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Antioxidant effect of *Citrullus lanatus* ameliorates fructose-induced placental aberrations

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Abstract

Background: Fructose consumption during pregnancy has been associated with exacerbation of placental oxidative stress. The hypoglycemic and anti-oxidant properties of *Citrullus lanatus* juice (CLJ) previously reported may provide remedy to the oxidative stress.

Objective: The study investigated the effects of *C. lanatus* juice on fructose-induced placental changes in Wistar rats.

Methods: Twenty pregnant rats were assigned into four groups (n=5) and treated from Gestation Day (GD) 1-21 with water (control), 10% Fructose (w/v), 50% CLJ (v/v) and Fructose + CLJ. All treatments were given *ad libitum*. Caesarean section was performed on GD 21 during which the pups and placentas were harvested and weighed. Blood glucose level, progesterone concentration, placental morphometric indices (weight, circumference and thickness), oxidative status (using spectrophotometer) and histology were assessed. Data were analyzed using ANOVA and $P<0.05$ was considered statistically significant.

Results: The weight and circumference of placentas of fructose group were lower ($p < 0.05$) than that of control. Placental thickness was higher ($p < 0.05$) in fructose group compared with control. Placental malondialdehyde was higher in fructose group ($p < 0.05$) and lower in fructose + *C. lanatus* group ($p < 0.05$) compared with control and fructose groups respectively. Placental histology showed severe and mild infarction of chorionic villi in the fructose and fructose + *C. lanatus* groups, respectively.

Conclusion: C. lanatus juice ameliorated fructose-induced changes in placental oxidative status and morphology. Thus, intake of *C. lanatus* juice may be beneficial for optimal and healthy development of placenta and fetus of mothers who experience sugar cravings during pregnancy.

Keywords: *Placenta, Fructose, Citrullus lanatus, Oxidative stress, Rats.*

Résumé

Contexte: La consommation de fructose pendant la grossesse a été associée à une exacerbation du stress oxydatif placentaire. Les propriétés hypo glycémiques et anti-oxydantes du jus de *Citrullus lanatus* (CLJ) précédemment rapporté peut apporter un remède au stress oxydatif.

Objectif: L'étude a étudié les effets du jus de *C. lanatus* sur les changements placentaires induits par le fructose chez les rats Wistar.

Méthodes: Vingt rates gravides ont été réparties en quatre groupes (n = 5) et traitées à partir du jour de gestation (JG) 1-21 avec de l'eau (témoin), 10% de fructose (w / v), 50% de CLJ (v / v) et Fructose + CLJ. Tous les traitements ont été donnés *ad libitum*. Une césarienne a été effectuée le JG 21 au cours de laquelle les souriceaux et les placentas ont été arrachés et pesés. La glycémie, la concentration de progestérone, les indices de morphométries placentaires (poids, circonférence et épaisseur), le statut oxydatif (à l'aide d'un spectrophotomètre) et l'histologie ont été évalués. Les données ont été analysées en utilisant ANOVA et $P <0,05$ a été considéré comme statistiquement significatif.

Résultats: Le poids et la circonférence des placentas du groupe fructose étaient plus faibles ($p <0,05$) que ceux du groupe témoin. L'épaisseur placentaire était plus élevée ($p <0,05$) dans le groupe fructose par rapport au témoin. Le malondialdéhyde placentaire était plus élevé dans le groupe fructose ($p <0,05$) et plus faible dans le groupe fructose + *C. lanatus* ($p <0,05$) comparativement aux groupes témoin et fructose respectivement. L'histologie placentaire a montré un infarctus sévère et bénin des villosités choriales dans les groupes fructose et fructose + *C. lanatus*, respectivement.

Conclusion: Le jus de *C. lanatus a* amélioré les changements induits par le fructose dans l'état oxydatif et la morphologie du placenta. Ainsi, la consommation de jus de *C. lanatus* peut être bénéfique pour le développement optimal et sain du placenta et du fœtus des mères qui éprouvent l'appétit excessif de sucrerie pendant la grossesse.

Mots-clés: *Placenta, Fructose, Citrullus lanatus, Stress oxydatif, Rats.*

Introduction

Women normally experience cravings for certain foods during pregnancy [1] and fructose-containing foods are the most commonly craved food items [2]. There have been reports on the negative effects of maternal consumption of fructose during gestation

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on maternal metabolic status [3], placental growth, fetal development and future health of offspring [4].

Fructose is a simple sugar found naturally in honey, fruits, and vegetables [5]. It is commonly used in the food industry to prepare chocolate, drinks, candy, ice-cream and fast foods [6]. Fructose intake has been shown to contribute to the increased prevalence of obesity [7- 8], diabetes [9] and metabolic disorders [10] which are all associated with an increase in systemic oxidative stress [11].

Oxidative stress in pregnancy is caused by increased metabolic activity of placental mitochondria [12]. This oxidative stress plays physiologic roles in the development of the placenta [13], embryo [14], and fetus [15]. However, aggravated placental oxidative stress alters placental morphometric indices and may result in pregnancy complications [16]. The physiological adaptations made by the placenta in response to maternal perturbations are generally believed to be responsible for the altered pattern of fetal development and the resultant predisposition of offspring to disease in the future [17]. Also, the contribution of oxidative stress in fetal origins of adult disease is supported by epidemiological evidence of placental oxidant indices in association with type 2 diabetes [18] and preeclampsia [19]. Thus, placental oxidative stress and altered placental morphometric indices are proposed links between intrauterine insults and disease pattern in adult life.

Citrullus lanatus (Watermelon) is a natural product originally from a vine of South Africa [20]. It consists of about 93% water; hence the name "Watermelon" [21]. The potent antioxidant and antidiabetic properties of *C. lanatus* juice have been reported [22, 23]. It was hypothesized in this study that concomitant consumption of *C. lanatus* juice along with fructose rich foods during pregnancy may prevent additional oxidative stress and its associated adverse placental and fetal effects. Thus, the aim of this study was to investigate the effects of *C. lanatus* juice on fructose-induced placental morphometric derangements and oxidative stress in Wistar rats.

Materials and methods

Experimental Animals

Twenty virgin female Wistar rats (120-150 g) and ten proven male breeders (250-300 g) were obtained from the Central Animal House, College of Medicine, University of Ibadan, Nigeria. They were acclimatized for two weeks and had access to pelletized feed and water *ad libitum* throughout the study. The female rats were paired with the proven male breeders at a ratio 2:1 (female: male). Mating was confirmed by the presence of sperm cells in the vaginal smear and the day on which sperm cells were observed was designated as Gestation Day (GD) 1 for each rat.

Preparation of C. lanatus juice and fructose solution

C. lanatus fruits were obtained from a farm in Egbeda, Oyo state, Nigeria. Identification was done at Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo state where a voucher specimen with FHI number 110505 was deposited. Each *C. lanatus* fruit was washed and cut into small pieces. The thick epicarp layer and the seeds were removed. The fleshy red-coloured endocarp was blended using an electric blender and passed through a sieve to obtain the juice on a daily basis. A 50% concentration was prepared daily by diluting *C. lanatus* juice with drinking water at ratio of 1:1 v/v [24]. A 10% fructose (Qualikem Fine Chemical P. LTD, India) solution was freshly prepared daily by dissolving 10 g of fructose in 100 ml of drinking water [4].

Treatments

The pregnant rats were assigned into four groups (n=5) on GD 1. They were treated from GD 1-21 by replacing their drinking water with fructose solution and/or watermelon juice as follows:

Group 1 - Control (water)
Group 2 - Fructose solution (10% w/v)

Group 3 - *C. lanatus* juice (50% v/v)
Group 4 - Fructose solution (50% v/v) + *C. lanatus* juice (10% w/v)

Measurement of body weight

Maternal body weight was measured every week using a weighing balance.

Blood glucose level measurement

On GD 21, blood was collected from rat tails by nipping with a pair of fine scissors. Blood sugar was estimated from a drop of the blood with a glucometer (Accu-Check Active, Germany).

Measurement of placental morphometric indices

Caesarian section was performed under thiopentone anaesthesia (50mg/kg i.p) on GD 21 [25]. The pups and placentas were harvested and weighed on an electric balance (Lisay, China). Placental volume was measured by water displacement method [26]. Placental circumference and thickness were measured using a digital Vernier calliper (Dial, India).

Homogenization of placenta

The placentas were washed in ice cold 1.15% KCl solution, blotted with filter paper and weighed. These were then chopped into bits and homogenized into volumes of the homogenizing buffer (pH 7.4) using a Teflon homogenizer. The resulting homogenates were centrifuged at 10,000 revolutions for 15 minutes in a cold centrifuge (4°C), to obtain the post mitochondrial fraction (PMF). The supernatant were collected and used for biochemical analyses.

Determination of protein concentration

The protein concentration of the supernatant was determined by means of the Biuret method described by Gornal *et al.* [27] using bovine serum albumin (BSA) as the standard.

Assessment of lipid peroxidation

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation as described by Rice-Evans *et al.* [28]. This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde, an end product of lipid peroxidation. Briefly, an aliquot of 0.4ml of the supernatant was mixed with 1.6ml of Tris- KCL buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75% TBA was added and placed in water bath for 45minutes at 80°C. This was then cooled in ice and centrifuged for 15minutes at 3000rpm. The resulted clear pink solution was measured at an absorbance of 532nm against a reference blank of distilled water. The MDA level was calculated according to the method of Adam-Vizi and Sergi (1982). Lipid peroxidation in units /mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5 M^{-1}Cm^{-1}$.

Estimation of superoxide dismutase (SOD) activities

The level of SOD activity was determined by the method of Mistra and Fridovich [29]. The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for SOD. Briefly, 1ml of the supernatant was diluted in 9ml of distilled water to make 1 in 10 dilutions. An aliquot of 0.2ml of the diluted supernatant was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. The reaction was started by addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Estimation of catalase activities

Catalase activity was determined according to the method of Sinha [30]. This method is based on the fact that dichromate in acetic acid is reduced to chronic acetate when heated in the presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. Briefly, 1ml of the supernatant was mixed with 49ml of distilled water to give a 1 in 50 dilution of the sample. The assay mixture contained 4ml of H_2O_2 solution (800 µmoles) and 5ml of Phosphate buffer in a 10ml flat bottom flask. One milliliter of properly diluted enzyme preparation was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1ml portion of the reaction mixture was blown into 2ml of dichromate acetic acid reagent at 60s intervals. The chromic acetate produced is measured calorimetrically at 570 nm for 3 min at 60s intervals after heating the reaction mixture in a boiling water bath for 10 min. Catalase activity expressed as µmol H_2O_2 consumed/ min/mg protein.

Estimation of reduced glutathione (GSH) level

The method of Beutler *et al.* [31] was followed in estimating the level of reduced glutathione (GSH). This method is therefore based upon the development of a relatively stable (yellow) colour when 5', 5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds present in reduced GSH. Briefly, 0.1ml of test sample (placenta homogenates) was diluted with 0.9ml of distilled water to give 1 in 10 dilutions. Then, 3ml of 4% sulphosalicyclic acid solution (precipitating solution) was added to the diluted test sample to deproteinize it. The mixture was centrifuged at 3,000g for 10 minutes. Thereafter, 0.5ml of the supernatant was added to 4ml of 0.1M phosphate buffer and finally, 4.5ml of Ellman's reagent was added. A blank was prepared with reaction mixture of 4ml of 0.1M phosphate buffer, 0.5ml of the diluted precipitating solution (addition of 3ml of precipitating solution and 2ml of distilled water) and 4.5ml of Ellman's reagent. All readings were taken within 5 minutes at 412nm, as colour produced is not stable following addition of Ellman's reagent. Reduced GSH, is proportional to the absorbance at 412nm.

Measurement of progesterone level

The progesterone ELISA kit (Calbiotech) was used for the quantitative measurement of progesterone level in the dam. The supernatant and progesterone enzyme conjugate were added to wells coated with anti-progesterone monoclonal antibody.

Progesterone in the sample competes with a progesterone enzyme conjugate for binding sites. Unbound progesterone and progesterone enzyme conjugate was washed off by washing buffer. Upon the addition of the substrate, the intensity of colour was inversely proportional to the concentration of progesterone in the samples. A standard curve was prepared relating colour intensity to the concentration of the progesterone.

Histology of placenta

A section of the placenta tissue fixed in 10% formalin was dewax in Xylene, then dehydrated in Absolute Alcohol, 95% and 70% Alcohol. Micro sections (about 4 µm) were prepared and stained with haematoxylin and eosin (H&E) dye according to Avwioro [32] and were examined under a light microscope by a Histopathologist who was ignorant of the treatment groups.

Statistical analysis

Data were presented as mean ± Standard Error of Mean (SEM). Means were compared using one-way ANOVA with Bonferroni post hoc tests. $P<0.05$ was considered statistically significant. All analyses were performed using Graph Pad Prism (version 5) software.

Results

There was no significant difference ($p>0.05$) in the body weight of dams among the groups (Fig.1). Serum progesterone level of dams showed no significant difference ($p>0.05$) among the groups (Fig. 2). Maternal blood glucose level at GD 21 was higher in the fructose group compared with the control group ($p<0.05$) and was similar in both the *C. lanatus* and fructose + *C. lanatus* groups when compared with the control group ($p>0.05$) (Figure 3). Placental weight and circumference were lower while placental thickness was higher in the fructose group in comparison with the control group ($p<0.05$) and this was similar in both the *C. lanatus* and fructose + *C. lanatus* groups when compared with the control group ($p>0.05$). The fetal weight on GD 21 was higher ($p<0.05$) in fructose group when compared with the control group and was similar in the *C. lanatus* and fructose + *C. lanatus* group in comparison with the control group ($p>0.05$). The feto-placental weight ratio was higher ($p<0.05$) in

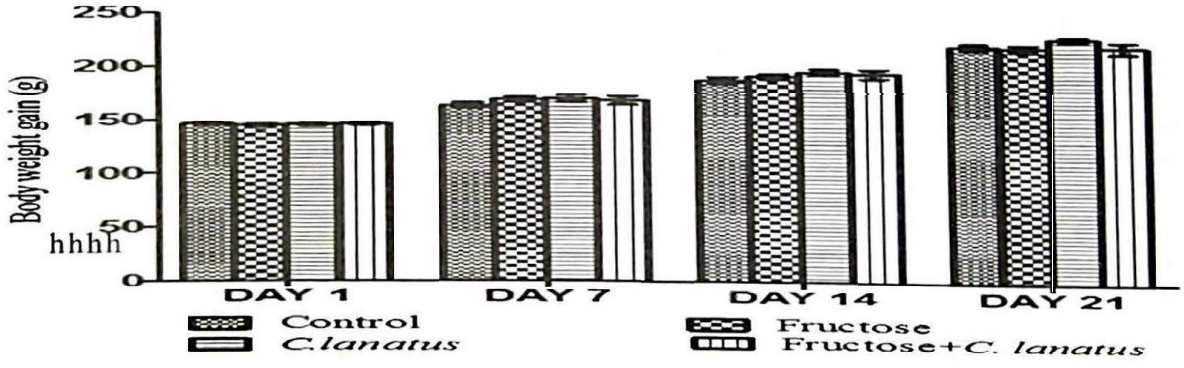

Fig. 1: Effect of fructose and *C. lanatus* juice on body weight gain of pregnant rats

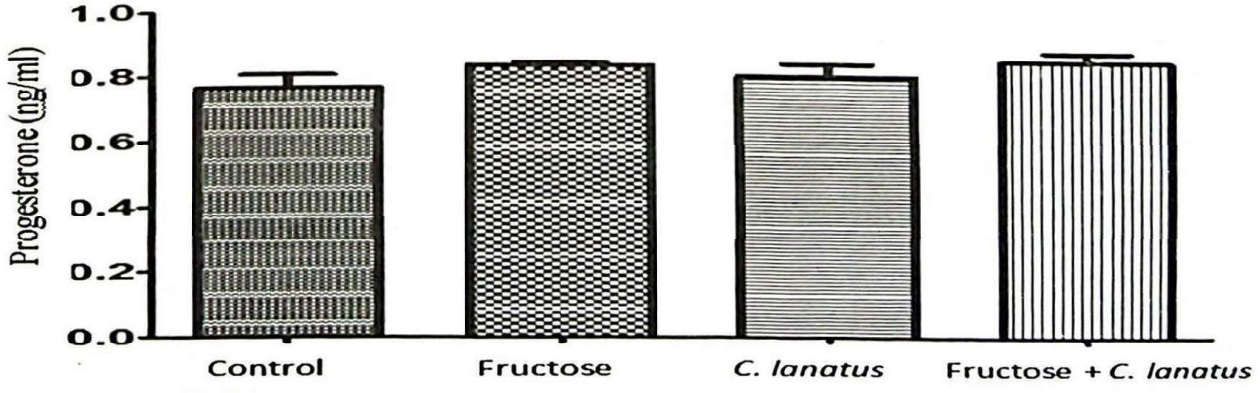

Fig. 2: Effect of fructose and *C. lanatus* juice on progesterone levels of pregnant rats

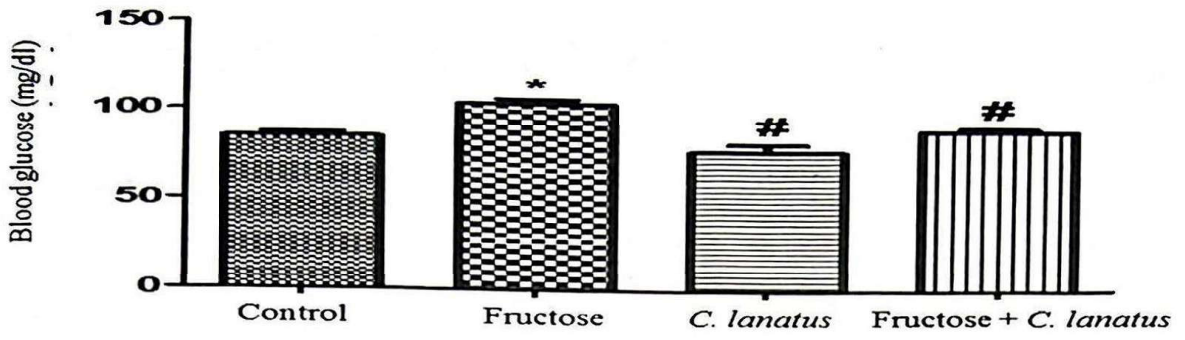

Fig. 3: Effect of fructose and *C. lanatus* juice on blood glucose level of pregnant rats
*P<0.05 when compared with control group. #P> 0.05 when compared with fructose group.

Table 1: Effect of fructose and *C. lanatus* juice on feto-placental morphometric indices.

Index	Control	Fructose	*C. lanatus*	Fructose + *C. lanatus* juice
Fetal weight (g)	3.5 ± 0.0	3.8 ± 0.0*	3.6 ± 0.1#	3.6 ± 0.0#
Placental Weight (g)	0.6 ± 0.0	0.5 ± 0.0*	0.7 ± 0.0#	0.6 ± 0.0
Circumference (mm)	4.8 ± 0.1	4.5 ± 0.0*	4.7 ± 0.1#	4.5 ± 0.0*
Thickness (mm)	2.5 ± 0.0	2.9 ± 0.1*	2.6 ± 0.1#	2.6 ± 0.0#
Volume (cm^3)	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
Feto-Placental weight ratio	5.8 ± 0.1	7.0 ± 0.2*	5.5 ± 0.1#	6.3 ± 0.2#

n=5, *p<0.05 when compared with control group, #p>0.05 when compared with fructose group.

Table 2: Effects of fructose and *C. lanatus* juice on placental oxidative status

	Control	Fructose	C. *lanatus*	Fructose + C. *lanatus*
MDA(U/mg protein)	0.001±0.0001	0.002± 0.0002*	0.0003±0.00001*#	0.001±0.00003#
SOD(U/mg protein)	5.8 ± 0.4	2.2 ± 0.3*	10.3 ± 0.9*#	6.1 ± 0.4#
Catalase (IU/L)	0.4 ± 0.01	0.4 ± 0.004	0.5 ± 0.003*	0.4 ±0. 0002
GSH(U/mg protein)	38.8± 2.1	25.5 ± 7.7	78.0 ± 4.4*	39.1±3.4#
Protein conc. (mg)	1.6±0.01	1.7±0.01	0.8±0.02*#	1.5±0.02

n=5, *P<0.05 when compared with control group, # P>0.05 when compared with fructose group
MDA = Malondialdehyde; SOD = Superoxide dismutase; GSH = Reduced glutathione

the fructose group than in the control group and was similar (p>0.05) in the *C. lanatus* and fructose + *C. lanatus* groups when compared with the control group (Table 1). Placental superoxide dismutase and reduced glutathione are lower in the fructose group compared with control ($p < 0.05$) and was similar in the fructose + *C. lanatus* group compared with the control group ($p > 0.05$). Placental malondialdehyde was higher in the fructose group and lower in the fructose + *C. lanatus* group compared with the control and the fructose groups respectively (Table 2). Photomicrograph of the control and *C. lanatus* groups showed normal chorionic villi, while the fructose and fructose + *C. lanatus* groups showed severe and moderate infarction of the chorionic, respectively (Fig. 4).

Discussion

Maternal fructose consumption alters placental growth and pattern of fetal development [4]. Fructose consumption causes oxidative stress and metabolic derangements in the placenta [33-34], both of which

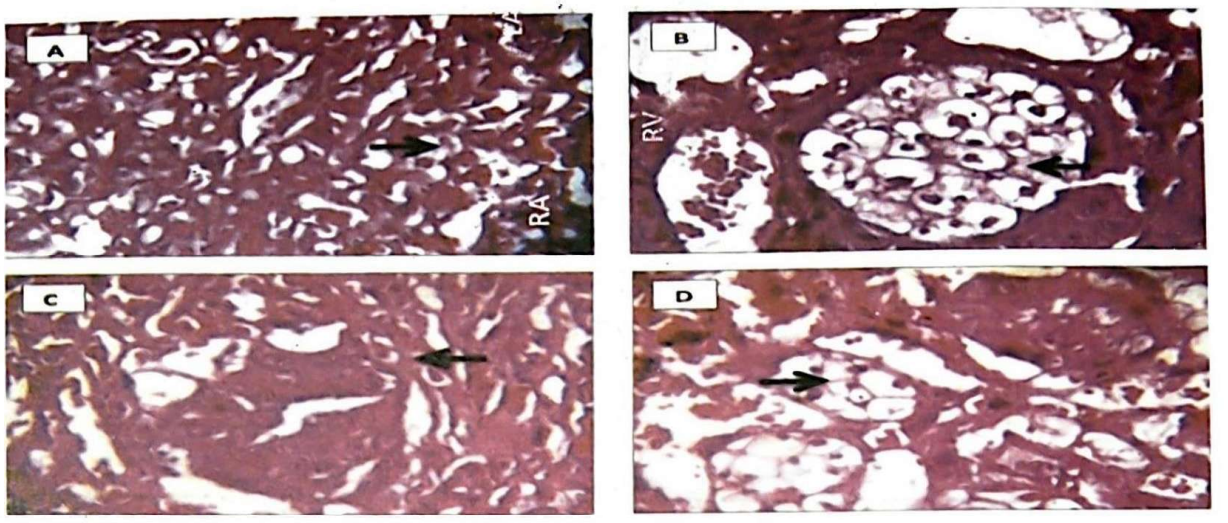

Fig. 4: Photomicrographs of placentas from pregnant rats given fructose solution and *C. lanatus* juice throughout gestation. H &E. Magnification X400. A = Control, B = Fructose, C = *C. lanatus*, D = Fructose + *C. lanatus*.

are detrimental to fetal development. There have been reports on the antioxidant and hypoglycemic effects of *C. lanatus* juice [23]. This study demonstrated the effects of *C. lanatus* juice on fructose-induced placental morphometric aberrations and oxidative stress in Wistar rats.

The higher maternal blood glucose level in the fructose group suggests that increased fructose consumption during pregnancy may cause hyperglycemia. This confirms results from previous studies that associated high consumption of fructose and fructose-rich foods with development of hyperglycemia and diabetes [3]. These conditions during pregnancy are known to affect fetal development with resultant susceptibility to health issues in adult life [3, 11, 35]. The rats which received both fructose and *C. lanatus* juice were normoglycemic suggesting that *C. lanatus* juice has the ability to prevent development of hyperglycemia in pregnant rats. Lycopene, a hypolipidemic agent present in *C. lanatus* may be attributed to this effect [36]. Maternal hyperglycemia throughout gestational period predisposes to an increase in glucose transport across the placenta [37]. Since maternal insulin does not cross the placenta [38], the pancreas of the fetus causes compensatory increase in insulin secretion thus stimulating increase in pup growth and adiposity which can result in the higher birth weight that was recorded in the fructose group. The birth weight of the group that had *C. lanatus* plus fructose was comparable with that of the control group. Again, the adiposity-countering effect of lycopene becomes evident.

Feto-placental ratio is often used as a proxy for placental efficiency and it is defined as the weight of fetus produced per weight of placenta [39]. In the present work, the observed increase in the fructose group feto-placental ratio might be due to low placental weight and high birth weight. Adequate fetal growth is dependent on nutrient transfer by the placenta [40] and evidence has shown that the placenta adapts anatomically and physiologically to changes in the environment in order to achieve optimal fetal growth [41]. These adaptations normally occur in response to maternal and/or fetal insults [41] and failure of such placental adaptations may result in high or low birth weight [42]. Thus, increased fetal weight might have resulted from the failure of placental adaptation. The failure of placental adaptation may also have contributed to the asymmetric increment in body weight. In addition, fructose consumption is reported to elevate uric acid production [43] which can lead to increased *de novo* lipogenesis [44] in the placenta and resulting in increased lipid traffic across the feto-placental unit causing high birth weight. Reduction in placental weight in dams fed with fructose has been reported [4] but why maternal fructose consumption causes reduced placental weight remains elusive. In the fructose + *C. lanatus* group, the placental weight, birth weight and feto-placental ratio were normal and comparable with that of control group. Again, the hypolipidemic effect of lycopene becomes evident.

Malondialdehyde (MDA) is an index of lipid peroxidation. The elevated malondialdehyde

levels observed in the placenta of fructose-fed dams in this study suggests the accumulation of superoxide radicals and hydrogen peroxide in the placenta thereby making it prone to oxidative stress [45]. As explained earlier, high fructose consumption during pregnancy may predispose to uremia with resultant oxidative stress. Oxidative stress is normal during pregnancy; however, exaggerated levels have been reported in pregnancies associated with complications. The low activity of superoxide dismutase in the fructose group placenta indicates that there was an accumulation of superoxide radicals in the placenta of fructose-fed dam. Superoxide dismutase (SOD) constitutes an important link in the biological defense mechanism through conversion of endogenous cytotoxic superoxide radicals to H_2O_2 which are harmful to polyunsaturated fatty acids and proteins [46- 47]. Catalase further detoxifies H_2O_2 into H_2O and O_2 [46].

Similarly, the reduction of catalase activity observed in placenta of fructose-fed dam clearly reflects the inability of the tissue to eliminate H_2O_2 produced by the inactivation of the enzymes probably due to the excess generation of ROS. Results suggest the reduction in the levels of these enzymes led to oxidative stress in the placenta of the fructose group. However, *C. lanatus* supplementation successfully preserved the levels of these enzymes. Reduced glutathione (GSH) is considered to be one of the most significant constituents of the antioxidant defense of living cells. The reduction of GSH may be one of the reasons for the increased vulnerability added to free-radical-induced damage.

Therefore, the decreased in GSH level observed in this study suggests that its toxic effects may expose the placenta to damage. *C. lanatus* supplementation successfully ameliorates the levels of these antioxidant compared to the fructose-fed group. The observed oxidative stress as demonstrated by increased MDA reduced SOD, catalase and GSH may be responsible for the severe infarction of chorionic villi seen within the frondosum layer as shown by the photomicographs of placenta.

However, supplementation with *C. lanatus* juice successfully ameliorated the fructose-induced placental oxidative stress and histological aberrations. This is in line with the studies of Oseni *et al.* [23] and Mohd *et al.* [24] which reported that watermelon juice decrease lipid peroxidation. This could be an indication that this medicinal fruit has the essential potentials to mitigate oxidative processes caused by maternal high fructose intake.

Conclusion

Citrullus lanatus juice modulated fructose-induced placental morphometric changes and oxidative stress in Wistar rats. Thus intake of *C. lanatus* juice may be beneficial for optimal and healthy development of placenta and fetus of mothers who experience sugar cravings during pregnancy.

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