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# Effect new simple media used for activation poor sperms for Holstein bulls born in Iraq

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***Abstract:*** *Objective: Elevate the percentage of motility in these poor motile ejaculates by using different concentrations of SMART media andexplore the best concentration of SMART media which gives best stimulation with or without the extender which used for manufactured frozen bull semen by Artificial Insemination Center – Iraq.*

***Methods:****A total of 44 ejaculates were studied during the period of this twoExperiments:Experiment 1 :Each semen samples were split further into 4 equal aliquots and one part no additiveT1-(control)each other's three parts were diluted with containing different SMART concentrations viz. T2 0.5ml, T3-1ml, T4-1.5ml, and incubation in water bath 37Cº and evaluation individual motility%, dead and abnormality % after 0 , 30, 45 and 60 minute of storage :Experiment 2: Samples of semen diluted with TFEG diluent were split further into 4 equal aliquots and each one was diluted with containing different SMART concentrations viz.no additive T1=TFEG (control) T2=TFEG+2.5 %,TFEG+T3-5%,T4=TFEG+10% after dilution artificial program that included the steps was followed dilution ,cooling package in straws 0.25 ml and freezing in liquid nitrogen and post –thawing in water bath 37C° during each steps conducted individual motility%, dead and abnormality% .*

***Results:****Experiment 1: Studies indicate that show that addition SMART media caused increase significant (p< 0.05) individual motility% and decrease dead and abnormality % for sperms of Holstein bulls born in Iraq during all periods of storage in water bath at 37 C° and the concentration 1ml of SMART was the best.*

*Experiment 2:Addition 10 % from SMARTto semen diluent gives the best concentration which improved sperm properties of bull semen during different steps of freezing semen (after dilution, cooling and post- thawing).*

***Conclusion:****Addnew simple media (SMART) caused improve significant (p< 0.05) in sperm properties of Holstein bulls born in Iraq during storage in water bath at 37 C° and sperm properties of bull's sperms dilution in TFEGand frozen in liquid nitrogen for 48hr period.*

## 1. Introduction

Successful outcome of A.I in cattle depends on a number of factors including, improvement semen quality and freezability of bull semen in resultant improving A.I,It may be critical to the success of fertilization, because it enhances the ability of sperm to detach from the wall of the oviduct, to move around in the labyrinthine lumen of the oviduct, to penetrate mucous substances and, finally, to penetrate the zona pellucida of the oocyte (Suarez &Ho 2003).Subjective motility evaluation is one of the most commonly used parameters to determine the quality of frozen-thawed semen for AI. Even though post-thaw sperm motility is a good indicator of viability, it is not always an accurate fertility predictor of an AI-semen dose (Roca et al 2015). Evaluations of sperm motility characteristics have been improved by the incorporation of the computer-assisted semen analysis (CASA) system, which measures several motility and motion parameters of

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spermatozoa that are closely related to fertility compared with subjective motility measurements (Kumar et al 2014, Sarsaifi et al 2015, Ledesma 2016 et al, Karunakaran et al 2017).Hyper activated motility has three main roles: to allow sperm to detach from the oviduct epithelium, migrate through the viscoelastic oviduct mucus in vivo and penetrate the layers of CC surrounding the oocyte in vitro and in vivo (Ho and Suarez, 2001, Suarez and Ho, 2003,( Shukla et al., 2012). Around the time of ovulation, the sperm acquire hyperactive motility and changes in the sperm head plasma membrane, thus enabling their detachment from the epithelium in the storage region of the female reproductive tract (Simons et al., 2014).Strong hyperactivated movements of the flagellum functionally aid the sperm's journey through viscous media such as the oviduct or fallopian tube mucus, and disperse the CC that surround the oocyte for access to the ZP (Suarez et al., 1991). The Kremer penetration tests a suitable assay for analyzing sperm ability to penetrate mucus, as it involves sperm penetration of the resembling viscous media methylcellulose. Sperm that are shown to penetrate methylcellulose in vitro are more likely to successfully bypass the cervical mucus, which is the first stage of sperm selection in vivo.Arginine prevents bilayer phospholipids membrane peroxidation under various

peroxidation situations through production of nitric oxide (NO) mechanism which protects structural and functional integrity of spermatozoa (Govil et al., 1992; Srivastava et al., 2000).Sperm hyper activation motility is promoted by exogenous addition of NO which may increase phosphorylation of flagella proteins (Thundathil et al., 2003; Harrison, 2004). First study on arginine was done in Iraq to stimulate in vitro the motility goat sperm (AL-Shaty, 2007) andbull sperm (AL-Ebady et al 2012 ).Artificial Insemination center in Iraq is responsible for processing of frozen semen in Iraq and the center depends on Holstein bulls born in Iraq to produce semen but for known individual variation in semen properties in these bulls which produce ejaculates with low quality, especially in sperm motility (Beran et al. 2012, Dolezalova et al. 2015,Prastowo1 et al 2019), and for this reason these poor ejaculates were excluded from processing in AI center which lead to economical loss.Moreover in Iraq many authors used different materials added in vitro toactivation poor motility of bull sperms such as arginine ,caffeine ,Pentoxifylline but no any study SMART media which gave good results in increasing motility and viability of poor motility for human sperm(Fakhrildin &Flayyih, 2011,Al Shammari et al 2015,Kadhim et al 2017).Therefore, this study was conducted as an attempt to:

1. Elevate the percentage of motility in these poor motile ejaculates by using different concentrations of SMART media with or without the extender which used for manufactured frozen bull semen by Artificial Insemination Center – Iraq.
2. 2-Explore the best concentration of SMART media which gives best stimulation of motility sperms especially during freezing in liquid nitrogen.

## 2. Materials & Methods

**Bulls:** This study was carried out at artificial insemination center of Abou-Ghareeb west of Baghdad, on (4) Holstein bulls born in Iraqi, semen was routinely collected from all bulls weekly with the aid of an artificial vagina, all bulls have the same age (3-4 years), and were kept under identical conditions of management, feeding and watering throughout the study period which start from January 2020 until March 2020.

**Ejaculates:**

Ejaculates range among 30- 40% of initial individual motility were taken weekly by using the artificial vagina method. A total of 44 ejaculates were studied during the period of this experiment. As a routine work in the AI center, after collection of semen, the sample was immediately brought to the laboratory, and placed in a water bath at (37-38˚C) for

macroscopically evaluation (volume, color, consistency) and microscopic evaluation (mass activity, individual motility, dead, abnormality and sperm concentration) according to the procedures which followed by artificial insemination center.

**Evaluation of semen characteristics.**

Mass activity: mass activity percentage was recorded immediately after semen collection by examining a drop of raw semen on a warm slide at 10x magnification under a microscope with attached stage warmer (temperature set at 37˚C), the score was calculated from 0 to 100(Evans and Maxwell 1987).

Individual motility: estimated by mixing one drop of raw semen with two drops of 2.9% sodium citrate solution on a warm slide at 37˚C, then the mixture covered by cover slide and examined under light microscope at 40x magnification, motility was scored on the basis of the percentage of spermatozoa with normal forward progressive movement, whereas those showing circling movements or those oscillating at one place were regarded as immotile (Chemineau et al1991).

Dead: dead percentage estimated by differential staining technique using Eosin-Nigrosin stain (Campbell et al., 1956; Douglas and Kenneth, 2013). The composition of the stain included Eosin-Y. 1.67 gm. and 10 gm. of Nigrosin in 100 ml of 2.9 % Sodium citrate buffer, the stain was matured and then used, The smears were prepared induplicate after mixing a small drop of neat semen with four drops of stain on a clean grease free microscopic slide at 370C. Two clean slide were prepared, one of which was used to prepared thin smear for the purpose of estimating the dead sperm percentage and the other for the abnormality.

Two Hundred spermatozoa were counted under the objective (40X) of a phase contrast microscope for estimating the percentage of live (unstained) spermatozoa, the pinkish (eosinophilic) and partially stained spermatozoa were classified as dead (Hafez and Hafez,2000).

Abnormality: The other slide that was attended by the smear was counted 200 sperm under oil immersion of a phase contrast microscope for estimating the percentage of abnormalities (Evans and Maxwell, 1987).

**3. Semen Processing (Dilution and Freezing):**

**Dilution of semen**

**Experiment 1:**

Each semen samples were split further into 4 equal aliquots and one part no additive T1-(control)each other's three parts were diluted with containing different SMART concentrations viz. T2 0.5ml, T3-1ml, T4-1.5ml, so as to have a final sperm concentration of 80 million sperms per ml, and incubation in water bath 37Cº and evaluation individual motility%, dead and abnormality % after 0 , 30, 45 and 60 minute of storage .

**Experiment 2:**

Semen samples were diluted with the basic control extender TFEG preparation as below:

1-Tris2.42 gr –Fructose 1 gr-Eggyolk 19 ml+ 1ml combination of antibiotic (Gentamycin 300µg, Tyrosine 150µg + Lico-Spectinomycine250 µg /ml)-Glycerol (6.4 ml) dissolve in double distal water 73.6 ml and this diluent (TFEG) which used by Artificial insemination center for frozen bull semen in Iraq.

2-Preparation of SMART media according to (Muhammad et al 2011) as below:

Ringer solution (Pharmaceutical solution industry, Jeddah, KSA; chemical composition per liter is bicarbonate 29 mmol/ l, sodium lactate3.2 g, sodium chloride 6.0g, potassium chloride 0.4 g and calcium chloride 0.27g) was taken within special nontoxic bottle and specific additives were added including 0.5 g phenol red (Panreac Quimica SA, Spain) and sodium pyruvate (0.01g; Prolabo, France)..(

Each semen dilution samples were split further into 4 equal aliquots and each one was diluted with containing different SMART concentrations viz.no additive T1=TFEG (control) T2=TFEG+2.5 %, TFEG+T3-5%, T4=TFEG+10%, so as to have a final sperm concentration of 80 million sperms per ml, and incubation in water bath 37Cº and evaluation individual motility%, dead and abnormality % and prepared for freezing process according to the following steps , and the assessments of semen quality were undertaken at semen dilution, cooling and freeze-thawing ,the parameters studying are individual motility, dead, abnormality percentage of sperms by using the same procedureswere used for evaluation these properties for neat semen which described above. Then after that, the dilution semen was frozen in liquid nitrogen according to followings steps:

**Steps of freezing dilution semen**

Cooling: after 30 minutes at room temperature, the all diluters semen were transferred into the cold cabinet, and in a controlled manner, allow to reach the stabilize degree 5C° in about 1-1.5 hour by adding in cold cabinet, ice cubes were added to the beaker when temperature of the water in the beaker reached 20C°, so should be bellow 5C° in a controlled manner, this can be done by the aid of sensitive thermometer to determine the degree of the temperature) and therefore evaluated the individual motility%, dead and abnormalities % .

**Equilibration and Packaging:** After 0.5hr when the temperature of dilution semen reaches 5C°package in straws 0.25ml and preservation at 5C° until end equilibration time.

Freezing: Straws for each treatment were racked horizontally on a special metal tray and frozen in liquid nitrogen vapor by placing the tray (4cm) above the liquid nitrogen level in the freezing tank for 9 minutes, then straws were dipped in the liquid nitrogen and stored at (-196 C°) for 48 hours.

**Thawing:** After 48 hr.one straw for each treatment and control was retrieved from the liquid nitrogen container and placed in a water bath at 37°C for 30 sec, used to evaluation individual motility%, dead and abnormalities %.

Statistical analysis. The experiment was conducted. Results are quoted as Mean ±

SE. Statistical analyses were carried out using the General Linear Model procedures (GLM) of SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Comparisons between values were analyzed by Duncan's multiple range test following an F-test in ANOVA (Duncan. 1955). Significance was set at ($P<0.05$).

## 4. Results

Results of Experiment 1: Effect different concentration of SMART media on sperm properties of Holstein bulls born in Iraq during storage in water bath at 37 C° summarized in Table 1 and Fig 1,2 and 3.

Individual motility : Results revealed that that the activation of bull sperms in vitro with all concentration T2 ,T3, and T4 of SMART media caused a significant ($P<0.05$) increase in the percentage of individual sperm motility compared to before activation in all incubation period 0, 30 ,45, 60 minute at 37 C ° both sperm activation ,and the concentration T3 ( 1ml) of SMART during 45 and 60min caused high significant ($P<0.05$) individual motility % compare with others treatment T2 and T4 , but the different no significant between all treatment during storage period 0 and 30 min, and among

T2 and T4 at periods 45 and 60 min. Overall mean of comparative between periods were show in Table 1 conducted that decrease significant (P<0.05) in individual motility % when increase period of storage semen until 60min which don't different significant from 45 min and Overall mean of comparative between treatment show that all treatment caused high significant (P<0.05) individual motility % compare with control but the differences no significant between all treatment of SMART (Table.1and Fig.1).

Dead sperms%:present study show that addition all treatment of SMART media improve viability of sperm after activation compare with before where decrease dead %significant (P<0.05) in T1 , T2 , T3, in other wise T3 give less significant (P<0.05) than T2 and T4 during period 30 , 45 ,60 min but the different no significant between all treatment during 0 min ,and among T2 and T4 during 30, 45 ,60min (Table .1).Overall mean of comparative between treatments were show that all treatments caused decrease significant (P<0.05) in dead % compare with control but T3 show least significant (P<0.05) than T2 and T4 but the differences no significant between T2 and T4 (Table.1,Fig.2).In addition to the Overall mean of comparative between periods were show in Table 1 revealed that60 min increase significant (P<0.05) in dead % compare with others period of storage but no significant different among 0 and 30 min and between 30 and 45 min .

Table1. Effect different concentration of SMART media on sperm properties of Holstein bulls born in Iraq during storage in water bath at 37 C°. (Mean ±SE)

| Semen properties | Incubation period (minute) | Before activation No additive T1 Control | After activation | | | Overall mean |
|---|---|---|---|---|---|---|
| | | | T2 0.5 ml | T3 1 ml | T4 1.5 ml | |
| Individual motility % | 0 | 30.32±2.65 B | 42.89±1.89 A | 45.43±3.01 A | 43.11±2.76 A | 40.43±3.42 a |
| | 30 | 20.20±3.09 B | 34.45±2.04 A | 35.31±1.89 A | 36.65±2.43 A | 31.65±3.84b |
| | 45 | 10.11±2.09 C | 23.67±3.88 B | 34.56±1.75 A | 24.89±2.08 B | 23.30±5.02 c |
| | 60 | 5.45±2.09 C | 19.54±2.76 B | 28.99±1.99 A | 20.03±1.54 B | 18.50±4.86 c |
| Overall mean | | 16.52±5.53 B | 30.13±5.28 A | 36.07±3.42A | 31.17±5.29A | |
| Dead % | 0 | 45.34±0.76 A | 33.89±0.65 B | 32.51±1.02 B | 31.78±0.21 B | 35.88±3.18c |
| | 30 | 53.03±0.87 A | 36.05±0.43 B | 32.05±0.41 C | 39.99±0.53 B | 40.28±4.54 bc |
| | 45 | 60.32±1.09 A | 40.32±1.03 B | 33.04±0.61 C | 41.05±1.44 B | 43.68±5.83 b |
| | 60 | 66.03±0.79 A | 65.32±0.64 A | 36.11±1.09 C | 54.03±1.05 B | 55.37±6.98 a |
| Overall mean | | 56.18±4.48A | 43.89±3.26B | 33.42±.91C | 41.71±4.59B | |
| Abnormality % | 0 | 20.05±1.91 A | 13.07±0.22 B | 11.56±1.91 B | 12.56±0.39 B | 14.31±1.93 c |
| | 30 | 25.88±0.87 A | 18.44±0.87 B | 13.00±0.49 C | 17.00±1.03 B | 18.58±2.69 bc |
| | 45 | 30.09±1.06 A | 20.98±1.02 B | 14.06±0.78 C | 19.03±0.54 B | 21.04±3.34ab |
| | 60 | 37.08±1.05 A | 26.65±0.56 B | 16.06±0.77 D | 21.04±0.59 C | 25.20±4.51 a |
| Overall mean | | 28.27±3.58A | 19.78±2.82B | 13.67±0.94C | 17.70±1.8 B | |

Within row different large letters for each parameter means significant at (p<0.05).
Within small letters in overall mean for Colum for each parameter differed significantly (p<0.05).

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Abnormality sperms %: Activation by using SMART causes decrease significant (P<0.05) in abnormality sperms% during all period of storage and observe T3 registeredhigh significant (P<0.05) compare with T2 and T4 at period 30 ,45 and 60 min, also T4 more significant (P<0.05) than T2 at 60 min (Table .1 and Fig .3) but the different no significant

**Fig : 1.Effect different concentration of SMART media on individual motility % of sperms for Holstein bulls born in Iraq during storage semen in water bath at 37 C°.**

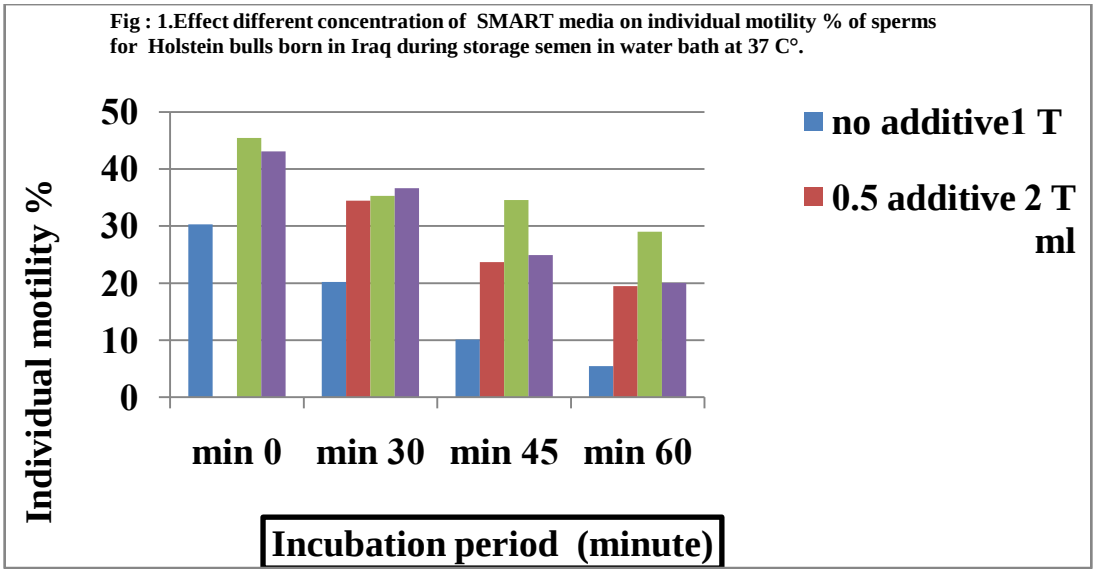
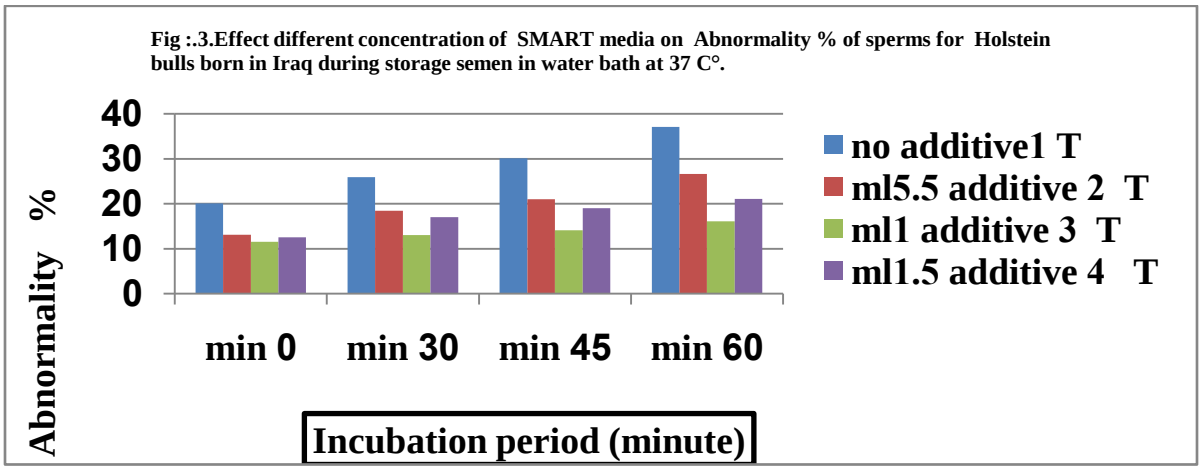



**Fig :.3.Effect different concentration of SMART media on Abnormality % of sperms for Holstein bulls born in Iraq during storage semen in water bath at 37 C°.**




among all treatment at 0 minute and between T2 and T4 at 30 and 45 min .Overall mean of treatment explained that additive activation SMART in different concentration to sperms of bulls caused reduced significant (P<0.05) the abnormality% compare with no additive and T3give the best significant (P<0.05) compare with others (Table.1).In addition to Overall mean of period of storage show that increase duration caused increase in the abnormality% of sperms ,but no significant between 0 and 30 min and among 30 and 45 min also between 45 and 60 min(Table1) .

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**Results of Experiment 2:** The results of semen analysis for pre and post additive different concentration (2.5, 5 and 10%) of SMART media to TFEG diluent in vitro sperm activation during step of freezing in liquid nitrogen (after dilution, cooling and post-thawing) were presented in (Table2 .and Fig .4, 5 and 6).

Individual motility %: It was showed significant (P<0.05) increased in the percentage of sperm motility of post-activation in T2, T3and T4- during cooling and post- freezing as compared with pre-activation T1- TFEG, but after dilution except T3 and T4 showed significant (P<0.05) increased in the individual motility %compare with T1 and T3 but the result in (Table 2, Fig .4) revealed the different no significant whether between T1 and T2 or

Table.2. Effect different concentration of SMART media additive to TFEGdiluent on sperm properties of Holstein bulls born in Iraq during frozen in liquid nitrogen (Mean ±SE)

| Semen properties | Steps of freezing | | Treatment | | | | Overall mean |
|---|---|---|---|---|---|---|---|
| | | | T1= TFEG Control | T2=TFEG + 2.5 % | T3=TFEG + 5 % | T4=TFEG + 10 % | |
| Individual motility % | After | Dilution | 44.12±2.65 B | 48.67±1.66 B | 49.83±1.91 A | 55.69±1.26 A | 49.57±2.38 a |
| | | cooling | 38.55±3.09 C | 50.85±2.96 B | 51.11±1.62 AB | 56.75±1.23 A | 49.31±3.83 a |
| | Post-thawing | | 20.11±2.09 C | 43.97±3.88 B | 44.06±1.60 B | 50.89±1.98 A | 39.75±6.74 b |
| Overall mean | | | 34.26±7.25 C | 47.83±2.03 B | 48.33±2.16 B | 54.44±1.80 A | |
| Dead % | After | Dilution | 30.54±1.06 A | 23.19±0.65 B | 22.58±0.02 B | 15.78±0.21 C | 23.02±3.01 ab |
| | | cooling | 32.03±0.87 A | 20.05±0.43 B | 19.05±0.41 B | 14.09±0.33 C | 21.30±3.80 b |
| | Post-thawing | | 35.32±1.09 A | 33.12±1.03 A | 23.04±0.61 B | 19.16±0.94 B | 27.66±3.89 a |
| Overall mean | | | 32.63±1.41 A | 25.45±3.93 B | 21.55±1.26 B | 16.34±1.49 C | |
| Abnormality % | After | Dilution | 19.85±0.68 A | 15.87±1.62 B | 13.46±1.01 BC | 10.11±0.11 C | 14.82±2.05 b |
| | | cooling | 25.33±0.35 A | 18.93±1.87 B | 19.00±0.65 B | 14.00±1.03 C | 19.31±2.32 b |
| | Post-thawing | | 34.88±0.66 A | 28.98±1.02 B | 24.63±0.67 C | 19.03±0.54 D | 26.88±3.35 a |
| Overall mean | | | 26.68±4.39 A | 21.26±3.95 AB | 19.03±3.22 BC | 14.18±2.58 C | |

Within row different large letters for each parameter means significant at (p<0.05).

Within small letters in overall mean for Colum for each parameter differed significantly (p<0.05).

among T3 and T4 through after dilution but the different no significant between T2 and T3 also among T3 and T4 in post- thawing in addition to individual motility %for T2did not differ significant compare with T3 through post-thawing. Results of overall mean which Show in Table. 2Which it is clear that T4 givehigh individual motility % and a way significant (P<0.05) compare with T1, T2 and T3 but the different no significant betweenT2 and T3. Moreover, overall mean for comparative among steps of freezing (Table .2) which explain that

**Fig . 4.Effect different concentration of SMART media additive to TFEG diluent on individual motility %of sperms for Holstein bulls born in Iraq during frozen in liquid nitrogen**

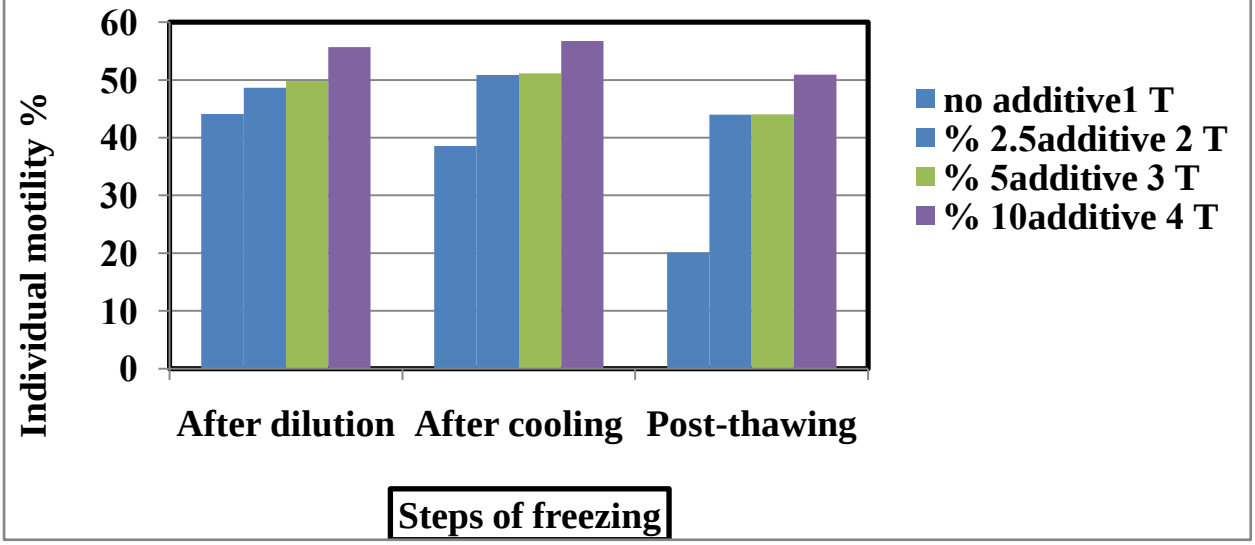
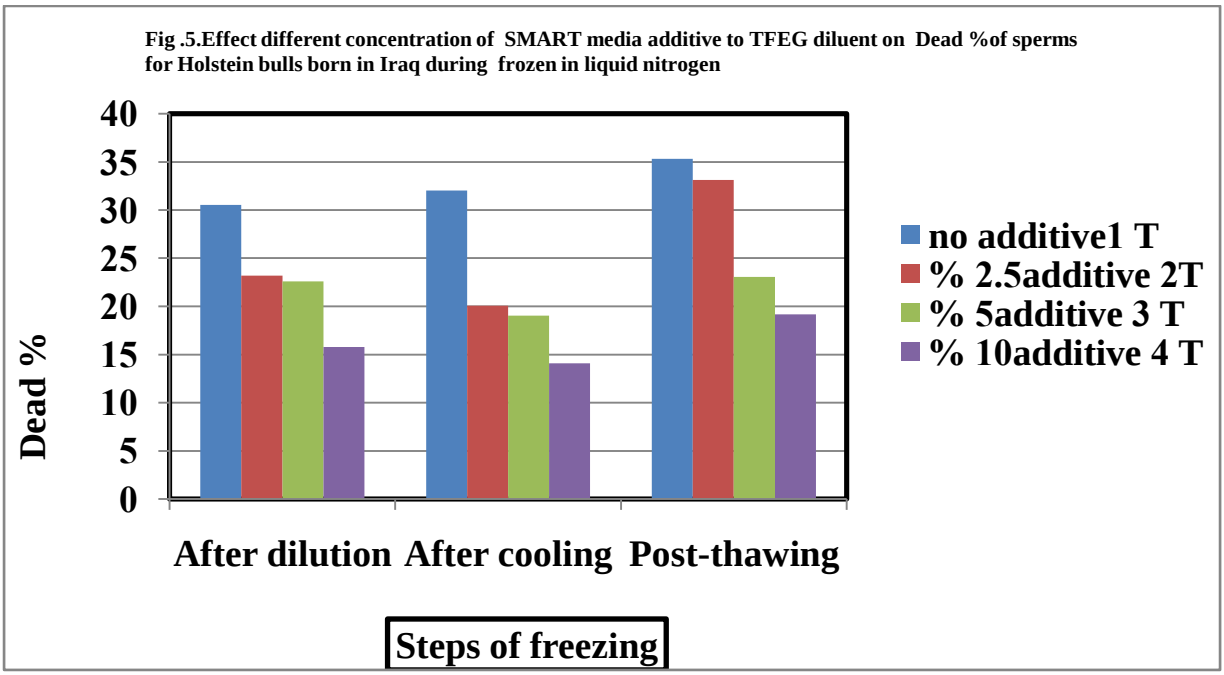



**Fig .5.Effect different concentration of SMART media additive to TFEG diluent on Dead %of sperms for Holstein bulls born in Iraq during frozen in liquid nitrogen**




generally post-thawing after freezing in liquid nitrogen caused decrease significant (P<0.05) in individual motility % compare with after dilution and cooling for steps of freezing semen.

Dead % of sperms: The effect treatments on dead percentage of bull sperm frozen in liquid nitrogen revealed in (Table .2 and Fig .5), in which, as comparing between treatments within each step observed. After dilution and cooling, the treatment T4 significantly($p< 0.05$) calculated decreased in dead % as compared with other treatments T1,T2and T3, inadditionT2 and T3show decrease significant ($p< 0.05$) in compare with T1 (Table .2 and Fig.5) but the changes no significant among T2 and T3.Post- thawing, results in this table showT3andT4 registered less significant ($p< 0.05$) in dead

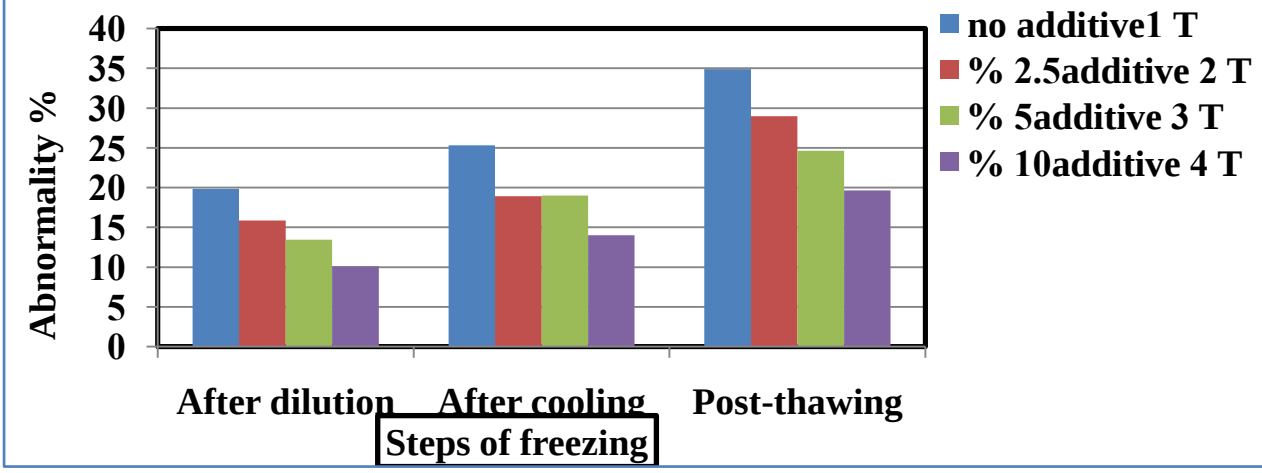
percentage of sperm compare with T1 and T2, but the differences no significant between T1 with T2 and T3 with T4.Results of overall mean which Show in (Table2),which it is clear that T4 give minimum dead sperms % significant (p< 0.05)compare with T2 and T3 were also give less dead sperm %significant (p< 0.05) among T1.Moreover overall mean for comparative among steps of freezing (Table .2) which definethat generally post-thawing post- thawing caused increase significant (P<0.05) in

dead% compare with after cooling but no found any differ significant compare between after cooling and dilution and also among after dilution and post –thawing.

Abnormality sperms%: The effect addition SMART on abnormality percentage of bull sperm frozen in liquid nitrogen observed in (Table .2 and Fig .6), in which, as comparing between treatments within each step revealed that: After dilution semen, the sperm abnormality percentage for T2, T3 and T4 (15.87,13.46,10.11) respectively were decreasesignificant (p< 0.05) compare with T1(19.85)and T4 recorded less abnormality sperms (p< 0.05) compare with 4T2,but the variation no significant between T2 and T3 and also among T3and T4 (Table 4-2).After cooling diluent semen in (Table 2 and Fig.6.)T4 which causes decrease significant (p< 0.05) in abnormal percentage in sperms where it was (14.00) compare with T2(18.93)and T3(19.00) were give less significant (p< 0.05)in abnormality percentage among T1 treatments, but the differences no significant between T2andT3.Abnormality percentage in sperms for Post-Thawing: Result revealed that the differences between all treatment significant (p< 0.05) where it observed thatT4(19.03) give least percentage better significant (p< 0.05) than T2(28.98) andT3(24.36)

were also recorded decrease in abnormality percentage significantly (p< 0.05) compare with T1(34.88) no additive ,but result show that overall mean of abnormality %for sperms in T4 (14.18) decrease significant (p< 0.05) compare with T1 (26.68)and T2 (21.26) ,also the different significant (p< 0.05) between T3 and T1but the differences no significant between T1andT2 , T2 and T3 ,T3andT4 (Table .2) which this table revealed that overall mean of post-thawing caused increased significant (p< 0.05) in abnormality sperms% compare with steps after dilution and cooling but the different between them no significant .

**Fig .6.Effect different concentration of SMART media additive to TFEG diluent on Abnormality %of sperms for Holstein bulls born in Iraq during frozen in liquid nitrogen**

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**5. Discussion**

Results of experiment 1and 2 show addition SMART media caused improve significant ($p< 0.05$) in sperm properties of Holstein bulls born in Iraq during storage in water bath at 37 C° andsperm properties of bulls sperms dilution in TFEGand frozen in liquid nitrogen for 48hr,these results agreement with (Fakhrildin &Flayyih, 2011,Al Shammari et al 2015,Kadhimet al 2017) for uses in activation human fresh sperms,Culture media provide the spermatozoa with needs that maintain optimal function of spermatozoa to give excellent results during preparation. Culture media are isotonic with semen to prevent any osmotic shock to spermatozoa and developing embryos during in vitro manipulation steps. It provides optimal buffering capacity, and maintains the pH within physiological levels to ensure sperm survival . Semen with normal viscose plays critical roles for sperm function and fertilization process but hyper viscosity caused negative effect on motility of sperms (Harchegania et al 2019), so may be SMART It had a role in reducing this effect .Fakhrildin &Flayyih, 2011concluded that the SMART medium was suitable for enhancement of sperm parameters of asthenozoospermic human using direct swim-up technique.In addition to contain SMART media mineral such as Na+ , K+ , Cahelp to increase motility of sperm and decrease dead and abnormality of bull sperms . The major components of bovine seminal plasma is made up of ions Na+ , K+ , Zn+ , Ca Demembranated bull sperm required higher levels of Ca2+ to hyperactivate than to activate (Ho et al. 2002). This indicates that the crucial site for the action of Ca2+ is the axoneme. Nevertheless, the means by which Ca2+ reaches the axoneme in the core of the flagellum to switch on hyperactivation are poorly understood. There is evidence for an intracellular Ca2+ store in the base of the flagellum of bull sperm. Receptors for inositol 1,4,5-trisphosphate (IP3), which releases Ca2+ from intracellular stores, were localized by antibodies to the base of the flagellum.

Furthermore, pharmacological agents known to release Ca2+ from stores were demonstrated to induce hyperactivation in bull sperm (Ho and Suarez 2001). Cyclic nucleotide-gated Ca2+ channels have been localized to bull sperm flagella (Wiesner et al. 1998); therefore, activation of cyclic nucleotide-gated channels by cAMP/cGMP may be one of the mechanisms providing Ca2+ entry. Various voltage-sensitive Ca2+ channels have also been localized to the flagellum (Wennemuth et al. 2003). A unique class of putative Ca2+ channel proteins, CatSper channels, are found only in spermatogenic cells and mature sperm. These proteins resemble voltage-sensitive channel proteins but also seem responsive to cyclic nucleotides (Ren et al. 2001; Quill et al. 2001). While CatSper has been shown to be involved in supporting sperm motility, it has not yet been demonstrated to regulate hyper activation.SMART media was consist from different ions such as calcium ,potassium , sodium, chloride ,more of , The major components of bovine The major components of bovine seminal plasma is made up of ions Na+ , K+ , Zn+ , Ca++, Mg++, Cl2, (Juyena & Stelletta, 2012).Bovine seminal plasma enhances sperm motility (Juyena & Stelletta, 2012; Poiani, 2006)and protects spermatozoa against immune attack in the female reproductive tract (Poiani, 2006) so deficiency of these ions caused poor motility .Therefore, compensating them with industrial additions of ions that have a role to improve motility and benefit ability sperm to protect for cooling and freezing bull sperms.

Period of storage liquid semen in water bath 37C° caused increase in sperm properties with increase period of storage. Mohamed et al 2012obtained demonstrated that 25°C has high sperm progressive movement as well as sperm viability compared to 4°C and 37°C. Sperm stored at 25°C showed normal morphological structure whereas there were morphological alteration at storage temperature of 4°C and 37°C.Many studies report that increase period of storage liquid

semen causes decrease in viability and fertility of sperms so used frozen semen especially bovine semen can used for long time until many years (Murphy et al 2018,Hahn ET AL 2019).Progressive motility of liquid bull semen was greater when stored at 158C than any other temperature. A previous study on liquid ram spermatozoa reported greater motility and viability at 58C in comparison with 158C (O'Hara et al. 2009, Murphy et al 2015).In contrast, this study has demonstrated that storage temperature had no effect on viability but had an effect on motility in liquid bull semen, up to 6 days after collection. In agreement with the present study, storage temperature has previously been reported to have no effect on viability or acrosomal status in human spermatozoa, but increased sperm motility at ambient temperatures, in comparison with storage above 328C (Thijssen et al. 2014). The sperm motility is supposed to be one of the most powerful indicators of fertility success in semen analysis. It is also the most useful information to provide appropriate treatments for infertility in male patients. Sperm motility is influenced by both length of time after ejaculation and temperature (Ouitrakul et al 2018). Many studies have been conducted to explain these factors that may affect the motility of sperm. Studies recommended that the optimal temperatures should be equal to the body temperature(Esfandiari et al 2002). With regard to sperm viability, some studies found an immediate decline after ejaculation, but the present study summarized that sperm viability could remain intact for two to three hours after ejaculation (Franken et al 2011).In the present study, the authors demonstrated that when semen sample was kept at laboratory room temperature prior to analysis, both, the sperm motility and the percentage of rapid sperms exhibit significant decline within one hour after ejaculation. Furthermore, sperm velocity continued to decrease progressively after one hour. The results in the present study are similar to the experiments carried out by ( Chomsrimek et al 2008). They also found that there was significant decline in the sperm motility after one hour. Similarly, study conducted by Visconti et al indicated that most sperm had decreased motility, and then became immotile after two hours of incubation ( Visconti 2012). The adenosine triphosphate (ATP), which was the main energy of the sperm, was rapidly consumed.

The ATP is formed by glycolysis enzyme and mitochondria in mid piece of sperm. After ejaculation, sperm acquires energy from nutrient molecules such as fructose, sorbitol, mannose, glucose, pyruvate, lactate, and hydroxybutyrate found in seminal fluid. Then the ATP is produced. It is then used in sperm motility and function(Visconti 2012). Therefore, the sperm become immotile after being out of energy. Interestingly, the authors' present data confirmed the similar changes of the direction of the motility and the vitality.

**6. Effect different steps of freezing on some semen properties:**

The result in this research (Table 4-1 and 4-2) indicated to significant ($p< 0.05$) decrease in the individual motility in poor ejaculates, while there are increasing percentages in dead%, and abnormalities% during the different stages of cryopreservation, these result approved with (El-Azab et al , 1998) on buffalo and (Hussian et al 2016,Al-Badrany et al , 2017 , Al-Badrany et al , 2020), in bulls, and resulting in harmful low PH which cause toxic on sperm cell (Ball & Peters, 2004). Maintenance of sperm function during freezing and thawing depends upon severalinterrelated factors that include cooling rate, equilibration period and freezing method (Bailey et al , 2000; Curry ,2000; Joshi et al ,2003 ; Anel et al ,2006). In the present study it was observed that controlled rate of cooling and freezing resulted in significantly higher sperm total and progressive motility, compared to uncontrolled rate of cooling and freezing. The fact that progressive motility is more affected by the freezing process than individual motility implies that these parameters measure different aspects of cell physiology and in particular, that the physiological basis for the progressive motility parameter is more sensitive to cryobiological damage (Anel et al , 2003). The controlled-rate cooling protocol, besides providing complete

automation in the cryopreservation process, might also protect spermatozoa against some adverse effect caused by minor fluctuation in temperature imposed by the transfer of cooled straws from cold cabinet to cell freezer as done in the uncontrolled cooling rate ram semen freezing protocol. Apart from identifying motile and static spermatozoa CASA can also categorize spermatozoa on the basis of velocity of each motile sperm, measure the mean sperm velocity and related sperm track dimensions (Joshi et al, 2003). The measurement of sperm velocity has been considered as an indirect indicator of mitochondrial function in spermatozoa. During cryopreservation spermatozoan mitochondria undergo damages (Peris et al, 2004). The freezing process negatively affects ($P < 0.05$) the sperm parameters (individual motility, dead and abnormality), agreement with (Üstuner et al, 2015).

**Concussions:** Addition 1 ml of SMART media which used for activation poor motility spermsforHolstein bulls born in Iraq during storage in water bath at 37 C°.Increase period of storage sperms in water bath at 37 C° led decrease in viability of sperms. Utilizing SMART media with concentrate 10 % caused improve significant ($p< 0.05$) in sperm properties of Holstein bulls born in Iraq after addition to TFEG diluent and frozen in liquid nitrogen for 48hr.

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**Authors' Contribution:**

All authors contributed equally in all the efforts for these articles

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