Effects of *Thymus vulgaris* L. essential oil and compounds on development and quality of bovine preimplantation embryos *in vitro*

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SUMMARY

Thyme oil is already being used in humans and animals for its various activities; however, little is known about its influence on preimplantation embryos. We aimed to investigate the effects of thyme oil on the growth, development and quality of embryos produced *in vitro*. A total of 266 blastocysts were harvested from 936 oocytes in 11 replicates. The addition of thyme oil significantly increased cleavage rate ($P<0.05$) morulae ($P<0.01$) and blastocyst ($P<0.001$) yield. Embryo quality parameters were found to be enhanced by antioxidant supplementation at atmospheric oxygen level ($P<0.001$). High oxygen tension during *in vitro* culture of bovine embryos is detrimental probably due to the accumulation of reactive oxygen species. Reduction of oxidative stress during *in vitro* production of bovine embryos may support subsequent development and improve the quality of blastocysts ($P<0.001$). Thyme oil can safely be used to decrease these adverse effects of oxidative stress in this regard. Moreover, in vitro culture of bovine embryos can be performed using carbon dioxide incubators instead of expensive ones.

KEY WORDS

Embryo; *in vitro* culture; antioxidant; oxidative stress.

INTRODUCTION

The oxygen tension in the lumen of the female reproductive tract is about one-third of the *in vitro* conditions. Suppression of oxygen tension to 5% positively affects the developmental competence of *in vitro* fertilized embryos, reduces the production of free radicals and ensures arrest of fewer embryos at 8-16 cell block. Moreover, high oxygen tension results extended 4-cell cycle in most embryos[1]. Embryos, cultured *in vitro*, are exposed to OS as a result of insufficient defence mechanisms. Nevertheless, surrounding cumulus cells isolate the oocyte from the extracellular media and thus the oocytes are not exposed to high oxygen. Therefore, extracellular antioxidant supplementation during in vitro maturation has no beneficial effect on cumulus-oocyte complexes (COC)[2]. COC can tolerate low levels of hydrogen peroxide (H_2O_2) exposure[3]. Moreover, antioxidant supplementation to the media of fertilization may reduce the number of embryos harvested due to the increased amount of H_2O_2. There are conflicting reports on whether antioxidant supplementation is necessary, especially at low oxygen concentrations, during embryonic development. Supplementation of various extracellular antioxidants to glucose-free media during the developmental period does not have a significant effect under low oxygen levels. The effect of oxygen tension on the development of in vitro cultured embryos varies depending on the culture media and the culture system. High oxygen tension may be more detrimental due to excessive free radical production as a result of increasing OS, particularly in defined and semi-defined culture systems[1].

Antioxidant supplementation to the culture media is considered the best strategy to reduce oxidation. Antioxidants reduce these harmful effects by preventing the formation of free radicals and reduce apoptosis by preventing the peroxidation of lipids and lipoproteins[4]. The most effective antioxidants are those which break free radical chain reaction. These antioxidants consist of aromatic and phenolic ring exchange H ion to free radicals[5]. Since phenolic compounds are considered potential antioxidants, their use in the prevention of oxidative stress-related diseases has been subjected to many researches[6]. Both synthetic and natural antioxidants counteract the detrimental effects of oxidative stress on reproduction. Synthetic antioxidants may have possible side effects in addition to their toxicity[7]. Thyme oil and compounds are promising in studies of replacing synthetic antioxidants with natural ones[8]. *Thymus vulgaris* L. is one of the strongest species that exhibit antioxidant activities among herbs and is now widely cultivated as tea, spice and herbal medicine[9]. Two main active compounds of thyme oil are thymol and carvacrol. Many reports are available regarding the antioxidant activities of thymol and carvacrol[6]. The phenolic content of thyme oil generates this effect[10]. Phenolic phytochemicals protect cell contents against free radical-induced damage by contributing to optimal health with their antioxidant and free radical scavenging abilities[6]. The in vitro antioxidant activities of plant extracts are due to their ability to scavenge free radicals or suppress singlet oxygen formation[11].

Oregano oil components provide protection against peroxide and mutagen-induced DNA damage[6]. Flavonoids and also phenolic phytochemicals such as carvacrol and thymol protect the cell against free radicals and oxidative stress but phenolic compounds can have both antioxidant and pro-oxidant effects dose-dependently[12].

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Currently, more than three-quarters of all IVF cycles in the world are performed under atmospheric oxygen conditions, albeit partially[13]. It is mostly hard to stabilize oxygen concentration in old generation incubators due to recurrent opening/closing of the solid frame and replacement of these incubators may slow over time because of high costs. To maintain continuity of in vitro processes and due to economic purposes, we also prefer to keep an old generation incubator at least for in vitro maturation and fertilization.

Preventing oxidative stress, which is a major problem particularly in embryo production from slaughtered cattle, and increasing the success in embryo production, as well as reducing the cost of incubators and developing natural tools, will lead to significant conveniences and developments in in vitro embryo production. From this point of view, the objective of this study was to investigate the effects of thyme oil and compounds on the development and quality of bovine embryos produced *in vitro*. Moreover, the potential of performing the whole process of in vitro embryo culture within carbon dioxide incubators was also investigated.

MATERIAL AND METHODS

Media for *in vitro* production of embryos were obtained as follows. Hepes–Tyrode's Lactate (TL), *in vitro* fertilization (IVF)-TL, sperm preparation (SP)-TL and potassium simplex optimized medium including amino acids (KSOM+AA) were purchased from Caisson (Sugar City, ID, USA) to prepare HEPES– Tyrode's albumin lactate pyruvate (TALP), IVF-TALP, SP-TALP and KSOM – bovine embryo (KSOM-BE) as previously described by Parrish et al.[14] and Loureiro et al.[15]. Thyme oil (W306509), carvacrol (W224511) and thymol (T0501) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Oocyte collection media (OCM) was tissue culture medium 199 (TCM-199) with Hanks' salts, L-glutamine, hepes and indicator of phenol red (Thermo Fisher Scientific) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 5% v/v fetal bovine serum (FBS). Oocyte maturation media (OMM) was TCM-199 with Glutamax™ and phenol red without hepes (Thermo Fisher Scientific) supplemented with 2.2 mg/mL sodium bicarbonate, 10% v/v FBS, 5 µg/mL gentamycin, 0.22 mg/mL sodium pyruvate, oestradiol and gonadotropins.

In vitro production of embryos

Ovaries were derived from a local abattoir in Hatay/Turkey (35° 52 – 37° 4 N, 35° 40 – 36° 35 E) and transported in saline (0.9% w/v NaCl) supplemented with penicillin/streptomycin, at 30-35°C within 2 h following the collection of the first ovary. *In vitro* production of embryos was performed as previously described[16]) but in brief, the follicular fluid including cumulus-oocyte complexes (COCs) from follicles (2-8 mm diameter) was aspirated using a 21-gauge needle attached to a disposable syringe and pooled into a sterile centrifuge tube. After allowing COCs to gravitate, supernatant follicular fluid was removed carefully by not disrupting the residue. Debris, including COCs, was then transferred into petri dishes containing OCM. Classification of COCs performed under a stereomicroscope (×10~40) according to the method of Boni et al.[17] and Tasripoo et al.[18]. COCs with at least 3 layers of compact cumulus cells surrounding a homogenous evenly granulated cytoplasm were classified as immature. Following classification, COCs were rinsed in Hepes-TALP and transferred into untreated 4-well dishes containing OMM (30-40 oocytes/well) for maturation (Day -1). *In vitro* maturation took place in a humidified atmosphere of 5% CO_2 in the air and lasted for 18-22 h. COCs were then rinsed in Hepes-TALP and transferred into IVF-TALP media. Frozen semen from a single bull was thawed and the swim-up method was used to separate the motile fraction of thawed semen[14]. Briefly, frozen-thawed semen was gently transferred to the bottom of a sterile centrifuge tube containing SP-TALP medium and incubated for 30-45 min under a humidified atmosphere of 5% CO_2 in the air. After that, the supernatant retarding the pellet was transferred into another sterile centrifuge tube and centrifuged at 400×*G* for 10 min. The supernatant was removed gently without disrupting the pellet containing live sperm cells (~100 µL). Sperm concentration was determined using a haemocytometer. The fertilization procedure was completed after the addition of diluted sperm, heparin and PHE cocktail (20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine in final concentration) solution into fertilization media containing oocytes. Sperm and oocytes were cultured together for about 8-12 h under conditions of humidified atmosphere and 5% CO_2 in the air. Putative zygotes were denuded of cumulus cells by gentle vortexing in HEPES-TALP at the highest speed for 3 min post-insemination and transferred randomly into KSOM-BE media supplemented with fatty acid free-bovine serum albumin (FAF-BSA) and gentamicin.

In vitro culture of embryos was performed under a humidified atmosphere of 5% CO_2 and 5% O_2 with the balance of N_2. Cleavage rates were assessed on Day 3 (Day 0 regarded as the day of *in vitro* fertilization). Blastocyst formation, morphology and quality parameters were assessed on days 7 and 8. Embryos were stained for 10 min in 1 µg/mL Hoechst 33342 according to the method of Moreira et al.[19] and the number of cells for each embryo was counted using fluorescent images of Hoechst-stained nuclei.

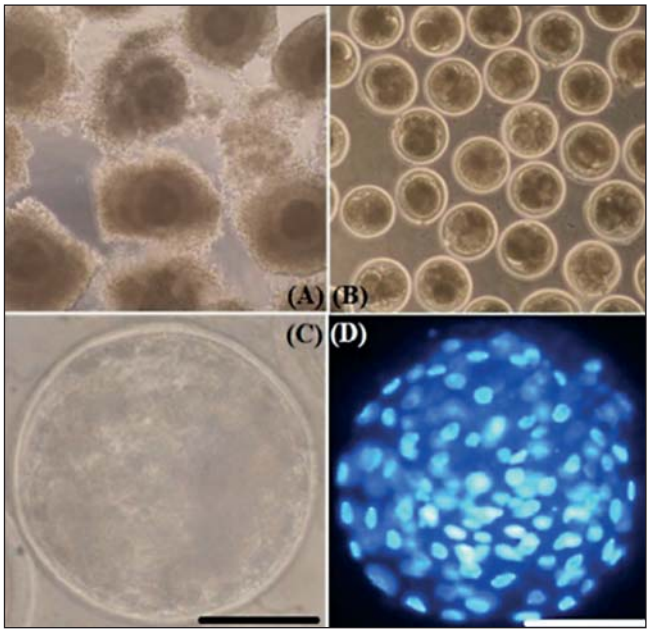

Figure 1 - Embryo quality assessment. Photomicrographs of (**A**) bovine oocyte, (**B**) Cleavage after 36-48 h, (**C**) d 8 blastocyst (scale bar = 100 µm) and (**D**) stained cells (scale bar = 100 µm) after Hoechst 33342 staining.

Experimental design

Solution of thymol at a concentration of 0.05 μM was prepared in ethanol. Designated treatment groups were [1] Control at low oxygen (5%) tension (CLO), [2] Control at high oxygen (20%) tension (CHO), [3] KSOM-BE supplemented with 0.05 μM carvacrol at low oxygen tension (CrvLO), [4] KSOM-BE supplemented with 0.05 μM carvacrol at high oxygen tension (CrvHO), [5] KSOM-BE supplemented with 0.05 μM thymol at low oxygen tension (TLO), [6] KSOM-BE supplemented with 0.05 μM thymol at high oxygen (THO), [7] KSOM-BE supplemented with 0.025% thyme oil at low oxygen tension (ThyLO) and [8] KSOM-BE supplemented with 0.025% thyme oil at high oxygen tension (ThyHO). Embryos were randomly assigned to the treatment groups and compared for embryo development and quality.

Statistical analyses

Data were collected in 11 replicates over days for the experiment. Data were arcsine transformed and analysed using a General Linear Model (GLM) for evaluation of differences between developmental stages of IVP embryos. Embryo diameters and blastomere counts were analysed using GLM. The model included as main effects oxygen tension (5 and 20 per cent) and the antioxidant resource (control, carvacrol, thymol and thyme oil). The interaction between the two tested effects was also considered in the following model;

$$Y_{ijk} = \mu + \alpha_i + ß_j + (\alpha ß)_{ij} + e_{ijk}$$

here μ is general mean, αi is the effect of oxygen tension, ßj is the effect of antioxidant, (αß)ij is the interaction between oxygen tension and antioxidant, and eijk is random error. Embryo quality scores were evaluated utilizing chi-square. All analysis was performed using SPSS software (v23.0, SPSS Inc., Chicago, IL). Data were presented as mean±standard error (SE). The p-value used to determine significance in all tests was 0.05.

Ethics

As stated by Animal Protection Act article 2c of the directive 2011/28914, embryos are not included in the list of organisms that require specific authorization, so no approval was requested.

RESULTS

The effect of oxygen tension on cleavage was significant in favour of low oxygen tension (p<0.01), in the present experiment. The number of cleaved embryos under low oxygen tension was 456 from 486 oocytes in all replicates (Figure 2). However, the effect of antioxidant supplementation to the culture media was not significant. Cleavage rates obtained for low oxygen tension was similar according to multiple comparisons of Tukey HSD (Table 1). Antioxidant supplementation to the culture media significantly enhanced the cleavage rate of embryos exposed to a gas phase of atmospheric oxygen (p<0.05). The number of cleaved embryos for high oxygen tension was 399 from 450 oocytes (Figure 3). Effect of antioxidant addition to the cul-

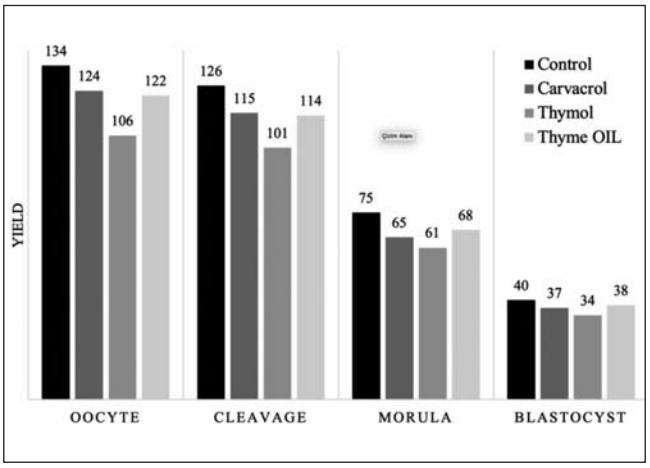

Figure 2 - Embryo yields under a controlled oxygen concentration.

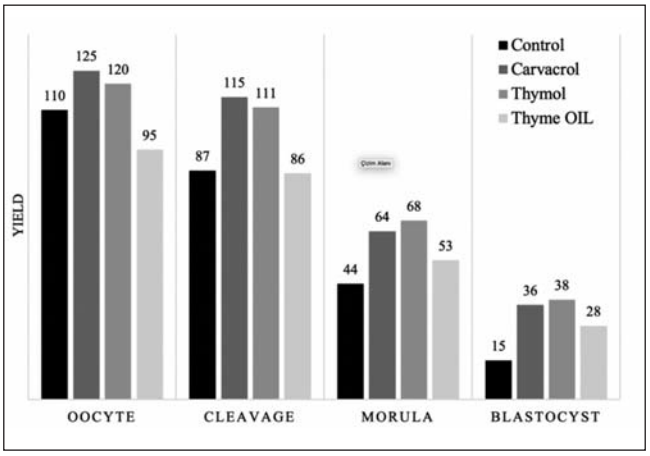

Figure 3 - Embryo yields under an atmospheric oxygen concentration.

Table 1 - Embryo counts during *in vitro* development.

		Control	Carvacrol	Thymol	Thyme Oil	SEM	P		
							AO	OT	AO × OT
Cleavage (%)	LO	93.13	92.91	95.44	93.65	0.96	0.01	0.00	0.023
	HO	78.98[a]	92.18[b]	92.58[b]	90.83[b]	1.47	9	2	
Morulae (%)	LO	59.72[a,b]	56.92[a]	60.41[b]	59.60[a,b]	0.76	0.00	NS	0.003
	HO	50.37[a]	55.99[b]	61.32[c]	61.49[c]	1.32	0		
Blastocyst (%)	LO	32.03	32.19	33.80	33.28	0.44	0.00	0.00	0.000
	HO	16.52[a]	31.24[b]	34.23[b]	32.30[b]	1.43	0	0	

LO: low oxygen tension; HO: high oxygen tension; AO: antioxidant; OT: oxygen tension; NS: non-significant; B: blastocyst; SEM: Standard error of the mean, different letters of superscript in a row represent different groups according to Tukey HSD.

ture media of IVP embryos under controlled and high oxygen tension found to be significant ($p<0.05$).

Antioxidant addition to the culture media did not result in any significant development rate under the condition of controlled oxygen tension. Nevertheless, a very slight increase was confirmed by thymol supplementation while a slight decrease was observed for those supplemented with carvacrol. The number of embryos developed to morulae was 269 under low oxygen tension (Figure 2), whereas it was 229 for atmospheric oxygen concentration (Figure 3). Development rates of embryos to morulae significantly increased by antioxidant addition under atmospheric levels of oxygen ($p=0.000$). Table 1 indicates an enhancement of antioxidant supplementation depending on oxygen tension ($p<0.01$).

An apparent decrease in blastocyst yield is noticeable from Table 1 at atmospheric oxygen concentration. The addition of antioxidants to the culture media significantly improved the blastocyst yield of IVP bovine embryos ($p=0.000$) at the atmospheric level of oxygen. Blastocyst yield for low oxygen tension did not enhance by antioxidant supplementation and was also similar in all groups. Blastocyst yield was 149 for low oxygen tension (Figure 2) and 117 at atmospheric oxygen concentration (Figure 3). According to these results, we can claim that the addition of thyme oil and/or compounds to the culture media of IVP bovine embryos significantly improved blastocyst yield depending on oxygen concentration ($p=0.000$).

Another remarkable parameter widely accepted other than blastocyst yield is embryo quality in *in* vitro embryo production. Embryo diameter, blastomere count and embryo quality scores were recorded during the experiment as qualitative and quantitative parameters in this regard (Table 2). The effect of oxygen tension on embryo diameter was significant and, the addition of antioxidants to the culture media improved embryo diameter under both low and high oxygen conditions ($p=0.000$). Best results were obtained by the addition of carvacrol and thymol independently of oxygen concentration. A dramatic decrease in embryo diameter was also recorded at atmospheric oxygen level.

Similar results were obtained for blastomere count, the addition of antioxidants to the culture media significantly improved blastomere numbers ($p=0.000$).

Maximum blastomere number recorded for thyme oil group, independently of oxygen concentration. Embryo quality score was significantly improved by the addition of antioxidants to the culture media ($p=0.000$).

DISCUSSION

The developmental ability of oocytes, derived from slaughtered cows is relatively low than those in vivo. The major challenge for scientists is to mimic in vivo conditions for in vitro embryo production. Disturbances in the redox state of the cell may cause toxic effects via the production of peroxides and free radicals and thus may damage protein, lipid and even DNA structure. Antioxidant supplementation to the media may prevent mitochondria and DNA damages[4].

In the present study, a significant decrease in cleavage rates was observed under the condition of atmospheric oxygen concentration. Oocytes rely on the energy provided by the mitochondria. Oxidative phosphorylation accounts for about one-third of ATP synthesis during the process reach up to cleavage[20]. Reactive oxygen species can initiate mitochondrial dysfunction[21]. ROS emerging as a by-product of mitochondrial metabolism can trigger oxidative damage and impair the ability to synthesize ATP. Reduced mitochondrial activity at this stage may result in the arrest of the oocyte, disrupted fertilization and impaired embryo development[4, 20, 22, 23]. Even though we disregarded individual oxygen consumption and ROS measurement, putative zygotes in the CHO group presumably suffered from oxidative stress.

Supplementation of culture media with thyme oil, carvacrol and thymol significantly improved the cleavage, morulae and blastocyst yields in vitro, independently of the oxygen tension (Table 1). Hydrogen peroxide does not interact directly with DNA. Crossing the biological membrane, H_2O_2 penetrates the cell nucleus and interacts with iron and copper ions to form OH radicals, which cause lesions and breaks in DNA. This protective effect of carvacrol and thymol against DNA lesions is due to the potent oxidant H_2O_2[24]. However, excessive production of hydrogen peroxide and superoxide anion can damage mitochondria. Chromatin condensation and fragmentation, apoptosis, disturbed cell division and embryonic arrest may occur as a result of protease and caspase activation, subsequently[25]. Reactive oxygen species can initiate detrimental effects and are able to pass through the cell membrane. This may result in the cell block of embryos only in IVF. Unfavourable conditions, such as OS, may challenge in vitro development of bovine embryos from one cell to blastocyst and result 8-16 cell block. This may have been perceived as a reason for the low morulae yield observed in this experiment. Metal chelators have a beneficial effect on embryo development[21]. Thyme oil and compounds seem

Table 2 - Blastomere number, diameter and quality score of blastocysts.

		Control	Carvacrol	Thymol	Thyme Oil	SEM	P		
							AO	OT	AO × OT
Diameter (μm)	LO	171.14^{a}	231.14^{c}	232.64^{c}	207.73^{b}	4.48	0.000	0.000	0.000
	HO	155.91^{a}	228.82^{c}	227.59^{c}	201.09^{b}	5.22			
Embryo quality	LO	1.8	1.8	1.73	1.80	0.34	0.000	NS	NS
	HO	2.0	1.8	1.77	2.41				
Cell count	LO	80.45^{a}	93.18^{c}	89.18^{b}	95.00^{c}	1.12	0.000	0.000	0.000
	HO	67.09^{a}	93.18^{c}	88.55^{b}	93.55^{c}	1.96			

LO: low oxygen tension; HO: high oxygen tension; AO: antioxidant; OT: oxygen tension; NS: non-significant; B: blastocyst; SEM: Standard error of the mean, different letters of superscript in a row represent different groups according to Tukey HSD.

to enhance morulae yield by reducing ROS formation due to its strong radical scavenger characteristic. Such antioxidants prevent lipid oxidation stimulated by Fe^{2+}/ascorbate and Fe^{2+}/H_2O_2 and thus aid to overcome this cell block[5].

Oxygen tension at the atmospheric gas phase resulted in a decrease in embryo diameter and blastomere count. However, antioxidant supplementation to the culture media under high oxygen tension enhanced diameter, blastomere count and also embryo quality (Table 2). A mechanism involving a cascade of metabolic reactions may cause the production of free oxygen radicals and such suboptimal conditions may result in arrested cell division and can influence programmed cell death. Higher oxygen tension triggers an upsurge of ROS in the cytoplasm of the embryo and thus a decrease in *in vitro* development[26]. The beneficial effect of thyme oil may partly occur due to the detoxification of highly diffusible reactive species. Antioxidant supplementation to the media permits embryo development, probably by limiting the peroxidation process. Germ cells are much more sensitive to ROS and OS than somatic cells. High ROS in culture media causes a decrease in embryo development and blastocyst formation[2]. Antioxidants serve to prevent the formation or circulation of free radicals and thus delay autoxidation. Most research exhibited that antioxidant supplementation is essential for better quality embryo production, increased longevity and blastulation[5].

Embryos may have different sensitivities to ROS at different developmental stages because of various defence mechanism thresholds. Both spermatozoa and embryos are sources of ROS, which may lead to oxidative damage and thus impaired embryo development in vitro. Blastocysts are more sensitive to free radicals[21, 27]. Today it is widely accepted that low oxygen tension is present in the mammalian genital tract but embryos are exposed to 20 per cent atmospheric oxygen during *in vitro* fertilization.

Reduced oxygen tension in *in vitro* embryo culture is related to better blastocyst formation and blastomere number in many species. Embryos expose decreasing amount of oxygen tension along their journey from the oviduct to the uterus, and finally lowest tension at blastocyst formation following compaction[28]. Gene expression, proteome, metabolism and genome are adversely affected in embryos under atmospheric conditions[23]. We can subjectively claim that OS is one of the environmental factors responsible for this critical drop in blastocyst yield by half. In corroborate with the results that report higher developmental rates of embryos cultured in vitro[22], we obtained high blastocyst yield under low oxygen tension and high oxygen tension by supplementation of thyme oil and compounds to the culture media.

It has been reported that the compounds of thyme oil can modulate DNA damage in a dose-dependent manner through peroxide and some genotoxins[6]. Hydrogen peroxide mainly causes DNA breaks through the formation of hydroxyl radicals by the Haber-Weiss reaction catalysed by ferric ions that can diffuse in cells and tissues. ROS pass through the cell membrane and cause changes in macromolecules in the cell, mitochondrial alterations, meiotic arrest of the oocyte, embryonic block and even apoptosis[29]. Phenolic compounds have the ability to chelate metal ions. The protective effect of flavonoids against H_2O_2-induced DNA damage is provided by the intracellular binding of iron[11]. In particular, thymol and carvacrol are effective in scavenging peroxyl radicals by reducing the peroxidation of phospholipid liposomes and the presence of iron and ascorbate[8].

Although optimum conditions have not been set down yet, controlled oxygen tension (~5%) stands for an accepted scientific fact for in vitro embryo production. Cumulus-cell matrix that surrounds oocyte during in vivo embryo development assist protection from intracellular pH alterations since such switch may cause impaired development. Our hypothesis here is that the beneficial effects of low oxygen tension are not essential when oocytes are enclosed in their cumulus cells. It has been reported that genes expressing proteins with antioxidant function are at a lower level at the 2-4 cell stage at 20% oxygen concentration. The effect here is due to the use of maternal storage antioxidants under OS conditions prior to genome activation in the embryo. However, it has been reported that the amount of antioxidants before the 8-cell stage is not induced by OS[30].

Herein, the addition of thyme oil and its compounds seem like an effective alternative to disburden the detrimental effect of oxidative stress at a gas phase of atmospheric oxygen. Regarding the complex composition of essential oils, we aimed to reveal the possible mechanism of action of thyme oil and its compounds on the reduction of OS.

In conclusion, carvacrol and thymol can be metabolized by cells during in vitro culture of bovine embryos in glucose-free semi-defined media, and can safely be used in terms of cyto- and genotoxicity to obtain improved developmental rate. Moreover, it was concluded that in vitro embryo culture with supplementation of thyme oil can also be performed in conventional carbon dioxide incubators.

In the present experiment, thymol and thyme oil increased the rate of bovine embryos developed into morulae and blastocyst in vitro. These results, in the first place, showed that herbal antioxidants can safely be used in terms of cytotoxicity dose-dependently and can enhance the environmental conditions of bovine embryos produced in vitro. Reduction of oxidative stress during in vitro production of bovine embryos may support subsequent development and improve the quality of blastocysts as a consequence. Further research on quantitative analysis of embryonic oxidative status would be crucial to acquire a precise definition of the anti-oxidative effects of thyme oil and compounds. Moreover, the effects of thyme oil and its compounds on reactive nitrogen species as well as reactive oxygen species should be the subject of further studies.

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