Effects of quinazolinones on Balb/C mice embryonic livers

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Heterocyclic compounds such as quinazolinones have variety of biological and pharmacological properties (anticancer, antiinflammatory, antimicrobial, antimalaria, etc.). Effects of two new quinazolinones viz., 4(3H)-quinazolinone-2-propyl-2-phenylethyl (QPPE) and 4(3H)quinazolinone-2-ethyl-2-phenylethyl (QEPE) were investigated on Balb/C mice embryos livers—the major organ of metabolism and detoxification of drugs and toxins. Histological and pathological studies demonstrated QPPE and QEPE as producers of toxic metabolites after biotransformation, creating necrosis, fatty changes, increase in the number of band cells, hepatocytes' diameters and alkaline phosphatase, in addition to sinusoid dilation, hemorrhages and hyperemia. Transmission electron micrographs showed lipid droplets in hepatocytes' cytoplasm, necrosis, vacuolization, cytoplasm disintegration, disfigured and swollen mitochondria, irregular and abnormal nuclei, nuclei with heterochromatin, condensed chromatins, myelin figures and autophages in injured hepatocytes. In conclusion, QPPE and QEPE make toxic components after biotransformation injuring membranes and creating inflammatory reactions. They also disturb metabolism of lipids pathways and cause the appearances of lipid droplets in hepatocytes.

Keywords: Embryonic liver, Quinazolinones, Teratogen

Teratogens are medications, chemicals, and infectious diseases or environmental agents that may interfere with normal development of embryos or fetuses which result in loss of pregnancies, pregnancy complications or birth defects. There are relatively common varieties of teratogens and the word teratogen is used to denote the result of a hazard assessment on a particular agent (for purpose of this guidance, a drug). The use of this term indicates that drugs have the capacities to produce abnormal development in an embryo or fetus under certain exposure conditions1,2.

Quinazolinones and condensed quinazolinones have potent CNS activities like analgesic, anti-inflammatory, sedative-hypnotic and anticonvulsant properties. Thiadiazol of quinazoline nucleus is also associated with diverse pharmacological activities such as antibacterial, antifungal, phosphodiesterase inhibitory, anti-inflammatory, platelet aggregation inhibitory, anti-hypertensive, anti-malarial, DNA repair enzyme, poly ADP-ribose polymerase-1(PARP-1), anticancer, anti-neoplastic and tubulin polymerization inhibitors3-9.

Liver is the major organ of metabolism and detoxification of foreign compounds entering the body and it is therefore exposed to a wide variety of exogenous and endogenous products including environmental toxins and chemicals present in foods or drinks10.

In continuation of previous studies11-19, the present study has been undertaken with an aim to investigate the effects of two new derivatives of quinazolinones [4(3H)-quinazolinone-2-propyl-2-phenylethyl (QPPE) and 4(3H)quinazolinone-2-ethyl-2-phenylethyl (QEPE)]20 on the histological, biochemical and intracellular structures of Balb/C mice embryonic livers21.

Materials and Methods

Balb/C mice22 (100, 3-4 months old ) originally obtained from Pars Company (Tehran, Iran), and randomly bred in Department’s animal house were housed at 24°±1°C, 50±0.5% RH and 12:12 h L:D controlled conditions. They were provided with lab chow and tap water. Virgin females (about 30 g) which mated males overnight were considered to be on day 0 of pregnancy12,13,17. The pregnancy was confirmed by presence of vaginal plugs.

QPPE and QEPE were obtained from Department of Chemistry, Shahid Beheshti University. The most effective dose of QPPE and QEPE was 100 mg/kg/body
weight. The Balb/C mice were divided into 4 groups of 25 animals each and on day 10 of pregnancy, the animals in each group received following treatments:

Gr 1: QPPE (100 mg/kg body weight, ip)\textsuperscript{19,20}
Gr 2: QEPE (100 mg/kg body weight, ip)\textsuperscript{19,20}
Gr. 3: Sham treated with 0.05% methyl cellulose\textsuperscript{23} (solvent, at 10 ml/kg body weight, ip)
Gr. 4: Control, received distilled water (10 mg/kg body weight, ip).

The treated mice were anaesthetized with chloroform on day 17 of pregnancy. The embryos were removed by cesarean section. The liver were excised for histopathological and cell structural studies. The band cells of livers of embryos of all four groups were counted in ten randomly chosen areas, using graded slides and the mean numbers were measured for each group.

**Statistical analysis**—The data were analyzed with statistical packages for social sciences (SPSS, version 15). For determining significant differences and differences in proportions to categorical variables between two or more groups, Kruskal-Wallis and Mann-Whitney U tests in addition to independent sample test and excel softwares were used for drawing histogrammes. Level of significance difference was $P<0.05$.

Liver sections (4 µm thick) from formalin-fixed samples were stained with H&E and Reticulin.

Immediately after sacrifice, ultra thin sections from 1 mm\textsuperscript{3} piece of the liver of 17 day old mice embryos from all the groups were fixed in 20 g/l glutaraldehyde and were examined under transmission electron microscope (TEM)\textsuperscript{24}.

**Results**

Morphological observations of randomly chosen 17-days old mice embryos from four groups showed abnormal embryos (thoracogastroshesis), underdeveloped embryos (Fig. 1A and B), enlarged disfigured placentas (Fig. 1C and D), severe hemorrhages in the neck region (Fig. 1E) and large swollen amelia embryo with hemorrhages in the neck region (Fig. 1F).

Light microscope observations demonstrated normal livers in 17-day old embryos of mice of control and sham groups with no changes (Fig. 2A and B) but an accumulation of lipid droplets (fatty changes), increase in the diameters of hepatocytes (Figs 2C, D and 3) and number of band cells (Figs 2E, F and 4), hyperemia, congestion and dilation of sinusoids (Fig. 2 G-L), hemorrhages (Fig. 2J and K), steatosis and necrosis (Fig. 2M and N), normal reticulum in the hepatocytes of livers of mice embryos from control and sham groups (Fig. 2O and P) and disintegrated reticulum (Fig. 2Q and R) were observed in the hepatocytes of embryos livers treated with QPPE (Fig. 2C, E, I, G, K) and QEPE (Fig. 2D, F, H J, L-N).

There were significant differences ($P<0.05$) between minimum and maximum weights and lengths of 17 days old embryos of Balb/C mice of control, sham and QPPE and QEPE treated groups (Figs 5 and 6).

Transmission electron microscopic studies revealed no changes in livers and hepatocytes of randomly chosen 17-days embryos of control and sham (treated with methyl cellulose) groups (Fig. 7A and B), but

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**Fig. 1**—Demonstrating abnormal (yellow arrow, thoracogastroshesis, A), underdeveloped embryo (B), enlarged disfigured placentas (C, D), severe hemorrhages in the neck (E, white arrow) and very large swollen embryo with hemorrhages in the neck (white arrow) of 17 days old embryos of mice treated with QPPE and QEPE (F)
Fig. 2—Light microscope studies of H & E (purple) and reticulin (yellowish green) staining showed normal livers in 17-day old embryos of Balb/C mice of control and sham groups (A, B) while accumulation of lipid droplets (fatty changes), increase in the diameters of hepatocytes (C, D) and number of band cells (E, F), hyperemia, congested and dilated sinusoidal capillaries (G-L), hemorrhages (J, K), steatosis, necrosis (M, N), normal reticular fibers in livers of 17-day old embryos of Balb/C mice of control and sham groups (O, P), and disintegrated reticular fibers (Q, R) in hepatocytes of livers of 17-days old embryos of Balb/C mice treated with QPPE (C, E, I, K, M, Q) and QEPE (D, F, H, J, K-N) were visible (I, J, 100×, others, 400×)
lipid droplets (Fig. 7C and D), myeline figures (Fig. 7D and E), disintegrated abnormal (round without cristae) large swollen mitochondria (Fig. 7E-H), cytoplasm disintegration (Fig. 7I and J), autophages (Fig. 7K-M), irregular and abnormal heterochromatin (Fig. 7G-I and M-O) were observed in the hepatocytes of livers of 17-day embryos mice treated with QPPE and QEPE.

Discussion

Teratogenic exposures can cause an embryo or fetus to develop abnormally. Factors like the gestational timing of the exposure, dose, route and nature of the agent itself determine whether an agent is teratogenic.

To induce malformations, teratogens must pass through placenta or reach the developing embryo through some other routes which make the phenomenon of teratogenesis applicable to all organisms including those in which embryonic development occurred outside the mother. Methyl cellulose (injected to the sham mice) had no teratogenic effects on the embryos of treated mice. Therefore, observation of high percentages of disorders mice embryos of experimental groups may be due to the injections of QPPE and QEPE or their active metabolites might have crossed the placenta creating abnormalities.

Mitochondria play central roles in cell metabolism and are major source of reactive oxygen species (ROS) production in the cell. Mitochondrial respiratory chains are small, highly reactive oxygen-containing molecules and are naturally generated in small amounts during body metabolic reactions. They can be toxic to the cells because they react with quite a few macromolecules such as proteins, lipids, DNA, and damage complex molecules.

Early signs of mitochondrial dysfunctions are productions of ROS, swelling of mitochondria, ATP depletion, failure of Ca\(^{2+}\) homeostasis, perinuclear clustering of organelles, activation of few proteases in particular calpains and cathepsins, lysosomal and ultimately plasma membrane ruptures.

Another major source of ROS especially in the liver is a group of enzymes such as cytochrome P\(_{450}\) mixed-function oxidases. Many different variants of these iron-
Fig. 7—Normal organelles such as nuclei and mitochondria in hepatocytes of 17-days old embryos of Balb/C mice of control and sham groups (A, B) but fatty changes (lipid droplets) (C, D), myeline figures (D, E), disintegrated abnormal (round without cristae) enlarged swollen mitochondria (E-H), cytoplasm disintegration (I, J), autophages (C, K, M), irregular and abnormal heterochromatins (G-I, N, O) were observed in the hepatocytes of livers of 17-days old embryos of Balb/C mice treated with QPPE and QEPE. (A, B, D, I, K, N, 7000×), (E, F, H, O, 30000×), (J, 20000×), (G, L, M, 12000×), (C, 4400×). [Nuclei=yellow arrows, mitochondria=blue arrows, lipid=red arrows, cytoplasm disintegration=orange arrows, RER=magenta arrows, myeline figure=green arrows]
containing enzymes exist some of which are responsible for removing or detoxifying a variety of compounds present in our environment and ingested (e.g., foods or drugs including alcohol). The biochemical reactions spurred (i.e., catalyzed) by cytochrome P₄₅₀ use molecular oxygen and small amounts of ROS are generated during these reactions.

ROS produce active groups after being affected by cytochrome P₄₅₀ and metabolized in livers and kidneys since quinazolinones have no active groups. Thus injuring cells and their organelles’ membranes create necrosis in hepatocytes. The results of the present study demonstrated necrosis and disrupted mitochondria. It is concluded that QPPE and QEPE could have damaged mitochondria producing ROS as a result causing hepatocytes’ injuries or QPPE and QEPE may have induced an increase in the activity of the enzyme cytochrome P₄₅₀ which metabolizes these drugs and other molecules and in turn generates ROS. Then QPPE and QEPE can increase production of ROS, enhance peroxidation of lipids, proteins, and DNA.

Myelin figures are conformational changes of membrane proteins and lipoprotein layers. Myelin figure formation is one of the features of drug toxicity. They appear as concentric membranous lamellar structures encapsulating thin bands of cytoplasm separated by electron lucent and elongated vacuolar spaces. Some myelin figures are observed enclosing dense round bodies resembling disintegrated mitochondria or secondary lysosomes.

By having the ability to metabolize glucose and fatty acids, liver is a main organ in energy homeostasis. Oxidation of fatty acids occurred in three subcellular organelles with β-oxidation confined to mitochondria and peroxisomes and CYP4A-catalyzed ω-oxidation occurring in endoplasmic reticulum. Disturbances in fatty acid oxidation and the failure of energetic processes in hepatocytes (different fatty acid oxidation systems) caused by genetic, toxic (including drug related) and metabolic perturbations also result in decreasing energy burning in the liver leading to the lipid storage in liver cells. Futher, chronic mitochondrial dysfunctions can lead to diseases characterized by lipid metabolism disorders and pathological triglyceride (TG) accumulations.

Liver is susceptible to secondary insults including a vulnerability to ROS, gut-derived endotoxins and adipocytokines such as tumor necrosis factor (TNF-α) and other cytokines. Fatty change is the most severe form of reversible cell damage. It is characterized by the presence of small and large vacuoles (microvesicular and macrovesicular) within hepatocytes. Droplets of fat press the nucleus and cytoplasm against the cell membrane. The disturbances of lipid peroxidation in hepatocytes are the consequences of oxidative stress and correlate with the stage of necroinflammatory changes of steatosis and necrosis. On the other hand, fatty acid overloads in hepatocytes acts both as a substrate and an inducer of microsomal cytochrome P₄₅₀ and fatty acid oxidation systems that generate ROS resulting in oxidative stress.

Oxidative stress that damages lipid peroxidation and mitochondria, occurs in chronic liver injuries such as metabolic disturbances, iron deposition and high fatty acid concentrations. Fatty acid cytotoxic activity is suggested to influence cell survival. Long-term accumulations of lipids may lead to liver lesions such as liver necrosis and fibrosis. Necrosis involves swelling and rounding-up of cells, loss of cell membrane integrity and spilling of cell content into the surrounding environment; cytoplasm disintegration is accompanied by occurrence of small irregular clumps of condensed chromatin in the nucleus. It is the end-result of bioenergetic catastrophe resulting from ATP depletion to a level incompatible with cell survival and was thought to be initiated mainly by cellular accidents such as toxic insults or physical damages. Liver cell necrosis is almost always accompanied by an inflammatory reaction presumably through the liberation of factors from dead cells. Cells that die by necrosis frequently exhibit changes in nuclear morphology.

Hepatic macrophages are in an activated state and able to secrete variety of products that may interact with hepatocytes and other liver cells. These products include cytokines such as TNF-α, interleukin-1 (IL-1) and interleukin–6 (IL-6), and ROS intermediates and proteolytic enzyme. Each of these products may be toxic to hepatocytes in several forms of toxic liver injuries.

Macrophages have been shown to be an essential cofactor for full hepatocellular necrosis to have occurred, thus not only may the hepatic infiltration with macrophage be important to the subsequent repair process, but these inflammatory cells may also be the source of some of the hepatocellular damages.
circularizing mononuclear cells. Once these cells are activated they may further release chemoattraction. Autophagy is the mechanism by which cells get rid themselves of damaged and superfluous organelles which capture the organelles targeted for autophagy and create double-walled structures that are isolated from the cytoplasm autophagy (removes excess and damaged organelles from the cell)\(^9\). It is therefore plausible that such a degree of cellular destruction could lead to cellular demise. Induction of autophagy may also cause necrotic cell death\(^6,14,42\).

Elevation of the band (neutrophilic) count is not specific for infection but may be secondary to inflammatory processes, tissue damages or necrosis, neoplasia, intoxication, metabolic abnormalities, hemorrhages and hemolysis or drugs\(^50\).

Hemorrhage and necrosis were observed in the present study. Liver sinusoids became dilated, blood congestion were also observed under the effects of QPPE and QEPE; presumably sinusoidal endothelial cell damage caused hemorrhage. On the other hand, hepatocyte toxicity could be created by several forms of cell injuries including hypoxia, hyperthermia, chemical toxicity and oxidative stress, all of which result in plasma membrane blebbing, cytoplasmic vacuolization and necrosis which is characterized morphologically by vacuolization of the cytoplasm, breakdown of the plasma membrane, dilation of cytoplasmic organelles (in particular mitochondria\(^51\)), swollen mitochondria and induction of inflammation around the dying cells attributable to the release of their contents and proinflammatory molecules. QPPE and QEPE injured membranes, caused inflammatory reactions after biotransformation. They also disturbed metabolism of lipids pathways, lipid droplets appeared in hepatocytes of livers of embryos of Balb/C mice treated with QPPE and QEPE.

References

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