Effects of Synthetic Food Color (Carmoisine) on Expression of Some Fuel Metabolism Genes in Liver of Male Albino Rats

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Abstract: Food additives were known since the old man from the ancient civilizations to introduce special color or taste in his food. Nowadays, there are over-use of synthetic chemicals as food additives to preserve foods, to improve characters and attract consumers especially children. Previous studies had reported many effects of overdoses from food additives, however, further scientific studies are needed in the molecular levels. The current work studied the effects of doses equivalent to the acceptable daily intake (ADI), 5 and 10 ADI folds of the synthetic food color (Carmoisine) on the experimental animals (male albino rats) for different periods. Gene expression of some fuel metabolism genes e.g. PPAR-alfa, ACo-A and CPT-1 were studied and supported by histological studies on rat liver. There were down-regulations of the studied genes which may leads to the conclusion that, carmoisine may decrease the fuel metabolism. The histological studies indicate also that high doses of carmoisine may affect the liver.

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1. Introduction:

The addition of colorants to make food more attractive is not a recent invention. Extracts from spices and vegetables were used as early as 1500 BC, in India and China for coloring skin. Wine was colored as early as 400 BC, and spices and condiments were being colored by AD 1400. Colorants derived from naturally occurring minerals, plants, and animals were prepared along with the spices that played such a prominent part in the development of early civilizations (Tannahill, 1973).

The advent of the use of food colorants in the late 1800S and early 1900S was unfortunately accompanied by their misuse in food adulteration. Before the development of synthetic colorants, dangerous mineral extracts were often used to color foods, frequently to disguise food of poor quality. Some of these deceptive practices included coloring of pickles with copper sulfate, cheese with vermilion and red lead, tea with copper arsenate, lead chromate, and Indigo, and Candy with lead chromate, red and white lead, and vermilion (NAS/NRC 1971 .

In 1956, the discovery of the first synthetic dye, mauve, by Sir William Henry Perkins prompted a search for other dyes. Further development of synthetic colorants then became attractive to the food industry because these colorants were superior to natural extracts in tinctorial strength, hue, and stability and was readily available in many hues. The addition of synthetic colorants to food in the United States was first legalized for butter in 1885, followed by the authorization to add colorants to cheese in 1896 (Newsome, 1986) .

Historically, synthetic colorants, also known as aniline dyes, were manufactured from coal tar derivatives. Although the colorants were highly purified before they were added to foods, the negative connotation of their association with coal tar resulted in much unfavorable publicity. As a result, synthetic colorants are no longer manufactured from coal tar derivatives but instead are developed form highly purified petrochemicals. The U.S. Department of Agriculture began to investigate the use of colorants in foods to establish principles for their regulation in the early 1900s. The first comprehensive regulation regarding food colorants was the Federal Food and Drug Act of 1906. Previously, 80 different colorants had been used in foods. Following establishment of the Federal food and Drugs Acts, the U.S. Department of agriculture eliminated all but seven of these colorants, on the basis of known composition and purity. This act and a series of food inspection decisions lead to establish of a voluntary certification program for food colorants with the food inspection decision of 1907. Subsequently, seven more colorants were added to the approved list. In 1938, The Federal Food, Drug, and Cosmetic Act established mandatory certification (21 CFR parts 73-74, 81-820) requiring submission of samples from each batch of colorant for evaluation of purity. Synthetic colorants that had previously been

known by common names were there given specific names to ensure distinction of those for use in foods from those for use in drugs or cosmetics. Three categories for designating colorant names. FD & C (Suitable for use in foods, drugs and cosmetics), D & C (suitable for use in drugs and cosmetics), and external D & C (Suitable for use in externally applied drugs and cosmetics) were defined (Branen *et al.*, 1990).

In 1960, the color additive amendments to the FD & C act were established, defining the term "color additive" as any dye, pigment or other substances made or obtained form a vegetable, animal or mineral or other source capable of coloring a food, drug or cosmetic or any part of the human body. Included in the two part amendments was a Delaney-type clause. Part (1) of the clause prohibited addition to food of any colorant found to induce cancer in human or animals. Part (2) of the clause permitted the use of current color additives under a provisional list in pending the completion of scientific investigations needed to determine their safety for permanence listing. Synthetic colorants were thus required to undergo premarketing safety clearance, and previously authorized colorants were reevaluated (Newsome, 1986).

The development and use of food colors in countries other than United States has been extremely varied. Havel and Smith (1980) reviewed development of regulations for use of food colorants in other countries. Legalization for food colorants in the United Kingdom was not established until the mid-1950s. Some countries do not regulate the use of food colorant, thus permitting the use of any colorants, while others prohibit the use of all synthetic colorants. The regulatory status of colorants in use outside as well as within the U.S. is reported in the International Life Science Institute Nutrition Foundations Catalog of Food Colors (ILSI/NF 1981) and updated periodically as new information becomes available.

Specific food products are expected to appear in certain color shades, and when deviations from these expectations occur, flavor perception is attended. The importance of color to the perception of the quality, odor, flavor, and texture of food is well documented. In studying how individuals react to sherbets of mismatched flavor and color, Hall (1958) found that white sherbets made with one of six test flavors (Lemon, Lime, Orange, Grape, Pine apple, and Almond) were confused, and flavor was difficult to identify.Also, when the sherbets were deceptively colored, most individuals mistakenly identified flavors. Color far outweighs flavor in the impression it makes on the consumer, even when the flavors are pleasant and the food is a popular one. It powerfully influences not only the consumer's ability to identify the flavor but also her or his estimation of its strength and quality. The use of food colorants aids in the production of food of preferred color values and provides significant functional advantages in a variety of situations colorants. It corrects for variation and irregularities resulting from storage, processing packaging, and distribution, assuming greater uniformity in appearance and hence acceptability. Color also, help preserve the identity or character by which foods are recognized (Hall, 1958).

The Certified Color manufacturer's Association (CCMA) has petitioned the FDA for approval of Carmoisine, a colorant similar in shade to FD and C Red No. 2. Carmoisine is widely used in food in Central and South America and Europe. However, colorants considered safe in one country may not be considered safe in other parts of the world. In the U.S. the toxicity of a colorant is tested under conditions similar to those under which it will be used. Therefore, since it is important that food colors be safe when ingested, animal feeding studies play a key role in their evaluation (Marmion, 1984). Present day toxicological testing, as suggested by the FDA, is listed in the RDA "Red Book" (FDA, 1982). Scientists in many other parts of the world, however, place emphasis on the effects of subcutaneous injection of the colorant. Because of the different mechanisms involved, the results of the studies conducted under these different test conditions vary and have resulted in much scientific and political debate.

Gerd and Lennart (1973) reported that "recurrences of urticaria could be prevented through the avoidance of food and drugs containing azo dyes and preservatives."

Feingold (1979) had two case reports illustrate the therapeutic response of congenital nystagmus to a diet eliminating synthetic food colors. A brief discussion of the hyperkinetic syndrome was offered with the proposal that a variety of neurologic and neuromuscular disturbances (grand mal, petit mal, psychomotor seizures, la tourette syndrome, autism, retardation, the behavioral component of Down's syndrome, and oculomotor disturbances) may be induced by identical chemicals, depending upon the individual's genetic profile and the interaction with other environmental factors. Juhlin (1981) and Twaroj (1983) reported that artificial food additives particularly, azo dyes and benzoate preservatives are a common cause of chronic urticaria and angioedema in both adult and children. Brozelleca *et al*. (1989) reported a reduction in body weight in female rats by high-dose of (FD and C red No, 40). Hong *et al*. (1989) reported that aspirin and food additives are known to induce bronchoconstriction, angioedema or urticaria in susceptible patients.

Van Bever *et al*.(1989) reported that children feed on food colors shown to have an adverse effect on a

daily Conner's rating of behavior. Osman *et al*. (1995) reported that synthetic food colorants administration increased the body weight gain until the fourth month but after that forth month a decrease in body weight as observed either in female or male mice, there were also an increase in organ/body weight. Gaunt *et al*. (1967) carried acute toxicity studies in rats and mice and a short term feeding study in rat. They reported an elevation in the relative kidney weight at the 1% level in females. Gaunt *et al*. (1969) reported that pig is less sensitive to Carmoisine than rat.

Rowe (1988) reported that subjects (children) were maintained on a diet free from synthetic additives and were challenged daily for 18 weeks with placebo (during lead-in and washout periods) or tartrazine or Carmoisine 50), each for 2 separate weeks. Two significant reactors were identified whose behavioral pattern featured extreme irritability, restlessness and sleep disturbance. One of the reactors did not have inattention as a feature. The findings raise the issue of whether the strict criteria for inclusion in studies concerned with hyperactivity" based on "attention deficit disorder" may miss children who indicate behavioral changes associated with ingestion of food colorings. Moreover, for further studies, the need to construct a behavioral rating instrument specifically validated for dye challenge is suggested. Booth (1993) illustrated the effectiveness of dietary advice in a young body with chronic idiopathic urticaria. An azo dye and preservative-free diet was initially advised, resulting in a total improvement in urticarial symptoms. Double-blind challenges confirmed the boy was intolerant to E127 (Erythrosine), E122 (Carmoisine), E128 (red 2G), and E102 (tartrazine) but not to E211 (sodium benzoate).

Murdoch *et al*. (1987) reported that ten healthy adults with a negative history of adverse reactions to foods and food additives were asked to exclude foods known to be high in histamine and food containing azo dyes and other coloring agents. He found that 200 mg tartrazine caused a significant increase in plasma and urinary histamine concentrations 30 min to 3 hrs after ingestion with a mean time of 100 min. Abdel-Rahim (1988) and Ibrahim *et al*.[38] reported pronounced increase in serum and liver transaminases activity of rats by ingestion of synthetic colorants. The load and species of food colorants ingested into animals for assimilation at any time may alter the activity of GOT and GPT followed by changes of overall protein metabolism.

Abou El-Zahab *et al*. (1997) found that administration of the synthetic colorants (Carmoisine, sunset yellow, indigocarmine, brilliant blue and brown chocolate HT) after 60 days induced damage to liver tissue as evidenced by a significant increase in AST, ALT and ALP in serum of rat groups. But, some researchers, (Ford et al. 1987 and Brozelleca and Hallagan 1988a, b) stated that Carmoisine and tartrazine caused insignificant changes in rat serum AST, ALT and ALP. Yet these contradictory results were recorded after long term toxicity studies which may indicate an adaptation mechanism on part of the liver. He, also stated that serum total protein exhibited significant increase in rats fed on colors containing Carmoisine after 30 and 60 days of diet supplementation, while albumin revealed significant increase in rats fed with color B (sunset yellow, tartrazine, Carmoisine and brilliant blue) after 60 days. In agreement with these findings, El-Sadany whose rats ingested the food colors indigocarmine and Carmoisine as well as Lord who administered tartrazine dye to the experimental rats. The accumulation of serum protein can be attributed to the stimulation of protein biosynthesis to produce the specific enzymes required for all processes. Amin *et al*. (2010) reported that Tartrazine and Carmoisine in low and high doses affect adversely and alter biochemical markers in vital organs.

El-Saadany (1991) pointed out that the ingestion of synthetic chocolate colorants (indigocarmine and Carmoisine) specifically increased RNA (not DNA) in rat liver cell homogenates. In connection with this, it is noted that their hydrolytic enzymes (DNase) in cytoplasmic as well as mitochondrial fractions were also stimulated to provide the necessary enzymatic machinery to cope with increased flow of ribnucleotides. Abou El-Zahab *et al*. (1997) stated that, the concentrations of ribonucleic acid (RNA) in liver cell homogenates exhibited a highly significant increase ($P < 0.01$) only on rats fed on diet supplemented with color C (brown chocolate HT and indigo carmine in addition to small fractions of Carmoisine, tartrazine, sunset yellow and brilliant blue) for 60 days, whereas DNA remained unchanged. Ali *et al*. (1988) also reported expected mutagenic effects of high doses from carmoisine and fast green.

Gaunt *et al*. (1988b) reported that the types and incidence of histological changes were comparable in control and test groups. A no-effect level of 0.5% Carmoisine was established in the diet of rats for 90 days, a level equivalent to 250 mg/kg/day. Shaker *et al*. (1989) noted an increase in hemoglobin content of rat administered chocolate brown color (0.1% w/w) consisting of tartrazine, noval coccine, Carmoisine and indigocarmine.

Abou El-Zahab *et al*. (1997) stated that, the marked discrepancies observed between the various research studies may be attributed to dose variations as well as the duration of colorant intake, where small doses for longer periods induced positive stimulatory effect on erythropoiesis, moderate duration and dose revealed inhibition and long term high dose

administrations produced no alterations. The total count in his study remained unchanged in all his experimental groups.

Tartrazine and carmoisine are an organic azo dyes widely used in food products, drugs and cosmetics. Therefore, Amin and his coworkers (Amin *et al*., 2010) evaluated the toxic effect of carmoisine on hepatic function, lipid profile, blood glucose, body-weight gain and biomarkers of oxidative stress in tissue. They administered carmoisine orally in two doses, one low (8 mg/kg bw) and the other high dose (100 mg/kg bw) for 30 days and concluded that carmoisine affects adversely and alter biochemical markers in liver at both doses.

In the liver, PPARα is a critical transcription factor for lipid metabolism, because several genes coding for enzymes involved with oxidation (either in peroxisomes or mitochondria) contain a functional peroxisome proliferator–responsive element in their enhancer regions (e.g., acyl-CoA oxidase, liver fatty acid–binding protein, cytochrome p 450A, hepatic lipoprotein lipase, and others) (Schoonjans *et al*., 1996).

The current study focus on the action of carmoisine (synthetic food color) in different doses for different periods on gene expression of the common genes controlling for fuel metabolism. It also supports the molecular studies with histological studies for the liver.

2. Material and Methods:

Chemicals: carmoisine ($C_{20}H_{12}N_2O_7S_2Na_2$) was ordered from Lobachemie PVL Ltd company (Mumbai, India).

Animals: A total of 30 Male Sprague-Dawley rats, 160 ± 10 g body weight (B Wt) were used in this study. Rats were kept on standard diet (Degrace *et al.*, 2003) and randomly divided in to 4 groups according to treatment. Control negative group (G0), group 1(ADI or acceptable daily intake) was treated with a dose equivalent to ADI (50mg/kg B Wt), group 2 (5xADI) was treated with 5 folds of ADI, group 3(10xADI) was treated with 10 folds of ADI. Three rats were taken from each group every 15 days for a period of 45 days.

Histological analysis: Animals were sacrificed. Removed liver slices were fixed in 10% neutral-buffered formalin and embedded in paraffin. Five µm thick sections were stained with hematoxylin-eosin, Alcian blue or Crossmon's trichrome stain for histological examination.

Semi-quantitive PCR: Total RNA from about 40mg liver was extracted using Biozol (Biolabs, USA) . The final amount of RNA was estimated by determining the optical density at 260 nm. First strand cDNA synthesis with total RNA was performed using reverse transcriptase (Jones *et al*., 1999). Subsequently, PCR-amplification was performed using specific primers and conditions (Table 1) for 30 of 1 min at 94 °C, 1 min at the annealing temperature indicated in Table 1, and 1 min at 72 °C. The final extension step was 5 min at 72 °C, PCR products were separated on 1.5% agarose gels visualized under UV light and analyzed using Alfa Ease FC software.

Statistical analysis: Data were expressed as mean ± standard deviation. Student's t-test was used to compare means. A level of $p<0.05$ was considered as statistically significant.

3. Results and Discussion:

Histological effects: In the control group there were normal hepatocytes with normal un-engorged hepatic sinusoids or any blood vessels, normal portal tracts (Figure 4), no alcianophilic hepatocytes (Figure 3A). The ADI treated groups didn't show deleterious effects especially for short period of treatment. However, the longer time of treating with ADI (for 30 and 45 days) resulted in swollen hepatocytes with fatty changes and some with congested hepatic blood sinusoids (Figure 6C). There were also, ballooned swollen hepatocytes that stores fat droplets as also was present mucoid degeneration of the hepatocytes with alcianophilia (5C).

Increasing the doses of carmoisine more than ADI (5xADI and 10xADI) for 15 days did not vary (Figure 6C) from those of 60 and 90 days fed of light doses. There were more degenerative changes of hepatocytes that revealed hepatic cells mucoid degeneration as shown in figure 6C.

Gene expression effects:

The levels of PPAR alfa gene expression fluctuated around that of the control group, because of the different doses along the various periods of treatments. After 15 days The expression levels were elevated more than that of the control group. The ADI at 15 days elevated the level of PPAR from 0.770 (control) into 0.910, the medium (5xADI) dose resulted in elevation into 0.913, and the high (10xADI) dose elevated the level from 0.770 (control) into 0.900. However, all the increments were non-significant.

At the 30 day group, only the ADI and medium doses were non-significantly increased from 0.772 (control) into 0.860 and 0.84 respectively. While, the high dose resulted in significant decrease in the expression level from 0.770 (control) into 0.670.

At the 45 day group, the ADI dose produced non-significant increase in level of expression from 0.780 (control) into 0.810. The medium and high doses resulted in significant decreases from 0.772 (control) into 0.770 and 0.690 respectively.

The expression pattern of ACO-A gene was similar to that of PPAR- alfa. After 15 days, there were non-significant increases in expression level due to treatments with ADI, medium and high doses from 0.630 (control) into 0.645, 0.640 and 0.637 respectively. However, at 30 day group there were non-significant increases when treated with ADI and medium from 0.670 (control) into 0.677 and 0.675 respectively. The high dose of carmoisine after 45 days resulted in significant decrease into 0.660.

Carnetine palmetoyl transferase-1 was expressed in heterogeneous pattern among the different periods of treatment. At the 15 day period, there were non-significant increases due to ADI, medium and high doses from 0.392 (control) into 0.410, 0.451 and 0.444 respectively. After 30 days, there were non-significant increases in response to ADI and high doses from 0.395 (control) into 0.511 and 0.472 respectively. Meanwhile, the increase in CPT-1 gene expression due to the medium dose at 30 day period was significant into 0.470.

After 45 days of treatment, CPT-1 gene was significantly decreased from 0.430 (control) into 0.400, 0.410 and 0.400 due to ADI, medium and high doses respectively.

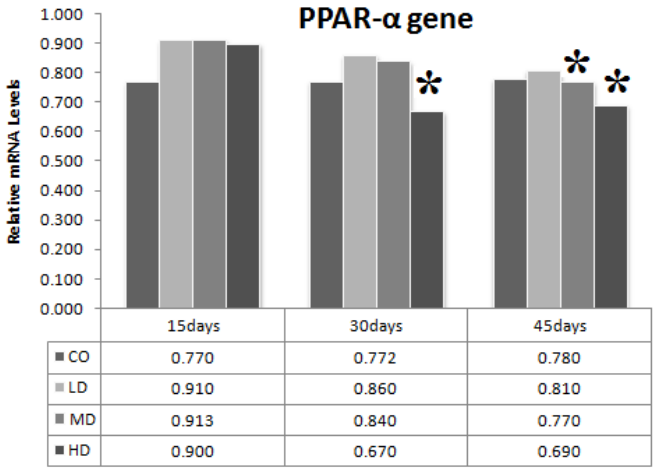
The down regulation of PPAR-α due to the high doses of carmoisine may lead to disturbance of fuel metabolism, which is manifested by the disturbance in some fuel metabolism genes e.g. ACo-A oxidase and CPT-1. The interpretation of our results could be supported by an explanation according to Videla and Pettinelli (2012), who summarized the expected consequences of disturbance in PPAR.

Videla and Pettinelli (2012) stated that, Liver PPAR-*α* downregulation and substantial enhancement in the hepatic sterol regulatory element binding protein-1c (SREBP-1c)/PPAR-*α* mRNA content ratio represent major metabolic disturbances between *de novo* lipogenesis and FA oxidation favouring the former, as a central issue triggering liver steatosis in obesity-induced oxidative stress and insulin resistance. The prosteatotic action of PPAR-*α* downregulation may be reinforced by PPAR- *γ* upregulation favouring hepatic FA uptake, binding, and transport, representing a complementary lipogenic mechanism to SREBP-1c induction leading to *de novo* FA synthesis and TAG accumulation. In addition, PPAR-*α* downregulation may play a significant role in enhancing the DNA binding capacity of proinflammatory factors NF-*κ*B and AP- 1 in the liver of obese patients, thus constituting one of the major mechanisms for the progression of simple steatosis to steatohepatitis. In the past, PPARs have been studied as drug targets for the management of Nonalcoholic fatty liver disease NAFLD in obesity and the broader metabolic syndrome (MetS) (George and Liddle 2008). However, PPAR-*α* agonists such as fibrates used to diminish steatosis and inflammatory scores in human steatohepatitis revealed poor effectiveness, thiazolidinediones have weight gain limitations, whereas that of dual PPAR-*αγ* agonists has safety concerns. Hepatic lipid metabolism was regulated via ligands of PPAR-*α* promoted the expression of genes encoding for proteins involved in FA oxidation at mitochondrial, peroxisomal, and microsomal levels, FA binding in cells, and lipoprotein assembly and transport and reduction in PPAR-*α* controlling FA oxidation (carnetine palmitoyltransferase-1a; CPT-1a), with the consequent enhancement in the hepatic SREBP-1c/PPAR-*α* ratios denoting a prolipogenic status. This condition may also involve diminution in TAG export from the liver via very-low density lipoprotein (VLDL) due to decreased production of apolipoprotein B-100, which is upregulated by LCPUFA n-3 and PPAR-*α* activation (Videla and Pettinelli 2012).

Table (1): The primer sequences and PCR conditions.

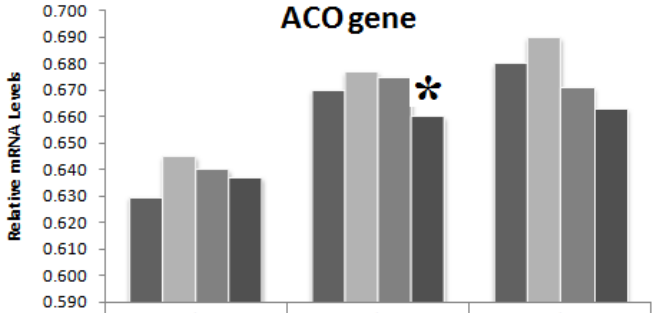
Gene name	5\primer	3\primer	T_{an}°C	Product size (bp)
PPARα	GGTCCGATTCTTCCACTGC	TCCCCTCCTGCAACTTCTC	62	404
CPT1	GAGACACCAACCCCAACATC	GTCTCTGTCCTCCCTTCTCG	55	295
ACO oxidase	AGCTTCACGCCCTCACTG	ACCACCCACCAACTTCCC	60	245

PPARα (Peroxisome proliferator-activated receptor alfa); CPT1 (carnetine palmetoyl transferase 1); ACo oxidase (Acyl Co-A oxidase).



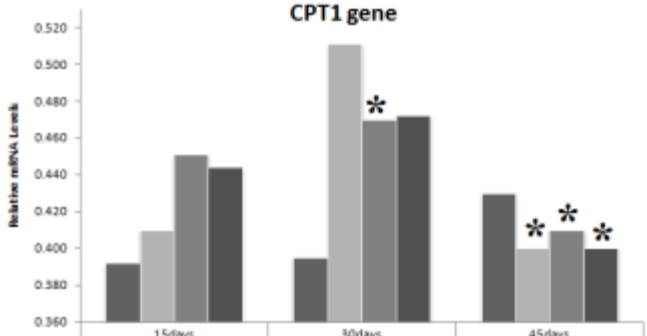
	15days	30days	45days
CO	0.770	0.772	0.780
LD	0.910	0.860	0.810
MD	0.913	0.840	0.770
HD	0.900	0.670	0.690

Figure 1: Histogram showing changes in expression level of mRNA for PPAR-α gene in relation to GAPDH due to treatment with carmoisine. Relative mRNA levels expressed in relative IDV (integrated density value) of PPAR-α/ GAPDH as measured by AlfaEaseFC software.



	15days	30days	45days
CO	0.630	0.670	0.680
LD	0.645	0.677	0.690
MD	0.640	0.675	0.671
HD	0.637	0.660	0.663

Figure 2: Histogram showing changes in expression level of mRNA for Acyl-COA gene in relation to GAPDH due to treatment with carmoisine. Relative mRNA levels expressed in relative IDV (integrated density value) of ACO A/ GAPDH as measured by AlfaEaseFC software.



	15days	30days	45days
CO	0.392	0.395	0.430
LD	0.410	0.511	0.400
MD	0.451	0.470	0.410
HD	0.444	0.472	0.400

Figure 3: Histogram showing changes in expression level of mRNA for CPT1 (carnetine palmetoyl transferase 1) gene in relation to GAPDH due to treatment with carmoisine. Relative mRNA levels expressed in relative IDV (integrated density value) of CPT1/ GAPDH as measured by AlfaEaseFC software.

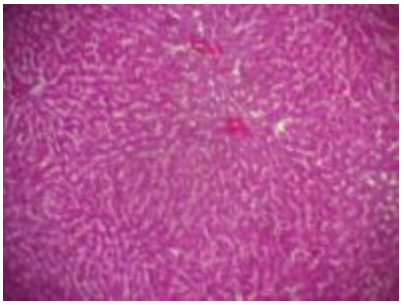
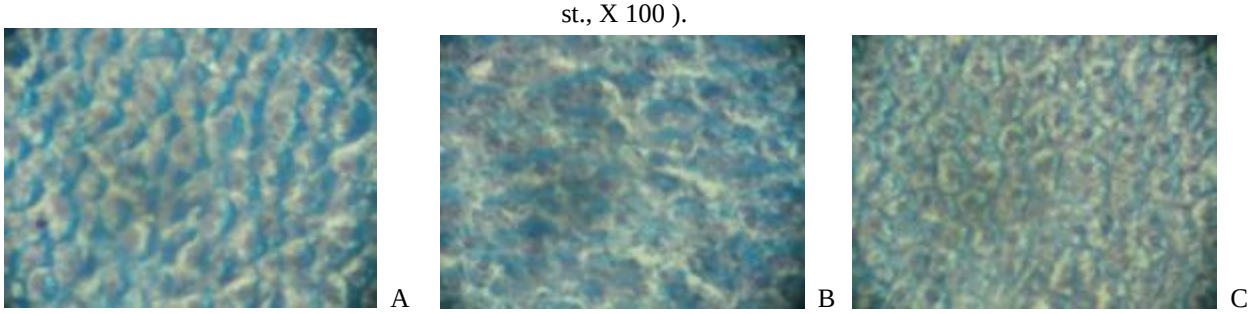
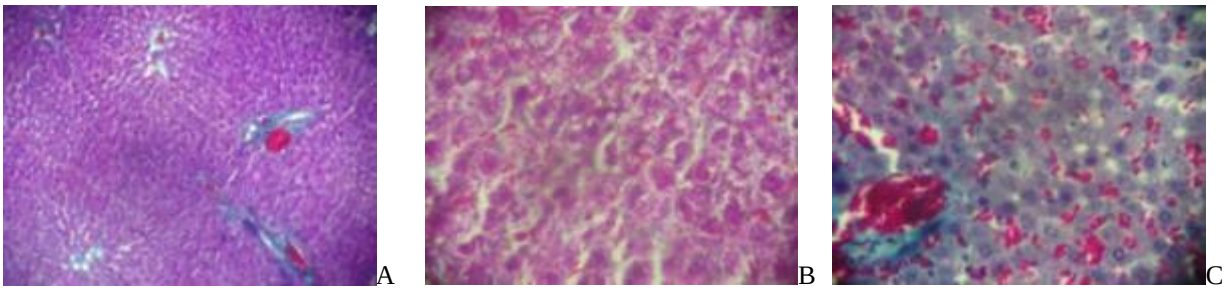


Figure 4: Liver section stained with routine stain, showing normal histology of liver in the control rat liver. (Hx& E. st., X 100).



A B C

Figure 5: Liver sections stained with Alcian blue stain X 400, showing: A) alcianophilia ; B) mucoid degeneration of the hepatocytes; C) ballooned swollen hepatocytes that stores fat droplets as also was present mucoid degeneration of the hepatocytes due to the effect of carmoisine on histology of liver.



A B C

Figure 6: Liver sections stained with Crossmon' s trichrome stain showing: A) fibrinoid deposition around some central veins and in the portal areas-X 100 ; B) swollen with fatty changes and some congestion of the hepatic blood vessels -X 400; C) congested hepatic blood sinusoids-X 400, due to the effect of carmoisine on histology of liver.

4. Conclusion:

From our studies we can conclude that, the expression levels of fuel metabolism proteins and enzymes e.g. PPAR-alfa, Acyl Co-A and Carnetine palmetoyl transferase-1 could be affected by the use of carmoisine especially the high doses for long time.

The high doses of carmoisine could be harmful to liver and lowers the expression level of some metabolic enzymes in it.

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