

EFFECT OF CHEMICALLY DEFINED LIPID MOLECULES ON BLASTOCYST DEVELOPMENT AND OUTGROWTH

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ABSTRACT

Introduction: Fatty acids play a diverse role in early embryonic development and implantation. This study examined the effect of a chemically defined additive containing saturated and unsaturated fatty acids on mouse fertilization and embryo development in vitro.

Objective: To observe the effect of a chemically defined addition of fatty acids on in-vitro fertilization, early embryonic development and blastocyst outgrowth

Study Design: F1 mice (4-6 weeks) were superovulated and 16 hours later oocytes were collected and prepared for IVF using spermatozoa from F1 males. Oocytes (n=355) were randomly allocated to global fertilization medium with (+) or without (-) chemically defined lipid (#11905-031, Life Global Technologies). Fertilization was checked (2-PN) 6-9hrs after insemination and embryos were grown up in blastocyst culture medium + lipid additive or controls. Embryo development was monitored daily until D5 when blastocysts were transferred to 96-well plates. Blastocyst outgrowths were imaged on D6, D7 and D9 using XY-Cyclone software. Data was analyzed (Chi-squares) to compare the effect of lipid on fertilization rate, blastocyst development and outgrowths.

Result: Oocytes (n=355) randomly allocated to control (n=170) or +lipid groups for fertilization showed no difference in fertilization rates in medium with (n=155) or without lipid. Addition of lipid to blastocyst medium resulted in a significant increase ($p < 0.05$) in blastocyst formation (73% vs 87%). but produced no difference in blastocyst hatching or outgrowth area.

Conclusion: A chemically defined lipid additive promoted embryonic development in vitro. Further studies are needed to examine the dose-response effect of lipid on fertilization and embryo development.

KEY WORDS: Blastocysts, Implantation, Fatty acids and In vitro culture.

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INTRODUCTION

Fertilization is the result of intercommunication of male and female gamete genomes [1]. From fertilization to blastocyst formation (4 to 6 days)

the human embryo undergoes remarkable changes in absorption and metabolism of the various components present in tubal fluid and uterine secretions. These metabolic changes

have a significant effect on embryo development [2]. For in vitro embryo development, the constituents of the culture medium are based on the constituents of these fluids [3-6]. An individual embryo's metabolic processes are associated with specific biomarkers of viability, which can be used to select a good quality embryo [7]. The pattern of utilization of metabolites such as glucose [8], oxygen [9,10], pyruvate [11], amino acids [12] and also lipids, which have a combination of fatty acids as their major constituent [13-15] known as "metabolomics" is useful to select a good quality embryo [16]. Along with all these mentioned metabolites, lipids are not only source of energy but also important in modifying and regulating cellular architecture and functional properties that affect cell-to-cell interactions, cellular propagation and transport [12]. The activities and actions of the proteins in cells are dependent on the lipid contents of the cell. Also the saturated and unsaturated fatty acid constituents of the lipid membrane [17] may modify the binding of effector molecules to the hormone receptor complex.

These fatty acids, which are the major components of the cell membrane lipid, are esterified to glycerophospholipids and glycerol and are essential to maintain the normal cellular function. Studies have shown that through this similar mechanism the varieties of saturated and unsaturated fatty acids in follicular and uterine fluid and embryos are important in fertilization and embryo development [12]. The same group has also reported that pre-implantation mouse embryos use exogenous fatty acids as a source for synthesis of lipids that are essential for embryo development and, by oxidation a source of energy. In vitro culture media, serum is the prime source of fatty acids and its presence or absence in vitro affects the morphology of bovine oocytes and further embryo development [18]. When BSA-bound fatty acids were removed along with a carbohydrate substrate from embryo culture a reduced the mouse embryo development [12]. Analysis of the oviduct and uterine fluid composition in sows revealed fatty acids were present in the endometrial secretions, however specific fatty acids were not identified [19]. The authors have concluded that

the main role of many unsaturated and saturated fatty acids in oocytes and in embryos is to supply energy. This is also seen in rabbit oocytes in the form of enhanced growth and continued development in salt based medium supplemented with unsaturated fatty acid in addition to saturated fatty acids such as palmitate, oleate, and stearate [20]. A study, using the bovine oocytes, has reported that total fatty acid content increases between day 11 and day 13 with a sharp rise in arachidonic acid by day 14, suggesting the start of prostaglandin synthesis is an important step in the process of implantation [21,22]. The mechanism behind that is prostaglandins the derivatives of arachidonic acid increases vascular permeability, stromal decidualization, trophoblast erosion that is the indispensable events of implantation [22]. This suggests that the lipid profile of an oocyte is dynamic and that embryo development is also sensitive to it [23]. Despite the conclusions from this introduction, there are still many aspects of the relationship between lipids, particularly free fatty acids, and oocyte quality, fertilization and embryo development that remain unknown. The aim of this project was-

- to observe the effect of culture medium containing a chemically defined mixture of lipids on fertilization and early embryonic development in vitro.

- to observe the direct effect of a chemically defined mixture of lipids on blastocyst outgrowth.

Data was analyzed (Chi-squares) to compare the effect of lipid on fertilization rate, blastocyst development and outgrowths.

METHODS

Media: KSOM handling media was used for oviduct and COC collection. Media was prepared fresh from 10 X concentrated stock solutions (prepared in house)²⁴ on blastocyst outgrowth. The medium used for fertilization was Global Fertilization media (Life Global Group, Brussels, Belgium) supplemented with (10%) HSA (Life Global Group Brussels, Belgium).

For embryo culture and for blastocyst culture was Cleavage Media and Blastocyst Media (Cook, Limerick, Ireland). The medium used for endometrial outgrowth was Blastocyst Culture Media

(Cook, Limerick, Ireland) with 10% FBS.

Test Material: Chemically defined lipid concentrate, (catalog number 11905-031, Life Global Technologies Melbourne VIC) was used in a concentration of $1\mu\text{l}$ for 1000 of media. The lipid was vortexed by vortex mixer (Ratek, Boronia, Victoria) before addition to the media.

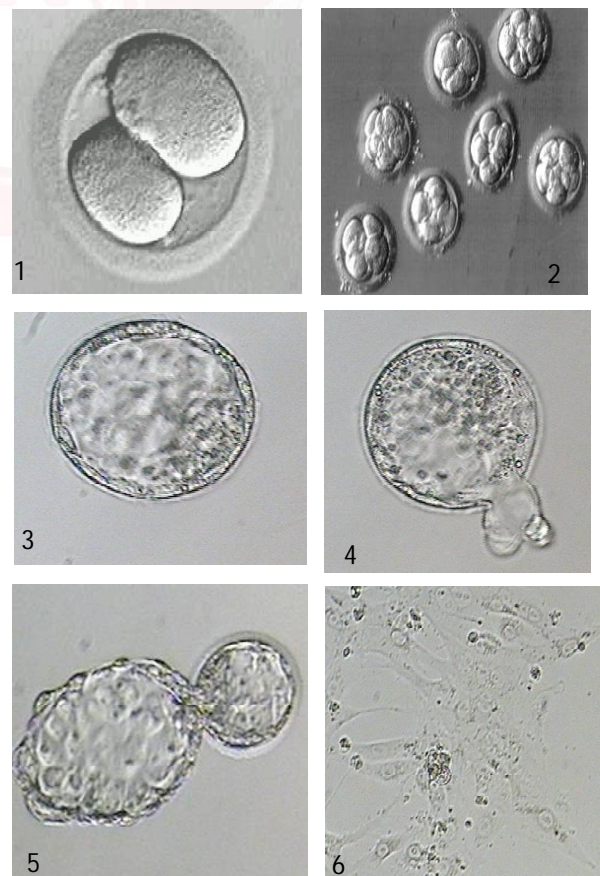
Animal and Ethics: Ethics approval for this research project was obtained from Monash Medical Centre Animal Ethics Committee (ACE Approval No A11/84). Four to six week old (C57BL/6X CBA) F1 female and (C57BL/6X CBA) F1 male fertile mice were sacrificed by cervical dislocation.

Experimental Protocol: Female mice were superovulated by intraperitoneal injections of 10 IU pregnant mare serum gonadotrophin (PMSG, Folligon, Bendigo) followed 46 hours later by 10 IU human chorionic gonadotrophin (hCG, Intervet, chorulon) Pre-equilibrated KSOM handling media was used for oviduct collection and embryo handling [24]. Culture media was pre-equilibrated at 37°C in CO_2 incubator (Sanyo, Gumma- Ken, Japan) [25]. Fourteen to sixteen hours after hCG injections oviducts and uterine horns were excised and placed into a sterile tube containing handling media at 37°C . The epididymides were removed, punctured with sterile forceps and spermatozoa were gently squeezed out into 0.5 ml pre equilibrated fertilization media in a tube. The tube was placed into an incubator at 37°C with 5% CO_2 in air for 30 mins to allow spermatozoa to disperse [26,27]. Groups of oocytes with associated cumulus masses were collected by puncturing the swollen ampulla and releasing them into a wash drop and then transferred into fertilization drop of test and control fertilization media dishes. Following dispersal of spermatozoa, they were thoroughly mixed and concentration was assessed using a haemocytometer. Volume for insemination of 0.5×10^6 motile spermatozoa was calculated and was added to fertilization drop in the without and with lipid fertilization dishes. Fertilization status of each oocyte was assessed at 5-6 hours after insemination by the presence of two pronuclei [26]. After 3 hours the best quality fertilized oocytes were extensively washed in culture micro-drops 1 & 2 and then transferred into drop 3 in both with lipid and

Table 1: Showing the dilution of chemically defined lipid concentrate in all medias used for experiment.

Fertilization Media			
	Global	HSA	Lipid
Control	4500 μl	50 μl	-
Test	4500 μl	500 μl 10% HSA/ μl	5
Cleavage Media			
	Cleavage Media	Lipid	
Control	5ml	--	
Test	5ml	5 μl (1:1000)	
Blastocyst Media			
	Blastocyst Media	Lipid	
Control	5ml	--	
Test	5ml	5 μl (1:1000)	
Endometrial Growth			
	Blastocyst Media	FBS	Lipid
Control	4500 μl	500 μl -10%	--
Test	4500 μl	500 μl -10%	5

Fig. 1: Representative images taken of the embryos on consecutive days showing developmental rate.



I- 2 cell embryo (day 1), II- Expanding Morulae (day 3), III- Blastocyst (day 4), IV- Hatching blastocyst (day 5/6), V – Wide hatching blastocyst (day 6), VI- Fully hatched attached blastocyst with TE, ICM outgrowth (day 7, 9).

without lipid cleavage culture dishes. Three to four embryos were transferred per drop in both the groups' cleavage culture dishes [4]. Embryos were cultured for up to 5 days in 30 μ l droplets of cleavage and then blastocyst culture media under paraffin oil in plastic culture dishes at 37°C in humidified atmosphere of 5% CO₂ in air. Embryo development was checked under a dissecting microscope at 80 magnifications and recorded each day until embryos reached the hatching blastocyst stage. Hatching blastocysts of with and without lipid media group were transferred individually into the wells of a 96 well outgrowth plate (Falcon, NY, USA). Images of the outgrowth plate containing hatching blastocysts were taken on the day of transfer on 5th or 6th day followed by 7th day and 9th day to check expansion of outgrowth by using eppendorf ICSI micromanipulator (Nikon, Tokyo, Japan) (Software used: Xyclone, Hamilton Thorne, USA). Outgrowth media was changed on 7th day [28] (Table 1 and Figure 1).

RESULTS

Total five replicas were performed to analyze the effect of chemically defined lipid on embryonic development and outgrowth. From all five replicates overall 355 oocytes were obtained and processed further for analysis. The data was transformed to produce the effect of chemically defined lipid on three different stages of development: Fertilization, blastocyst development and outgrowth of blastocysts. Representative images of all the developmental stages are shown in figure 3. The data was transformed to produce effect of chemically defined lipid on three different stages of development: Fertilization, blastocyst development and outgrowth of blastocysts.

Effect on fertilization: Of 355 oocytes, 170 and 185 oocytes in without lipid culture media and with lipid culture media were processed for fertilization. In our third replicate instead of normal motility and normal sperm count we observed remarkably less fertilization rate, this was seen in all other experiments groups who utilizes the same male mouse. Of the total oocytes processed for fertilization in both the culture medias 61.7% (105) in the "without lipid" media and 57.8% (107) in the "with lipid"

culture media were fertilized ($p > 0.05$).

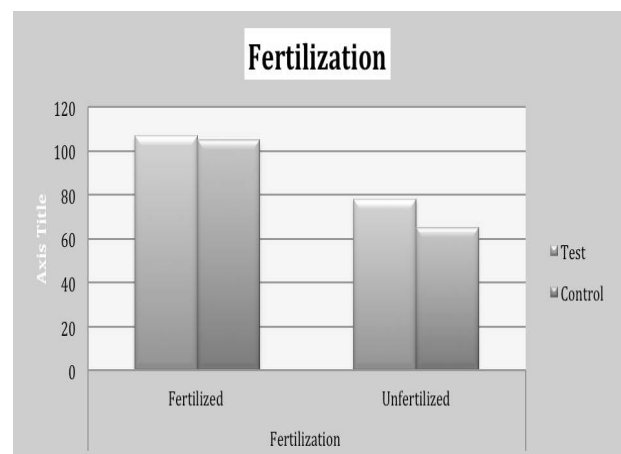
No significant difference was seen on fertilization rate in fertilization with lipid and fertilization without lipid media (Table 2 and Figure 2).

Table 2: Effect of lipid on the total number of oocytes and number of fertilized oocytes.

Replicate Number	Study group	No. of Oocytes found	No. of fertilized Oocytes	No. of unfertilized Oocytes	F Fertilization Rate
1	Fertilization Media	5	4	1	83.30%
	Fertilization Media+ Lipid	5	5	0	100%
2	Fertilization Media	38	23	15	60.50%
	Fertilization Media+ Lipid	42	24	18	57.10%
3	FertilizationMedia	43	10	33	23.20%
	FertilizationMedia + Lipid	47	9	38	19.10%
4	FertilizationMedia	41	35	6	85.30%
	FertilizationMedia + Lipid	48	38	10	79.10%
5	FertilizationMedia	43	33	10	76.70%
	FertilizationMedia + Lipid	43	31	12	72%
Total control		170	105	65	61.70%

Chi-square without Yates' correction for Two Tailed chi-square = 0.568 degrees of freedom = 1 probability = 0.451

Fig. 2: Graphical representation of the effect of chemically defined lipid on fertilization rate of mouse oocytes (Test = Fertilization media with chemically defined lipid, Control = Fertilization media without chemically defined lipid). $p > 0.05$.



Effect on blastocyst development rate: Out of the fertilized group total 73% (77 out of 105) in without lipid and 87% (93 out of 107) in with lipid culture media blastocysts were formed. The difference seen in blastocyst formation rate in blastocyst with lipid and blastocyst without

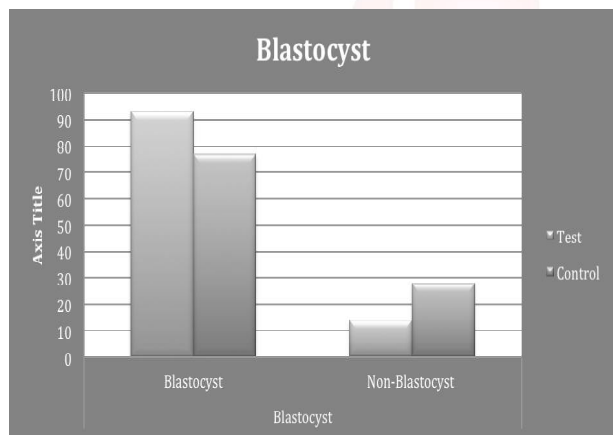
lipid media was statistically significant ($p < 0.05$), (Table 3 and Fig 3).

Table 3: Showing the total number of blastocyst formed fertilized oocytes.

Study Group	Blastocyst		Total no. Of 2 cells
	Blastocyst	Non - blastocyst	
Test	93	14	107
Control	77	28	105

Chi-square without Yates' correction chi-square = 6.15 degrees of freedom = 1 probability = 0.013

Fig. 3: Graphical representation of effect of chemically defined lipid on blastocyst development rate in vitro (Test = Blast. with Chemically defined lipid media and Control = Blast without Chemically defined)



Effect on blastocyst outgrowth rate: Of the total number of blastocyst formed 38% (40) in without lipid culture media and 51% (55) in with lipid culture media underwent wide hatching. No significant difference was seen on hatching blastocyst rate in blastocyst with lipid and blastocyst without lipid media ($p > 0.05$; Table 4 and Fig 4).

Fig. 4: Graphical representation of effect of chemically defined lipid on blastocyst hatching rate in vitro (Test = Blast. with Chemically defined lipid media and Control = Blast without Chemically defined)

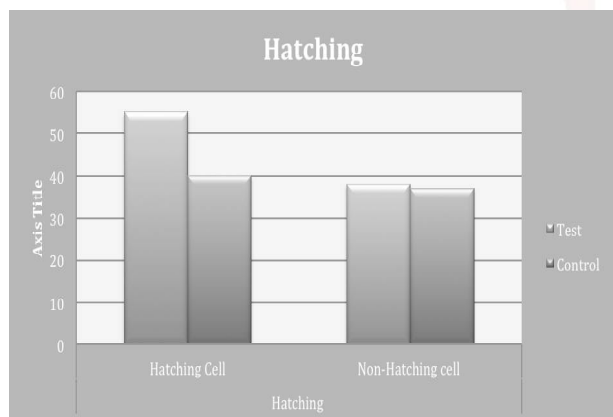


Table 4: Total number of hatching blastocyst from total number of blastocysts in control and lipid media

Group	Hatching blastocysts		
	Hatching blastocysts	Non-Hatching blastocyst	
Test	55	38	93
Control	40	37	77

Chi-square without Yates' correction chi-square = 0.884 degrees of freedom = 1 probability = 0.347

Effect of chemically defined lipid on blastocyst qualitative grading: This was analyzed by performing grading analysis between media with lipid and without lipid. In media with lipid all blastocyst were transferred on day 5th and very few on day 6th. No significant difference was seen in both media with lipid and media without lipid groups ($p > 0.05$). In both groups most of the blastocyst were in the wide-hatching stage on the day of transfer. Also the TE and ICM score were grade one in both groups.

On day 7th all blastocysts in both study groups were fully hatched and 100% attachment was seen in both groups.

On day 9th all blastocysts in both groups were fully hatched with a TE score of one and an ICM score of 2 and 3

Effect of chemically defined lipid on blastocyst outgrowth: This was analyzed by calculating endometrial out growth areas for each embryo in both study groups was although an extensive growth was observed from blastocysts in both the study groups the results between the two groups was not statistically significant.

DISCUSSION

Fatty acids are typically stored in the oocytes and in the follicular and endometrial fluid in the form of triacylglycerol (TG), esters of glycerol arranged as non-differentiated lipid which are used as source of metabolic energy [23]. The endogenous fatty acid contents of oocytes, follicular and uterine fluid are different from species to species; the oocytes and embryos of mice contain a low level of endogenous lipid [29], whereas human oocytes and cat oocytes contain an intermediate and high level of fatty acids respectively [30,31]. It has been reported

that the fertilizing quality of oocytes and subsequent embryonic development is affected by changes in fatty acid present in the lipid content of the follicular fluid, by altering the lipid metabolism [32]. Based on all the different roles of essential and non-essential fatty acids we have observed the combined effect of saturated and unsaturated fatty acids present in the chemically defined lipids on fertilization, blastocyst development and hatching of blastocysts.

In this study, we found that a chemically defined lipid mixture containing essential and non-essential fatty acids with antioxidant DL-alpha-tocopherol acetate did not effect on fertilization rates in the mouse. Little data is available about the effect of chemically defined lipid on mouse oocyte fertilization and embryo development. Most of the studies have been on bovine oocytes and have used either saturated or unsaturated individual fatty acid. The unsaturated fatty acid linolenic acid when added in vitro bovine maturation medium showed an increase in the number of oocytes that reached the metaphase II stage and these MII oocytes exhibited more active mitogen activated protein kinase activity. The resulting embryos were of better quality, with higher proportions at the cleavage stage [33]. Another study reported that exogenous addition of oleic acid, an unsaturated fatty acid in BSA (VBSA) and FAF BSA, inhibited fertilization of mouse oocytes. Also the addition of palmitic acid in BSA (VBSA) and FAF BSA also reduced the fertilization rate of mouse oocytes [34]. Palmitic acid a saturated fatty acid is readily taken up by bovine oocytes [35] and it has been observed that bovine oocytes mature more slowly in the presence of palmitic and stearic acids and have significantly reduced fertilization and cleavage rates [34,36]. The action of oleic acid (unsaturated fatty acid) has been reported to protect the oocyte from adverse effect of saturated fatty acids such as palmitic and stearic [32]. One of the major contents of the chemically defined lipid used in this study was cholesterol. Previous studies have shown that high cholesterol in oocytes causes increased lipid accumulation leading to alterations in mitochondrial membrane potential and an increase in apoptosis of ovarian cells because of lipotoxicity [37,38]. Studies using

mice on high fat diet have shown that signs of lipotoxicity such as lipid accumulation, increased the incidence of apoptosis and endoplasmic reticulum stress leading anovulation, and significantly decreased in vivo fertilization rates [39]. The mechanism behind this effect is that lipid accumulation causes an increased in free fatty acids resulting in oxidative damage and formation of cytotoxic and highly reactive lipid peroxides. These in turn affect functional changes in endoplasmic reticulum and ultimately result in apoptosis [40,41]. Also mitochondria when exposed to high free fatty acids result in oxidation by mitochondria reactive oxygen species forming lipid peroxidase affecting mitochondrial function leading to apoptosis [42]. In relation to our finding that there was no statistically significant effect of lipid on fertilization rate may relate to the combine action of unsaturated fatty acid on saturated fatty acid. As the saturated fatty acids are rapidly used as energy source in cellular biosynthesis process and unsaturated fatty acids are required for delivery of fatty acids to inner mitochondrial membrane to maintain normal cellular function and growth [43]. Also the data shows that the concentration and the ratio in which these fatty acids and cholesterol are present in the media also affect fertilization.

Our second observation relates to blastocyst formation rate, which was significantly higher when lipids were added to the blastocyst media. These experiments demonstrate that both essential and non-essential fatty acids support growth of 2-cell mouse embryos to viable morulae and then blastocyst stage. This result is consistent with the observation of several studies performed on mouse embryo revealing that the capacity of mouse embryos to synthesize lipids and sterols is reduced up to eight-cell stage and thereafter increases significantly [34,44]. Our finding is also similar to the results from previous studies in which fatty acids have been shown to have a beneficial effect on in vitro rat embryo development [45]. The energy required for embryo development is provided by the endogenous fatty acids along with carbohydrate metabolism. Thus the beneficial effect of fatty acids might

be explained by their utilization as in absence of carbohydrate substrate and for the synthesis of phospholipids and glycolipids required for the formation of cellular membranes [46]. Fatty acid may be used by developing embryos in the production of water for blastocoel fluid by beta oxidation (mitochondrial) [47]. In mice this is followed by cortical localization of cytoplasmic droplets and mitochondrial attachment to the cell surface [48]. Also the compaction of cells, which leads to blastulation, depends on the lipid present in the plasma membrane [49]. Studies using sheep, cattle and pig oocytes with intact zona pellucida have revealed that triglyceride was the most fatty acid rich lipid fraction in oocytes from all the three species but the most consistent was phospholipid [48]. Some unsaturated fatty acids like linolenic acid, linoleic acid and oleic acid have promoting effects on blastocyst formation [23], while others, like palmitic acid, stearic acid, which are saturated fatty acids, inhibit blastocyst development [15]. Despite the facilitatory and inhibitory effects of unsaturated and saturated fatty acids, our study found that chemically defined lipids containing a combination of both fatty acids promoted blastocyst formation in the mouse. This suggests a critical relationship between saturated and unsaturated fatty acids at the different stages of embryonic development and that the appropriate concentrations of fatty acids are essential for the embryo growth.

Our third observation was that there was no significant difference in the hatching blastocyst rate between the chemically defined lipid treatment group and controls. Previous studies have reported that total fatty acid content in uterine fluid is increased between day 11 and day 13 with a sharp rise in the arachidonic acid by day 14. This suggests that during implantation fatty acid concentrations rise denoting the start of prostaglandin synthesis and that this is a crucial step in maternal –fetal embryonic interaction [21].

In relation to our finding, a study by [18] has shown that total fatty acid content does not affect hatching rates in bovine blastocysts but that there is a decrease in the total fatty acid contents of 2 cell bovine embryos. . Oocytes, exposure to elevated saturated fatty acids

affects blastocyst quality and amino acid metabolism [50]. Number of blastocyst derived from the in vitro matured oocytes exposed to high levels of saturated fatty acids decreased in association with an increase in apoptosis. This effect is because of saturated fatty acid like palmitic acid, stearic acid have a negative effect on cell viability [51,52]. These observations showing different effects of unsaturated and saturated fatty acids on blastocyst hatching and outgrowth (implantation) suggest that the ratio of saturated and unsaturated fatty acid concentrations is crucial for the blastocyst implantation. Majority of the previous studies have examined the effect of saturated and unsaturated fatty acids on fertilization and embryo development, most have used bovine or porcine oocytes tested with single saturated or unsaturated fatty acids; few have used combination of saturated and unsaturated fatty acids, or the mouse as the study species. This is the first study to report on the effects of a combination of eight different saturated and unsaturated fatty acids with cholesterol. These components may have had stimulatory and inhibitory effects on the end points studied, and even the surfactants and solvents should also be considered as inhibitory factors. Thus it is promising that no major negative effects were observed with this mix and thus could be considered for future research considering the use of this chemically defined lipid in culture media.

CONCLUSION

We conclude that the chemically defined lipid used in this pilot study promotes early embryonic development without any obvious adverse effects on any facet of embryo development. However to confirm our results, additional studies are needed on the various aspects such as an increased number of replicates for the analysis, dose-related experiments to examine the effect of increased dose of chemically defined lipid on fertilization and also embryo development. Also, because these studies were done using the same sample of chemically defined lipid, we would like to perform experiments to compare the effect of fresh and aged samples of the chemically defined lipid to see

the effect on oocytes and embryos of oxidation due to air exposure [53].

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Conflicts of Interests: None

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