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Impairment of Placental Angiogenesis in Rats Treated with Saffron: The Role of Low Dose Gamma Irradiation

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ABSTRACT

The current study is an endeavor to estimate how much the teratogenic effect of saffron (Crocus sativus L) on pregnant rats upon exposure to low doses of gamma radiation. Pregnant rats were received 50 mg saffron extract/kg body weight (every 2 days over 18 days) and exposed to 3Gy of gamma radiation delivered as 0.5Gy every 3 days over 18 days. Transcription factor HIF-1, sFLT-1, a soluble like tyrosine kinase with antiangiogenic properties, significantly increased in placental and fetus tissues of rats received saffron treatment compared to control rats. Plasma VEGF and placenta VEGF receptors (FLT-1) endure significant decreases in pregnant rats treated with saffron and significant alteration in the NO concentrations in both of placenta and fetus tissues. Deranged antioxidant GSH concentration and GSHPx activities, and pro-oxidant MDA, were markedly observed. Besides, we observed increases in LDH level in the placenta, fetus tissues, and maternal blood plasma. Unexpectedly, the severity of changes in biochemical parameters are less pronounced in rats treated with saffron and exposed to low dose gamma radiation. It could be concluded that saffron at a certain stage and certain doses could exert considerable toxicity symbolized serious life threats during pregnancy and low dose gamma irradiation could oppose this harmful action.

INTRODUCTION

Placental angiogenesis is an ordinary physiological process strongly regulated during pregnancy. Angiogenesis in the placenta causes proliferation, migration, and differentiation of endothelial cells in the preexisting trophoblastic micro vessel like angiogenesis in any other organs [1]. In several pregnancy complications, disturbed placental angiogenesis is the most conjoint pathological sign that appeared [2].

Vasculogenesis during embryogenesis started in extraembryonic mesoderm allantois by the formation of angioblast (endothelial progenitor cells [3]. Convincing evidence suggests that vascular endothelial growth factor (VEGF) is essential for all steps of placental vascular formation and development. Down-regulation of VEGF gene or interruption of genes encoding VEGF receptors give rise to embryonic lethality because of atypical blood vessel formation during embryogenesis.[4] NO which serves as an effective vasodilator factor in the placenta produces in response to potent stimulation of endothelial cells of a placental artery by VEGF [4-5].

Placental angiogenesis has been implemented in hypoxia circumstances. The hypoxia-inducible factor 1 (HIF-1) is the arbitrator of hypoxic conditions elaborated in the transcription of several oxygen-dependent genes that encrypt for proteins allied with angiogenesis and cell metabolism. HIF-1 has two domains: HIF1-α, hypoxia-inducible, and HIF-1β, constitutively expressed during low oxygen conditions. During the growth of the placenta (in early gestation) HIF1-α is highly expressed. However, overexpression of HIF1-α is associated with many inflammatory disorders, like preeclampsia. HIF1-α highly expressed during hypoxia regulates the angiogenic proteins like VEGF and PIGF which induce angiogenesis. When these proteins concentration changed, angiogenic imbalance led to damage of endothelial cells and the starting of preeclampsia [6].

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Plentiful interest has been suggested exploitation placental angiogenesis as a goal for the progress of diagnosis tools and probable therapeutics for pregnancy difficulties built on the knowledge of placental angiogenesis in normal and anomalous pregnancies. Studies on placental angiogenesis afford advanced vision for a good understanding of normal placental biology which paves to improvement in the diagnostic tools for pregnancy difficulties and the compactions of miscarriage, premature birth, and fetus abnormalities [6].

Saffron (Crocus sativus L), a perennial stemless herb supposed to have some properties with significance in traditional medicine like antispasmodic, eupeptic, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac, and abortion [7]. Several pharmacological studies confirmed antioxidant, anticancer, anticonvulsant, anti-inflammatory and antitumor effects, radical-scavenging and learning- and memory-improving properties of saffron [8-9]. The active constituents of saffron safranal (a volatile agent), crocetin, and its glycoside crocin dye material handle its pharmacological activities [10]. As saffron and its active components are extensively used in traditional medicine and modern pharmacology, and because of its echo as safe food and medicinal agent, the probability of gathering saffron uses and exposure to radiation or/and pregnancy becomes largely to pay attention. In the present study, toxicity of embryos and the angiogenesis of the placenta have been investigated in pregnant rats exposed to low doses of gamma irradiation and treated with saffron.

Currently, the use of radiation in medical practice is accredited extensively. The usage of medical radiation modalities engaging in low doses, such as CT scans, has increased vastly over the past decades [11] despite negative consequences determined for human health post-exposure to ionizing radiation. One of the most common risks is the exposure of pregnant women, which affects embryo/fetus and developing, for instance, structural and function brain defects. Radiotherapy or medical imaging during pregnancy frequently results in abortion, delay of maternal therapy, or pre-term delivery [12]. The recompenses of existing medical exercise can still be upgraded for pregnant women and their unborn child, thus a better understanding of radiation effects, especially in the low-dose range, is impressive [13].

Based on the above, the current work was directed to evaluate the response of placental angiogenesis to saffron treatment for exposure to low dose gamma irradiation and the effect of these milieus during pregnancy. To fulfill these goals, we determined the angiogenic, transcriptional factors, and growth protein in the placenta and fetus tissues. The Redox tone tissue damages marker besides the apoptotic marker's caspase 3 and granzyme B were estimated.

MATERIALS AND METHODS

This study has been executed in the National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt.

Animals

Female Swiss albino rats weighing 100-150 g were attained from the breeding unit at the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt. Rats (10 animals/cage) were housed and kept under appropriate environmental surroundings (controlled air, temperature, and relative humidity). Mice were provided with pellet food and free admittance to water.

Animal experimentations were consistent with the guidelines of ethics by Public Health Guide for the Care and Use of Laboratory Animals (National Research Council),15 following the recommendations for the proper care and use of laboratory animals approved by the Animal Care Committee of the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

Chemicals

All chemicals used in the present investigation were purchased from Sigma Chemical Company, St. Louis, U.S.A, and of analytical grade. Capsules of Saffron supplement (50 mg/ capsule) were purchased from Raintree (Carson City, NV).

Saffron preparation

Each capsule content comprised 100% pure Saffron leaf/stem powder without binders and was suspended in DMSO (50 mg/ml). After incubating for 5min, the suspension was centrifuged, and the supernatant was filtered to remove any remaining particles. The solution was freshly prepared before treatment.

Irradiation procedures

The whole body γ-irradiation was applied using Canadian Gamma Cell-40 (137Cs) biological irradiator at the NCRRT, Cairo, Egypt. The dose rate during the period of the experiment was 0.006 Gy/s. Rats were irradiated with a fractionated dose of 0.5Gy every 3 days for 18 days, according to the experimental design.

Work design

An entire number of 32 female Swiss albino rats were randomly disseminated into 4 equally size groups:

Group 1: (Pregnant, P): Group of pregnant rats received a vehicle of Saffron (DEMSO) Group 2: Irradiated Pregnant (RP): Group of pregnant rats exposed to 0.5 Gy whole-body gamma radiation every other 3 days up to the total dose of 3Gy after 18 days from initiation of the experiment. Group 3: Saffron Pregnant (SP): Group of pregnant rats injected intraperitoneally with Saffron (50 mg Saffron /kg body weight every other 2 days for 18 days starting from the initiation of the experiment and during the time of pregnancy (from zero time). Group 4: Saffron Pregnant Irradiated (SPR): Group of pregnant rats injected intraperitoneally with Saffron (50 mg Saffron /kg body weight every other 2 days and exposed to 0.5Gy whole-body gamma irradiation every other 3 days up to the total dose of 3Gy for 18 days starting from the initiation of the experiment and during the time of pregnancy.

Samples preparation

One day after complete all treatments; rats were anesthetized using diethyl ether. The blood was collected on heparinized tubes by using a syringe for heart puncture and the collected blood was preserved at -20ºC for biochemical analysis. Animals were immolated and undergo necropsy. The placenta and fetus were collected, rinsed in ice-cold isotonic saline, blotted dry with a filter paper, and preserved at -20ºC for ongoing biochemical analysis.

Tissue samples preparation for biochemical analysis

A 10% (w/v) tissue homogenate (placenta or fetus) was prepared on an ice bath by homogenizing a unit of weight of fetus or placenta tissues in 10 volumes of ice-cold isotonic. An aliquot of the 10% homogenate was centrifuged at 3,914 g at 4ºC for 10 minutes, and the supernatant was separated and stored at -20oC for the analysis of HIF1alpha, sFLT-1α, FLT-1, GSH, GSHPX, Caspase3, granzyme B, MDA, NO and LDH. Besides, in maternal plasma, we determined VEGF and LDH.

Biochemical Analysis

The GSH content was determined photometrically at 412nm using 5, 50-dithiobis-2-nitro benzoic acid [14]. Glutathione Peroxidase (GSHPx) activity (U/mg protein) was assayed according to the method of Gross et al [15]. The activity of GSHPx expressed as GSH consumed per min per mg protein. Lipid peroxidation product, malondialdehyde (MDA), was measured (nmole/g wet tissue) by thio barbituric acid assay, which is based on MDA reaction with thio barbituric acid-forming thio barbituric acid reactive substances (TBARS), a pink-colored complex exhibiting a maximum absorption at 532nm [16].

The transducer of several genes responsible for the angiogenesis and vascularization hypoxia-inducible factor (HIF-1α) was estimated using a commercial ELISA (enzyme-linked immunosorbent assay) KIT (R&D systems/ Bio techno, UK). HIF-1α protein standards were used to establish the standard calibration curve. From the standard curve, the limit of detection, the limit of quantification, and the coefficient of variation was determined. Results were expressed as pg/ mg protein.

Tissue samples were assayed for sFLT-1α, a soluble tyrosine kinase receptor. A polyclonal anti-sFLTR-1 antibody and a sFLTR-1-HRP conjugate are used to apply the competitive enzyme immunoassay technique of My Biosource ELISA Kits. The assay sample and buffer are poured together in plate wells pre-coated with sFLTR-1, HRP conjugate. After one hour of incubation, the wells were decanted and washed five times. Thereafter, wells were incubated with HRP enzyme substrate. A blue-colored complex was formed (product of the enzyme-substrate reaction). After adding a stop solution, the blue color was turned to a yellow solution. The intensity of color was measured spectrophotometrically at 450nm in a micro-plate reader. The sFLTR-1 from samples and sFLTR-1-HRP conjugate compete for the anti-sFLTR-1 antibody binding site therefore, the intensity of the color is inversely proportional to the sFLTR-1 concentration. A standard curve was plotted relating the intensity of the color (O.D.) to the concentration of standards. The sFLTR-1 concentration in each sample was calculated from this standard curve.

Abcam Simple Step ELISA® technology is a single-wash 90 min sandwich ELISA designed for the quantitative measurement of FLT1 (VEGF-R1) protein in rat EDTA plasma (sensitivity 0.11 ng/ml). This ELISA Technique employs capture antibodies conjugated to an affinity tag that is recognized by the monoclonal antibody-coated the Simple Step ELISA® plates.

A commercially available sandwich enzyme-linked immunosorbent assay (ELISA) got from R & D Systems in Europe (Abingdon, UK), applied to assay VEGF. The sensitivity of the assay was 9.0 pg/ ml. All samples were assayed in duplicate.

Nitric oxide (NO)was measured (nmole/g wet tissue) as the stable end product, nitrite, conferring to the method of Mar Miranda et al. [17]. The test is based on the reduction of nitrate by vanadium trichloride acidic Griess reaction. The diazotization of sulfanilic acid with nitrite at acidic pH and subsequent coupling with N- (10 naphthyl)-ethylenediamine produced an intensely colored product that is measured at 540nm.

The cleaved caspase-3 level was assayed using a Human/rat Cleaved caspase (Asp175) DuoSetR IC ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) whereas granzyme B level was measured by rat Granzyme B ELISA kit (Ray Biotech, Norcross, GA, USA).

Lactate dehydrogenase (LDH) was assessed by ELISA kits (R&D Systems) following the manufacturer's instructions for the enzyme-linked immunosorbent assays via ELISA micro-plate reader (DV990 BV 416; Gio.DE VITA and CO., Rome, Italy).

Statistical analysis

Results were expressed as a mean value ± SE (n=8 rats). Statistical package of social science (SPSS) version 20.0 for windows was used to analyze of data. By using One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range were. Differences between groups were significant when $P \leq 0.05$.

RESULTS

Redox status

The data collected showed that GSH concentrations and GSHPX activities were declined significantly ($P<0.05$) in the placenta's tissue and fetus of rats exposed to gamma radiation (RP) associated with significant increases in MDA contents as compared to untreated pregnant rats (P) (Figure 1: C-D). While in the group of rats treated with saffron (SP), the concentration of GSH and the activity of GSHPX displayed significant increases concurrently with recording the significant decrease in MDA level compared to rats of the (P) group. However, in the group of rats treated by saffron and exposed to gamma radiation (SRP), the data obtained pointed to significant improvement in the values of the 3 parameters even in placenta or fetus tissues when compared with RP or SP rats' group despite significant changes from their control level in rats of (P) group (Figure 1: A- F).

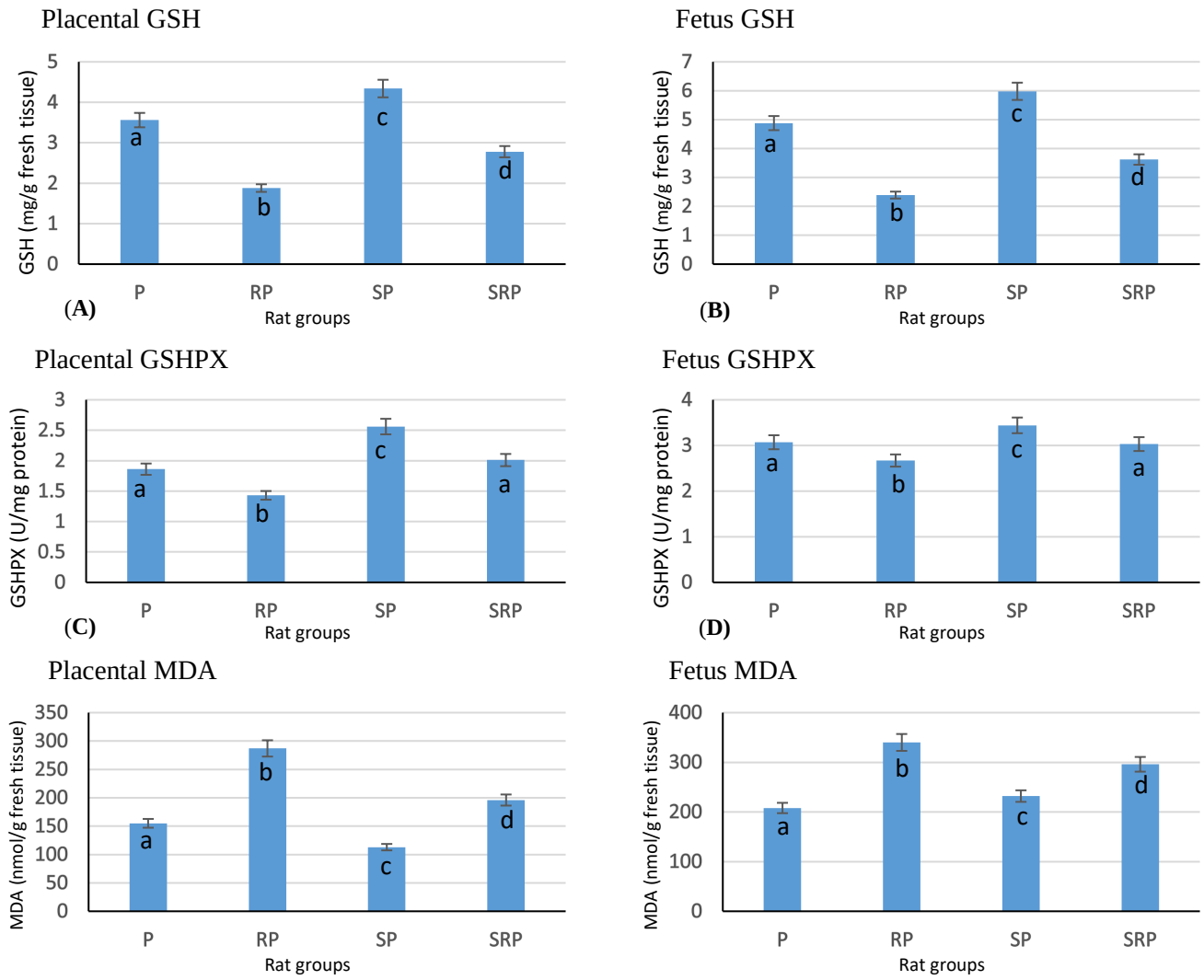

Fig. (1): Influence of saffron or/ and irradiation on the redox status in the tissue of placenta or fetus of pregnant rats.
P: pregnant rats, RP: irradiated pregnant rats, SP: Pregnant rats treated by saffron, and SRP: pregnant rats treated with saffron and exposed to gamma irradiation. Column tagged with dissimilar letters within the same histogram indicated significant difference values at P≤0.05.

Transcriptional and antiangiogenic factors: HIF-1a, sFLT-1

The data obtained revealed significant increases (P≤0.05) in the expression of HIF-1α and sFLT-1 proteins in both placental and fetus tissues in the group of pregnant rats treated with saffron (SP) when compared with untreated pregnant rats. In pregnant rats exposed to gamma radiation (RP), there are no significant changes when compared with normal pregnant rats. However, in rats received saffron and exposed to gamma radiation, the increases in the expression of transcriptional Factor HIF-1α, and sFLT-1 were less manifest in both placenta and fetus tissues compared to rats of the P group (Figure 2: A- D).

Angiogenic Factors: VGEF, FLT-1, and NO

The results pointed to significant decreases displayed in maternal plasma VEGF and placental FLT-1 decreases compared to pregnant untreated rats (P group). Also, there are no remarkable changes observed for these parameters in pregnant rats only exposed to gamma radiation compared to untreated pregnant rats. Whereas, in rats received saffron and exposed to gamma radiation, decreases in plasma VEGEF and placental FLT-1 were less manifested compared to rats in the P group (Figure 3: A- B). Besides, the data exemplified in Figure (3: D-C) demonstrated that the exposure of pregnant rats to whole-body gamma radiation during the period of pregnancy does not induce any remarkable changes in NO content of placenta or fetus tissue compared to untreated pregnant rats (P). Whilst saffron administration induced significant elevation(P≤0.05) in the NO content of both placental and fetus tissues comparing with rats in the P group. In rats of the SRP group, although the changes are significant when compared to P group, the changes are less manifested comparing to the SP group.

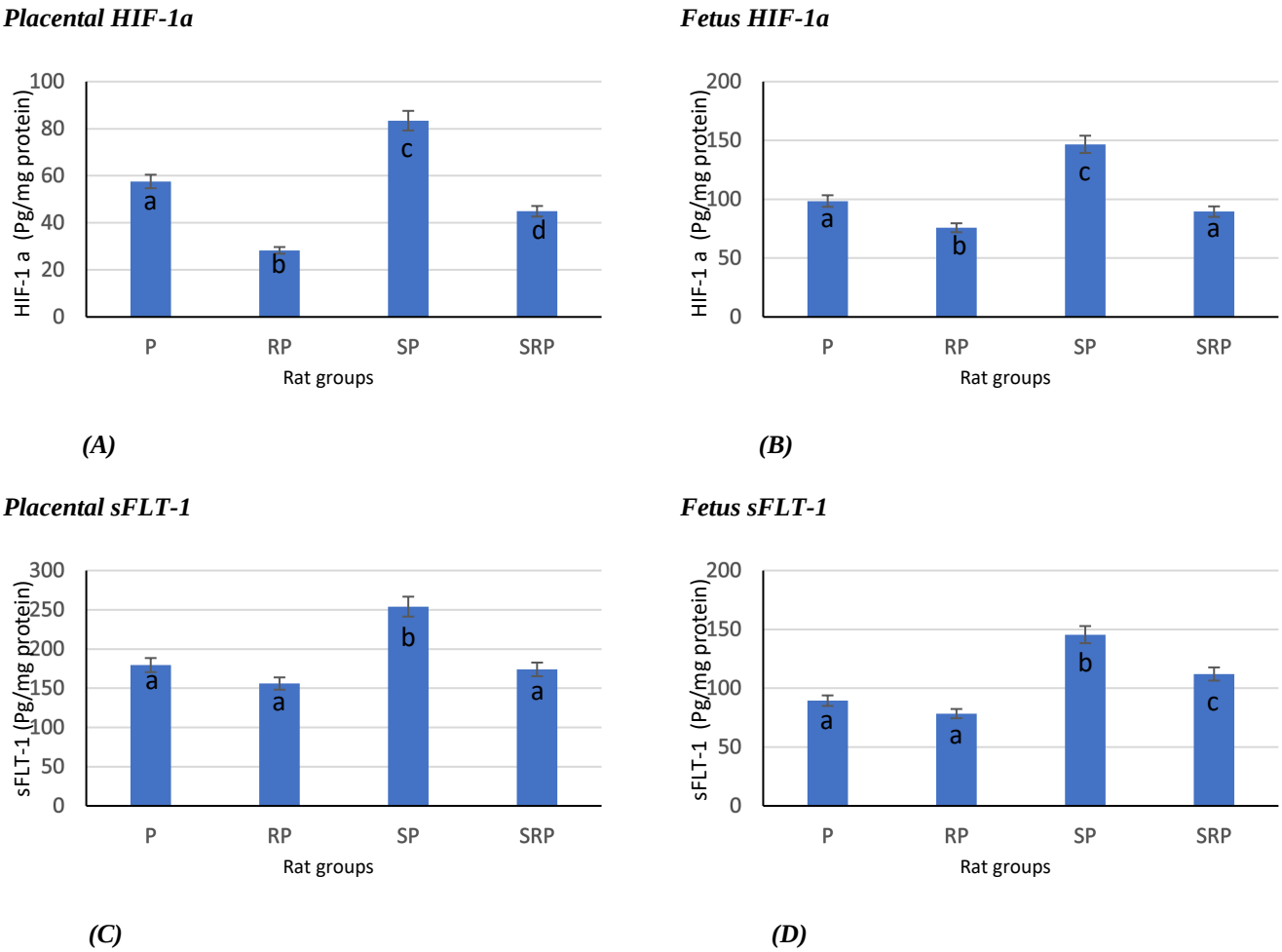

Fig. (2): Influence of saffron or/ and irradiation on the expression of HIF-1α, sFLT-1in the placenta and fetus tissues of pregnant rats. P: pregnant rats, RP: pregnant irradiated rats, SP: Pregnant rats treated by saffron, and SRP: pregnant rats treated with saffron and exposed to gamma irradiation. Column tagged with dissimilar letters within the same histogram indicated significantly different values at P≤0.05.

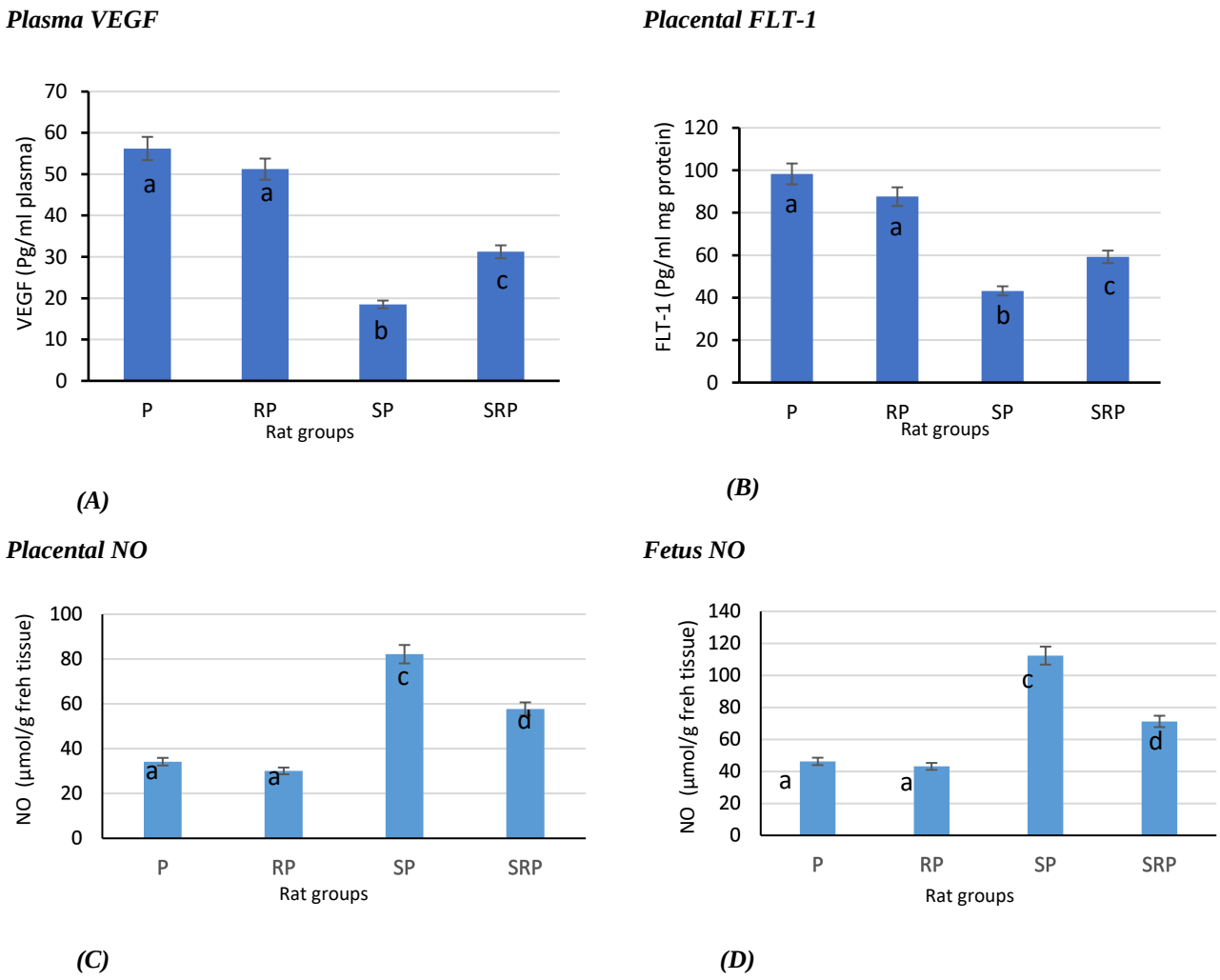

Fig. (3): Influence of saffron or/ and irradiation on the expression of VEGF (maternal plasma), FLT-1 (placenta), and NO (placenta and fetus) of pregnant rats. P: pregnant rats, RP: pregnant irradiated rats, SP: Pregnant rats treated by saffron, and SRP: pregnant rats treated with saffron and exposed to gamma irradiation. Column tagged with dissimilar letters within the same histogram indicated significantly different values at P≤0.05.

Apoptotic factors: Caspase3 and Granzyme B

The protein expression of apoptotic regulators (cleaved caspase3 and granzyme B) displayed significant decreases (P≤0.05) in the RP group as compared with the P group in both placenta and fetus tissues. While significant elevation in protein expression of the 2 regulators was observed in the SP group comparing with the P group even in placenta or fetus tissues. Besides, significant changes of caspase3 and granzyme B contents in placenta or fetus tissues of the SRP group were observed compared to the P group, and these changes seemed approximately in the median between changes in the SP group and that in the RP group (Figure 4: A-D).

Tissue damage sign (LDH)

The results for Lactate dehydrogenase (LDH) protein changes in the placenta or fetus tissue and maternal blood plasma were shown by Figure (5: A-C). It is clear, there were no significant variations in LDH protein concentration of RP rats when compared to rats of the P group, even in the placenta, fetus tissue, or maternal plasma. However, in the group of rats supplemented by saffron SP, a significant increase in LDH protein was recorded in all sites of observation when compared to its equivalent value in P group. Also, these increases in LDH proteins were less manifest in the SRP group when compared to the SP group, despite significant variation from the control level.

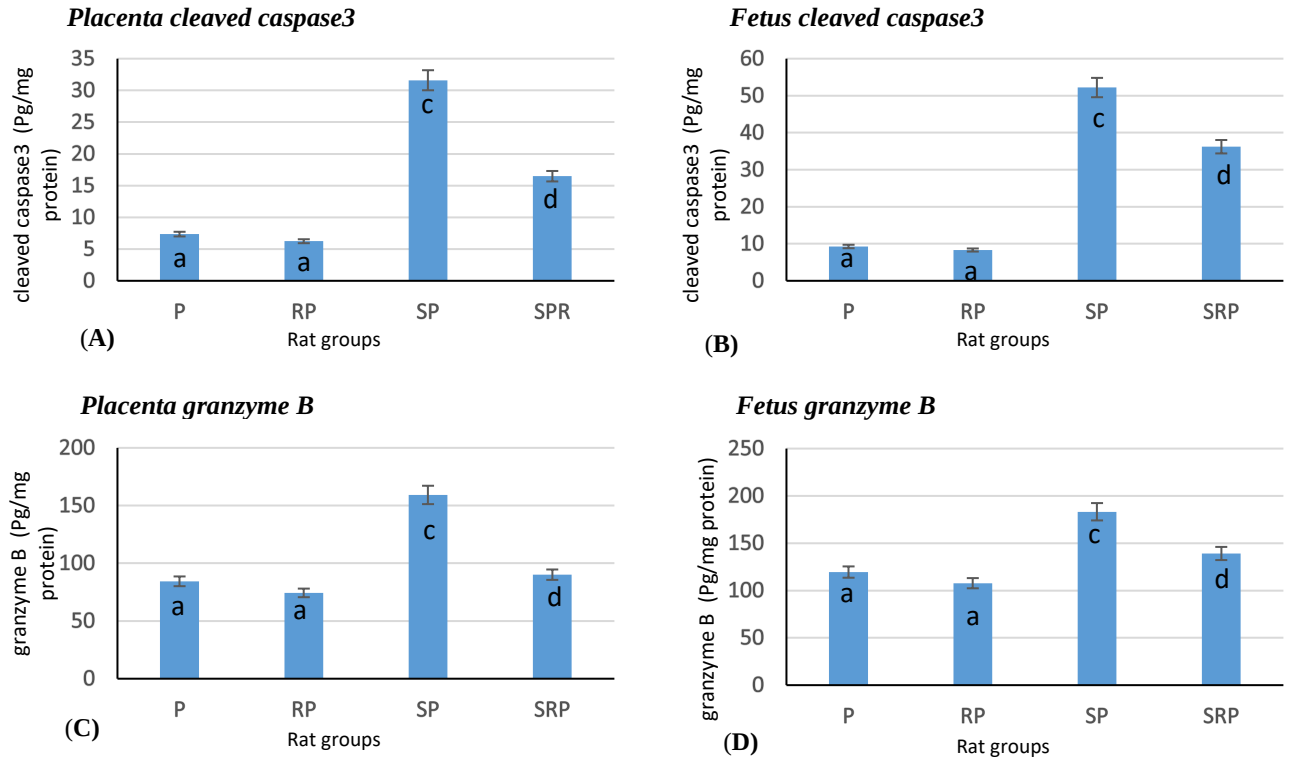

Fig. (4): Influence of saffron or/ and irradiation on the expression of cleaved caspase3 and granzyme B in placenta or fetus tissues of pregnant rats. P: pregnant rats, RP: pregnant irradiated rats, SP: Pregnant rats treated by saffron, and SRP: pregnant rats treated with saffron and exposed to gamma irradiation. Column tagged with dissimilar letters within the same histogram indicated significantly different values at P≤0.05.

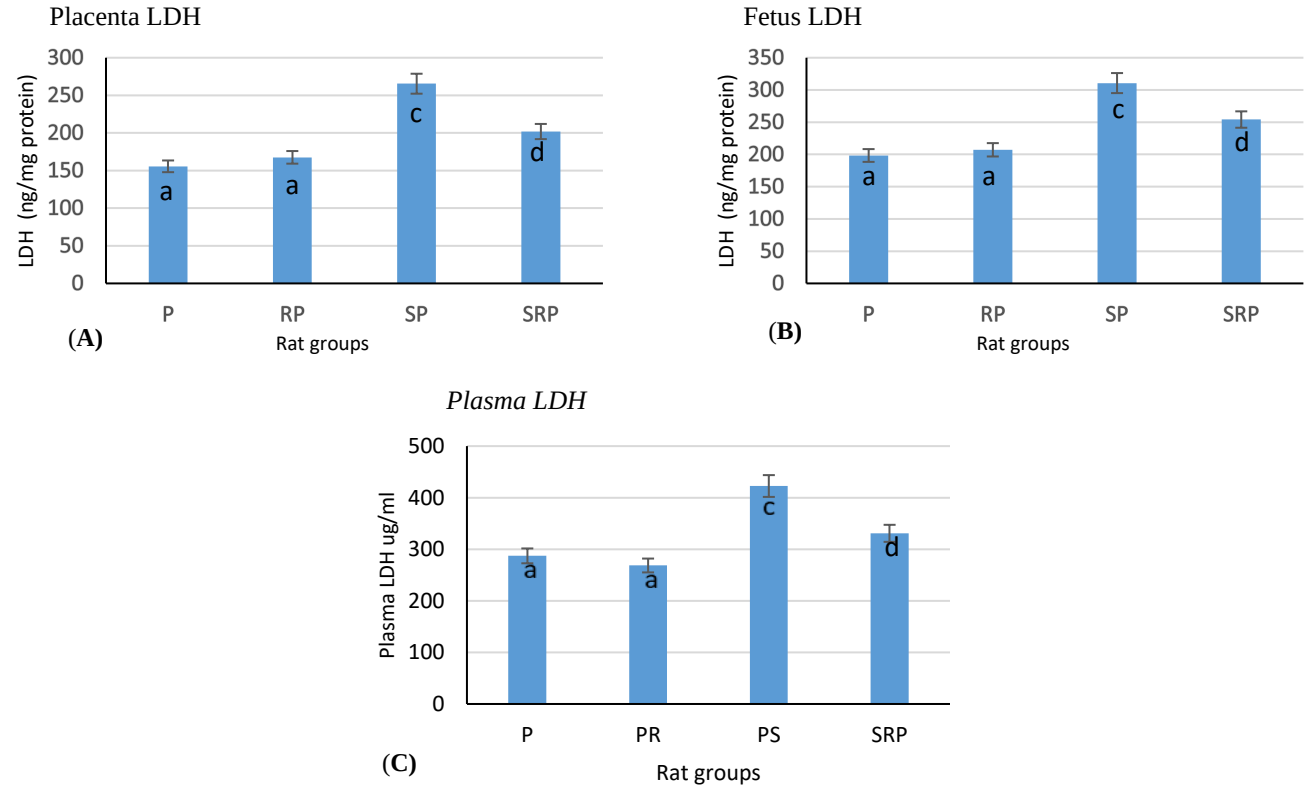

Fig. (5): Influence of saffron or/ and irradiation on the LDH protein expression in placenta or fetus tissues of pregnant rats.P: pregnant rats, RP: pregnant irradiated rats, SP: Pregnant rats treated by saffron, and SRP: pregnant rats treated with saffron and exposed to gamma irradiation. Column tagged with dissimilar letters within the same histogram indicated significantly different values at P≤0.05.

DISCUSSION

Recently, dependence on natural compounds for medical purposes and health maintenance is one of the basic endeavors to sustenance and improve human life. Saffron is one of these natural compounds which attract great attention. Many pieces of evidence documented that saffron's main component such as crocin, crocetin, and safranal could inhibit neurons erosion and depression, have antitumor effects might be because of its capability to induce inhibition of nucleic acid, scavenging free radical, affecting the expression of topoisomerase 2, induce programmed cell death, reduce cancer incidence and significantly increase the lifelong of the animal. Basheeruddin and Inamdar (2010) [10] stated that saffron has cancer preventive and anti-genotoxic potential, which qualify it to play a fundamental role in cancer chemotherapy as an adjuvant.

However, several pieces of evidence have emerged during the actual use of saffron pointed to definite complications which might drive to serious problems. We tried in the present study to shed light on certain complications and avoid saffron erroneous effects in case of incessant consumption of saffron and its active components. Hence, the pivotal aim was to examine the teratogenic effect of saffron on pregnant rats upon exposure to low doses of gamma radiation.

Pregnancy is associated with momentous risks of difficulties that can affect the health of mother and newborn. One of the stern risks is the imbalance of the oxidative/antioxidant, which is manifested by discrepancies of the antioxidant defense system. Pregnancy is a physiological situation categorized by the brittleness of defense in contradiction of pro- oxidizing agents, which causes a disorder of oxidizing/antioxidant status [18].

These instabilities in redox status can be accountable for significant materno-fetal difficulties. High levels of lipid peroxidation products (MDA) which are observed during pregnancy almost come from the lipid peroxidation process occurred in the placenta. MDA produced are mainly secreted on the maternal side, pass through maternal blood, triggering damages in other tissues, and remain in maternal circulation for some time [19]. In a normal pregnancy, the natural antioxidant can cope with this increase in the production of ROS. However, the disturbances of maternal antioxidants during pregnancy can increase reactive oxygen species which could act as primary or secondary messengers on both cell death and growth. The oxidative stress raised because of disturbances in the balance between antioxidants and reactive oxygen species could alter several reactions that affect fetal growth.

During completion of the intact investigations, we observed significant modifications of redox status (MDA, GSH concentration, and GSHPx activity) in placental and fetus tissues of rats that received saffron and exposed to low doses of gamma irradiation. These changes were less pronounced compared to pregnant rats exposed to saffron (Figure1). The results got might be attributed to the antioxidants aptitudes of saffron active constituents which could play a vital role in the adjustment of redox tone in both placental and fetal tissues during normal pregnancy or during exposure of pregnant animals to gamma radiation. Rahaiee et al.,[20] depicted that spices comprising phenolic and flavonoid compounds possess antioxidant activities and are commonly consumed as antioxidants food supplements. The antioxidant activities of saffron could be ascribed to its phenolic content and to its active ingredients such as safranal, crocin, crocetin, and carotene [21]. The saffron extract of C. sativus flower contains many polyphenol components which might successfully decrease the activity of free radicals and provide a robust defense for the different organs in contradiction of various oxidative damages under a dose-dependent behavior [22].

Despite saffron could protect the fetus against oxidative stress, the challenge here is the neutralization of ROS plenty, which might be required to exert~~ing~~ a central role in the fetus growth where ROS act as primary or secondary messengers on cell death and ~~on~~ growth. This vital and direct role of ROS on growth is because of the redox status that turns on the arrangement of certain transcription factors that affect pathological cellular signaling during proliferation which detour the flawed differentiation towards apoptosis [23]. Also, our data revealed increases of HIF-1α and sFLT-1 in both placenta and fetus tissues in rats supplemented with saffron compared to untreated pregnant rats. There are no detectable changes recorded in pregnant rats exposed to gamma radiation for the 2 parameters, even in placenta or fetus tissues. Whereas, significant decreases of HIF-1a and sFLT-1 are observed in the SRP group compared to the SP group (Figure 2: A-C). Tel et. al., [24] reported that the transcription factor HIF-1α (hypoxia-inducible factor-1α) accomplished a certain important function during placental development. HIF-1α arbitrates adaptive responses to oxidative trauma by

nuclear translocation and regulation of gene expression. Mitochondrial variations are serious for this adaptive response.

As ordinary pregnancy is characterized by a state of oxidative stress in which placental mitochondria generate reactive oxygen species [25], the saffron might bother this operation because of overproduction of HIF-1a that sync the highest concentration of ROS. Thus, placental and fetal tissue alteration of hypoxia hallmarked by overexpression of HIF-1a paved to pregnancy disorder in rats received saffron supplement because of angiogenic instabilities which handles the development of placental cord and substantially the fetal development. Also, Khalil et al. [26] reported that the antiangiogenic soluble fms -like tyrosine kinase 1 (sFLT-1) which is a non-membrane associated variant of VEGF receptor binds the angiogenic factors VEGF (vascular endothelial growth factor) and PlGF (placental growth factor) and prevent them from doing normal functions. Therefore, the rises in sFLT-1 in rats supplemented by saffron might alter the process of blood vessel growth through alteration of angiogenic/ antiangiogenic balances present during normal pregnancy and causing circulating low level of free VEGF and PIGF. Gayatri et al. [27] showed that the abnormal concentration of HIF-1α, the preliminary factor for the hypoxic conditions, causes the inequality in the expression of the angiogenic protein. Increasing tissue oxygen accompanied with saffron treatment may induce part of the antiangiogenic effect, as hypoxia is one of the most important stimulators of angiogenesis [28]. The hypoxia stimulated the expression of sFLT-1 in normal pregnancies where, despite the placental growth in a hypoxic milieu, leads to a 20-fold rise in sFLT-1 expression [29]. The placenta is assumed to be the major source of sFLT-1 during pregnancy [30]. It seemed that the HIF-1α increases in rats who received saffron extract could be contributed to the increased expression of sFLT-1 in both placental and fetus tissues.

The placenta is one of the richest sources of both pro-angiogenic and anti-angiogenic factors. Vascular endothelial growth factor (VEGF) is the most important aspect for all steps of placental vascular formation and development. VEGDF stimulates endothelial expression of proteases that break down the extracellular matrix and release endothelial cells from anchorage, permitting them to migrate and proliferate [31]. The current study showed that the administration of saffron to pregnant rats significantly decreases the concentration of VEGF in maternal plasma and VEGFR in the placenta compared to untreated pregnant rats. No significant changes in rats exposed to gamma-ray as applied in the present study. Interestingly, the changes for these parameters are less expressed in the SRP group of rats when compared to SP rats, despite significant differences from P rats (Figure 3: A-B). These results might premise irregular pregnancy with rats treated with saffron. Ferrara [32] and Shalaby et al. [33] stated that deactivation of a single VEGF allele or interruption of genes encoding VEGF receptors such as VEGFR1 causes embryonic lethality because of anomalous blood vessel development during embryogenesis, signifying a crucial role of VEGF/VEGFRs in vascular formation. Zheng et al. [4] reported that VEGF powerfully arouses placental artery endothelial production of nitric oxide (NO). Nitric oxide, one of the most multifunction signaling molecules at systemic and cellular levels, is actively taking part in trophoblast invasion and placental development [34]. Our results pointed to the increased level of NO in placental and fetus tissues of pregnant rats supplemented by saffron, while the level of NO decreased in rats exposed to gamma radiation compared to pregnant rats. Despite significant increases recorded in pregnant rats treated by saffron and exposed to gamma radiation compared to pregnant rats, the increases are less pronounced compared to the PS group (Figure 3: C-D). The increases in NO in pregnant rats treated with saffron could show pregnancy problems. Herr et al. [35] stated that vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (FLT-1) (as signaling molecules which are expressed during angiogenesis) exert their effects in part through NO synthesis. High NO levels can prevent angiogenesis and its effect on cell survival and proliferation depends on its concentration. Tikvica et al. and Myatt [38-39] reported that some pregnancy diseases, such as IUGR and PE, display a high concentration of placental NO and nitrosative stress deprived of acceptable placental vascularization.

Besides, our data reveals that the apoptotic regulators caspase3 and granzyme B endures significant increases in both placental and fetus tissues of rats supplemented with the saffron extract. In rats treated with saffron and exposed to gamma irradiation, the increases are less manifested compared to saffron treated rats (Figure 4: A-D). Barlett [40] showed that apoptosis is essential for development, including neural development, reduction of oocytes at birth and shaping of fingers and vestigial organs. Some cells quickly proliferated during development undergo apoptosis, the process necessary for many steps in development, including neural

development, reduction in egg cells (oocytes) at birth, and the shaping of fingers and vestigial organs in humans and other animals [40]. Therefore, understanding the mechanisms involved in physiological and in disturbed or dysregulated apoptosis may lead to the development of alternative methods of preventive treatment of various developmental abnormalities.

The development of obstetric complications and underlying pathogenesis of different diseases of the fetus could be attributed to the disruption of apoptotic regulatory mechanisms which results in apoptosis inhibition or hyper-activation [41].

The apoptotic regulators caspase 3 and Granzyme B are notably over-expressed in both placental and fetal tissues of pregnant rats treated with saffron, pregnant rats exposed to gamma radiation, or rats treated with saffron and exposed to gamma radiation. However, the increase is less pronounced with rats treated with saffron and exposed to gamma radiation. The increases in apoptotic regulators accompanied with decreases in angiogenic regulators might be mutually pointed to pregnancy problems because of saffron. Saffron reestablished the superoxide dismutase, catalase, and malondialdehyde levels and increased the expression of active caspase 3 and granzyme as observed in our results. These results are consistent with the finding of Fikry et al. [42] who reported that saffron administration enhanced the formation of the active form of caspase3 and inhibited the formation of MMP9. Cleaving of caspase 3 plays a vital role in the induction of apoptosis. Caspase activation occurs through the release of cytochrome c from the mitochondria. The mitochondrial outer membrane permeability and cytochrome c release are directly activating effector caspase (caspase 3 and 7) which execute the apoptotic program [43]. The increased granzyme in the PS group triggers caspase-independent target cells. Granzyme enforces mitochondria to generate reactive oxygen species (ROS) that disrupt the transmembrane potential but does not perambulate the mitochondrial outer membrane leading to mitochondrial damage which is critical to granzyme-induced cell death[44].

The assessment of LDH protein expression in placenta and fetus tissues revealed increases in LDH expression in both placenta and fetus tissues of pregnant rats supplemented with saffron extract compared to pregnant rats. No significant changes were observed in PR and PRS rats. Exposure to the low dose of gamma radiation in sync with supplementation with saffron extract could reduce by the certain way the damages induced by saffron on placental and fetus tissues (Figure 5: A-C). LDH is expressed widely in body tissues, an enzyme found in almost all living cells. It is a sign of common impairment and disease because it is released during tissue damage. He et al. [45] reported that the low birth weight of infants is associated with an increase in serum LDH levels. However, Qublan et al. [46] stated that there is no significant overtone between pregnancy disorders and LDH level. In our study, the increases in placental and fetus tissues, and plasma LDH, could prove the existence of certain pregnancy difficulties which might be related to saffron intake.

Based on all the previous, it could be suggested that diet including saffron could be dangerous on maternal or fetus health and increase the incidence opportunity of miscarriage or incomplete development of the embryo. This could be attributed to the effect of saffron in placental angiogenesis and apoptotic regulators, and that joined to the disturbances of redox tone and NO concentration in placental and fetus tissues. The synchronized exposure to the low dose of gamma irradiation and saffron supplementation could reduce the effect of saffron in the certain process as observed in the current study. It could be recommended avoiding using saffron during pregnancy stages.

Authors' Contributions:

The 2 authors have participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript, conducting the experiments and NMElF wrote the manuscript and GElT contributed in reformatting the main document following the author instruction.

Declaration of conflicting interests

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