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The Effects of Fructose on the Developmental Competence, Biochemical Biomarkers and Apoptotic Gene Expression by Murine Oocytes

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THE goal of the current study was to investigate the effects of fructose supplementation in the culture medium on *in vitro* maturation, and the developmental competence of murine oocytes, biochemical markers and the expression of apoptotic and HIF-1α in cumulus oocyte cells (COCs). Murine oocytes were matured in IVF media supplemented with 1.25, 2.75 and 5-mM fructose. The developmental parameters (MI, MII, GVBD, degenerated and activated cells) were examined with NO, GPX, GSH, MDA, and TAC biomarkers. The expression of mRNA for apoptotic genes by COCs were evaluated. The low dosage improves significantly ($P<0.05$) the developmental competence markers than the control and higher doses of fructose. In vitro-matured mouse oocytes with 1.25 mM fructose showed a significant increase ($P<0.05$) in NO level in the In-vitro matured when compared with the control and other groups. The GPX concentrations in the in-vitro matured oocytes showed a significant decrease ($P<0.05$) with 5mM fructose. The MDA levels in the vitro-matured oocytes were higher ($P<0.05$) when the murine oocytes were exposed to 2.75 mM of fructose. The TAC in the oocyte showed no changes ($P<0.05$) when the oocytes were exposed to both 1.25 mM. Moreover, the supplementation of IVM media showed a down regulation of apoptotic and HIF-1α genes when compared with the control and high dosage supplementation ($P<0.05$). Altogether, the supplementation of IVM media with lower dosage of fructose improves the developmental competence markers in the murine oocytes via its effects on oxidant and antioxidant activity and downregulation of apoptotic and HIF-1α genes.

Keywords: Fructose, Developmental competence, Murine, Apoptotic genes, and Oxidative stres.

Introduction

The oocyte-granulosa cell complex is the most basic component necessary to sustain oocyte growth and development. In order to evaluate the mechanisms promoting nuclear and cytoplasmic maturation in mammalian oocytes, a culture system was developed using these complexes. Nuclear and cytoplasmic maturation are two stages of oocyte maturation [1, 2]. Oocytes resume meiosis from GV breakdown to prophase-I enter metaphase-I, and move through anaphase-II / telophase-I to the metaphase-II (MII) stage during nuclear maturation [3]. The process of cytoplasmic maturation also involves

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further alterations occurring within the oocyte, including as the buildup of mRNA and proteins, remodelling of the cytoskeleton and organelles, and adjustments to cellular metabolism [2, 3].

The capacity to complete pre-implantation progress is known as cytoplasmic maturation, whereas nuclear maturation is the continuation of the first meiosis and the extrusion of the first polar body (PB1) [3, 4]. It is well known that in-vitro matured oocytes have lower developmental capability than in vivo matured oocytes, owing to insufficient cytoplasmic maturation.

In vitro maturation (IVM) of oocytes has the opportunity to be a beneficial tool for both clinical infertility treatment and animal assisted reproductive technologies [5] . The IVM has been and continues to be a significant research technique for determining factors that definitely effect oocyte competency developmental.

Indeed, the great majority of studies on oocyte competency regulation are conducted in vitro. These studies show that the cumulus significantly affects oocyte biology, including (but not limited to) the supply of small molecular weight molecules necessary for controlling meiotic maturation and oocyte metabolism; the acquisition of cytoplasmic signaling mechanisms that enable the oocyte to develop later [5].

Although numerous studies, IVM-derived oocytes from most species have a slightly inferior developmental result than those obtained via *in vivo* maturation [6] . The contact between the cumulus cells and the oocyte is essential for oocyte competence and has been the topic of numerous studies [7]. However, when compared to *in vivo* maturation, this communication is enhanced during in-vitro maturation. As compared to *in vivo* produced COCs, the cumulus cells of an in-vitro matured COC would have significantly altered gene expression patterns, indicating defective features of cumulus cell function.

In vitro embryo development is greatly influenced by the conditions in which human oocytes are cultured during *in vitro* maturation and fertilization. Many research teams have developed various mammalian embryo culture mediums [8]. Single-step medium is one of these media that is used often nowadays for a variety of tasks, including in vitro maturation or *in vitro* culture of mammalian embryos that were created either through in vivo or in vitro fertilization [3,8,9].

The energy substrate is one of the most significant elements in any culture medium for optimum embryo growth *in vitro*. In a single-step media for IVM of mammalian oocytes and subsequent IVC of *in vitro* generated embryos, glucose was introduced as an energy source [8, 10]. Fructose is capable of entering the glycolytic pathway. Furthermore, fructose is abundant in bovine reproductive systems [10-12] and successfully increases embryonic development in hamster bovine, porcine [13], and human embryos [14]. However, in both hamsters and bovine blastocysts, fructose can increase overall cell counts. Furthermore, fructose's embryotrophic effect is shown by the expression of the fructose-transporter gene in bovine embryos [15]. Altogether, the current study aimed to investigate the effects of fructose on in vitro maturation, and the developmental competence of murine oocytes, biochemical markers and the expression of apoptotic and HIF-1α in cumulus oocyte cells (COCs)

Material and Methods

Experimental animals

The current study was carried out at Mansoura University's Reproductive Biology Research Center, Department of Theriogenology, Faculty of Veterinary Medicine. The Committee for Research Ethics of Mansoura University's Faculty of Veterinary Medicine in Egypt has accepted all experimental procedures (PhD/29). All the procedures of biosecurity and biosafety for research work in laboratories were followed [16]. The mice were purchased from a medical research center (MERC), Faculty of Medicine, Mansoura University, 35516, Mansoura, Egypt.

Fifty sexually mature female mice (8-12 weeks old; approximately 25 g body weight) obtained from the Mansoura university experimental research center (MERC). The animals were kept under a 12-h light/dark cycle and a temperature of 21.0 ± 0.5 C., with access to water and food ad libitum to induce superovulation, 48 hours before to the scarification of mice, each mouse received a 10 IU intraperitoneal injection of pregnant mare serum gonadotropin (PMSG, Gonaser®, HIPRA, Spain) [17] .

Chemical materials

All chemicals in the present study were purchased from Sigma Aldrich. In addition, all supplies were purchased from Falcon.

Handling media

Earl's balanced salts (Cell culture medium EBBS)

The major objective of buffered salt solutions is to maintain pH and osmolarity in cell culture applications while also providing necessary inorganic ions (PAN-BIOTECH GMBH). These solutions are typically used to clean tissues and cells. In cell and tissue treatment, they are frequently coupled with other treatments.

G_MOPS™

The media has amino acids and antibiotics, and it has the same osmolality and pH as Vitrolife culture media [18]. (https://blog.vitrolife.com/togetheralltheway#::text=All%20content%20%. C2% A9% 20 Vitrolife % 202018IS) pH stable handling media developed to support the handling and manipulation of oocytes and embryos outside the incubator. G-MOPS are handling medium that allows operations such as ICSI to be performed outside of the CO_2 incubator without worry about pH changes, even in the most time-consuming conditions.

Multipurpose Handling Medium (MHM)

The first dual buffered solution to ensure the safety of gametes or embryos throughout IVF operations such as oocyte handling, embryo transfer, micromanipulation, and gamete washing. It is not necessary to utilize a CO_2 incubator. To regulate pH and support optimal cell growth, the MHM™ solution contains valuable amino acids as well as a unique mixture buffering system of HEPES and MOPS.

Maturation media

Global® total® Single-step medium

The oocytes, associated cumulus cells, and sperm function best in a protein-supplemented solution that is a bicarbonate-buffered and contains all 20 amino acids as well as glucose, lactate, and pyruvate.

Human - and -globulins* (0.6 mg/ml), LifeGlobal® Protein Supplement* (4.4 mg/ml), Sodium Chloride, Sodium Pyruvate, Potassium Chloride, Calcium Chloride, Potassium Phosphate, Magnesium Sulfate, Sodium Bicarbonate, Sodium Lactate, Glucose, Amino Acids, EDTA, Phenol Red, and Gentamicin Sulfate* (10 µg/ml), *from a therapeutic-grade source.

Oocytes recovery after ovaries collection

The abdomen was opened after cervical dislocation in mice, and the ovaries were found and removed from the body. The ovaries were then freed of any remaining fat and rinsed in warm sterile physiological saline (0.9% NaCl) to remove any blood stains [19] . After that, the ovaries were put in a sterile 60 mm Petri dish with G-MOPS™ Plus Medium (Vitrolife, Sweden) to recover the oocytes. According to MontiRedi, the release of cumulus-oocyte complexes (COCs) from the ovaries were achieved by gently puncturing the antral follicles with a sterile insulin syringe needle under a SZ61 zoom stereo microscope (Olympus, Japan). COCs that had been released were identified and sorted according to their morphological characteristics. Only high-quality COCs of oocytes that showed uniformly granular ooplasm and were surrounded by compact multi-layers of follicular cells were used [20].

Fructose preparation (in-vitro study)

Fructose extract (≥99%) was purchased from Sigma_Aldrich Scientific Inc.(Fructose; Sigma-Aldrich,57-48-7) (Cairo, Egypt). For preparing the Fructose stock solution, 0.09 mg of fructose were dissolved in 1mL of IVM maturation media to achieve a concentration of 5 mM.

Fructose stock solution was immediately aliquoted, protected from light, and stored in a refrigerator (4℃) till the day of use. A fructose concentration of 1.25, 2.75 and 5mM/ml was used in the present study. These concentrations were employed according to Tsujii et al. [15], and our preliminary observations. Fructose working solutions were freshly prepared every time via diluting the stock solution with appropriate amounts of the IVM medium.

According to Su et al. [21] the COCs were randomly cultured in groups of 20–25 oocytes in a 50 µl droplet of prewarmed IVM media covered with sterilized mineral oil for 17 h at 37°C in 5% CO_2 in air with a maximum humidity (95%).

Assessment of the rates of mature (MII), MI, GVBD, activated and degenerated oocytes:

After 17 hours of oocytes incubation in Global total medium, oocytes were denuded from surrounding cumulus cells by gentle pipetting many times. Maturation rate was judged based on the breakdown of the germinal vesicle with formation of MII spindle and/or extrusion first polar body. The rate of MI, GVBD, degenerated and activated oocytes was calculated by dividing their total number by the total number of cultured oocytes [22].

Biochemical analysis

Each group had four samples examined. Each sample contained 20-25 oocytes containing

COCs in 100 µl of IVM medium. The oocytes were pelleted by centrifugation at 3000 rpm for 10 minutes at 4°C. The cell pellets were gently washed twice with 500 µl of cold phosphate-buffered saline (PBS). The pellets were disrupted in 800 µl of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH = 8.0) by passing them through three cycles of repeated freezing and thawing followed by vertexing. Lysed oocytes were centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant was decanted and utilized for biochemical analysis.

Intracellular oxidative biomarker levels of NO [23] and MDA [24] within the in-vitro matured oocytes were determined calorimetrically using commercially available kits (Biodiagnostics , Egypt) per manufacturer's instructions.

TAC [25], GSH [26], and GPX [26, 27] intracellular antioxidant biomarker levels inside in-vitro matured oocytes were measured using colorimetric test kits (Biodiagnostics, Cairo, Egypt) according to manufacturer's instructions.

Gene expression analysis

All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). These primers include Bax , caspase 3, P53 and HIF 1α expression genes as apoptotic and in mice oocyte. The sequences of the primers are provided in Table 1. The procedures for mRNA extraction from the Oocytes were performed using Trizol reagent (Thermo Fisher Scientific, UK) according to the manufacturer's instructions for RNA extraction from the Oocytes [28]. The HisenscriptTM cDNA synthesis kit was used to convert one microgram of isolated RNA into cDNA (Intronbio, South Korea). The freshly generated cDNA strand served as a template for gene expression studies involving angiogenic proteins and water transporters. The Pikoreal real-time PCR system was used for the PCR (Thermo Scientific, Lituania). The PCR cycling conditions were as follows: initial denaturation at 94 °C for 9 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 40 seconds, and elongation at 72 °C for 1 minute, followed by a final elongation step at 72 °C for 9 minutes. The 2-ΔΔct technique was used to calculate fold expression relative to the control group for gene expression analysis against B-actin as a housekeeping gene.

Statistical analyses

The run of Statistics analyses will be according to Elmetwally [29] .The normality of quantitative data (matured oocytes number, and mRNA of HIF-1α and apoptotic genes) will be determined using normal probability plots and the Kolmogorov-Smirnov test, which will be generated using SAS's UNIVARIATE procedure. The mean error of the mean will be used to express all experimental data (SEM). A one-way analysis of variance (ANOVA) will be used to determine the effect of Fructose on the oocyte maturation, and biochemical markers changes followed by Duncan's multiple comparison test. SAS® will be used for statistical analysis (version 9.2, SAS Institute, Cary, NC, USA). For all analyses, $P \leq 0.05$ was defined as significant.

TABLE 1. Sequences of forward and reverse primers used for qPCR quantitation.

Gene	Forward primer	Reverse primer	Accession numbers
BAX	AGACAGGGGCCTTTTTGTTAC	GAGGACTCCAGCCACAAAGAT	XM_015094220.1
Caspase 3	GAATGTCAGCTCGCAATGGTAC	AGTAGTCGCCTCTGAAGAAAC-TAG	NM_001293722.1
P53	CCCCTGAAGACTGGATAACTGT	GACAGGCACAAACACGAACC	NM_030989.3
HIF 1α	GCAACTAGGAACCCGAACCA	TCGACGTTCGGAACTCATCC	NM_024359.2
B actin	CCCGCGAGTACAACCTTCTT	AACACAGCCTGGATGGCTAC	NM_031144.3

Results

The effect of varying Fructose concentrations in IVM global® total media on the developmental competence of in-vitro matured mouse oocytes:

Among the four tested concentrations of fructose, the addition of 1.25 mM fructose to the IVM medium revealed a significant increase in the MII% (53.92±7.078%) when compared to the control; 2.75 and 5mM of fructose supplementation (37.06±1.57, 36.76±3.77 and 24.47±1.50%, respectively, p< 0.0001) (Table 2). On the other hand there was non-significant difference in the MII percentage yield when supplementation of medium by 2.75mM fructose as well as in the control group (36.76±3.77% and 37.06±1.57 %, respectively; p>0.05) (Table 2).

The MI % in the current study exhibited significant increase with 1.25 mM fructose (36.16±4.43%, p< 0.0001) when compared with the control and other treatments (20.41±1.16, 26.16±1.55 and 21.97±5.01 %, respectively, p< 0.05) (Table 2). Conversely, GVBD % was decreased significantly (P< 0.0001) with supplementation of 1.25 mM of fructose (3.57±0.88 %, p< 0.0001) in comparison to control, 2.75 and 5mM of fructose (Table 2; 16.65±0.64 ,10.76±3.29 and 21.63±1.62 %, respectively, p< 0.0001).

The degenerated oocytes percentage decreased significantly when the maturation medium with 1.25mM fructose (2.78±0.30%, p< 0.0001) than without, with 2.75 and with 5mM of fructose supplementation (18.24±2.34, 9.70±2.90 and 14.21±4.39%, respectively, Table 2). The percentage of activated oocyte percentage showed a significant decreased when the maturation media supplemented with 1.25 mM fructose (8.16±1.72 % p< 0.0001) in comparison to supplementation of maturation media with 0, 2.75 and 5mM of fructose (9.03±1.61, 16.37±4.62 and17.93±3.70%, respectively, Table 2).

Effect of adding different concentrations of Fructose to IVM global® total media on oxidative stress and antioxidant biomarker levels of In-vitro matured mice oocytes:

In vitro-matured mouse oocytes with 1.25 mM fructose showed a significant increase in NO level in the In-vitro matured when compared with the control and other treatment groups (1.25 mM: 76.39±1.05 mmol/L; 0 mM:.54.39±0.44; 1.25: 51.94±1.60 and 2.75: 44.38±0.59 mmol/L; 5 mM: 51.94±1.60; mmol/L; P < 0.0001), (Table 3).

The GPX activity in the in-vitro matured oocytes (Table 2) showed a significant decrease with 5mM fructose (113.33±27.83nm) when compared to control treatment (783.77±5.53. nm/L); 1.25 mM fructose (394.70±3.60nm/L); and 2.75 mM fructose (1945.08±7.38 nm/L, P < 0.0001) (Table 3).

In the current study the GSH concentrations in the vitro-matured oocytes exhibited a significant increase in the control treatments (4.74±0.47 mmol/L) (Table 2) when compared to the other treatments (3.36±0.05 mmol/L; 3.85±0.47 mmol/L; 3.78±0.20 mmol/L,P< 0.0001 for 1.25, 2.75 and 5 mM fructose respectively) (Table 3).

In regard to MDA levels in the vitro-matured oocytes was investigated to be higher when the murine oocytes exposed to 2.75mM of fructose in cultural media (Table 3: 10.65±0.27 mmol/L, p<0.0001) when compared with 1.25 mM of fructose (9.52±0.12 mmol/L,p< 0.0001); control (7.86±0.04 mmol/L, p< 0.0001) and 5 mM fructose (8.80±0.09 mmol/L, P < 0.0001).

TABLE 2. Effect of adding different concentrations of Fructose to IVM global® total media on developmental competence of In-vitro matured mice Oocytes.

Treatment	n	MII%	MI%	GVBD%	Degenerated%	Activated%
Fructose (0 mM) control	168	37.06±1.57[a]	20.41±1.16[c]	16.65±0.64[b]	18.24±2.34[a]	9.03±1.61[b]
Fructose (1.25 mM)	109	53.92±7.078[a]	36.16±4.43[a]	3.57±0.88[d]	2.78±0.30[d]	8.16±1.72[b]
Fructose(2.75 mM)	103	36.76±3.77[b]	26.16±1.55[b]	10.76±3.29[c]	9.70±2.90[c]	16.37±4.62[a]
Fructose (5 mM)	106	24.47±1.50[c]	21.97±5.01[c]	21.63±1.62[a]	14.21±4.39[b]	17.93±3.70[a]

Means with different superscripts (a, b, c) are significantly different (P < 0.05). Values are mean ± SEM. Abbreviations: MII= metaphase II; MI= metaphase I, GVBD= germinal vesicle break down; TiO2NP= titanium dioxide nanoparticle.

TABLE 3. Effect of adding different concentrations of Fructose to IVM global® total media on oxidative stress and antioxidant biomarker levels of In-vitro matured mice oocytes.

Treatment	n	NO	GPX	GSH	MDA	TAC
Fructose(0Mm)control	168	54.39±0.44[b]	783.77±5.53[b]	4.74±0.47[a]	7.86±0.04[c]	0.16±0.001[c]
Fructose(1.25 mM)	109	76.39±1.05[a]	945.08±7.38[a]	3.78±0.20[b]	9.52±0.12[b]	0.16±0.001[c]
Fructose(2.75 mM)	103	44.38±0.59[d]	394.70±3.60[c]	3.85±0.47[b]	10.65±0.27[a]	0.35±0.026[a]
Fructose(5mM)	106	51.94±1.60[c]	113.33±27.83[d]	3.36±0.05[c]	8.80±0.09[c]	0.19±0.009[b]

Means with different superscripts (a, b, c, d) are significantly different (P < 0.05). Values are mean ± SD. Abbreviations: n= number of oocytes; NO= nitric oxide; GPX= Glutathione peroxidase (GPx); GSH = Glutathione; MDA = Malondialdehyde acetate; TAC = Total antioxidant capacity; TiO2NP= titanium dioxide nanoparticle.

The TAC levels in the in-vitro matured oocyte showed a significant increase when the oocytes were exposed to fructose at a concentration of 2.75mM (0.35±0.026 mmol/L, p< 0.0001) (Table 3). The TAC levels were (Table 3: 0.19±0.009 mmol/L) when the oocytes were exposed to 5 mM fructose in the maturation media. On the other hand, the TAC in the oocyte showed no changes when the oocytes were exposed to both 1.25 mM and 0 mM of fructose in the maturation media (Table 3: for both concentrations: 0.16±0.001 mmol/L).

Effect of adding different concentrations of Fructose to IVM global® total media on the mRNA expression of the apoptotic and hypoxia-inducing factor genes in the In-vitro matured mice Oocytes:

The supplementation of the IVM global total media with different concentrations of fructose showed a significant effect (P<0.05) on the expression of apoptotic and HIF-1α mRNA by the mice COCs (Fig. 2b). The mRNAs expressions were investigated to decrease with the supplementation of 1.25 mM fructose than the control and other doses of fructose supplementation. The mRNA for BAX and caspase 3 were found to be highly expressed in COCs groups treated with 2.75 and 5 mM of fructose (P<0.05) (Fig. 1a, b) than the control and 1.25 mM fructose supplementation. The expression of P53 mRNA showed no significant effects between COCs treated with 2.75 and 5 mM of fructose (P>0.05) (Fig. 2a). At the same time, the supplementation of IVM media with the lower concentration of fructose showed a significant decrease of P53 mRNA expression. Regarding the expression of HIF 1α, the supplementation of IVM media with high concentrations of fructose significantly decreased (p>0.05) the expression of HIF-1α mRNA. The lower concentration of fructose showed no significant effects (p<0.05) (Fig. 2b).

Discussion

Effect of adding different concentrations of Fructose to IVM global® total media on developmental competence of In-vitro matured mice Oocytes (In-vitro study)

The environments in which oocytes are cultured during IVF and IVM are crucial to the developmental competence of the immature oocytes and embryos in-vitro. The current study found that the addition of fructose to the cultural medium of in-vitro maturation medium plays an important role in increasing the percentage of developmental competence of murine oocytes (MI; MII; GVBD and degenerated oocytes) at a concentration of 1.25 mM. Moreover, the higher concentration of fructose in the cultural medium decreases the percentage of degenerated oocytes and increases the percentage of oocyte activation. As noted, the increase of degenerative oocytes is not a desirable sign for the development of embryos in-vitro [15,30]. The fundamental regulator of oocyte metabolism and viability is the percentage of acid metabolism, and maturing oocytes in a media deficient in these regulators causes the oocyte to lose the ability to control its metabolism. It is generally known that during certain developmental stages, fructose is digested more actively than glucose. This shows that the metabolism and maturational capacity of oocytes are primarily influenced by the incorporation and oxidation of glucose and fructose [15].

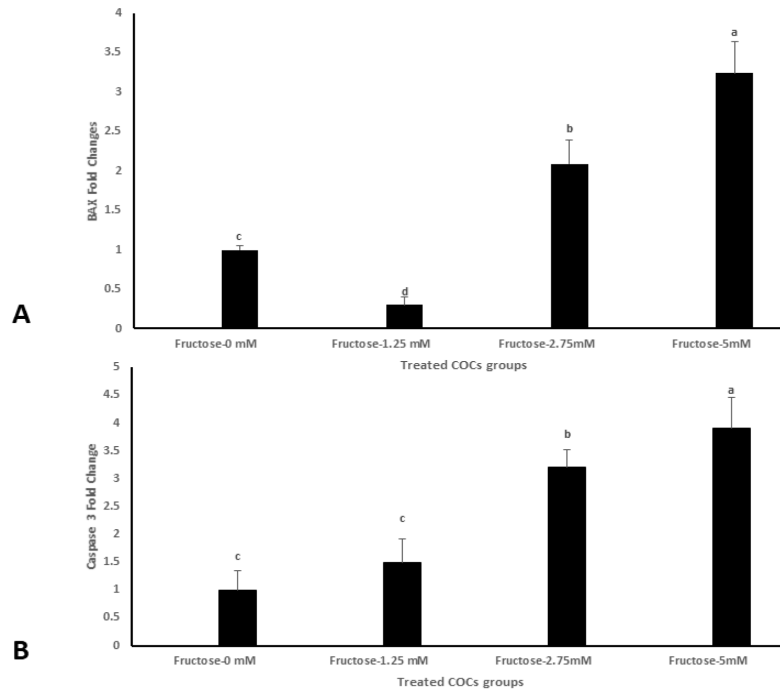

Fig. 1. Effect of adding different concentrations of Fructose to IVM global® total media on the expression of BAX (A) and Caspase 3 (B) by murine COCs. Different superscript letters ($P < 0.05$) indicate significant effects. All quantitative data are presented as means and SEM.

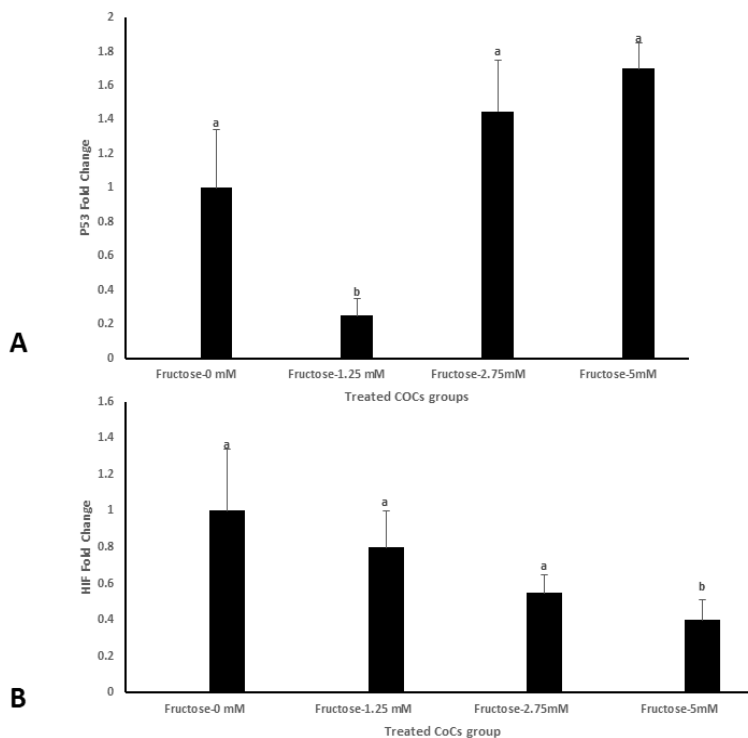

Fig.2. Effect of adding different concentrations of Fructose to IVM global® total media on the expression of P53 (A) and HIF-1α (B) by murine COCs. Different superscript letters ($P < 0.05$) indicate significant effects. All quantitative data are presented as means and SEM..

The improvement of the developmental competence in the current study when the maturation medium was supplemented by fructose is also attributed to the ability of fructose to enter the glycolytic pathway [31]. Furthermore, fructose in the current study increased the MI and MII. These results are similar to previous studies that indicates the ability of fructose to increase the total cell numbers [32]. Furthermore, the fructose transporter gene is expressed in bovine embryos, emphasising the embryotrophic function of fructose. According to earlier research, adding glucose and fructose to mTALP medium enhanced the spermatozoa of boars' potential to produce sperm [32, 33]. The requirement of Glucose for fertilization is due to the need of both cumulus cells and the oocytes. Cumulus cells are thought to create a significant amount of pyruvate in vivo, according to Gardner and Leese's hypothesis [34]. Therefore, the oocytes surrounded by the cumulus cells' ovulation are depending on the nutrients in the oviduct. Additionally, Brown and Whittingham [35] have demonstrated that the presence of pyruvate and glucose increases the rate at which 1-cell mouse embryos cleave within the first 24 hours.

The results of the current study are similar to that recorded by Wen et al., 2020 [36] in porcine oocytes. The addition of 1.25mM fructose to the culture medium greatly improved blastocyst development in pig transgenic cloned embryos. The presence of Glut5, which has a high affinity for fructose, suggests that the early embryo is capable of transferring this energy substrate, as well as its cryoprotective effects on many sections of mature oocytes [15,36]. Additionally, other research linked the positive benefits of fructose to the use of 14C-glucose in frog oocytes, and they found that at different embryonic stages, 1.25mM fructose was digested more vigorously than glucose [37].

On the other hand, Shin et al. 2017 [38], found that adding 2.75-3 mM fructose to the IVM medium resulted in a higher nuclear maturation of immature pig oocytes. Also when 5 mM of fructose were added to the IVM media of mouse oocytes, the nuclear maturation rate was decreased. In pigs, it was investigated that the oocytes exposed to media were supplemented with 5-5.5mM fructose to improve developmental competence and increase the ability of oocytes to reach MII [39].

The final stages of gamete maturation, fertilization and the start of embryonic development occur in the fallopian tube since the success rate of the various stages is very high under physiological conditions [40]. A good understanding of the oviductal physiology would provide useful data for the advancement of embryo IVP systems. Nitric oxide (NO), a very basic but virtually ubiquitous chemical involved in numerous intra- and extracellular signalling mechanisms, has been researched as a potential means of enhancing embryo IVP systems [41]) Mammalian oocytes spend the majority of their development, up until the gonadotropin surge of ovulation, imprisoned in the prophase of the first meiotic cycle. Both cumulus cell-enclosed GV-stage oocytes and denuded GV-stage oocytes, which were obtained from immature mice primed with pregnant mare's serum gonadotropin (PMSG), underwent spontaneous hormone-independent maturation and advanced to the metaphase II (MII) stage in vitro when cultivated in an appropriate medium. However, the developmental competence of oocytes matured in vitro without cumulus cells [42].

In the current study, the levels of No in the oocytes was investigated to increase when supplementing the maturation media with 1.25 mM fructose other than the other fructose concentrations and a control group. This indicates the beneficial activity of fructose during the early stages of development may be via its ability to increase the concentration of NO that has a potent antioxidant capacity. Furthermore, it was previously assumed that NO's actions were mainly mediated by the activation of soluble guanylyl cyclase (sGC), which creates varying levels of cyclic guanosine monophosphate (cGMP), which in turn activates cGMP-dependent protein kinases (PKGs), cGMP-gated cation channels, and cGMP-regulated phosphodiesterase (PDEs) [43]. Interestingly, NO in a dual manner is influences mouse cumulus cell-enclosed oocytes' *in vitro* meiotic maturation.

In the present study, the supplementation of maturation media with fructose at a concentration of 1.25 mM exhibits a significant increase in the GPX when compared with the control and other fructose-treated oocytes. The primary biological function of the GPX family of peroxidase-active enzymes is to guard the body against oxidative damage [17, 20]. Of note, antioxidants are the compounds which suppress the formation of ROS or oppose its actions and so maintain the function of the oocytes in-vitro. There are two types of antioxidants: enzymatic and non-enzymatic. Enzymatic antioxidants such as superoxide dismutase

(SOD), catalase (CAT), different peroxidases, and peroxiredoxins (PRDXs), including glutathione peroxidases (GPXs), are the components of enzymatic antioxidants. Peroxides can be converted to water and alcohol by these enzymes [44]. So, the present results prove the crucial effects of fructose that have antioxidant capacity via its effects on the levels of GPX in the cultural media.

Glutathione is a potent antioxidant that protects the cells from the detrimental effects of ROS. It is well-known that GSH contributes significantly to the cellular defense against dangerous substances of both endogenous and foreign origins [45]. Glutathione (GSH), a significant non-enzymatic antioxidant in oocytes, is an antioxidant that can reduce the effects of ROS [46]. It ensures proper microtubule formation, stops ROS from damaging tubulin assembly, and keeps the spindle functioning normally during meiosis [47]. Decreased intracellular GSH levels can also cause apoptosis to rise and embryo growth to be postponed [47].

In the current study, we investigated the effects of fructose supplementation to the maturation media on the levels of GSH. The fructose increased the levels of GSH and that means its protective effect during the process of IVM and embryo production. In bovines, stimulation of glutathione synthesis of in vitro matured oocytes has beneficial effects on embryo development and freezability [45]. In the current study, the MDA levels increased significantly with the supplementation of the maturation media with different concentrations of fructose. Malondialdehyde is a byproduct of polyunsaturated fatty acid peroxidation and is frequently brought on by high ROS concentrations [48]. Furthermore, it has been found that high MDA values are linked to DNA damage and low in vitro fertilization consequences [49].

In the present study, we found that MDA level is increased significantly in the groups of the oocytes treated with 1.25, 2.75 and 5mM fructose than control group. This agrees with [50] who found that there was an increase in liver lipid peroxidation in the group of rats fed a 60% fructose diet for 10 weeks. Another study found that MDA levels rise in a hamster pancreatic -cell line as a result of lipid peroxidation caused by fructose (Suzuki et al. 2000). The MDA may increase due to many factors such as inflammation, toxicity, exposure to mutagens and stress [51].

When the in-vitro developed oocytes were treated to fructose at a concentration of 2.75mM, the TAC levels increased significantly. TAC in the oocyte, on the other hand, showed no alterations when exposed to 1.25 mM and 0 mM fructose in the maturation media. Total antioxidant capacity, which includes both enzymatic antioxidant systems (such as glutathione peroxidase and superoxide dismutase) and nonenzymatic antioxidant systems (such as vitamin C, glutathione, hypotaurine, and taurine) found in oviductal and follicular fluids, can be used to assess embryo and oocyte protection against ROS [52].

The levels of these antioxidants would be a demonstrator of the severity of oxidative stress. The findings in the present study suggest an important role for fructose supplementation in the maturation media in preserving the integrity of polyunsaturated fatty acids structure within oocytes. Consistent with the present investigations, TAC was significantly elevated in the follicular fluids of those follicles in which their oocytes were successfully fertilized [53].

In the current study, the concentration of 1.25, 2.75 and 5mM fructose resulted in a lower intra-oocyte glutathione (GSH) content than control group, and this is may be due to fructose diminished reduced GSH in rats when given a single oral dose of fructose [54]. On the other hand the results of this study don't match those of [38,55], who found that adding 2.75-3 mM fructose to the IVM medium resulted in a higher intra-oocyte glutathione (GSH) content than control,1.25-1.5 and 5-5.5mM fructose groups and this is may be due to species oocytes difference or type of media used.

Nitric oxide is a signaling molecule that is involved in many processes and regulates several biological functions. The NO has been demonstrated to play a role in reproductive system physiology, where it modulates the activity of reproductive organs in both sexes [56]. Experiment data from multiple animal species imply the presence of an intraovarian NO-generating system in females, which could be involved in follicular development control. NO also has a favorable influence on follicle development and selection in relation to angiogenic processes, and it may have a function in modulating steroidogenesis in ovarian cells..

In the present study, we find that level of NO is decreased in 1.25 and 2.75mM fructose groups than control and 5mM fructose group. This result as [37] the same with who reported that Fructose feeding has been shown to diminish the production of nitric oxide by lowering the activity and expression of NO in human.

Our findings show that 1.25 mM fructose was comparable to 2.75 and 5 mM fructose in supporting in vitro oocyte maturation and might be employed in conjunction with glucose as an alternate energy source for in vitro mammalian oocyte maturation.

Oxidative stress occurs when the equilibrium between prooxidants and antioxidants shifts toward excess free radical production [57].

Oxidative stress affects all key cell components, including lipids, proteins, carbohydrates, and DNA. The oxidation of unsaturated fatty acids, known as lipid peroxidation, is one of the most damaging outcomes of free radical attack. Malondialdehyde is a byproduct of lipid peroxidation (MDA). It can be used as a cumulative marker of lipid peroxidation because it is a stable end product [58].

The effect of fructose on the expression of apoptotic genes and hypoxia inducing factor was studied for the first time in the current study. The supplementation of IVM media with low dose of fructose 1.25 mM fructose decreased mRNA expression of apoptotic genes compared to the control and higher fructose dosages. BAX and caspase 3 mRNA were observed to be more abundant in COCs treated with high dose fructose than in the control and low dose fructose groups. However, no significant differences were found between COCs treated with different higher concentrations of fructose. However, there were no significant effects of fructose supplementation of IVM global total media on mRNA HIF-1α expression. In women, the small dosage supplementation of fructose to the in-vitro maturation media suggests positive expression of HAS2, PTX3, GREM1, and VCAN that correlated with good quality oocytes and can be used as an indicator among PCOS women. These genes are responsible for cumulus expansion, cumulus cell apoptosis, and glucose metabolism [59]. The higher dose of fructose was reported to reproduce some of the features of metabolic syndrome, most changes were caused by oxidative stress and insulin resistance [54]. The regulation of the apoptotic genes resulted in the normal expansion of COCs and production of good quality embryos. The increased fold changes for the apoptotic genes and may HIF-1α indicate a bad quality of oocytes and hence decreased the yield of embryos after *in vitro* maturation [59,60]. Of note, a good balance of cumulus growth and apoptosis is required for optimal ovulation. Cumulus cell apoptosis causes poor oocyte results, embryo fragmentation, and impaired blastocyst development [59,61]. These results suggest that the low concentration of fructose would produce a good quality COCs that the higher ones due to the effects of the higher concentration of fructose alters the expression of apoptotic genes and HIF-1α.Other studies proved that women who were successful in becoming pregnant had a considerably lower percentage of apoptotic cells than those who were unsuccessful [62]. The same results were proved also in equine and suggested that the quality of oocytes and pronucleus formation after ICSI was related to the granulosa cell apoptosis [63].

Altogether, the supplementation of global total media (IVM) with small dosage fructose was found to improve the developmental competence of murine oocyte via its effects on biochemical changes as well as the changes in the expression of apoptotic and hypoxia inducing factor 1 genes expression by COCs. Further studies are indicated to illustrate the signaling pathways affected by fructose supplementation and lead to improvement of embryo yield in murine experimental models.

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Conflict of interest

There are no competing interests.

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تأثير الفركتوز علي الكفأة تطور البويضات والمؤشرات الحيوية والجينات المسئولة عن موت الخلايا المبرمج في بويضات الفئران

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أجريت هذه الدراسة لمعرفه تأثير اضافه الفركتوز الي ميديا نضج البويضات في المختبر (IVM) علي الكفاءة التنموية لبويضات الفئران ، والدلائل البيوكيميائية و الجينية المسببة لموت الخلايا المحيطة بالبويضات. تم نضج بويضات الفئران في الميديا المضاف اليها ١٫٢٥ و ٢٫٧٥ و ٥ ملي مول من الفركتوز. تم فحص الكفاءة التنموية للبويضات (MI ، MII ، GVBD ، الخلايا المتدهورة والمنشطة) باستخدام الدلائل البيوكيميائية NO و GPX و GSH و MDA و TAC. ,والدلائل الجينيةBAX ، Caspase 3 ؛ تم تقييم P53 و HIF-1α . تحسن الجرعة المنخفضة (١٫٢٥ ملي مول) من الفركتوز بشكل ملحوظ (P <0.05) علامات الكفاءة التنموية مقارنةً بالجرعات الضابطة والجرعات الأعلى من الفركتوز (٢٫٢٧ ، ٥ ملي مول). أظهرت بويضات الفأر الناضجة في المختبر مع ١٫٢٥ ملي من الفركتوز زيادة معنوية (P <0.05) في مستوى NO في المختبر النضج عند مقارنتها بمجموعة التي أظهرت تركيزات GPX في البويضات الناضجة في المختبر انخفاضًا كبيرًا (P <0.05) مع ٥ ملي فركتوز. تم فحص مستويات MDA في البويضات الناضجة في المختبر لتكون أعلى (P <0.05) عندما تعرضت بويضات الفئران إلى ٢٫٧٥ ملي مول من الفركتوز. لم يظهر TAC في البويضة أي تغييرات (P> 0.05) عندما تعرضت البويضات لجرعة أقل من الفركتوزو المجموعة الضابطة. علاوة على ذلك ، أظهرت ميدياIVM انخفاضا لجينات موت الخلايا المبرمج و HIF-1α عند مقارنتها مع مكملات الفركتوز الضابطة والجرعة العالية (P <0.05). في الختام ، فإن اضافة الفركتوزالي ميديا نضج البويضات بجرعة منخفضة يحسن علامات الكفاءة التنموية في بويضات الفئران من خلال تأثيره على نشاط الأكسدة ومضادات الأكسدة وكذلك التعبير المنظم لجينات موت الخلايا المبرمج و HIF-1 α. حكم ومجموعات العلاج الأخرى.