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CYTOLOGICAL STUDY ON RAT ISOLATED HEPATOCYTES AND POSSIBLE PROTECTION OF GINGER (EXTRACT AND NANOPARTICLES) AGAINST ACETAMINOPHEN TOXICITY

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ABSTRACT

Ginger is one of most important medicinal plants which used in variable diseases. Recently, ginger nanoparticles have been isolated from ginger extract and exhibited anti-apoptotic activity, hepatoprotective effect against alcohol and may effectively reduce colitis. Our study aimed to compare between the effect of ginger extract and ginger nanoparticles at concentration of 60 µg/ml against hepatotoxicity caused by acetaminophen (APAP; N-acetyl-para-aminophenol) at concentration of 0.1 mg/ml using primary isolated rat hepatocytes. Cytotoxicity was determined by assessing cell viability and leakage of cytosolic enzymes, such as (ALT& AST). Oxidative stress was investigated by measuring levels of MDA and catalase enzymes. The cytopathological lesions were detected by light microscope. Exposure of isolated rat hepatocytes to APAP caused cytotoxicity and oxidative injury, manifested by loss of cell viability and significant increase of enzymes leakages. Also, APAP caused progressive depletion of catalase content and increase in intracellular MDA accumulation, in addition to alteration in histological structures of the cells. Pretreatment of hepatocytes with either GE or GNPs ameliorated the hepatotoxicity, oxidative stress and enzymatic leakage induced by APAP. However, GNPs were more effective compared to ginger extract pre-treated hepatocytes.

Key words: Acetaminophen, Ginger extract, Ginger nanoparticles, Isolated hepatocytes, Cytology, Rats.

INTRODUCTION

Liver is considered as one of an important organs which responsible for the metabolic process in our bodies (Adam et al., 2016). The continuous using of synthetic drug could induce oxidative stress and tissue injury of multiple organs including liver (Rashid et al., 2016). One of these drugs is acetaminophen (APAP) which used in treatment of fever and reducing of pain (Kandemir et al., 2017). At a high dose, APAP could induced liver injury which confirmed by using lab animals and isolated hepatocytes in multiple studies (Woolbright & Jaeschke, 2017). Acetaminophen caused liver toxicity by production of N-acetyl-p-benzoquinonemine (NAPQI), which exhibit mitochondrial oxidative stress, apoptosis, necrosis and DNA fragmentation (Ramachandran & Jaeschke, 2017).

Recently, herbal medicines are got more attention by scientists especially after appearance of antibiotic resistant bacteria and increasing of the side effects of synthetic drugs (Mekuriya & Mekibib, 2018). One of most popular herbal plant is ginger, which has variable pharmacological activities such as anti-inflammatory, cardiovascular activities (Yang et al., 2014), anti-cancer activities (Dhanik et al., 2017), and heptoprotective effects (Hamed et al., 2012 ; Gabr et al., 2019). Also, ginger used to manufacture of artificial nanoparticles such as Fe3O4, ZnO Ag, and Au in process named by “green synthesis”(Dhayalan et al., 2017 ; Stan et al., 2017).

Ginger has veterinary importance, it can increase the rate of feed up take by improving the appetite of animal (Shams Al-dain and Jarjeis, 2015). Also, ginger has the ability to reduce the recovery time in equine after racing event and hard exercise (Mekuriya & Mekibib, 2018). On the other hand, ginger can promote the weight gain and performance of poultry (Khan et al., 2012) and being helpful in treatment of Mycoplasma gallisepticum (Sutardi et al., 2015). As well as, protecting the fish from microorganisms, including bacteria causing skin lesions such as E. coli and Staphylococcus aureus and fungi (Shakya, 2015).

Ginger (Zingiber officinale Roscoe), an indigenous plant in tropical Asia and probably in southern China and India, has a long life, cane-like plant with annual leafy stalks (Hassan & Hassan, 2018). The pharmacological properties of ginger are attributed to its active ingredients such as gingerols and shogaols, which exhibit a strong anti-inflammatory and anti-oxidant activities (Dhanik et al., 2017). Shogaols are the dehydrated form of gingerols (Wang et al., 2017). In spite of the structure similarity of both shogaols and gingerols (Sirichote et al., 2008), Shogaols exhibit stronger antioxidant than gingerols due to it was absorbed faster and the t1/2 was longer than gingerols (Li et al., 2019).

Natural nanoparticles which isolated from edible plant act in a way like mammalian exosomes, they are containing protein, lipids and miRNA. Moreover, natural nanoparticles are easily produced relative to synthetic NPs (Zhang et al., 2017). In addition, Isolation of natural nanoparticles from plant can provide a safe method to drug delivery within the body without induced side effects such as synthetic nanoparticles (Abdu et al., 2017). Ginger nanoparticles (GNPs) is one of natural nanoparticles which isolated from ginger extract are characterized by stability in stomach (Zhang et al., 2017), and easily distributed from the intestine to the liver which consider the targeted organ by GNPs (Zhang et al., 2015). Ginger nanoparticles did not induce changes in the body weight, blood parameters and histological structures of different organs (Zhang et al., 2018). On the other hand, GNPs have been reported to exhibit hepatoprotective effect (Zhuang et al., 2015; Bakr et al., 2019), and treatment of intestinal disease (Zhang et al., 2017), and colitis (Zhang et al., 2018). Thus, in our in vitro study we assessed the hepatoprotective properties of isolating ginger nanoparticles relative to ginger extract using acetaminophen as a hepatotoxic compound and compare the results with our previous in vivo study (Bakr et al., 2019), to examine the possibility of using GNPs in drug delivery without causing any side effects.

MATERIAL AND METHODS

Animals

This study was performed by using five Sprague Dawley adult male rats (120 - 150g) were obtained from VACSERA Company (Egypt). All methods used in this protocol were accepted by the Institutional Animal Care and Use Committee (IACUC) as defined in the Cairo University guidelines (Approval ID: CUIJIS5617). The animals were housed in polyethylene plastic cages at ambient temperature of (25 ± 2°C), humidity
(40-50%), and a light- dark cycle of 12 hours. Rats were adapted for 7 days before start of the study supplied with pelleted balanced diet and tap water *ad libitum*.

**Chemicals**

Acetaminophen was purchased as prepared finished powder from Sigma Chemical Co. (Saint Louis, USA).

**Preparation of ginger extract**

Fresh ginger roots were purchased from local market. The dried roots of ginger were compressed into powder by using blender. 125 g of the powder were macerated in 1 L of distilled water for 12 hr at room temperature and were then filtered through a 5 µm filter to get the final extract. The concentration of the extract is 24 mg/ml (Sakr *et al.*, 2011). We used this stoke to prepare GE at concentration equal to 60 µg/ml based on the previous *in vitro* study (Pashaei-Asl *et al.*, 2017).

**Preparation of ginger nanoparticles**

Quantity of washed roots equal to 200 gm were powdered by using a mixer at the highest speed for 10 min. After that, ginger juice was then centrifuged in sequence as following (1,000 g for 10 min/ 3,000g for 20 min /10,000g for 40 min), then pellet was resuspended in 3 ml of PBS and referred to as microparticles. The supernatant was then spin at 150,000g for 90 min by using ultra speed centrifuge followed by resuspension of the pellet in 3 ml PBS. After that a freeze- drying process was made to remove water from nanoparticles after it is frozen and placed under vacuum to keep NPs stable for long time, this process named by (lyophilization). We used this stoke to prepare NPs at concentration of equal to 60 µg/ml (Zhuang *et al.*, 2015).

**Characterization of nanoparticles: Morphology and Size**

Lyophilized (unhydrated) ginger nanoparticles morphology, diameter, and size distribution were determined using scanning electron microscopy (SEM) (XL-30 ESEM- FEG SEM, FEI Company, USA). Average diameters of 500 particles were determined from SEM images (n=3) using image analysis software (ImageJ, National Institutes of Health, version 1.5a, ImageJ.nih.gov) (Mahmoud *et al.*, 2019).

**Isolation of hepatocytes**

The hepatocytes were isolated from rat by a collagenase two-step perfusion technique as described by Abdel-Aleem *et al.* (2014). Rat was anaesthetized by using ketamine (100 mg/kg ip). The abdominal cavity was incised to reveal the liver which perfused in situ with calcium perfusion. The abdomen was then open and the liver was perfused with calcium chloride containing 5 mM calcium chloride, for 10 min. The freshly isolated hepatocytes were filtrated through cotton gauze and centrifuged for 2 min at 600 rpm. Isolated hepatocytes were washed twice by low-speed centrifugation followed by resuspension in HEPES-bicarbonate buffer containing 0.5% bovine albumin (pH 7.4). After that the cells were suspended Trypan Blue in Krebs Hanseliet buffer to assess the viability albumin (~85–90%).

**Experimental Protocol**

In this study, the concentration of acetaminophen (APAP) was adjusted to 7 mM (0.1 mg/ml) (Abou-Zeid *et al.*, 2016). Ginger extract and ginger nanoparticles were prepared in phosphate buffer saline (PBS) at 7.4 PH at concentration of 60 µg/ml (Pashaei-Asl *et al.*, 2017). The concentration of freshly prepared isolated hepatocytes was 5×10⁶ cell/ml, the cells were divided into 6 groups (2 ml of the suspended cells/tube). The groups were treated as following, G1: served as control negative, G2: treated with GE only, G3 treated with GNPs only, G4: treated with APAP only, G5: treated with APAP + GE, G6: treated with APAP + GNPs. Ginger extract and ginger nanoparticles were preincubated with hepatocyte 30 minute prior to acetaminophen exposure in group 5 and 6 respectively.

**Assess the viability of cells**

Evaluation of cell viability was assessed by using trypan blue at two time points (0 and 120 minutes), by using the light microscope (Abdel-Aleem *et al.*, 2014)

**Determination of enzymes leakage percent**

ALT and AST were assessed using reagent test kits purchased from SPECTRUM co. (Germany) according the method described by Alaa *et al.* (2016).

**Determination of oxidative stress in liver homogenate**

Lipid peroxidation (MDA) and catalase activity (CAT) were assayed by the method described by Weng *et al.* (2019).

**Cytopathological evaluation of isolated hepatocytes**

Ten µl of cell suspension from all groups at 120 min were placed on a glass slide and left to dry at room temperature. The cells were fixed by adding 10µl of absolute ethanol on the dried film and left to dry at room temperature. The slides were stained with hematoxylin and eosin according to (Kienan, 1999) and examined using BX43 light microscope and captured using Olympus BX 43 microscope linked to Olympus DP 27 CellSens dimensions software (Kienan, 1999).

**Statistical analysis**
One-way analyses of variance [ANOVA] was used to determine statistical significance followed by Tukey post-tests. Statistical analyses were performed using Software Graph Pad [*p <0.05, **p <0.01, ***p <0.001].

RESULTS

Nanoparticles characterization

The morphology of GNPs is shown in (Fig.1) GNPs demonstrated a spherical morphology. The average diameter of GNPs measured from SEM images were 102.3 to 998.3 nm. This is in agreement with previous data of Zhuang et al. (2015).

Cell viability

Cell viability was assessed by using trypan blue. The results were obtained by counting viable and died cells of three fields under the light microscope at two time points (0 and 120 minutes) where died cells proved by presence of trypan blue inside their cytoplasm however viable cells were impermeable to trypan blue. A significant decrease in cell viability was observed after exposure to APAP compared to control cells. Neither GE nor GNPs exposure induced any significant change in the percentage of viability from the control. On the other hand, concurrent preincubation of the cells with GE or GNPs before exposure to APAP inhibited the decrease in the cell viability caused by APAP alone (Fig.2).

Determination of in vitro cytotoxicity

Determination of cytotoxicity and damage of cell membrane was evaluated by measuring the level of both ALT and AST enzyme at different point times (0, 60 and 120 min.). Exposure of hepatocytes to APAP resulted in a significant increase in the leakage of ALT and AST enzymes from hepatocytes compared with other groups especially at 120 min (P<0.001). Pretreatment of hepatocytes with GE or GNPs ameliorated the effects of APAP on enzyme leakage which become more notable at 120 min., but it was more significant in group which preincubated with GNPs (Fig. 3&4). On the other hand, neither GE nor GNPs caused any change in the enzyme’s leakage at any time points.
Figure 3. Showing the mean values of ALT in all groups at different point times; (*P<0.05, **P<0.01, ***P<0.001).

Figure 4. Showing the mean values of AST in all groups at different point times; (*P<0.05, **P<0.01, ***P<0.001).

Assessment of oxidative stress-induced by acetaminophen in isolated hepatocytes was done by measuring cellular MDA and CAT levels. APAP caused significant increase in MDA content from isolated rat hepatocytes compared to control and other groups at 0, 60 and 120 min. (P≥0.05, P≥0.01 and P≥0.001, respectively). Neither GE nor GNPs alone showed any effect on MDA content at all time points. However, concomitant incubation of cells with GE and APAP or GNPs and APAP led to decrease the level of MDA relative to G4 (Fig. 5).

Figure 5. Showing the mean values of MDA in all groups at different point times; (*P<0.05, **P<0.01, ***P<0.001).

On other hand, exposure of hepatocyte to APAP induced drop in CAT level when compared with control hepatocyte at 60 and 120 min. While preincubated cell with GE and GNPs prevent the depletion in CAT level which induced by APAP exposure. Moreover, there were no change in CAT levels in GE and GNPs treated cells in group 2 and 3 respectively (Fig. 6). The pre-incubation of GNPs with APAP treated hepatocytes achieved the best improvement of the hepatic oxidative status when compared with GE.
Cytopathological analysis

On cytological level, control group (G1) showed normal hepatocytes which may be present as small groups (2-6 cells) or single cells. Normal hepatocytes are round or polygonal cells which have either one or two basophilic round/oval nuclei with fine dispersed chromatin, and granular cytoplasm which surrounded by intact cell membrane (Fig. 7A&B). The presence of debris and leukocytes in the background may be occurred. There were not any cytopathological changes in neither GE nor GNP's treated hepatocytes in (Fig.7C&D).

Acetaminophen treated hepatocytes (G4) showed cytopathological changes such as pyknosis, necrosis, foamy cytoplasm due to presence of multiple round clear vacuoles and cell lysis (Fig. 8).
Fig. 8 Hepatocytes of APAP treated group (G4) showing, (A) Hepatocellular cytoplasm appears foamy due to presence of multiple round clear vacuoles, nucleus appear pyknotic and move to peripheral side. (B) Two hepatocytes, one of them showing pyknotic nucleus (arrow) and the other one showing necrosis with complete lysis of nucleus. (C) Three hepatocytes (arrows) showing necrosis and complete lysis of nucleus. (D) Nucleus undergo lysis associated with complete ballooning of hepatocytes. All slides stained with H&E.

While hepatocytes treated with GE and APAP in (G5) showed pyknosis (Fig. 9A), vacuolar cytoplasm (Fig. 9B), hydropic degeneration associated with lysis of nucleus (Fig. 9C), and necrosis (Fig. 9D). On the other hand, minor cytological cytopathological changes were observed in GNPs+APAP treated hepatocytes. Most of hepatocytes appeared with normal cellular structure (Fig. 9E), while the other cells showed minor vacuolation (Fig. 9F).

Fig. 9 (A) Hepatocytes of GE+APAP treated group (G5) showing pyknotic nucleus with deep eosinophilic cytoplasm. (B) Single hepatocyte from G5 showing single vacuole with pyknotic nucleus and irregular cell membrane. (C) Hepatocytes from G5 showing vacuolar degeneration of cytoplasm (arrow), moreover the nucleus undergoes lysis. (D) Two hepatocytes from G5, one of them showing necrosis (arrow), and the other one showing single vacuole with pyknotic nucleus. (E) Single hepatocyte from GNPs+APAP treated group (G6) showing normal eosinophilic granular cytoplasm with central nucleus. (F) Single hepatocyte from GNPs+APAP treated group (G6) showing minor vacuoles in cytoplasm. All slides stained with H&E.

**DISCUSSION**

Liver is an important organ which included in metabolism, detoxification and production of plasma proteins such as clotting proteins. Isolated hepatocytes provide a simple model to study liver disease and evaluated the hepatotoxicity, in addition to metabolism of xenobiotics (Guguen-guillouzo, 2002; Khater et al., 2020). Another characteristic is that, isolated hepatocytes are stable under different conditions. So, isolated hepatocytes are the system of choice to study the hepatoprotective effect of some plant extracts. In general, chemically induced liver damage is more readily investigated in tissue culture than in whole animals (El-tawil & Moussa, 2006).

This study aimed to evaluate the protective effect of ginger in both extract and nanosize form against cytotoxicity induced by acetaminophen on isolated hepatocytes. Since there are few studies evaluating the *in vitro* protective effects of ginger nanoparticles and ginger extract on primary
isolated liver cells, acetaminophen was adjusted to 7 mM (0.1 mg/ml) to hepatocytes to induce marked cytotoxicity according to (Abou-Zeid et al., 2016). Ginger extract and ginger nanoparticles were prepared at concentration of 60 μg/ml (Pashaei-Asl et al., 2017). Isolated hepatocytes were preincubated with GNPs and GE before exposure to APAP with 30 min to evaluate their hepatoprotective effects.

Our study showed a significant reduction in cell viability and increase in ALT & AST leakage percent from the hepatocytes which exposure to APAP compared to control cells. These results are attributed to the ability of APAP to induce acute hepatocellular damage through a complicated consequences which include: (1) Transformation of cytochrome P450 into reactive compound which lead to reduction of glutathione ad bind with cellular protein; (2) production of ROS which cause damage of the cell membrane; (3) raised oxidative stress, combined with changes in calcium homeostasis causing change in mitochondrial permeability and loss its ability to produce ATP; (4) loss of ATP which leads to necrosis (Hinson et al., 2010; Imam et al., 2020).

Concurrent preincubation of the cells with GE and GNPs before exposure to APAP inhibited the decrease in the cell viability caused by APAP alone but it was more significant in hepatocytes incubated with GNPs. These results were attributed to the ability of ginger to preserve the structure of the cell membrane (Hasan et al., 2016). On the other hand, there was non-significant variation between GE and GNPs treated cells (G2&G3) when compared with control cells (G1) in decreasing viability with increasing AST and ALT enzymatic leakage.

Exposure of primary isolated rat hepatocytes to APAP significantly increased the MDA content and decreased the CAT levels. The hepatotoxic effect of acetaminophen is due to its conversion to N-acetyl-p-benzoquinone-imine (NAPQI) which binds with glutathione. Once the depletion of glutathione store happened, the production of ROS and induction of oxidative stress occurred (Bunchornwatakul & Reddy, 2013). Our study revealed that preincubation of the cells of (G5&6) with both GE and GNPs lead to adjustment the levels of MDA and CAT. These results were attributed to phenolic active ingredient presents in ginger such as [6]-gingerol, [6]-shogaol which have a strong antioxidant activity in vitro because of their ability to be donors of hydrogen atoms and to scavenger of the free radicals (Syafitri et al., 2018). On other hand, ginger was reported to decrease the gene expression of anti-oxidant enzymes such as GSH, SOD, and MDA (Jeena et al., 2013) and decrease the expression of both superoxide production and catalase (Ahmed et al., 2008).

On the cytological level, APAP treated hepatocytes showed morphological and pathological changes such as pyknosis, fatty degeneration, and necrosis. Acetaminophen could induced cellular alteration by up-regulation of proinflammatory cytokines such as caspase-3, caspase-1, TNF-α and interleukin-1β (Lin et al., 2014). Moreover, Kupffer cell reported to induce inflammatory mediator in addition to the role of infiltrated neutrophils in production of proteases and superoxide anion radicals which caused cell damage (Tachihamaya et al., 2011). but in our in vitro study we cannot suggest the interaction with the other cells. However, we can evaluate the primary effect of early APAP toxicity on the isolated hepatocytes depending on mitochondrial dysfunction theory (Abou-Zeid et al., 2016). Reactive metabolites of acetaminophen induce modification of mitochondrial proteins and increased the cytosolic calcium and peroxynitrite levels within the cells, which lead to formation of extra covalent bonds with cellular proteins producing mitochondrial dysfunction, increase the mitochondrial membrane which followed by releasing of the ingredients of ginger nanoparticles into the cytoplasm, resulting in cellular necrosis. Also, oxidative activity of acetaminophen could induce disturbance in microvilli which causing cell ballooning (Sakr & Salaby, 2012). Moreover, we could assess the possible mechanism of APAP toxicity under normal physiological media with estimation of the different pathological change and cellular interaction from our previous in vivo study (Bakr et al., 2019).

Ginger extract and ginger nanoparticles improved the histological architecture of hepatocytes by preservation the integrity of cellular membrane due to its antioxidant effect (Gholampour et al., 2017). In our study, Ginger nanoparticles showed more hepatoprotective effect against acetaminophen than ginger extract. It may be attributed to the small size of nanoparticles which makes them proper for therapeutic effects. Several study reports that, nanoparticles less than 100nm can enter cells, at less than 40 nm they can enter cell nucleus, and less than 35 nm they can pass the blood brain barrier (Dutta & Brahmacary, 2009).

Additionally, GNPs serve as stronger antioxidant against free radical compared to ginger extract. This result is attributed to shogaol which regulate the genetic expression of many antioxidant and detoxification enzymes (Abdu et al., 2017). Shogaols which carried by GNPs are in bind form in contrast the shogaol carried by GE which present in free form. So, less amount of shogaol carried by GNPs required for obtaining the equal effects on the hepatocytes compared with GE (Zhuang et al., 2015). Besides, shogaols in the GNPs have an critical role in the production of Nrf2 (Nuclear factor erythroid2 related factor 2) which regulate H2O2 production (Li et al., 2016). Reactive metabolites of acetaminophen induce modification of mitochondrial proteins and increased the cytosolic calcium and peroxynitrite levels within the cells, which lead to formation of extra covalent bonds with cellular proteins producing mitochondrial dysfunction, increase the mitochondrial membrane which followed by releasing of the ingredients of ginger nanoparticles into the cytoplasm, resulting in cellular necrosis. Also, oxidative activity of acetaminophen could induce disturbance in microvilli which causing cell ballooning (Sakr & Salaby, 2012). Moreover, we could assess the possible mechanism of APAP toxicity under normal physiological media with estimation of the different pathological change and cellular interaction from our previous in vivo study (Bakr et al., 2019).

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Ginger nanoparticles was confirmed in vitro by using fluorescent and flow cytometry (Zhang et al., 2018). Additionally, GNPs serve as stronger antioxidant against free radical compared to ginger extract. This result is attributed to shogaol which regulate the genetic expression of many antioxidant and detoxification enzymes (Abdu et al., 2017). Shogaols which carried by GNPs are in bind form in contrast the shogaol carried by GE which present in free form. So, less amount of shogaol carried by GNPs required for obtaining the equal effects on the hepatocytes compared with GE (Zhuang et al., 2015). Besides, shogaols in the GNPs have an critical role in the production of Nrf2 (Nuclear factor erythroid2 related factor 2) which regulate H2O2 production (Li et al., 2016). Reactive metabolites of acetaminophen induce modification of mitochondrial proteins and increased the cytosolic calcium and peroxynitrite levels within the cells, which lead to formation of extra covalent bonds with cellular proteins producing mitochondrial dysfunction, increase the mitochondrial membrane which followed by releasing of the ingredients of ginger nanoparticles into the cytoplasm, resulting in cellular necrosis. Also, oxidative activity of acetaminophen could induce disturbance in microvilli which causing cell ballooning (Sakr & Salaby, 2012). Moreover, we could assess the possible mechanism of APAP toxicity under normal physiological media with estimation of the different pathological change and cellular interaction from our previous in vivo study (Bakr et al., 2019).

CONCLUSION

from our study we could conclude that, transforming the natural particles of ginger to nanoparticles (nanosizing) is supposed to give good therapeutic results by reducing the parameters of liver toxicity and providing a safe method for drug delivery especially to the liver (Xiao et al., 2014), counter to artificially synthesized nanoparticles which reported to deliver small doses of drugs to specific cells, but with limitation such as in vivo toxicity and limited production range (Zhang et al., 2017). Critically, GNPs exhibited excellent biocompatibility relative to ginger extract in vitro and in vivo according our pervious study (Bakr et al., 2019).

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