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Optimisation of Total RNA Extraction from Bovine Oocytes and Embryos for Gene Expression Studies and Effects of Cryoprotectants on Total RNA Extraction[1]

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Abstract—Gene expression is required for understanding bovine oocytes meiotic maturation as well as the potential of embryonic development. In the present study a standardized reagent protocol for total RNA extraction was designed for bovine oocytes and embryos, which is considered specific and less expensive. For such purpose oocytes ($n = 795$) recovered from about 80 ovaries were divided in three groups: Group 1 modified Trizol® (MTP, $n = 355$); Group 2 Guanidinium thiocyanate protocol (GNTC, $n = 140$) and Group 3 Commercial Kit protocol (CKP, $n = 60$). Oocytes belonging to group 1 ($n = 100$) and 3 ($n = 20$) were subjected to vitrification using two cryoprotectants 1,2 propandiol (PROH) or Dimethylsulfoxide (DMSO). The 240 remaining oocytes were divided into 3 groups in which 100 were used, in fresh, for in vitro fertilization, and 140 oocytes were vitrified using PROH ($n = 70$) and DMSO ($n = 70$) as cryoprotectants, being then fertilized in vitro after thawing. Embryos were used nine days after fertilization. Gene amplification (SDHA, GAPDH and DNMT1) was performed in oocytes, and gene quantification (DNMT1) in in vitro produced embryos at the stage of blastocyst ($n \approx 10$). Efficiency of the extraction was further compared. The purity of all samples to different protocols ranged from 1.10 to 1.25 for GNTC protocol; from 2.05 to 2.63 for the CKP and from 1.50 to 2.11 for the developed MTP, being the last one nearest to the expected purity levels for RNA samples (1.7–2.0). On average, for 30 fresh oocytes, from spectrophotometer readings, total RNA concentration was 127.8 ± 9.3 ng μL^{-1} for MTP, against 46.4 ± 9.5 ng μL^{-1} from CKP and 47.6 ± 12.9 ng μL^{-1} for GNTC protocol. Using the MTP to evaluate RNA in 30 vitrified/thawed oocytes, resulted in a total RNA concentration of 61.3 ± 3.3 ng μL^{-1} and 40.0 ± 12.4 ng μL^{-1}, respectively for DMSO and PROH. Regarding total RNA concentration and purity, in blastocyst stage, more purity was observed in DMSO as compared to PROH (1.8 vs. 1.2) ($p < 0.05$). Better results were also observed on the MTP for gene amplification when compared with the other protocols. For gene quantification, the proposed protocol quantified DNMT1 gene with PCR efficiency (0.933) after normalization against GAPDH and SDHA. Amplification and quantification of genes proved specificity and efficiency of the MTP over the other protocols.

Keywords: total RNA extraction, bovine oocytes and embryos, gene amplification and gene quantification

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INTRODUCTION

To study gene expression in samples with small number of cells and tissues, many different techniques have been developed. Normally a single mammalian cell contains 10–30 pg of total RNA out of this around 85% is ribosomal RNA (rRNA), 15–20% is transfer RNA (tRNA) and 1–5% is messenger RNA (mRNA) [1]. Noteworthy, 35% of mRNA was found in the nucleus [2, 3]. In this way, there are several difficulties in isolation of total RNA from embryos and oocytes because of limiting quantity of cell and consequently RNA [4], which in bovine oocytes ranges from 0.7 to 5.3 ng in different stages of oocytes development [5, 6]. Hence a stabilized protocol is necessary to extract total RNA with standard number of oocytes instead of using variable amount of bovine oocytes and embryos and further to analyse the function of reagents as Guanidinium thiocyanate (GNTC) and reagents in the commercial kit protocols in total RNA extraction. A biochemical aspect which plays a major role in the oocytes growth is the mechanisms of Ca^{2+} homoeostasis during growth and maturation [7]. The increased calcium (Ca^{2+}) levels helps to maintain nucleic acids in aqueous phase instead dissolving in phenol phase, by co-precipitating with nucleic acids in precipitation step of isopropanol in RNA extraction [8]. The quality of oocytes also has to be taken into consideration as only the good ones have more possibility to develop as embryos [9]. As oocytes are normally evaluated by morphological criteria, they are

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considered as good quality if they are compact, with several layers of cumulus cells and granulosa adhering to cumulus [10].

There are different manual and kit protocols for the extraction of total RNA, like GNTC/phenol (Guanidinium thiocyanate) method, Trizol® and different commercial kit protocols. Trizol® reagent is one of most common chemical solutions used in the extraction of RNA, DNA, and proteins included in many of the kit products. Guanidinium thiocyanate and chloride are most effective protein denaturants [11]. Guanidinium chloride is a strong inhibitor of ribonuclease so it is introduced as deproteinization agent for extraction of RNA by Cox [11]. Later on chloride was replaced by phenol, to extract ungraded RNA from ribonuclease rich tissues like pancreas [12], hence GNTC/Phenol (Guanidinium thiocyanate) method is specific to large amount of tissues. The guanidinium method has been used not only in RNA isolation but also in DNA from eukaryotic cells; however the protocol to RNA differs from DNA and varies according to the type of tissues from which nucleic acids are retrieved [11, 13, 14]. Single step method of RNA extraction, known as Trizol®, was introduced by [15], which is a chemical combination of guanidinium thiocyanate, phenol and chloroform, providing high yield and purity RNA results. Trizol® protocol was designed to eliminate ultracentrifugation step in GNTC/phenol and chloride method and it is more specific in RNA isolation because it maintains the RNA integrity during tissue homogenization and breaking down cells components [15, 16]. In all aerobic organisms *SDHA* (Succinate dehydrogenase flavoprotein subunit A) gene functions as a membrane bound component of both citric acid cycle and respiratory chain [17]. The physical and catalytic properties of succinate dehydrogenases are from phylogenetic sources of bovine [17]. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) is also known as G3PDH which catalyses the six step of glycolysis and helps in breakdown of glucose for energy and carbon molecules [18]. GAPDH acts as a link between metabolic state to gene transcription by moving between cytosol and nucleus [19]. The cellular location of GAPDH is cytosol since the glycolysis takes place in cytosol. *GAPDH* gene is highly stable and constitutive expressed at high levels in most of tissues and cells [20]. For this reason *GAPDH* and *SDHA* genes are chosen as reference genes. *DNMT1* gene (DNA methyltransferase1) was chosen to test the efficacy of the protocol, to make the comparison between the housekeeping genes and normal genes.

In the experiment 1, the aim is to establish a standard protocol for the extraction of total RNA from a minimum number of fresh and vitrified bovine oocytes and granulosa cells, enabling the amplification of two housekeeping genes *SDHA* and *GAPDH* and the *DNMT1* gene. The experiment 2 is performed to test the efficiency of this protocol over embryos at the blastocyst stage through gene quantification of *DNMT1*.

MATERIALS AND METHODS

Chemicals

The chemicals and reagents used in the experiment were obtained from Sigma–Aldrich (USA). All the RNA extraction chemicals are from Ambion® Life Technologies and all the PCR reaction mixtures are from Fermentas Company (USA).

Collection of Ovaries

Around 80 to 100 ovaries were obtained from a local abattoir from adult animals, trimmed of adhering tissue and transported to the laboratory in Dulbecco's phosphate buffered saline (DPBS) at a temperature ranging from 34 to 37°C within 2 h post slaughtering. All ovaries were rinsed once with 70% alcohol and followed by a wash with fresh DPBS upon arrival at laboratory.

Experimental Design

In the present study a renewed protocol was developed aiming to test it aptitude against to CKP and GNTC protocol in total RNA extraction from fresh bovine oocytes. Further to evaluate the new protocol, total RNA extraction was performed in vitrified oocytes using two cryoprotectants: PROH (1,2 propandiol) or DMSO (Dimethylsulfoxide) (Experiment 1). To test the newly developed protocol, total RNA extraction was also performed in embryos at the blastocyst stage followed by gene quantification (Experiment 2).

Recovery of Immature Oocytes

Cumulus oocytes complexes (COCs) were collected by aspiration from antral follicles (2–8 mm diameter) with 18 gauge needle. Good quality COCs ($n = 795$) based on their morphological appearance, in which covered by at least four layers of compacted cumulus cells and evenly granulated ooplasm, were washed twice in TCM-199 (Tissue Culture Medium-199) supplemented with 2% FBS (Foetal Bovine Serum), 0.3 mg mL^{-1} glutamine and 50 μg mL^{-1} gentamycin and randomly assigned for non-vitrified control (fresh COCs) ($n = 655$) and vitrified ($n = 140$) oocytes groups.

Immature Oocytes Vitrification/Thawing

The vitrification method was based on the protocol used by [21], with some modification using one of the two cryoprotectants: PROH or DMSO. Briefly, group from 20–40 immature oocytes were initially equilibrated for 5 min in holding medium (TCM-199 medium with Hepes, supplemented with 1.5 M PROH or DMSO, 0.1 M sucrose, 20% FBS and 50 μg/mL gentamycin).

After equilibration, oocytes were transferred to vitrification solution (2 M PROH or DMSO in TCM-199 Hepes medium with 0.1 M sucrose, 20% FBS and 50 μg/mL gentamycin) for 30 s at room temperature. The oocytes were then loaded in a French mini straw (FMS) and immediately plunged into LN2 for storage.

For thawing, the straws were removed from LN_2, held in air for 5 s and transferred quickly to a water bath at 37°C for 30 s. The contents of straws were emptied into TCM-199 Hepes medium supplemented with 20% FBS, in a stepwise manner of serial dilution (0.5, 0.1 and 0 M) of sucrose for 5 min in each step. After thawing, the oocytes were washed twice in DEPC (dietilpirocarbonate) water and the granulosa cells were mechanically separated from the oocytes for denudation. Then the denuded oocytes and the granulosa cells were stored in RNAse free tubes in −80°C.

In Vitro Embryos Development Using Fresh Oocytes

Some of fresh oocytes were subjected to in vitro maturation, fertilization and embryonic development, according to [22]. Briefly, washed oocytes were matured in TCM-199 supplemented with 10% FBS, 5 μg/mL of FSH-LH ("Stimufol", Belgium), 1 μg/mL estradiol-17β, 0.15 mg/mL glutamine, 22 μg/mL Na-pyruvate and 50 μg/mL gentamycin and 20 μg/mL of nystatin. After 24 h of maturation under 5% CO_2 in a humidified atmosphere at 38.5°C, oocytes were placed for insemination in fertilization TALP medium briefly, thawed semen were washed three times by centrifugation, twice in sperm-TALP medium (4 mL for each) and final washing in IVF-TALP medium supplemented with 10 μg/mL heparin, 6 mg/mL BSA (EFAF), 22 μg/mL Na-pyruvate and 50 μg/mL gentamycin and 20 μg/mL of nystatin. After removing the supernatant, sperm pellet was homogenized with 0.25–0.5 mL of remaining IVF-TALP medium for adjusting the sperm concentration to 1×10^6 sperm/mL. Oocytes and sperm were cultured in 50 μL of fertilization medium (10–15 oocytes/droplet) for 22–24 h at 38.5°C in 5% CO_2 in air. Presumptive embryos were denuded by vortexing, washed and cultured in TCM-199 with Hepes supplemented with 3 mg/ml BSA (Fr. V), 22 μg/mL Na-pyruvate, 10 μg/mL NEAA (MEM, Non-essential amino acid), 20 μL/mL EAA (BME, Amino acid) and 50 μg/mL gentamycin and 20 μg/mL of nystatin in incubator at 38.5°C in 5% CO_2 in air. Cleavage rate was determined after 3 days of fertilization (day 0) and the embryonic development was evaluated at day 6 of culture until blastocyst stage. Further the blastocyst stage samples were washed twice with DEPC water and stored in RNAse free tubes at −80°C.

Total RNA Extraction with Three Different Protocols

The commercial kit protocol (PureLink® RNA Mini Kit) was used according to the fabricant and samples ranging from 10 to 30 oocytes per tube, granulosa cells and vitrified oocytes had the total RNA extracted and they were re-suspended in 50 μL of DEPC-treated water. The GNTC protocol were processed with samples ranging from 30 to 60, and total RNA extraction as follow: (1) 100 μL of denaturing solution (4 M Guanidinium thiocyanate, 0.02 M of sodium citrate), 0.72 μL of 14.4 M beta-mercaptoethanol, 10 μL of 2 M sodium acetate (pH 4.0), 100 μL of phenol saturated with water (pH 5.5) and 20 μL of chloroform:isoamyl alcohol are added to the sample and vortex it for 3 min vigorously; (2) the samples were centrifuged at 12000 g for 5 min; (3) the upper phase (aqueous phase) were transferred into the RNAse free tube; (4) 1 μL of 2 mg mL^{-1} of glycogen, 100 μL of isopropanol added into each sample and mixed by inversion; (5) the samples were centrifuged for 12000 g for 30 min at 4°C; (6) the supernatant was removed to a new tube and the RNA pellet was washed twice with 200 μL of 75% ethanol in 0.1% DEPC-treated sterile water; (7) the pellet was air dried for 15 min and further dissolved in 20 μL of DEPC-treated water.

The MTP developed for total RNA extraction were performed on samples ranging from 10 to 70 of oocytes per tube, vitrified oocytes and granulosa cells by (1) adding 100 μL of Trizol® to the samples, pass it in the vortex and incubate for 3 min; (2) adding 50 μL of chloroform to the RNAse free tubes and invert them for 15 s incubating at room temperature for more 3 min; (3) the samples were centrifuged at 12000 g for 30 min at 4°C; (4) the aqueous phase was transferred in to a new tube; (5) 2.5 volumes of isopropanol added to the aqueous phase collected; (6) the tubes were centrifuged at 12000 g for 30 min at 4°C; (7) the supernatant was discarded and the pellet washed with 150 μL of 70% ethanol and centrifuged at 7500 g for 5 min; (8) the pellet was dried in an incubator for 30 min at 37°C and further dissolved in 20 μL of DEPC water.

All the total RNA samples from the three protocols were stored at −80°C, all these samples were preheated at 60°C for 5 min and evaluated using a spectrophotometer (NanoVeu GE Company), based on the spectrophotometer reading of the samples stated in Tables 2 and 3 further cDNA synthesis was performed.

cDNA Synthesis

Total RNA samples stored at −80°C until were reverse transcribed into cDNA in a total volume of 20 μL, with Revert Aid™ H Minus First Strand cDNA synthesis Kit according to the manufacture's protocol [23]. Three micrograms of total RNA were used for reversed transcribed reaction using an oligo dT12-18 (1.0 μL or 500 ng) primer and the same volume was used from the reverse transcription reaction to perform the qPCR. The first step of the reverse transcription was incubation of the RNA samples at 65°C for 5 min with 1.0 μL of oligo$(dT)_{18}$. Primers and 5.0 μL of Nuclease free water. Followed it was added into each reaction 4.0 μL of 5X Reaction Buffer, 1.0 μL

Table 1. Primers used for PCR and real-time PCR in the present study

Gene	Sequence (5'–3')	Product length, bp	T_m, °C
GAPDH	Forward-GCACAGTCAAGGCAGAGAAC Reverse-TACTCAGCACCAGCATCACC	109	54
SDHA	Forward-CTGCAGAACCTGATGCTTTGTG Reverse-ACGTAGGAGAGCGTGTGCTT	188	55
DNMT1	Forward-AGCAATGGGCAGATGTTCCA Reverse- ATCTCGCGTAGTCTTGGTCG	268	54

of RiboLock™ Inhibitor (20 u/μL), 2.0 μl of 10 mM dNTP mix, and 1.0 μL of RevertAid™H Minus M-MuLV Reverse Transcriptase (200 u/μL) to the incubated samples and all this reaction mixture was subjected for incubation at 45°C for 1 h and 70°C for 5 min for reverse transcriptase inactivation. The cDNA reaction mixture was prepared according to manufacturer's protocol [23]. All the reverse transcribed (cDNA) samples were treated with RNAse H (Thermo Scientific) for 30 min at 37°C by adding 2.3 μL of 10X reaction buffer and 0.7 μL of RNAse H, *E. coli* (Fermentas). The cDNA samples were stored in –20°C for further polymerase chain reaction with the housekeeping and DNMT1 genes.

Gene Amplification

Suitable forward and reverse primers (Table 1) were designed by using Primer plus 3 software [24] for *GAPDH* (GenBank ref. NM_0010434034.1), *SDHA* (GenBank ref. NM_174178.2) genes and *DNMT1* gene (GenBank ref. NM_182651.2).

The PCR amplification of *GAPDH*, *SDHA*, and *DNMT1* was performed in a total volume of 20 μL, with 1X PCR buffer with 3.0 mM of $MgCl_2$, 250 μM of dNTP mixture (adenine, cytosine, timine and guanine), 10 pmol μL^{-1} of each primer (forward and reverse), 1 unit of *Taq* DNA polymerase (Fermentas, Thermo Scientific), 2.5 μL of cDNA and water enough to complete the total volume. The reaction was done in 35 cycles, with 30 s of denaturation at 94°C, 30 s of annealing at 54°C and 45 s of extension at 72°C. The cycles had an initial denaturation at 94°C during 3 min, followed by a final extension at 72°C for 10 min and used for electrophoresis. Briefly, 3% of TAE (Tris acetate plus EDTA buffer) agarose gel was prepared with 120 μL of 1 × TAE buffer, 3.6 g of agarose, 12 μL of SYBR safe (Invitrogen). The gel loaded with 8 μL of PCR products with 2 μL of loading dye solution. As weight molecular marker, 6 μL of 1 Kb DNA ladder plus (Gene Ruler, Fermentas) was loaded and kept for gel running for 30 min at 120 volts. Gel photographs were taken for further analysis with a transiluminator equipment (UVI tech, UVI Doc) and the intensity of the bands were measured.

Gene Quantification

Total RNA extraction was conducted in blastocyst stage by modified Trizol® protocol, the spectrophotometric reading were taken which was followed by cDNA synthesis and quantitative real-time PCR (qRT-PCR). The gene quantification protocol was performed using the ABI Prism 7500 (PE Applied biosystems) in 96 micro-well plates and the Thermo Scientific Absolute Blue QPCR SYBR Green Low ROX Mix (Thermo Scientific ABgene® UK). All samples, including the external standards and non-template control, were run in triplicate. The reaction conditions had been established through a series of preliminary optimization experiments including the calibration curves. Each 25 μL final volume reaction contained 1X Blue QPCR SYBR Low ROX (which includes Thermo-Start™ DNA polymerase and 3 mM of $MgCl_2$ in addition to Blue dye and ROX dye), 1 μM of each forward and reverse primer, water and template cDNA. Template cDNA corresponds to the stage of blastocyst samples. Water for a non-template control was included to confirm the absence of contamination in the reaction mixture. The reaction was initiated by activation of Thermo-Start™ DNA polymerase at

Table 2. Spectrophotometric readings of fresh oocytes by different protocols

Number of Oocytes	Method of total RNA extraction	Purity 260/280 nm	Concentration, ng/μL
30	GNTC	1.175	47
50	GNTC	1.25	104.5
60	GNTC	1.10	59.25
10	MTP	1.50	46.25
20	MTP	1.55	79.2
30	MTP	1.50	152.8
40	MTP	1.66	77.75
70	MTP	1.50	199.2
25	CKP	2.12	26.8
10	CKP	2.63	24.4
25	CKP	2.06	31.2

Table 3. Total RNA concentration and purity of the blastocyst stage samples by using MTP

Embryos at Blastocysts stage	Purity 260/280 nm	Concentration, g/μL
12 blastocysts fresh	1.81	92
11 blastocysts fresh	1.56	164
11 blastocysts DMSO	1.82	308
12 blastocysts DMSO	1.82	324
9 blastocysts PROH	1.24	288
11 blastocysts PROH	1.26	588

95°C for 15 min, followed by 40 three-step amplification cycles consisting of 15 s denaturation at 95°C, 30 s at 54°C and 30 s at 72°C. A final dissociation stage was run to generate a melting curve for verification of amplification product specificity. The quantification was carried out using the comparative cycle threshold (Ct) method, with the results expressed in relation to endogenous reference genes and a control group. The gene quantification was performed on the reference genes (*GAPDH* and *SDHA*) and *DNMT1* required gene.

RESULTS

Experiment 1: Efficiency of the MTP over Kit and GNTC Protocol

All spectrophotometer readings of fresh oocytes samples by different total RNA extraction protocols are reported in Table 2 which was subjected to gene amplification. Basing on the ratio of absorbance at 260 and 280 nm the purity of the samples to different protocols ranged from 1.10 to 1.25 for GNTC protocol; from 2.05 to 2.63 for the CKP and from 1.50 to 2.11 for the MTP. With the total rna concentration from all samples (Table 2) for the three protocols an average was calculated for 30 oocytes, being: 127.8 ± 9.3; 46.4 ± 9.5 and 47.6 ± 12.9 ng μL^{-1} respectively for MTP, CKP and GNTC. Further, to demonstrate potency of MTP in vitrified oocytes, the total RNA extraction was performed using this new protocol, comparing with the CKP only, since the GNTC turn up the worst results in the first part of the experiment 1. Spectrophotometer readings notifies that good purity levels were seen in vitrified oocytes with DMSO (ranging from 1.93 to 2.27) when compared with vitrified PROH oocytes (ranging from 1.47 to 2.08). Samples after being checked by triplicate spectrophotometric readings, the total RNA samples were subjected to CDNA synthesis followed by gene amplification. Typical amplification results of housekeeping genes (*GAPDH* (109 bp), *SDHA* (188 bp)) and *DNMT1* (268 bp) are shown in Fig. 1 to 4. Figures 1 and 2 represent results from the amplification of fresh oocytes extracted by GNTC, MTC and CKP. In Fig. 1, samples 1 to 3 of GNTC protocol have a total RNA concentration of 47.0, 104.5 and 59.25 ng μL^{-1}, respectively for sample 1–3. In samples 4 to 8 of MTP total RNA concentration was 46.25, 79.2, 199.2, 77.75 ng μL^{-1}, respectively for sample 4–7. In Fig. 2, working with the CKP, total RNA concentration was 24.4, 31.2, 24.0 and 21.2 ng μL^{-1} respectively for samples 1–4.

Further the amplification was performed on *DNMT1* gene (Fig. 4) to test the viability of the MTP over CKP in general gene instead of housekeeping gene. The amplification of *DNMT1* gene (268 bp), with total RNA extracted of fresh oocytes and granulosa cells using the MTP and CKP are shown in Fig. 3 in which samples 1 to 3 of MTP had total RNA concentration as follows: sample 1 with granulosa cells recovered from 10 oocytes having concentration of 104.5 ng μL^{-1}, sample 2 of 20 oocytes with concentration of 79.2 ng μL^{-1} and sample 3 with 77.75 ng μL^{-1} concentration from 40 oocytes. Samples marked as 4 to 7 of CKP had total RNA concentration as follows; sample 4 of 30 oocytes with concentration of 24.0 ng μL^{-1}, sample 5 with concentration of 21.2 ng μL^{-1} from 40 oocytes, sample 6 with granulosa cell collected from 30 oocytes with concentration of 34.4 ng μL^{-1}

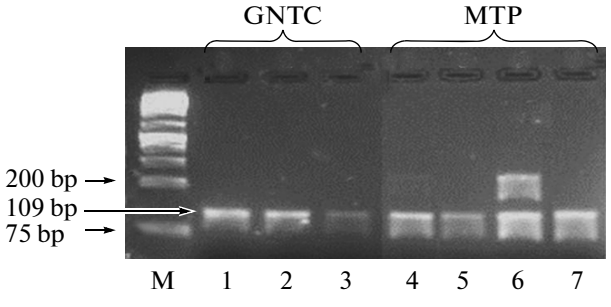

Fig. 1. Amplification of *GAPDH* gene (109 bp) with fresh oocytes using GNTC and MTP. The numbers 1 to 7 are the samples: 1 (30 oocytes); 2 (50 oocytes); 3 (60 oocytes); 4 (10 oocytes); 5 (20 oocytes); 6 (70 oocytes); 7 (40 oocytes), M represent markers.

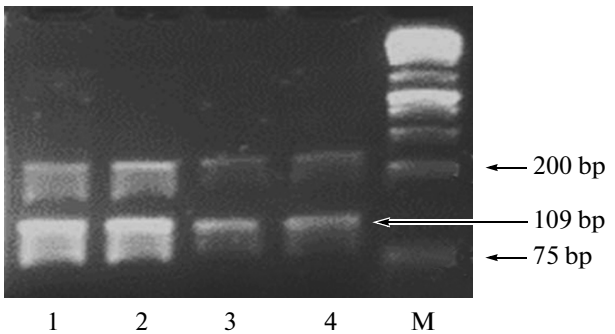

Fig. 2. Amplification of *GAPDH* gene (109 bp) with fresh and vitrified oocytes using CKP. The numbers 1 to 4 are the samples: 1 (10 oocytes); 2 (25 oocytes); 3 (30 oocytes); 4 (30 oocytes), M represent markers.

and sample 7 with concentration of 38.0 ng μL^{-1} from granulosa cells recovered from 40 oocytes.

Experiment 2: Productivity of Modified Protocol in Total RNA Extraction in Embryos

After attaining the results from the experiment 1, the MTP was evaluated in bovine embryos at the blastocyst stage. Table 3, shows that RNA purity ranged from 1.24 to 1.82 and concentration from 92 to 588 ng/μL. Nonetheless, blastocysts treated with PROH represented a less purity level of 1.24 and 1.26, respectively for 9 and 11 blastocysts. Further these same samples were subjected to cDNA synthesis followed by real time PCR. *DNMT1* gene PCR efficiency was 0.93 and after normalization against *GAPDH* and *SDHA*, the relative quantification of this gene was up regulated in DMSO (1.33) with respect to control and down regulated in PROH (0.79).

DISCUSSION

After analysing the results of experiment 1 the developed MTP had proven its efficiency by attaining better purity and total RNA concentration over other protocols. From the Table 2 the spectrophotometer readings of the three protocols, confirm MTP is nearest to the expected purity levels for RNA samples (1.7–2.0) [25], being CKP's purity more than 2.0 and GNTC less than 1.5. As per the total RNA concentration for the average of 30 oocytes representing high total RNA concentration (127.8 ± 9.3 ng μL^{-1}) was recorded in MTP over the other two protocols. If calculations are done for 10 oocytes, it would be possible to obtain enough total RNA using the MTP and the CKP (46.25 ng μL^{-1} vs. 24.4 ng/μL^{-1}) but results would be null for the GNTC protocol. These results clearly demonstrate a better performance of MTP and it would be not possible to work with so few oocytes using GNTC protocol. Also for the purity levels, GNTC protocol was the worst and it may be due to usage of high concentration of different chaotropic agents (guanidinium thiocyanate, sodium citrate and phenol) leading to disruption of the three dimensional structures in macro molecules such as proteins, DNA and RNA [26]. Research developed by Tsygankova et al. [27] recommended that after deproteinization procedure of proteins from the "aqueous phase" precipitation of nucleic acids can be forced by ethanol and then to remove impurities, dissolve the resulting precipitate by 2-methoxyethanol. Further, the nucleic acids can be precipitated by cetavlon followed by washing with 0.01 m sodium acetate by triple redissolution of nucleic acids with ethanol. Such procedure provides absolute purity and nativeness of isolated preparations of nucleic acids and conservation of their biological activity from any biological source.

Low total RNA concentration levels were observed in CKP, possibly of too much washing of materials with different wash buffer using spin cartridge, where most of the RNA is clued to their membranes, leading to the loss of RNA instead of only impurities [28]. Testing the MTP and CKP protocols on vitrified oocytes had given new result. Usage of DMSO in total RNA extraction may give better performances when compared with normal total RNA extraction. Calculation carried out for 30 oocytes, show that, on average, total RNA concentration of DMSO was 61.3 ± 3.3 ng μL^{-1} being 40.0 ± 12.4 ng μL^{-1} for PROH. This difference can be due as DMSO accelerates the activity of isopropanol during the precipitation of nucleic acids extraction procedure [29].

Gene amplification results explains more depth analysis of the MTP, CKP, and GNTC protocols when compared with spectrophotometer readings and also proves how best is the MTP over the other two protocols. As it can be observed in Fig. 1 samples 1 to 3 of GNTC protocol had light bands even though more number of oocytes (30 to 60) was used during the extraction. Reason behind for these light bands can be explained by the poor RNA purity levels of the samples

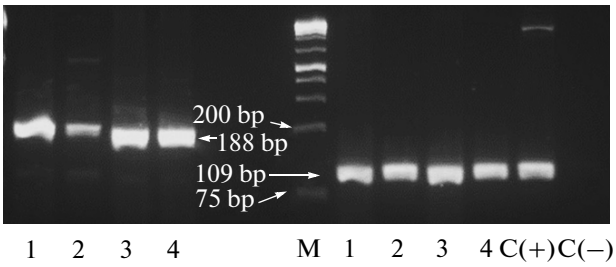

Fig. 3. Amplification *SDHA* gene of (188 bp) and *GAPDH* gene with fresh oocytes ranging from 20 to 30 by MTP. Samples 1 (30 oocytes), 2 (25 oocytes), 3 (20 oocytes), 4 (30 oocytes) with positive (C(+)) and negative control (C(−)), M represent markers.

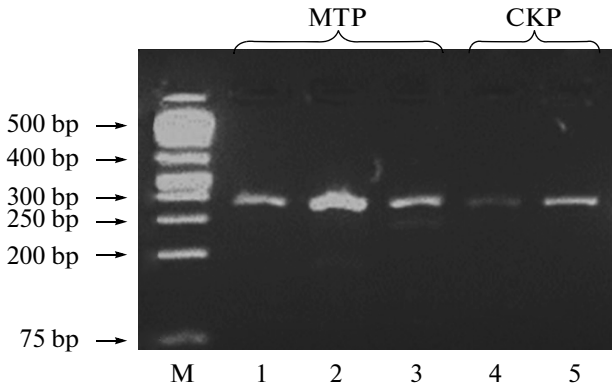

Fig. 4. Amplification of *DNMT1* gene (268 bp) with fresh oocytes and granulosa cells by MTP and CKP. The numbers 1 to 5 are the samples: 1 (granulosa from 10 oocytes); 2 (20 oocytes); 3 (40 oocytes); 4 (30 oocytes); 5 (40 oocytes); M represent markers.

Table 4. Efficiency of three protocols in the different conditions

RNA extraction Protocols	Modified Trizol protocol	GNTC protocol	Commercial kit protocol
Safety	Less bio hazardous	More bio hazardous	Less bio hazardous
Efficiency, ng/μL	Very good	Good	Very good
Specificity of bands	Bright and specific bands are observed	Specific band are observed but light	More non-specific bandsare observed
Amount of Tissue (Fresh oocytes) required	10–30	30–60	25–30

(ratio from 1.10 to 1.25), indicating more DNA and phenol contamination [30]. The major disadvantage of GNTC protocol was the usage of larger amounts of toxic components like phenol, beta-mercaptoethanol, and guanidium isothiocyanate reagent, leading to the contamination of samples affecting thus the RNA purity levels. Higher amount of phenol leads to less adsorption of RNA to aqueous phase during the phase separation process of the RNA extraction. It happens because less amount of guanidium isothiocyanate leads to the increase strong repulsion forces between the negatively charged nucleic acids and the hydroxyl groups of phenol causing loss of nucleic acids in phenol (i.e. more number of nucleic acids resides in phenol phase) [31]. The same authors described that Guanidium isothiocyanate acts as bridge between phenols, nucleic acids and also decrease the repulsion forces between them. The other disadvantages of GNTC protocol was time consuming for preparation as well as the use of bio hazardous chemicals components.

The amplification results from commercial kit protocol (Fig. 2) had more number of non-specific bands for samples (1–4) when compared with PCR amplification results of other protocols. These non-specific bands are maybe due to the required RNA binding to the walls of spin cartage while washing the samples and also as the purity levels of some of the samples were more than 2.0 (i.e. 2.27 and 2.7) showing protein contamination. Loss of total RNA material was observed while transferring the total RNA to new vials and also some molecules of total RNA can remain in the silica cartridge with a low volume of washing buffered supplied. In the other way if a high amount of washing buffer is used, it can lead to a high diluted final sample. Loss of total RNA leads to reduced concentration of mRNA; hence total RNA concentration levels of CKP samples were low. Besides being a good protocol for high amount of tissues, CKP does not work well with few oocytes being expensive, especially when the total RNA extraction is not a routine in the laboratory. The sample 4 (Fig. 1) was amplified with brighter band even though the oocytes used in total RNA extraction was very few (10 oocytes), showing the efficiency of the MTP. Samples 6 (Fig. 1) had shown a non-specific band which can be explained by high amount of RNA concentration 199.2 ng μL^{-1}, or by protein contamination. As housekeeping genes (*GAPDH* and *SDHA*) were always being expressed and had indemnity of amplification, the PCR amplification of these genes, were evaluated using the total RNA samples isolated by the MTP. As no non-specific bands and no negative control are observed in Fig. 3, it proves that MTP is more specific to bovine oocytes by obtaining specific bands with minimum oocyte's number (i.e. 25–30 oocytes).

After over all analysis of the first part of the experiment, GNTC protocol had supposed to be inefficient towards MTP and CKP, so for the further experimental part only MTP and CKP protocols were studied. The amplification results of *DNMT1* gene (Fig. 4) had proven the viability of MTP over CKP. Samples 1 to 3 of MTP had very bright bands when compared with the samples 4 and 5.

Gene expression of *DNMT1* of blastocysts produced from vitrified oocytes using DMSO as cryoprotectant shoes up regulation in comparison with control (C) and down regulated to PROH vitrified ones. After analysing the results of both experiments (1 and 2) for the three different protocols, evaluation was done based on cost, time, and efficiency. Concerning, cost estimation the purposed protocol revealed to be cheaper than the other two protocols, being however very similar to the GNTC protocol. Based the time consumption, the CKP and MTP was about 3 hours for each, while for the GNTC protocol, the double time was necessary. From the efficiency of the three protocols based on purity, concentration, toxicity of chemicals and efficiency in gene amplification, the modified protocol was considered efficient over the other protocols (Table 4). For bio hazardous chemicals, GNTC protocol was much complex when compared with MTP and CKP. As far as number of cells is concerned, the new purposed protocol gives good results with a minimum of 10 oocytes, while for the other protocols much more number is required.

In summary proposed protocol here presented can be considered more effective than GNTC and CKP after analysing the spectrophotometric, PCR amplification and gene quantification results. For the total RNA extraction in bovine oocytes and embryos, the MTP is more efficient and viable over other protocols.

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