

Research Article

EFFECT OF MATERNAL CAFFEINE AND RETINOIC ACID INTAKE ON LIVER DURING PRE- AND POSTNATAL DEVELOPMENT OF MICE, *MUS MUSCULUS*

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ABSTRACT

The present study was conducted to evaluate the effect of caffeine and retinoic acid as the active metabolite form of vitamin A on the liver at pre- and postnatal periods of development. In either the pre- or the postnatal period of study, caffeine was able to perturb carbohydrates, increase calcium and decrease iron those accompanied with down regulated expression of TGF β 2 in hepatic tissue as compared to control. Treatment with retinoic acid at both doses in the present study either separate or combined with caffeine upregulate calcium against suppression of iron. Moreover, TGF β 2 show biphasic that severely down regulated during gestation and extended to express intranuclear at postnatal in liver tissue. Decreased iron overload of liver with concomitant down regulation of TGF β 2 expression represent the most important points of this study that protect the liver of young age from oxidative damage and suppress the gene-dependent extracellular matrix deposition. These data may contribute to the therapeutic uses of these substances in preventing the progression of liver damage in hepatitis diseases.

Keywords: Caffeine, Retinoic acid, TGF β 2

INTRODUCTION

Caffeine is a methylxanthines of secondary plant metabolite derived from purine nucleotides (Ashihara and Crozier, 1999). The most well known methylxanthines are caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), which occur in tea, coffee, cacao and a number of other non-alcoholic beverages of plant origin. In 2001 the Committee on Toxicity of Chemicals in Food, UK, concluded that although caffeine intake >300 mg/day might be associated with low birth weight and spontaneous miscarriage, the evidence was inconclusive (COT, 2001). In pregnancy, caffeine is the most widely consumed xenobiotic with the potential to adversely affect the developing fetoplacental unit. Caffeine intake of ≥ 300 mg/day has been associated with fetal growth restriction (Grosso *et al.*, 2001; Bracken *et al.*, 2002; Clausson *et al.*, 2002). The amount of caffeine and metabolites available to the fetoplacental unit depends on the maternal caffeine metabolism and clearance, which shows marked variation between individuals because of genetic and environmental factors (Rasmussen *et al.*, 2002; Grosso *et al.*, 2006; Boylan *et al.*, 2008). The mean caffeine intake throughout pregnancy was recommended to be reduced than the limit of 300 mg/day as recommended (COT, 2001; Bech *et al.*, 2007). However, the consumption of caffeinated beverages during pregnancy is quite common (Brent *et al.*, 2011). Both clinical data and animal experiments indicate that caffeine has the potential to induce reproductive and embryonic toxicity (Vik *et al.*, 2003; Momoi *et al.*, 2008; Huang *et al.*, 2012). Epidemiological investigations also indicate that caffeine intake during pregnancy is associated with impaired fetal growth that

preferentially and adversely affect fetal skeletal (Bakker *et al.*, 2010). In addition, prenatal caffeine exposure before and during pregnancy were found to increase the incidence of fetal absorption and still birth in mice, also the bodyweights and body lengths of the liveborn offspring were severely retarded (Huang *et al.*, 2012). However, other studies report that caffeine intake during pregnancy is not associated with changes in birth weight or length (Clausson *et al.*, 2002; Bech *et al.*, 2007).

Moreover, the requirement of the developing mammalian embryo for retinoic acid is well established. Retinoic acid (RA) belongs to the retinoid family and includes the isoforms all-trans retinoic acid (ATRA), 9-cis retinoic acid (9-cis-RA), and 13-cis retinoic acid (13-cis-RA). Retinoic acid, the active form of vitamin A, can be generated from retinol and retinyl ester obtained from food of animal origin, and from carotenoids, mainly β -carotene of vegetables and fruits. The mammalian embryo relies on retinol, retinyl ester and β -carotene circulating in the maternal bloodstream for its supply of vitamin A. RA is involved in various physiological processes, such as embryonic development, reproduction, vision, cell growth, differentiation, apoptosis, inflammation and maintenance of epithelial tissues (Marletaz *et al.*, 2006; WHO, 2009; Tanumihardjo, 2011; Zhou *et al.*, 2011; Zhou and Qin, 2012). It has been shown also that intake marginally above the recommended dietary intake with low clearance during pregnancy is associated with embryonic malformations. The classical signs of hypervitaminosis A occur in skin, nervous system, musculo-skeletal system, circulation, and internal organs, as well as in the fetus following an excessive dietary intake or an intake of drugs containing large doses of specific retinoids (Blomhoff and Blomhoff, 2006).

From the above mentioned data, it is clear that both of caffeine and vitamin A sharing:- 1) A rapid absorption and decreased clearance during pregnancy and in new born 2) Ease of placental transfer without degradation 3), Adverse effects on fetoplacental unit, skeleton and visceral organ. So, the aim of the present study was to find out further details relationship effects to resolve the controversy between the psychoactive-ergogenic food item (caffeine) and hypervitaminosis A in developing embryos and neonates as their dams consume these substances during pregnancy and lactation. Also, early feed pups were included as they become fed independently.

MATERIALS AND METHODS

Caffeine: Caffeine, anhydrous pure crystals (Merck, C₅H₁₀N₄O₂, 194.2 g/mol) was obtained commercially. Stock solution in saline was prepared and renewed as required during the experimental period.

Retinoic acid (the active metabolite of vitamin A): Retinoic acid (C₂₀H₂₈O₂, 300.4 g/mol), the active metabolite of vitamin A in the form of 13-cis form (Isotretinoin) product of Sigma 500 mg/package was obtained. In olive oil (Wang *et al.*, 2007), stock solution was prepared and renewed as required during the experimental period.

Animals and experimental design: Immature mice, *Mus musculus*, after weaning were obtained from animal house of Assiut University. Animals were acclimatized in laboratory under normal light and temperature conditions with free access of food and water. Males and females were kept separately till maturity in cages. For the prenatal study at days 14, 18 of pregnancy, one male was mixed with two females in several cages. After the insurance of vaginal plug following copulation in one round of gestation, the pregnant females were classified into six groups from the 7th day of gestation (the first day of the organogenesis in mice) (Stromland *et al.*, 1991), G1 a control (intraperitoneally vehicle treated), G2 administered orally with caffeine at dose (2 mg/100 g bw), G3, G4 intraperitoneally injected with retinoic acid at doses 2, 4 mg/kg bw, respectively. G5, G6 a combined groups caffeine-administered at morning (2 mg/100 g bw) and at evening intraperitoneally injected with retinoic acid (2, 4 mg/kg bw), respectively. Caffeine dosing of pregnant females was carried out according to Case *et al.*, (1996). Meanwhile, isotretinoin dosing was carried out at 2, 4 mg/kg bw, a range of doses that induce different abnormalities on injected at the seventh day of gestation (Sulik *et al.*, 1995). Treatment of pregnant females with caffeine was conducted daily, while retinoic acid treatment was conducted at day after the other day for either the separate or combined treatments from the 7th to the 18th day of gestation. Pregnant females at day 14 and at day 18 of pregnancy for different groups were dissected.

For postnatal study, from pups delivery up to six week of age after birth, newborns were classified into six groups. G1 control (intraperitoneally vehicle treated), G2 caffeine-administered through lactating dams at dose (2 mg/100 g b.w) up to the 3rd week of pups age and then through oral administration up to the six week (the period of independent feeding). G3, G4 intraperitoneally injected with retinoic acid at doses (2, 4 mg/kg b.w), respectively for six weeks. G5, G6 combined groups treated with caffeine and retinoic acid.

Caffeine-administered through suckling up to the third week and then by oral administration up to the 6th week. Treatment of pups with caffeine was conducted daily, while retinoic acid treatment (2, 4 mg/kg bw) was conducted at day after the other day for either the separate or combined treatments similar to the treatments of groups, G3 and G4, respectively. At the 3rd and 6th week of postnatal period, pups of various experimental groups were dissected and fixed for histological, histochemical and immunohistochemical investigations.

Histological and histochemical study: For prenatal study, mice embryos at E14 and E18 of previous groups were fixed in Carnoy's fixative, dehydrated in ethyl alcohol, cleared in methyl benzoate and infiltrated with paraffin wax. Serial sections of embryos at 7 μ thick in paraffin was mounted on glass slides and dried at 40°C. Sections were stained with Haematoxylin and Eosin for general histology, Periodic acid Schiff's (PAS) reaction for polysaccharide detection, Prussian blue for iron detection, Alizarin red S-stain for calcium detection (Drury and Wallington, 1976), Acridin orange/Ethidium bromide stain for nuclear fluorescence (Kasibhatla *et al.*, 2006). For postnatal study at the end of 3rd (suckling period) and the end of the 6th week (independent feeding), liver of different groups were processed for section preparation. Mounted sections were stained with the different stains as indicated above in addition to Masson's stain for collagen at the 6th week postnatal. Sections were dehydrated in ascending grades of ethanol, cleared in xylene and mounted with DPX. Selected sections of the developing liver at 14, 18 day of gestation were photographed and processed as required. Also, sections of the liver at the 3rd and the 6th week were selected, photographed and processed for evaluation in different groups compared to control. Also, the percentage of binucleated hepatocytes at the 3rd and the 6th week were evaluated and compared to control. The comparison of this last evaluation was made by counting cells with binuclear nucleus in 500 hepatocytes per section in various treatments. Counting and imaging were made using a light microscope (Axiolab Standart 20, Carl Zeiss, Jena, Germany) coupled to a video camera (AxionCam, Carl Zeiss, Jena, Germany).

Immunohistochemistry of TGFβ2: In immunohistochemical study, deparaffinized Superfrost/Plus slides-mounted sections of embryos at 14, 18 day of gestation and those of liver at the 6th week postnatal of the different experimental groups were retrieved for re-antigenicity using 10 mM citrate buffer at pH6 in 100°C for an hour (Buchlowalow and Bocker, 2010). After cooling at room temperature, sections were treated for 10 minutes with 0.3% hydrogen peroxide block and then with protein block (phosphate buffer solution, pH 7.6, with 0.5% BSA, 0.5% casein and less than 0.1% sodium azide) for 10 minutes to block nonspecific background staining, then sections were incubated with primary antibody (Rabbit Anti-human TGF β2 polyclonal antibody, Spring Bioscience, USA) and then washed using phosphate buffer and incubated with secondary antibody, Biotinylated Goat Antipolyvalent (Antipolyvalent HRP DAB detection system, Spring Bioscience, USA) according to the manufacture protocol. Reactions and colour were visualized by using chromogene mixed with 3, 3'-diaminobenzidin (1:10) (DAB substrate, chromogen, Spring Bioscience). In all cases, negative control sections

in which the primary antibody not applied to tissue sections were carried out. Sections were dehydrated in ascending grades of ethanol, cleared in xylene and mounted with DPX mounting media.

Processing: In either the histological, histochemical or the immunohistochemical preparations, sections were examined microscopically to evaluate the effect of caffeine, retinoic acid and the co-administration of both in experimental groups compared to the control. Sections of the developing liver of pre- and postnatal periods of study were selected, photographed and processed for evaluation in different groups compared to control. Male embryos were considered for prenatal period of study based on serial sectioning and staining with H&E at gonadal region to identify the testicular tissue. While males of postnatal study were selected following dissection.

RESULTS

14 Days old embryos

At E14 of gestation in control, the liver has the characteristics of hematopoietic tissue in Hematoxylin and Eosin-stained sections. Three types of cells, hemopoietic, hepatoblasts and immature megakaryocytes are distinguished. High density of hematopoietic cells is present throughout the developing organ. Among the hematopoietic tissue, proliferative hepatoblasts are scattered that characterized by large basophilic vesicular euchromatic nuclei of variable size and shape making it difficult to appreciate individual cell shape. The hematopoietic cells present at this stage are nucleated of the erythroid lineage that contains uniformly hyperchromatic nuclei scattered among the hepatoblasts. The third type of cells that can be easily distinguished in Hematoxylin and Eosin-stained sections is the immature megakaryocytes that are characterized with their large size and regular outlines (Figure 1A). In acridin orange/ethidium

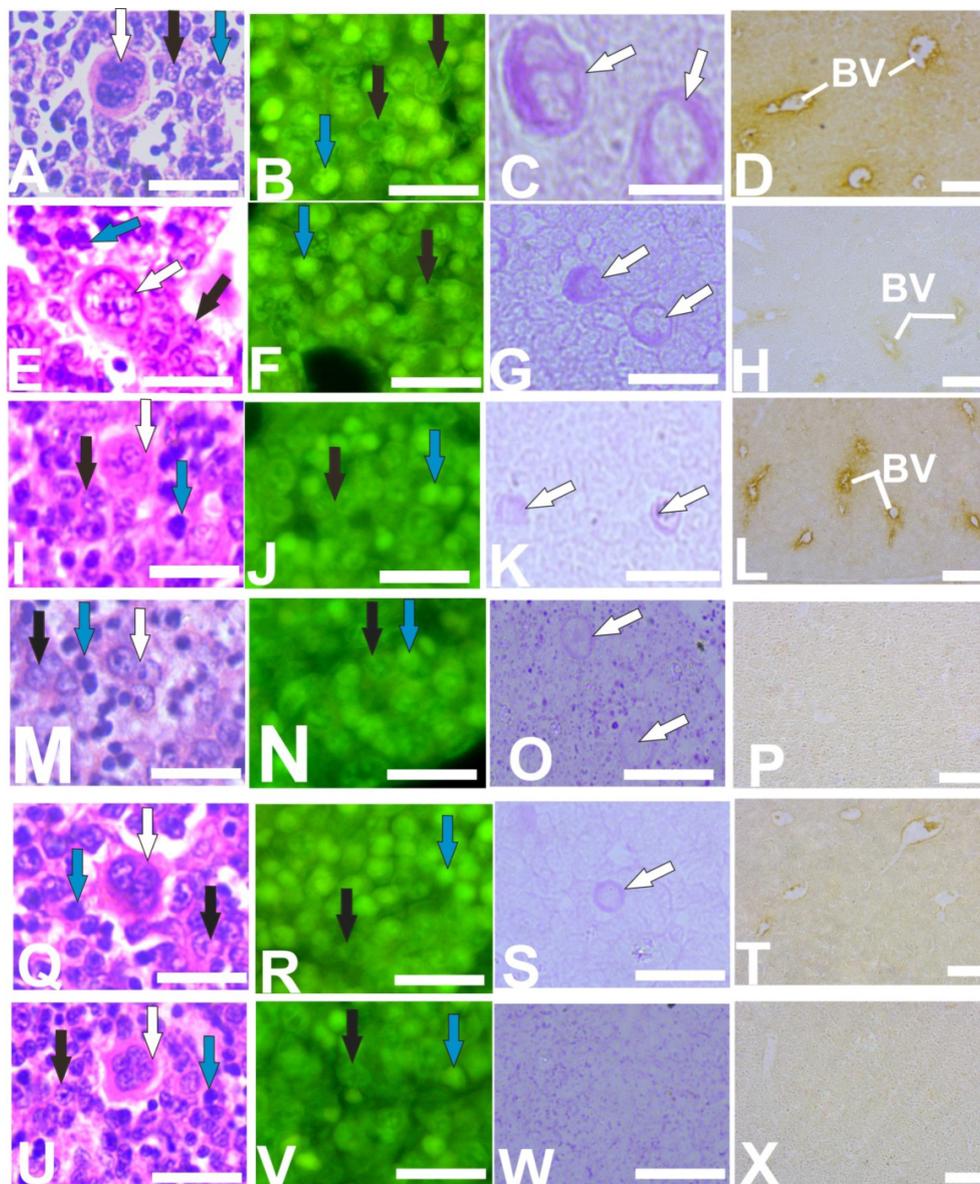


Figure 1: Photomicrographs of liver sections at 14th day of gestation. A-D Control, E – H Caffeine treated, I – L Retinoic acid treated at 2 mg/kg b.w, M – P Retinoic acid treated at 4 mg/kg b.w, Q – T Combined treatment at 2 mg/kg b.w with caffeine, U–X, Combined treatment at 2 mg/kg b.w with caffeine. Megakaryocytes (white arrows), hepatoblasts (black arrows), hemopoietic progenitors (blue arrows), blood vessels (BV). H&E; Acridin orange/Ethidium bromide; PAS and Immunostain of TGFβ2 from left to right, respectively. Scale bar 20 μm.

bromide-stained sections proliferated hepatoblasts have variable fluorescences while the hematopoietic cell nuclei are dense and homogeneously fluorescent (Figure 1B). PAS-stained sections revealed the positive cytoplasm of megakaryocytes (Figure 1C). TGF β 2 stained section revealed intense expression around the developing blood vessels against relatively pale expression throughout the developing parenchyma (Figure 1A and Figure 1D).

In caffeine treated embryos, the developing liver revealed the existence of hyperchromatic hemopoietic and vesicular euchromatic nucleated hepatoblasts similar to those observed in control. The most observed effect of caffeine treatment is a distorted nuclei of the megakaryocytes compared to control (Figure 1E). Acridin Orange/Ethidium bromide-stained sections revealed a well fluorescence of hepatoblasts and hyperchromatic hemopoietic cells similar to control (Figure 1F). Decrease of PAS-stained cytoplasm of the distorted megakaryocytes and severe downregulation of TGF β 2 expression were noted (Figure 1G and 1H) as compared to control.

In retinoic acid treated embryos, the effect seems to be a dose-dependent. Megakaryocytes are the most affected of the constituent cells (hyperchromatic hemopoietic and hepatoblasts) in H&E stained tissue. In 2 mg/kg b.w treated with retinoic acid, embryos distorted nuclei of megakaryocyte were observed against the hyperchromatic hemopoietic cells and hepatoblasts, which look like to those observed in control (Figure 1I). Acridin orange/ethidium bromide stained sections revealed a little effect concerning the fluorescence of hepatoblasts that appeared dark against a well fluorescence of hemopoietic cells (Figure 1J). The effect of 2 mg/kg b.w of retinoic acid is best manifested in PAS stained sections since a decreased stainability of megakaryocytes was noted (Figure 1K) as compared to those observed in control and caffeine treated embryos. In contrast to caffeine effect, TGF β 2 expression was best detected around the developing blood vessels (Figure 1L). In 4 mg/kg b.w of retinoic acid treated embryos, deformed megakaryocytes (Figure 1M), decreased fluorescence of both the hepatoblasts and hemopoietic cells (Figure 1N), accumulation of PAS positive granules and negatively stained macrophages were noted (Figure 1O) as compared to those of control, caffeine and 2 mg/kg bw treated embryos, respectively. In addition, the dose dependent effect of retinoic acid is best manifested in immunostained sections since severe downregulation of TGF β 2 expression was observed around the developing blood vessels (Figure 1P) similar to caffeine effect.

In combined treatment of caffeine and retinoic acid at dose 2 mg/kg b.w megakaryocyte with branched cytoplasm and well organized nuclei, hyperchromatic hemopoietic cells and hepatoblasts are well observed (Figure 1 Q). However, depressed fluorescence of hepatoblasts (Figure 1R), PAS-staining of megakaryocytes (Figure 1 S) and severe downregulation of TGF β 2 expression (Figure 1T) were noted. In combined treatment with 4 mg/kg b.w lobulated megakaryocyte nuclei (Figure 1U), depressed tissue fluorescence (Figure 1 V), decrease in PAS-positive tissue granules (Figure 1W) were the most observed differences

as compared to the effects of 4 mg/kg b. w of retinoic acid treated embryos. Also, the expression of TGF β 2 was severely inhibited (Figure 1X).

18 Days old embryos

At 18th day of gestation, H&E stained sections of control embryos revealed a decline in erythropoietic activity as the hepatocytes gain contact with each other and continue to form the hepatic cords. At this age of development the hematopoietic populations have been restricted to reduced hematopoietic islands, individual cells and regressed megakaryocytes scattered throughout the developing parenchyma of the liver (Figure 2A). PAS-stained sections of control revealed regularly distributed macrophages throughout the hepatic parenchyma. The contents of these cells are heavily stained that make it difficult to differentiate between their nuclei and cytoplasm (Figure 2A/1). While the hepatocytes are stained with a little PAS-positive inclusions. Iron and calcium and TGF β 2 expression homogeneously detected throughout the developing hepatic parenchyma (Figures 2A/2,3,4) respectively. In caffeine treated embryos hepatocyte vacuolation, congested blood vessels and sinusoids were the most observed effects (Figure 2B) compared to control. PAS stained sections revealed inhibition of macrophage content (Figure 2B/1). Also, caffeine treatment results in decreased iron (Figure 2B/2), increased calcium (Figure 2B/3) and down regulated expression of TGF β 2 (Figure 2 B/4).

Retinoic acid treatment was found to provoke decrease in hemopoietic cells, hepatocyte vacuolation and nuclear pyknosis in a dose-dependent manner (Figures 2C and 2D), induced PAS positive contents throughout the hepatic parenchyma (PI 2 C/1, D/1) and decrease in iron content (Figure 2C/2 and 2D/2) and calcium (Figure 2C/3 and 2D/3) while TGF β 2 expression was not changed (Figure 2C/4 and 2D/4) as compared to control.

In combined treatments, hepatocyte vacuolation and nuclear pyknosis were less observed (Figures 2E and 2F) compared to caffeine or retinoic acid treatments. PAS stained sections revealed intense reticular framework between hepatocytes at G6 (PI 2 F/1) compared to the different treated groups. In addition, reduced iron (Figures 2 E/2 and 2F/2), calcium (Figures 2E/3, 2F/3) and down regulation TGF β 2 expression (Figure 2 E/4, F/4) were noted.

Liver of suckling period

At the end of suckling period (3 weeks post natal) in which the newborn receive the food through lactating control and treated mother's the liver was studied at histological and histochemical levels. In control, the characteristic organization of the hepatic parenchyma was noted. Hepatic cords are radiated around central vein those interspersed with sinusoids. Kupffer's cells and lymphocytes are recognized within the lumen of sinusoids (Figure 3A). Binucleated hepatocytes are recognized that represents about 20% of the constituting cells. In all treatments the lobular organization was the same as observed in control (Figures 3B–3F). The most observed difference was confined to the percentage of binucleated hepatocytes in caffeine and in the combined treated groups with the two doses of retinoic acid. The

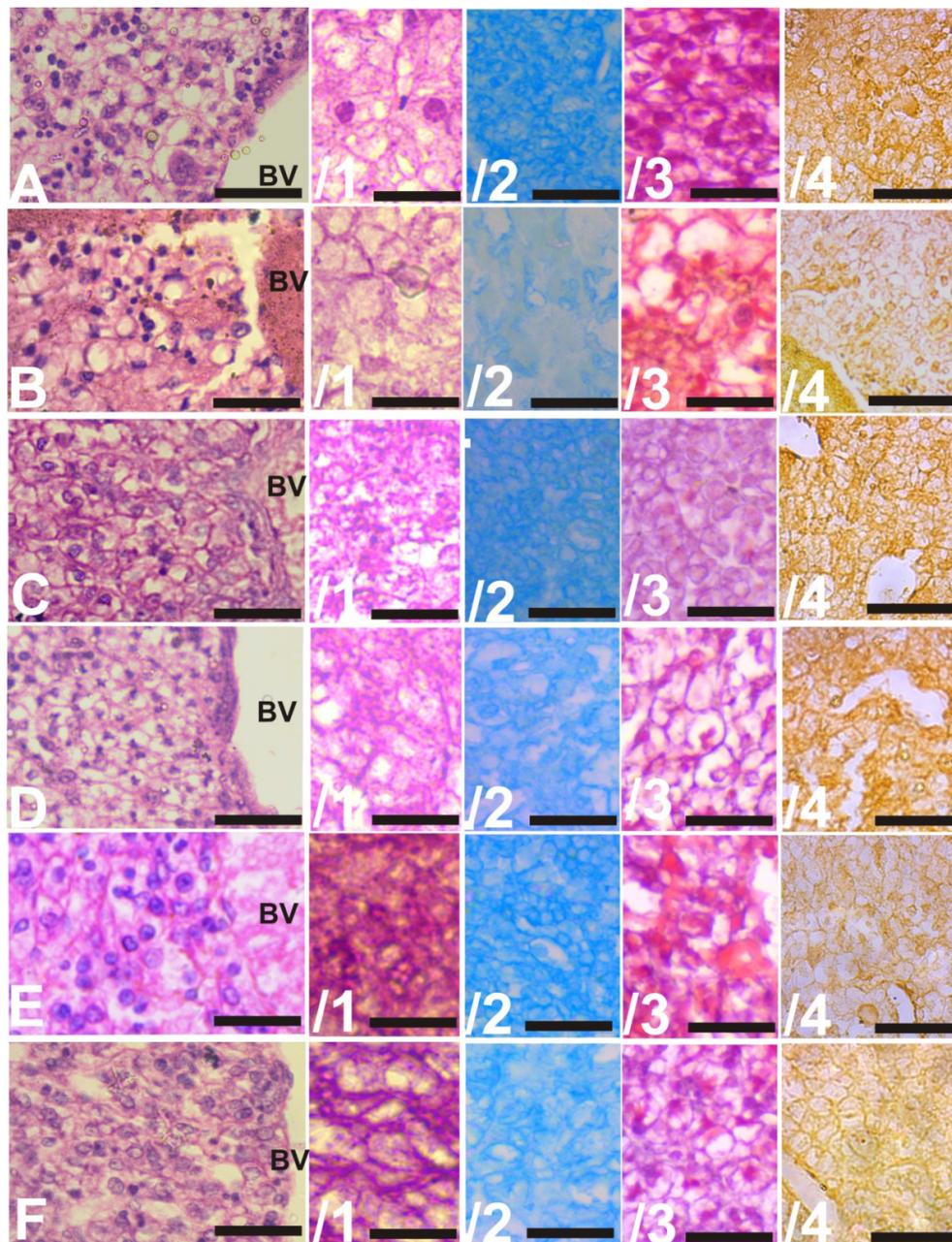


Figure 2: Photomicrographs of liver at 18th day of gestation. A - /4 Control, B - /4 Caffeine treated, C - /4 Retinoic acid at 2 mg/kg b.w treated, D - /4 Retinoic acid at 4 mg/kg b.w treated, E - /4 Combined treatment at 2 mg/kg b.w with caffeine, F - /4 Combined treatment at 4 mg/kg b.w with caffeine. H & E; PAS, Prussian blue; Alizarin Red S stains and Immunostain of TGFβ2 from left to right, respectively. Scale bar 20 μm.

recorded percentages were 12, 11, 10%, respectively. Meanwhile the recorded percentages of the binucleated hepatocytes in retinoic acid treatments at 2, 4 mg/kg b.w of treated mother's look like to that of control that represents 18, 20%, respectively. In PAS stained sections caffeine was found to provoke carbohydrate deposits (Figure 3H) in contrast to retinoic acid at both doses of treatments (Figures 3 I and 3J) those are negatively stained similar to control (Figure 3G). PAS positive deposits in combined treated groups appeared to be retinoic acid dependent. At 2 mg/kg b.w of retinoic acid treatment combined with caffeine carbohydrate deposits appeared (Figure 3K) similar to that of caffeine treatment only. Meanwhile negatively stained deposits at 4 mg/kg b.w of retinoic acid treatment combined with caffeine (Figure 3L)

similar to both of the control (Figure 3G) and retinoic acid treatments at both doses (Figures 3H and 3I), respectively was noted.

Liver of independent feeding

At independent feeding, that begins after weaning up to the 6th week postnatal during the direct treatments the liver was studied. In control the binucleated tetraploid hepatocytes percentage increased (30%) compared to that observed (20%) at the 3rd week of postnatal during suckling. Decreased percentage of the binucleated hepatocytes was encountered in caffeine treatment (14%) and in combined treatment with caffeine and both doses of retinoic acid (15, 11%, respectively) at the same age as compared to control. In retinoic acid

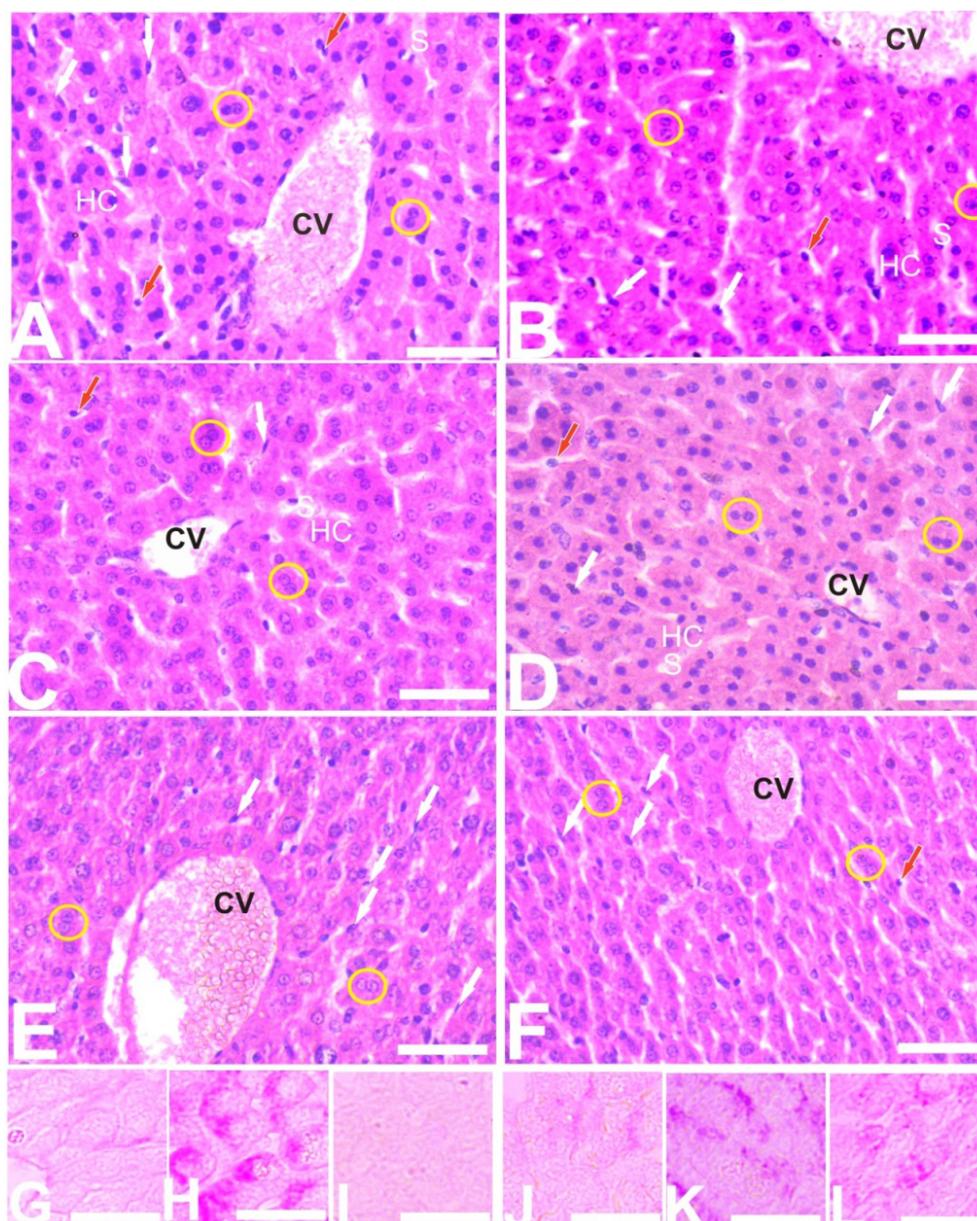


Figure 3: Photomicrographs of liver sections at the end of the 3rd week of suckling. Normal organization in control and all treated groups is shown. A (Control), B (Caffeine treated), C,D (Retinoic acid treated at 2, 4 mg/kg b.w of lactating mother's), E, F (Combined treatments with caffeine and retinoic acid at both doses, respectively). Central vein (CV), sinusoids (S), hepatic cords (HC). Kupfer's cells (white arrows), lymphocytes (red arrows), binucleated hepatocytes (ellipses). H&E stain Scale bar 20 μ m. G-L: PAS stained sections showing PAS-positive hepatocytes in caffeine (H) and in combined treatment at 2 mg/kg retinoic acid with caffeine (K) compared to control (G) and other treatments. PAS stain Scale bar 20 μ m.

treatments, the percentage of binucleated hepatocytes was severely decreased at 2 mg/kg b.w (5%) as compared to the percentage at 4 mg/kg b.w (15%) and to that of control at both the 3rd and the 6th week. The decreased percentages of binucleated hepatocytes were markedly correlated with hepatocyte hypertrophy and reduced of sinusoidal lumen in all treatments.

Histological examination of H&E stained liver sections revealed the general architecture of hepatic parenchyma in both of the control and all treated groups (Figure 4). Lobular organization, central vein, radiated hepatic cords, sinusoids and complementary cells (Kupffer's and lymphocytes) were recognized. The most observed effect of treated groups including infiltrated foci near the central vein and deeper

in the hepatic parenchyma of caffeine treatment was noted (Figure 4B), dose-dependent induction of giant nuclei that accompanied with cell hypertrophy in retinoic acid treatments (Figures 4C and 4D) and vacuolation in combined treatments with caffeine and retinoic acid (Figure 4 E, F) as compared to control (Figure 4A).

In acridin orange/Ethidium bromide stained sections, regular hepatocyte nuclei were noted throughout the liver tissue that intensely fluorescent in control (Figure 4A insert). In caffeine, in addition to infiltrated foci around the central vein and deeper in hepatic parenchyma, pale fluorescence of some hepatocyte nuclei were noted against a well fluorescent inflammatory cells (Figure 4B insert) as compared to control. Retinoic acid treatments results in a dose dependent giant

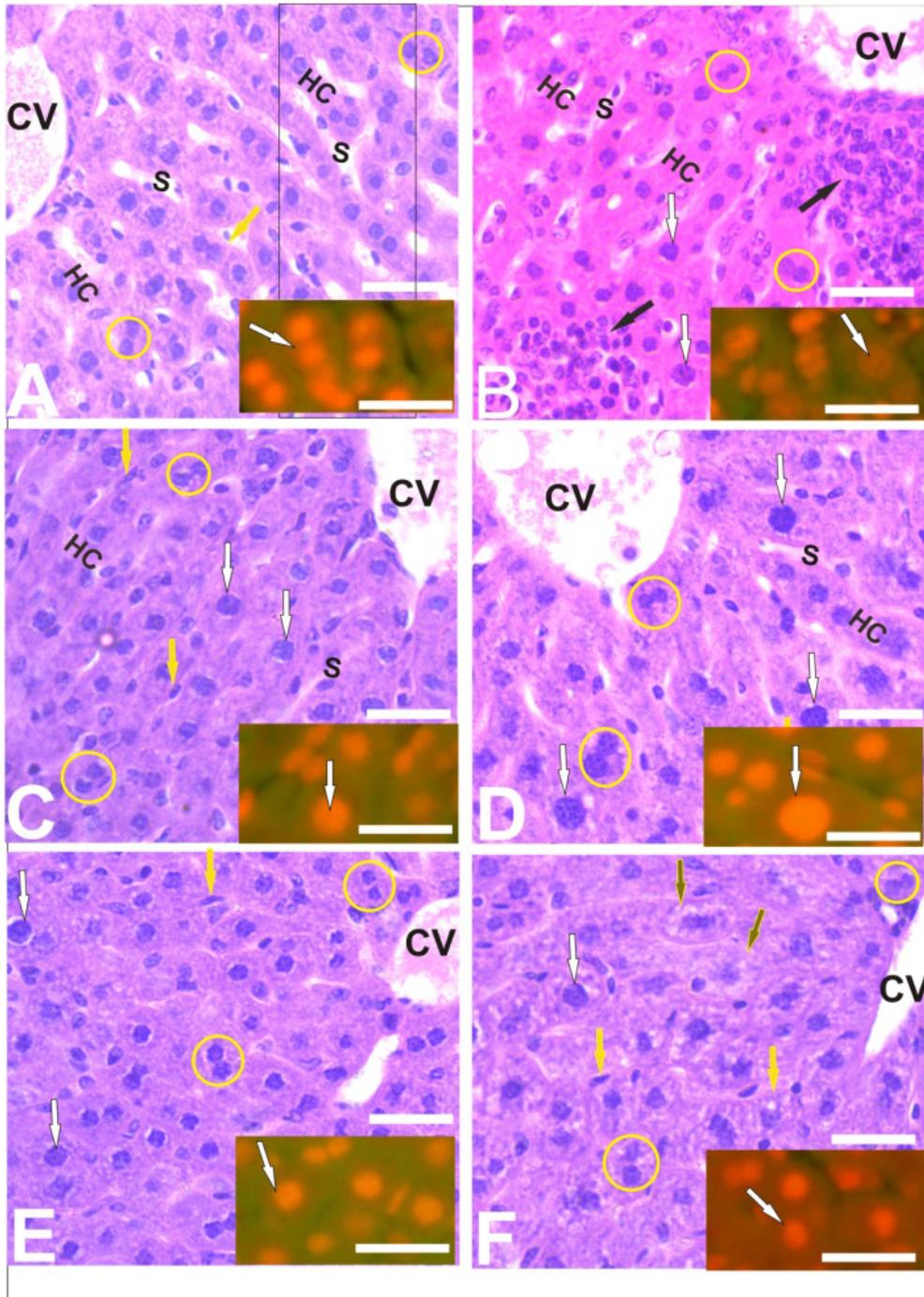


Figure 4: Photomicrographs of liver sections at 6th week postnatal showing hepatic cords (HC), sinusoids (S) and central vein (CV) of the hepatic lobules. Foci of infiltrated cells (black arrows), induced polyploid giant hepatocyte nuclei (white arrows), Kupffer's cells (yellow arrows), binucleated polyploid hepatocytes (ellipses) are indicated. A: Control, B: Caffeine treatment, C: Retinoic acid treatment at 2 mg/kg b.w, D: Retinoic acid treatment at 4 mg/kg b.w, E, F: Combined treatments with caffeine and retinoic acid 2, 4 mg/kg b.w, respectively. H&E stain Scale bar 20 µm. Inserts: Polyploid giant nuclei induction is shown in retinoic acid treatment at 4 mg/kg b.w compared to control and the other treatments. Acridin orange /Ethidium bromide stain. Scale bar 20 µm.

nuclei induction with well fluorescence (Figures 4C and 4D inserts) in contrast to caffeine treatment. Giant nuclei induction was concomitant with a well-recognized Kupffer's cell that lines the sinusoidal cavity. Combined treatments with caffeine and both doses of retinoic acid don't reveal either the paleness or giant nuclear induction (Figures 4E and 4F inserts) as compared to caffeine or retinoic acid treatments.

In Masson's stain for collagen detection, thicker wall of

central veins was noted in caffeine (Figure 5B) and retinoic acid treatment (Figure 5D) at 4 mg/kg b.w as compared to both the control (Figure 5A), retinoic acid treatment (Figure 5C) at 2 mg/kg b.w and in combined treatment with caffeine and retinoic acid at both doses (Figures 5E and 5F), respectively. In addition, caffeine treatment results in fibrotic foci (Figure 5B) in the inflammatory foci that observed in H&E stain. From the other hand, retinoic acid treatment at 4 mg/kg b.w however, did not initiate the fibrotic foci it

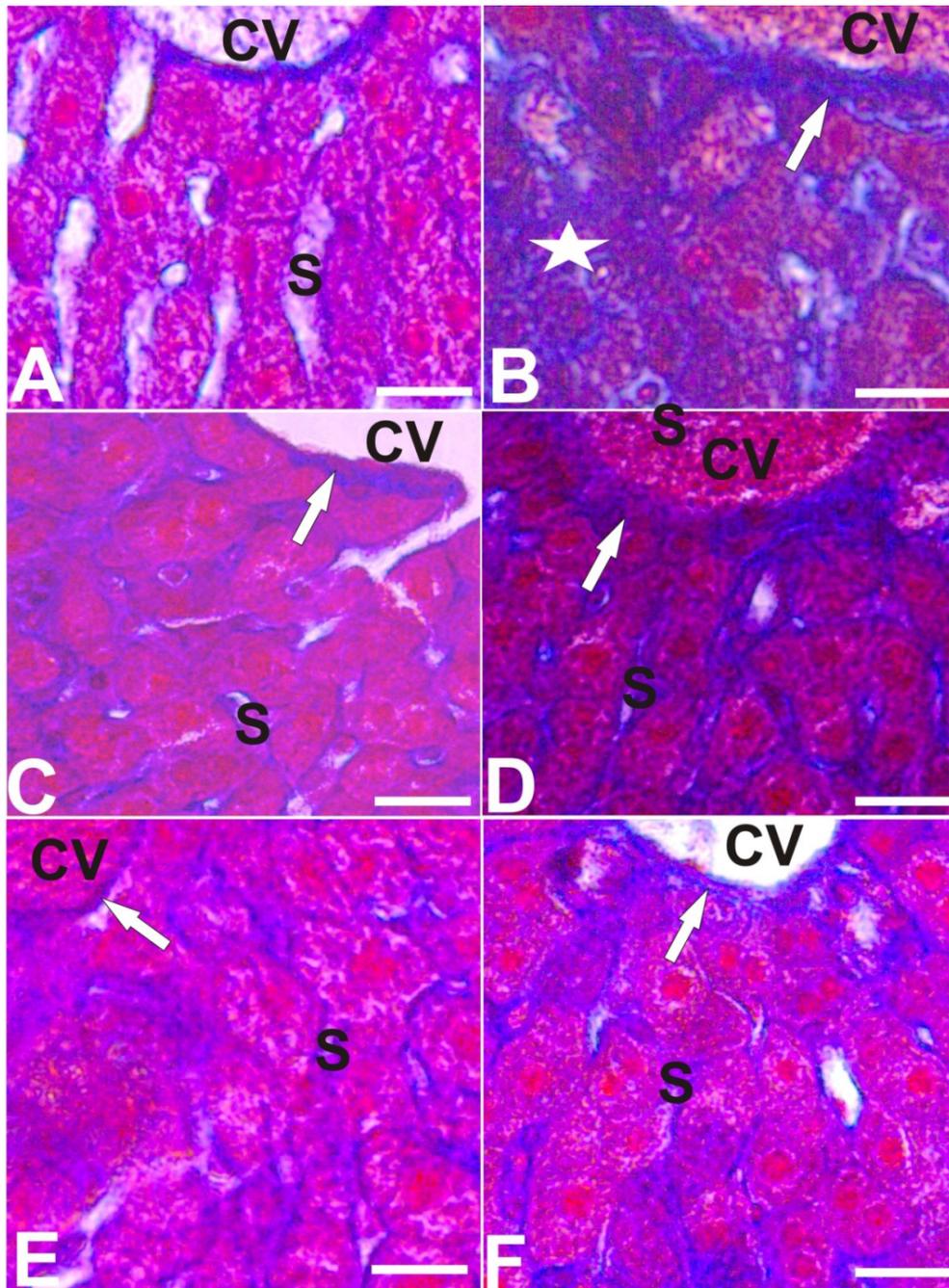


Figure 5: Photomicrographs of liver sections at the 6th week postnatal showing thickening in the wall of central vein in caffeine- (B) and retinoic acid treatment at 4 mg/kg b.w (D) compared to control (A), retinoic acid treatment at 2 mg/kg b.w (D) and to the combined treatments (E, F) (white arrows). Fibrotic foci (star) and heavily stained collagen in sinusoids were noted in caffeine and in 4 mg/kg b.w of retinoic acid treatments. Central vein (CV), (S) sinusoids. Masson's stain. Scale bar 15 μ m.

induce intense collagen stainability around the hypertrophoid hepatocytes (Figure 5D) as compared to either the control or to the combined treatments.

PAS stained sections revealed uniform distribution of carbohydrates within the hepatocytes of control (Figure 6 A). Caffeine (Figure 6B), retinoic acid at 2 mg/kg b.w (Figure 6C) or in combination treatment with retinoic acid at 2 mg/kg b.w (Figure 6E) result in decreased carbohydrate store of hepatocytes as compared to control. In contrast, positive carbohydrate detection was noted in retinoic acid at 4 mg/kg b.w (Figure 6 D) and in combined treatment with caffeine (Figure 6F) however, the carbohydrate content was decreased as compared

to control. Calcium detection in Alizarin red S stained section revealed high stainability of calcium in the hepatic parenchyma in caffeine treatment (Figure 6H). Increased stainability of calcium was also noted in retinoic acid treatment at 4 mg/kg b. w (Figure 6J) and in the combined treatment with caffeine and retinoic acid at both doses (Figures 6L and 6M) as compared to control (Figure 6G) and retinoic acid treatment at 2 mg/kg b. w (Figure 6I). In contrast to calcium detection, prussian blue stained sections for iron revealed a decrease in iron in hepatic parenchyma in caffeine (Figure 6 N), retinoic acid at both doses separately (Figures 6O and 6P) or in combination with caffeine (Figure 6Q and 6R) as compared to control (Figure 6M).

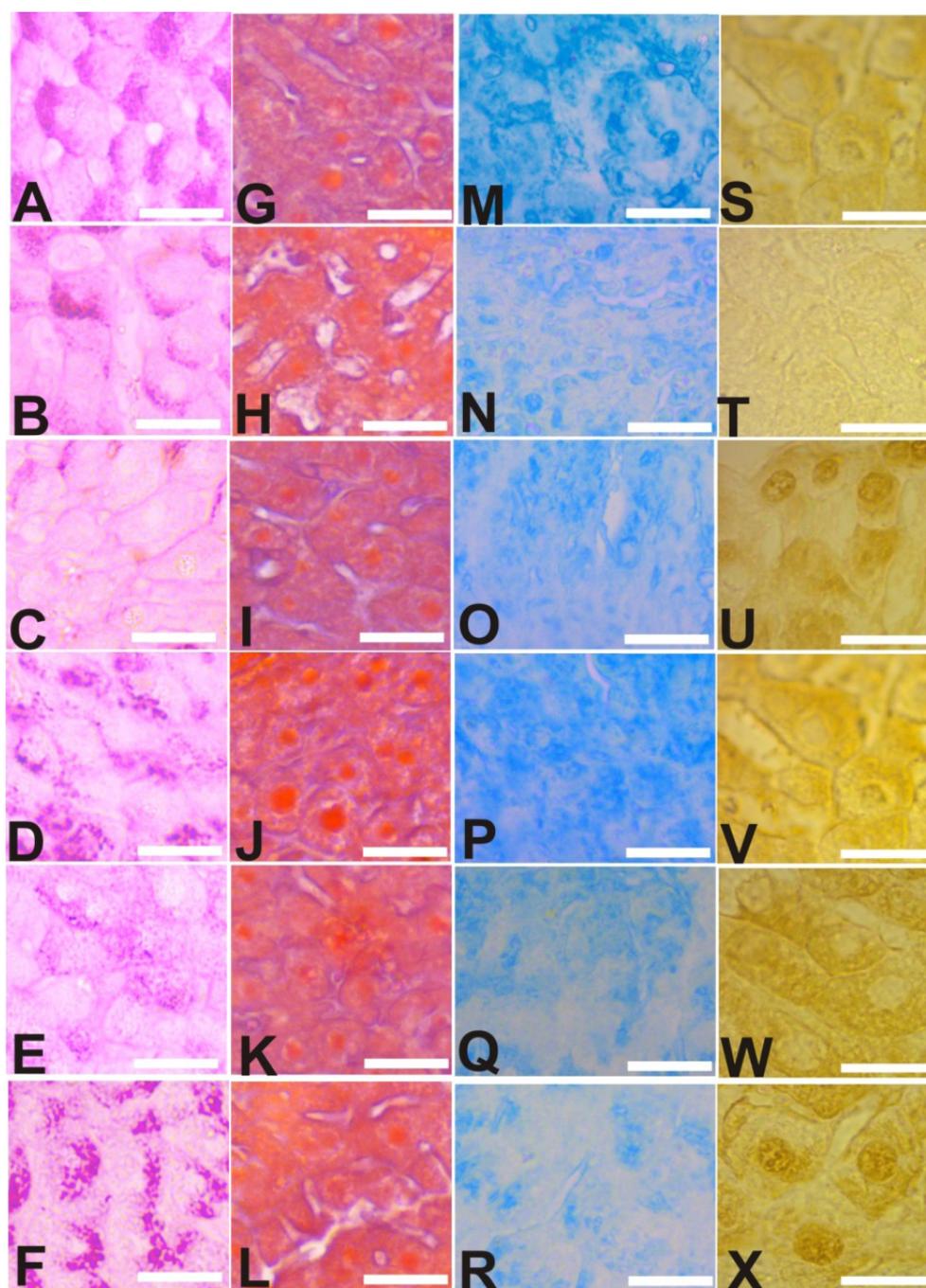


Figure 6: Photomicrographs of liver sections at 6th week postnatal: A–F: PAS stained sections showing decreased carbohydrate in caffeine (B), retinoic acid at 2 mg/kg b.w either separately (C) or combined with caffeine (E) that relatively detected in retinoic acid at 4 mg/kg b.w (D) and recovered in combined treatment with caffeine (F) compared to control (A). G–L: Alizarin Red S stained sections to show the increased calcium in caffeine (H), retinoic acid at 4 mg/kg b.w (J) and in combined treatments with caffeine at both doses (K, L) as compared to control (G) and retinoic acid at 2 mg/kg b.w (I) that appeared similar to control. M – R: Prussian blue stained sections to show decreased iron in caffeine (N), retinoic acid at 2 mg/kg b.w (O) and in combined treatments with caffeine at both doses (Q, R) as compared to control (M) and retinoic acid at 4 mg/kg b.w (P) that appeared higher compared to other treatments. Scale bar 20 μ m. S–X: Immunostained sections to show severe downregulation of TGF β 2 expression in caffeine (T) that upregulated in retinoic acid treatments (U, V) and differentially expressed in combined treatments with caffeine in dose-dependent of retinoic acid (W, X) as compared to control. Scale bar 10 μ m.

Concomitant changes in TGF β 2 expression was noted since caffeine treatment results in complete inhibition (Figure 6T), upregulation in retinoic acid treatments (Figures 6U and 6V) and differential cytoplasmic/neucleus expression in combined treatments with caffeine and retinoic acid (Figures 6W and 6X) compared to the faint expression of control (Figure 6S) that show faint expression.

DISCUSSION

The present study revealed that the fetal liver at 14th day of gestation at histological level can be compared to adult bone marrow, hyperchromatic hemopoietic progenitor and the polyploid megakaryocytes (the platelet forming cells) were well observed. Caffeine and retinoic acid dosing during

pregnancy results in altered and dose-dependent deformation of megakaryocyte differentiation and function as expressed in H&E and PAS stained sections. Concomitant fluorescence depression of hepatoblastes and downregulated expression of TGF β 2 in the developing liver were noted that relatively recovered in combined treatment with caffeine and retinoic acid in a dose-dependent manner in the prenatal period of development. Studies on the development of hematopoietic system have revealed the initiation sites of hematopoiesis and proposed a migration of stem cells during development of the mouse embryo (Huang and Auerbach., 1993; Medvinsky and Dzierzak, 1996; Cumano *et al.*, 1996). Cells with long-term marrow repopulating activity have been detected in the yolk sac and the aorta-gonadomesonephric region at days 9 to 10 of gestation for the first time in an embryonic life of mice (Muller *et al.*, 1994; Yoder *et al.*, 1997). Hematopoietic stem cells are believed to migrate into the liver around day 11, and subsequently into the bone marrow and spleen, whereas the fetal liver remains as a main organ of definitive hemopoiesis during the embryonic period as has been noted in the present study. It has been also reported that the fetal liver hematopoietic stem cells have a greater proliferative capacity than do adult bone marrow stem cells (Rebel *et al.*, 1996; Harrison *et al.*, 1997). The frequency of hematopoietic stem cells in day 14-fetal liver cells is comparable to that in adult bone marrow cells similar to the observed histology as indicated in the present study that regressed at 18th day of gestation as the development proceed and the liver acquired the characteristic cellular architecture of hepatocytes at 18 day of gestation. The observed effects of either caffeine or retinoic acid treatments indicates their ability to cross the placenta to exert their effects. In this context, Ikeda *et al.*, (1982) and Kimmel *et al.*, (1984) concluded that no placental barrier to caffeine and unusually high levels of caffeine have been reported in premature infants born to women who are heavy caffeine consumers as has reported (Khanna and Somani, 1984). The authors also reported that the cytochrome P450 1A2 which is the principal enzyme involved in caffeine metabolism is absent in the placenta and the fetus. For retinoic acid excess during development, also results in major embryonic defects which often overlap with those observed in retinoid deficiency (Morriss-Kay and Ward, 1999). Patterns of retinoid accumulation in embryonic liver indicate the onset of vitamin A storage occurs by midorganogenesis that exceeds the retinoids in placenta of the developing concepts (Shah *et al.*, 1987) and exerts their effects as indicated in the present study concerning specially the differentiation of megakaryocytes at 14th day of gestation and altered the stain ability of polysaccharides and macrophages at 18 day of gestation. The defective differentiation of megakaryocytes in either caffeine or retinoic acid treatments was recovered in co-treatments with caffeine and retinoic acid. Megakaryocytes in these treatments are similar to those observed in control since the hallmarks of megakaryocyte maturation that include polyploidization and expansion of cytoplasm were noted as reported by Patel *et al.*, (2005) and Richardson *et al.*, (2005). Also, regardless the altered reactivity of megakaryocytes toward PAS and the down regulated expression of TGF β 2 at the prenatal period of development, proper differentiation of megakaryocytes were noted in co-treatments which may

indicate the involvement of other growth factors of the transforming superfamily that need further investigation.

At postnatal during suckling and after weaning in control increased percentages of binucleated hepatocytes from the 3rd to the 6th week were noted. Decreased percentages of binucleated hepatocytes were observed in all treatments with concomitant polyploid giant nuclei induction compared to control. Binucleation was considered as an interesting feature in adult hepatocytes that begins from the neonatal liver (Celton-Morizur *et al.*, 2010). In this context, weaning was found to increase the amount of circulating insulin that induce incomplete cytokinesis to generate binuclear hepatocytes during liver maturation (Celton-Morizur *et al.*, 2009, 2010; Gentric *et al.*, 2012). Hence, the observed decrease in binucleated hepatocytes in various treatments during suckling or after weaning suggests that cells under caffeine, retinoic acid or combined treatments failed to divide properly and tend to polyploidization as clearly indicated in H&E and acridin orange stained nuclei of hepatocytes at the 6th week postnatal. Polyploidy, the state of having greater than a diploid DNA content (tetraploid, octoploid, etc.), is a widespread physiological phenomenon observed particularly in plants, fish, and amphibians (Otto and Whitton, 2000). Although it is less common in mammals, some tissues including heart muscle cells, platelet progenitor megakaryocytes, and liver parenchyma develop a certain degree of polyploidy during their normal life cycle. The appearance of polyploid cells is often associated with late fetal development and coincides with terminal differentiation and restricts hepatomrgalogenesis (Gerlyng *et al.*, 1993; Gupta, 2000). Polyploidy is a characteristic feature of mammalian hepatocytes (Celton-Morizur and Desdouets, 2010). During postnatal growth, the liver undergoes substantial changes, including gradual polyploidization. This process generates the successive appearance of tetraploid and octoploid hepatocytes with 1 (mononucleated; e.g., 4n, 8n) or 2 nuclei (binucleated; e.g., 2 \times 2n, 2 \times 4n). In adults, 70% of all hepatocytes in rodents and 40% in humans are tetraploid (Seglen, 1997; Toyoda *et al.*, 2005). Interestingly, in the adult liver, the generations of polyploid cells were re-induced following a variety of cellular stressors. Polyploid cells were detected in response to stress and injury, as has been described for heart muscle cells; VSMCs during hypertension; and thyroid cells in hyperthyroidism (Auer *et al.*, 1985; Vliegen *et al.*, 1995; Hixon *et al.*, 2000; Storchova and Pellman, 2004). Moreover, in many human carcinomas, cells with polyploidy, DNA content arise as an early step in tumorigenesis and precede the formation of a neuploid cells (Ganem *et al.*, 2007). Liver re-growth after partial hepatectomy or following oxidative damage and metabolic overload (copper/iron) is associated with a pronounced increase in the proportion of cells that are polyploid (Sigal *et al.*, 1999; Gorla *et al.*, 2001).

In the present study, giant nuclei inductions in either caffeine, retinoic acid or in the combined treatments reflect their ability to suppress cell proliferation through inducing incomplete cytokinesis resulting in polyploidy. For many years, caffeine has been generally believed to suppress cell proliferation (Levi-Schaffer and Touitou, 1991). Caffeine has also been shown to inhibit ultraviolet B (UVB)- induced skin

cancer in mice (Lou *et al.*, 1999), suppressed epidermal growth factor (EGF)-induced malignant cell transformation (Nomura *et al.*, 2005) and perturb key cell cycle regulatory proteins (Bode and Dong, 2007). Also, retinoids have been shown to exert an anticarcinogenic effect through suppression of the cell cycle, induction of apoptosis, suppress liver regeneration and/or differentiation. Meanwhile giant nuclei were noted in BrdU incorporation indicating polyploidy induction similar to the observed findings in the present study indicating DNA replication in endomitosis of polyploid induction (Ledda-Columbano *et al.*, 2004). Also, RA induces cell cycle arrest and differentiation exerts in different cell including ES, F9, or HL60 cells (Rochette-Egly and Chambon, 2001; Altucci *et al.*, 2007; Mongan and Gudas, 2007) and in the present study in both caffeine and retinoic acid either in the separate or in the combined treatments resulting in polyploidization a mechanism by which hepatomegally and direct mitosis in proliferative disease can be controlled.

The present study revealed that caffeine administration from the 3rd to the 6th week during the independent feeding induces inflammatory cell infiltration and deposition of collagen. A drawback effect of caffeine that is controlled in co-treatment with retinoic acid at both doses used in the present study. In contrast, beneficial effects of caffeine were reported in preventing liver dysfunctions in several investigations (Tanaka *et al.*, 1998; Wells, 2000; Gressner *et al.*, 2002; Ruhl and Everhart, 2005; Gressner *et al.*, 2008). Still, however, conflicting data about the effect of caffeine were reported. In the present study the inflammatory infiltrated foci that detected closely to the blood vessels or deeper in hepatic tissue with collagen deposits can be attributed to the chronic administration, the young age at which the treatment was conducted and the species differences. In this context, various studies previously reported that RA regulates the expression of ECM and plays a significant role in fibrotic diseases (Wang *et al.*, 2007; 2008; Hisamori *et al.*, 2008; Aguilar *et al.*, 2009; Ye *et al.*, 2010; Xiao *et al.*, 2011; Zhou *et al.*, 2013). However, divergent and contradictory effects are reported in the literature that can be attributed to the RA doses used in various studies for RA treatment in fibrotic disease.

Concomitantly, in either the pre- or the postnatal period of study, caffeine was able to perturb carbohydrates, increase calcium and decrease iron those accompanied with down regulated expression of TGF β 2 in hepatic tissue as compared to control. Treatment with retinoic acid at both doses in the present study either separate or combined with caffeine upregulate calcium against suppression of iron. Moreover, TGF β 2 show biphasic that severely down regulated during gestation and extended to express intranuclear at postnatal in liver tissue. In chronic administration of caffeine to young rats an increase in intestinal calcium absorption, which compensated for the urinary loss was reported (Yeh and Aloia, 1986; Yeh *et al.*, 1986). Also, Massey and colleagues (Massey and Wise, 1984; Massey and Hollingbery, 1988; Bergman *et al.*, 1990) showed that caffeine-induced diuresis increased urinary calcium loss acutely. So the increased absorption of calcium in young age during caffeine administration contributes to the elevation of circulating calcium with consequent upregulation in hepatic tissue

before excretion. In contrast, decreased hepatic store of iron in caffeine administration was noted. This observation is in accordance with earlier study since a cup of coffee reduced iron absorption from hamburger meal by 39% (Morck *et al.*, 1983). Retinoides were also included in calcium and iron metabolism in bone as the main site of calcium deposition and liver as the main organ of iron store. In this concern, calcium mobilization resulting in bone fragility (Melhus *et al.*, 1998) and depletion of hepatic store of iron were reported in retinoid administration (Tsuchya *et al.*, 2009). So, in co-treatments calcium overload and iron depletion were noted accompanied with down regulation of TGF β 2 expression regardless the intranuclear expression noted in the present study. Retinoic acid is so antagonize the down regulated effect of caffeine on TGF β 2 gene may be in part due to its receptor interference with TGF β (Pendaries *et al.*, 2003).

In conclusion, the present study contribute to the benefits of both caffeine and retinoic acid as the active metabolite of vitamin A during pregnancy, suckling and independent feeding of young age. Decreased iron overload of liver with concomitant down regulation of TGF β 2 expression represent the most important points of this study that protect the liver of young age from oxidative damage and suppress the gene-dependent extracellular matrix deposition. These data may contribute to the therapeutic uses of these substances in preventing the progression of liver damage in hepatitis diseases.

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