 7 days. After 14 days of treatment, blood samples were collected, the b.wt change, tumor indices and biochemical parameters were investigated. PGS effects used for EAC treatment are carried out by kidney function. Serum urea and creatinine were evaluated to reflect any clinical changes as well as histological alterations of renal tissues were investigated. The kidney functions were improved in EAC-bearing mice treated with PGS, as evidenced by ameliorating their histological structure. The study showed that PGS has a moderate antitumor effect on EAC- bearing mice.

1. Introduction

Cancer is a multicellular disease that can arise from all types of cells and organs with multiple factors. Cancer, the second killer disease of the world and its management is a major socio-medical problem. Although, several chemotherapeutic approaches have been taken to tackle the complexity of different cancer, the incidence and mortality rate for most form of cancer still remain high. Toxicity and resistance may represent the potential obstacles to successful clinical development. Search for novel and safe drug from natural plant products are ventured worldwide (Baskar et al., 2012). Experimental cancer models have been critical in cancer drug discovery because they act as predictors of treatment success or failure (Wong et al., 2012). Ehrlich ascites carcinoma is similar to human tumors and is most sensitive to chemotherapy as it is undefined and has a rapid growth rate (Kabel et al., 2013).

Chemotherapy is often regarded as the primary therapeutic option for many types of malignancies, whether with or without surgery (Mcree et al., 2011). Chemotherapeutic agents used in current clinical practice have significantly reduced mortality/morbidity while also improving patient quality of life (Bae and Park, 2011). Cisplatin is a highly effective chemotherapy medication used for many types of solid tumors (Tang et al., 2021). It is commonly thought that the key biochemical mechanism of cisplatin entails binding the drug to the DNA in the cell nucleus and subsequent interference with normal transcription and/or replication mechanisms (Wu et al., 2019). Selective and more efficient new drugs are urgently needed to address this problem; natural products (NPs) represent an available source of new anticancer drugs, especially from plants, microbes and marine organisms (Newman and Cragg, 2015). NPs have anti-cancerous potential due to the occurrence of natural antioxidants carrying out as reducing agents, free radical scavengers, and quenchers of singlet oxygen. They can reduce or minimize the toxic side effect of chemotherapy and radiation treatment by reinforcing their cancer killing action (Indap et al., 2006; Wamidh and Adel, 2010). Numerous research

have investigated new synthetic compounds or in natural agents as pharmacological models for antitumor activity (El-Naggar et al., 2011; El-Naggar et al., 2015; El-Naggar et al., 2020). Skin secretions from amphibians have garnered attention because of their potential for drug development (Conlon et al., 2014). The parotoid glands of the genus Bufo are situated on the sides of the head, as well as the neck or shoulder areas (Perry, 2000). Proteins, peptides, poisonous substances, steroids, alkaloids and biogenic amines depending on the species and other chemical components are among the substances released by these glands (Sciani et al., 2013). PGS released from the skin involved in defense against predators and germs (Kowalskia et al., 2018).

PGS of bufonid toads are a plentiful source of bioactive substances having cytotoxic, cardiotoxic, and hemolytic effects (Kowalskia et al., 2018). PGS of toads has been used clinically as antimicrobial, local anesthetic, and antineoplastic agent (Baldo et al., 2015). Hundreds of peptides have been discovered from amphibian skin (Conlon et al., 2014; Oelkrug et al., 2015). PGS of bufonid toads are a plentiful source of bioactive substances having cytotoxic and hemolytic effects (Kowalskia *et al*. 2018). The crude extracts of PGS of *Rhaebo guttatus* and *R. marina* showed strong toxic effects on rat breast carcinoma (Oliveira et al., 2019). In addition, peptides that isolated from PGS showed anticancer activity (Oelkrug et al., 2015). Previous study by Giri et al. (2018) reported that Indian toad skin extract (*Bufo melanostictus*, Schneider) inhibited EAC cells growth in EAC-bearing mice.

2. Materials and Methods

2.1. Chemicals

Cisplatin (Cis), Urea, Creatinine, were purchased from Bio-diagnostic Company, Egypt.

Collection of toads and preparation of parotoid gland secretions

Between November and January 2021, one hundred toads (*B. regularis*) were collected from fields in Egypt's Monufia Governorate's Sadat City. The toads were carefully placed into wooden cages before being moved to the Tanta University Faculty of Science's Zoology lab. Toads washed with distilled water, the latero-dorsal region of the parotoid macro-glands was gently squeezed by hand, and PGS were collected and put in a beaker. The toads were released to nature after sample collection. The collected secretions were lyophilized after being dissolved in distilled water, sonicated, stored at -20 ˚C and dried under decreased pressure. The crude extracts were weighed and kept for further processing (Zoggel et al., 2012).

EAC-cells expansion and inoculation

The first inoculum of EAC cell line was purchased from the Department of Tumor Biology, National Cancer Institute, Cairo University. The cell line was intraperitoneal (i.p.) inoculated 1×10^6 cells / mouse. Cells were grown in the peritoneal cavity of mice and transferred every ten days to new animals. Mice were monitored daily for signs of tumor progression, including the amount of abdominal distension and signs of illness and distress. The volume of ascites fluid was determined by needle (18–22 gauge) aspiration. Withdrawal of ascites fluid was performed under aseptic conditions. To run the experiment on EAC-bearing mice, individual mouse was inoculated with 1×10^6 EAC-cells (El-Naggar et al., 2016).

Animals and experimental protocols

Forty healthy female Swiss albino mice (average weight of 18–20 g) were obtained from the Animal facility of Cairo University, Egypt. Allowed for acclimating for 1 week in the animal house conditions of the Faculty of Science, Tanta University, before being divided into groups. Target values for temperature and relative humidity were about 22 ± 1ºC and 55 ± 5% respectively, light- dark (day/night) cycle was achieved. Mice were given drinking tap water and normal experimental pelleted animal food *ad libitium*. All the experiments were done in compliance with the guiding principles for the care and use of the laboratory animals at the Faculty of Science, Tanta University.

Mice were randomly divided equally into four groups (n = 10) according to body weights to minimize the standard errors between groups as follows.

Gp 1 was kept as the negative control. Mice in Gp 2 were i.p inoculated with EAC-cells (1×10^6/ mouse) and was kept as the positive control Mice in Gps 3 and 4 were i.p injected with EAC (1×10^6/ mouse) followed by Cis (2 mg/kg) in Gp 3 was i.p injected daily for 7 days. While mice in Gp 4 were injected with (1/10) of LD_{50} of PGS daily for 7 days. On the day 14, the total body weight changes were assessed, all mice were sacrificed. Blood samples were collected from retro-orbital venous plexus under anaesthesia using heparinized microhematocrit tubes (Prasanna et al., 2017). Each blood sample was collected in clean tubes containing disodium salt of EDTA (Abdallah et al., 2020) for hematological examination (n=6)/ group. The sera were separated and frozen at -20 ˚C until used for determination of kidney function tests.

Estimation of kidney functions urea and creatinine levels

Urea and creatnine activities in serum were assayed by using commercial kit according to the method of Vijay and Kiran (2018).

Preparation of the kidney for histopathological study

The liver specimens (0.5 cm^3) from all groups will be collected and immersed in 10% neutral buffered formalin. The samples will be dehydrated in ascending graded series of ethanol, cleared in xylene, and impregnated, and embedded in paraffin wax. Sections of 5-7µm will be cut by using Leica Microtome (RM 20352035; Lecia Microsystems, Wetzlar, Germany) and mounted on glass slides. Paraffin sections will be stained by hematoxylin and eosin (H&E) stain according to Suvarna et al. (2019). The stained sections will be examined with a BX50/BXFLA Microscope (Olympus, Tokyo, Japan).

Statistical analysis

Group's data expressed as means ± S.D. were analyzed by t-test while percentage data were analyzed by SPSS software. $P < 0.05$ was considered as significant value for all statistical analyses in this study.

3. Results

Effect of the treatment with Cis or parotoid gland secretions on hematological parameters

As compared to control group, all other experimental groups except EAC-bearing mice group didn't show significant alterations in the total red blood cells (R.B.Cs), hemoglobin (Hb) level, hematocrit (Hct) %, and platelets count ($P \geq 0.05$). EAC-bearing group showed significant decrease in the total count of R.B.Cs, Hb level, Hct % value, and platelets count ($P \leq 0.05$) (Table 1). The total white blood cells (W.B.Cs) count and their differential were significantly increased in EAC-bearing mice alone ($P \leq 0.05$). EAC-bearing mice that had treated with Cis and PGS showed significant decrease in total W.B.Cs when compare with EAC-bearing mice group ($P \leq 0.05$) (Table 2 and Figure 1).

Effect of Cis or parotoid gland secretions treatment on urea and creatinine levels

The levels of urea, and creatinine in EAC-bearing mice were significantly increased when compared to the control group ($P \leq 0.05$). The treatment of EAC-bearing mice with PGS, led to a significant decrease in urea and creatinine levels when compared to EAC-bearing mice ($P \leq 0.05$) (Table 3 and Figure 2).

Table 1. The total R.B.Cs count, Hb levels, Hct value and the total platelets count in different groups.

Groups	R.B.Cs ($\times 10^6$/Ul)	Hb (g/dl)	Hct (%)	Platelets ($\times 10^3$/Ul)
Ctrl	8.9 ± 0.75 [a]	13.5 ± 1.93 [a]	44.5 ±1.8 [a]	776.2 ± 63.8 [a]
EAC alone	5.9 ± 0.68 [b]	9.5 ± 0.79 [b]	32.5 ± 2.8 [b]	610.3 ± 72.6 [b]
EAC/Cis	7.6 ± 1.82 [a]	12.5 ±1.89 [a]	38.3 ± 3.1 [a]	710.5 ± 38.2 [a]
EAC/PGS	7.5 ± 0.87 [a]	12 ± 0.91 [a]	37.5 ± 3.2 [a]	695.7 ± 58.4 [a]

The values represented means ± S.D; **Ctrl:** Control; **PGS:** Parotoid gland secretions; **EAC:** EAC-bearing mice; **Cis:** Cisplatin; **R.B.Cs:** Red blood cell counts; **Hb:** Hemoglobin; **Hct:** Hematocrit. P value ≤ 0.05 was considered to be statistically significant. Means that do not share a letter are significantly different.

Table 2. The total W.B.Cs count and their differential percentages in different groups.

Groups	W.B.Cs ($\times 10^3$/Ul)	Lymph. (%)	Neut. (%)	Mon. (%)
Ctrl	7.9 ±1.68 [a]	78.5 ± 6.4 [a]	20.2 ± 2.4 [a]	1.2 ± 0.09 [a]
EAC alone	19.5 ± 0.89 [b]	68.1 ± 7.6 [b]	30.6 ± 2.8 [b]	1.2 ± 1.00 [a]
EAC/Cis	9.6 ± 2.01 [a]	67.3 ± 4.8 [a]	31.5 ± 3.3 [a]	0.98 ± 0.81 [a]
EAC/PGS	12.2 ± 0.86 [b,c]	71.4 ± 4.2 [a]	26.5 ± 2.9 [a]	2.1 ± 3.3 [a]

The values represented means ± S.D; **Ctrl:** Control; **PGS:** Parotoid gland secretions; **Cis:** Cisplatin; **W.B.Cs:** White blood cells; **Lymph:** Lymphocyte; **Neut:** Neutrophil; **Mon:** Monocyte. P value ≤ 0.05 was considered to be statistically significant. Means that do not share a letter are significantly different.

Table 3. Levels of urea and creatinine in the different groups.

Groups	Kidney function parameters	
	Urea (mg/dL)	**Creatinine (mg/dL)**
Ctrl	44.2 ± 4.1 [a]	0.67 ± 0.25 [a]
EAC alone	86.5 ± 8.3 [b]	1.35 ± 0.12 [b]
EAC/Cis	58.2 ± 5.9 [c]	0.9 ± 0.09 [c]
EAC/PGS	65.5 ± 6.8 [c]	1.05 ± 0.19 [b, c]

The values represented means ± S.D.; **Ctrl:** Control; **PGS:** Parotoid gland secretions; **EAC:** EAC-bearing mice; **Cis:** Cisplatin. P value ≤ 0.05 was considered to be statistically significant. Means that do not share a letter are significantly different.

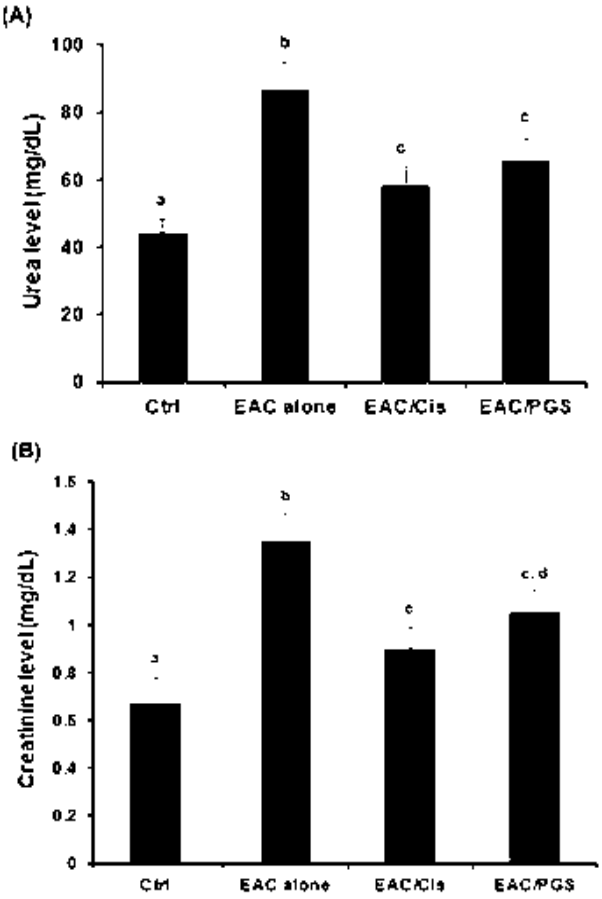

Fig. 2 (A and B). Serum levels of urea **(A)** and creatinine **(B)** in the different groups of mice.

Effect of parotoid gland secretions on kidney sections of different groups

The kidney section of the control mice (Gp1) showed renal Bowman's capsule, proximal and distal convoluted tubule (PCT-DCT). The Bowman's capsule had an outer mantle layer and an inner mantle that surrounds a mass of blood capillaries known as glomerulus (Figure 3). In EAC- bearing mice (Gp2), renal tissues showed marked histological alterations. These alternations include pyknotic nuclei, hemorrhage in interstitial tissue, congested blood vessel, degeneration and vacuolation of renal tubules, several degenerated glomerulus, degenerated distal convoluted tubules (Figure 4). The renal tissues of EAC- bearing mice treated with Cis (Gp3) showed normal renal histological structure with normal glomerulus, proximal convoluted tubules, and distal convoluted tubules compared to Gp2. Moreover, few degenerated glomerulus and little hemorrhage in interstitial tissue were observed. (Figure 5). In EAC-bearing mice treated with PGS (Gp4), renal tissues showed normal renal histological structure with normal glomerulus and tubules. In addition, few degenerated glomerulus and little tissue hemorrhage were observed (Figure 6).

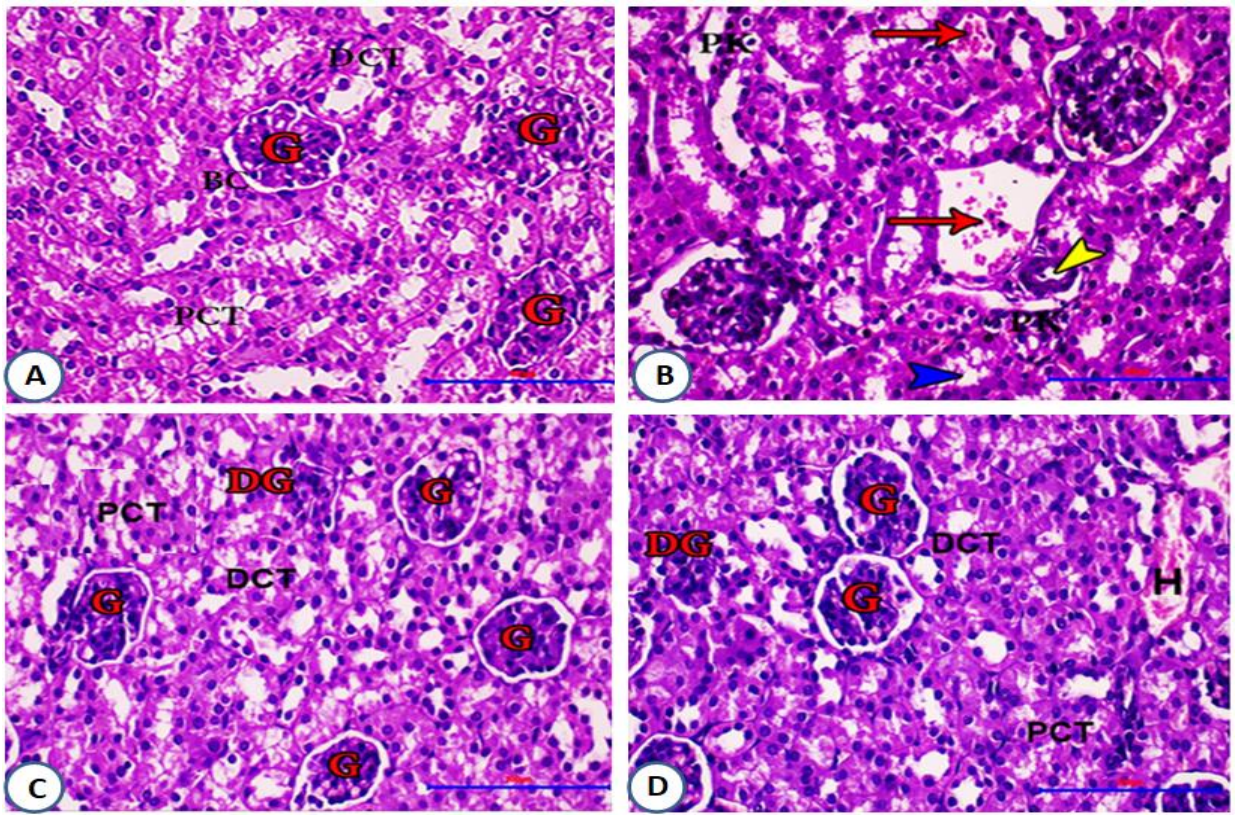

Figure 2. Photomicrograph of kidney section of the control mice showing the showing the structure with normal glomerulus (**G**). (H&E stain) (X40) (A). Kidney section of EAC-bearing mice showing Pyknotic nuclei (PK), hemorrhage in interstitial tissue (red arrows), congested blood vessel (yellow arrowhead), degeneration and vacuolation of renal tubules (blue arrowheads). (H&E stain) (X400) (B). Kidney section of EAC-bearing mice treated with Cis showing normal renal histological structure with normal glomerulus (**G**), few degenerated glomerulus (**DG**), proximal convoluted tubules (**PCT**), and distal convoluted tubules (**DCT**). (H&E stain) (X200) (C). Kidney section of EAC- bearing mice treated with PGS showing normal renal histological structure with normal glomerulus (G), proximal convoluted tubules (PCT), few degenerated glomerulus (DG), and distal convoluted tubules (DCT), and little tissue hemorrhage (H). (H&E stain) (X200) (D)

5. Discussion

In the current study, there are significant decreases in total RBCs count, platelets count, Hct and Hb percentage in EAC-bearing mice. This could be due to the suppressive effect of EAC toxin on the bone marrow and erythropoiesis, these findings is consistent with the previous study obtained by Hashem et al. (2020), who reported that hematological parameters of EAC- bearing mice after 12 days showed a significant reduction in total RBCs count, platelets count, Hct and Hb concentration. Treatment of EAC- bearing mice with Cis restores these alternations close to the control mice. Treatment of EAC- bearing mice with PGS showed an improving in the total RBCs count, platelets count, Hct and Hb concentration when compared to EAC- bearing mice alone, however there are no significant changes when compared to EAC- bearing mice that had treated with Cis.

Current data showed that there were increases in total WBCs and neutrophil percentage in EAC-bearing mice when compared to control group. This could be due to inflammatory response or stress due to proliferation of EAC cells (Badr et al., 2011). This result agreed with the data obtained by Hashem et al. (2020), who reported that there was significant elevation in total leukocytic and granulocytic counts in EAC-bearing mice group. As compared to EAC-bearing mice, treatment of EAC group with Cis restores these alternations close to normal. This could be due to the inhibitory effect of chemotherapy on both of EAC cells and bone marrow (Salem et al. 2011). This result agreed with the data obtained by Hashem et al. (2020). Treatment of EAC-bearing mice with PGS showed an improving in the total number of WBCs and in lymphocytes and neutrophils percentages as compared to EAC-bearing mice. This could be due to antitumor effect of PGS. The results showed that EAC-bearing mice showed elevating in urea and

creatinine levels. This could be attributed to muscle necrosis catabolic effect of the tumor and the elevation in urea production, this result agreed with the data obtained by Saleh et al. (2022). Treatment of EAC- bearing mice with Cis decreased the serum level of urea compared to EAC- bearing mice, these findings is consistent with the data obtained by Saleh et al. (2022) and Salama et al. (2022). However, EAC- bearing mice treated with PGS led to an improvement in serum creatinine and urea levels. Current study revealed that section of kidney of EAC-bearing mice showing several histopathological signs. While after treatment with Cis or PGS exhibit some sort of improvement, this evidenced by the decrease in urea and creatinine levels in the current study. A previous study reported that using the natural products for treatment of EAC-bearing mice were able to improve the improper liver and kidney tissue architecture and eliminate its toxicity (Lin et al., 2020)

Conclusion

This study reported the impact of PGS that were obtained from the Egyptian toad (*B. regularis*) and showed its antitumor effect on kidney functions.

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