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1 **Original Research Paper**

Revised

2 **Dimethyleacetamide improves the cryosurvivability of Indian red jungle fowl (*Gallus***
3 ***gallus murghi*) sperm**

4

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17 **Running Title:** Cryopreservation of Indian red jungle fowl sperm with DMA

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26 ABSTRACT

27 It was hypothesized that dimethyleacetamide (DMA) can be used as an alternate to glycerol
28 for cryopreservation of Indian red jungle fowl semen. Four concentrations of DMA (4%, 6%,
29 8% and 10%) in extender were compared with previously optimized cryopreservation
30 protocol based on 20% glycerol (control) for Indian red jungle fowl. Sperm motility, plasma
31 membrane integrity, viability, and acrosome integrity were assessed at the stage of post-
32 dilution, cooling, equilibration, and freeze-thawing. The whole experiment was
33 repeated/replicated for five times independently. Sperm motility, plasma membrane integrity,
34 viability and acrosome integrity were recorded highest ($P<0.05$) at post-dilution, cooling,
35 equilibration, and freeze-thawing in extender having 6% DMA compared to control and other
36 experimental extenders. The highest ($P<0.05$) recovery rates of all aforementioned
37 parameters were also recorded in extender having 6% DMA; thus, 6% DMA was further
38 compared with control (20% glycerol) for fertility after artificial insemination. Eggs were
39 collected for five days after artificial insemination with semen cryopreserved in extender
40 containing 6% DMA and control. The higher no. of fertilized eggs, fertility, no. of hatched
41 eggs, hatch (%) and hatchability were recorded with semen cryopreserved in extender having
42 6% DMA compared to control. It is concluded that 6% DMA maintained higher post-thaw
43 quality and fertility of Indian red jungle fowl semen and is a better replacement of glycerol.

44
45 **Keywords:** Cryo-damage; Motility; Fertility; Acrosome; Sperm viability; Indian red jungle
46 fowl

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51 1. Introduction

52 Cryopreservation provides safety net against the stochastic events of nature;
53 continuously at work and playing role in the deletion of valuable genetic resource [1]. During
54 the last few decades, increasing human population has resulted in extreme exploitation and
55 selection pressure in poultry birds that pushed indigenous or local species/breeds in the
56 background, disposing of valuable genes [2,3]. According to Domestic Animal Diversity
57 Information System, about 50% of the poultry species have been enlisted in endangered
58 category [4]. The Indian red jungle fowl is a wild sub-species of genus Gallus, and is
59 considered as one of the ancestor of domestic chicken [5-10]. The population of Indian red
60 jungle fowl is facing a notable threat in its native range due to habitat destruction, poaching,
61 inbreeding and genetic hybridization [11]. The cryo-banking has great potential for
62 application in *ex situ in vitro* conservation of this precious Gallus sub-species [12]. Semen
63 cryopreservation is the most feasible method in birds as cryopreservation of oocyte or
64 embryo is not possible because of large size, high lipid content and polar organization [13],
65 and alternative methodologies such as primordial germ cells methodologies are too invasive
66 for at risk populations [14, 15].

67 It is generally accepted that avian sperm has low surface to volume ratio and
68 elongated tail that makes it more vulnerable to osmotic, chemical, thermal and physical
69 stresses during cryopreservation process compared to mammalian sperm [16,17]. Freeze-
70 thawing process not only causes irreversible damage to mitochondria, mid-piece and
71 acrosome of sperm [18], but also induces physical and chemical changes that alter
72 physiological processes. Sperm may lose ATP due to energy metabolism [19] and
73 glycoproteins or glycolipids necessary for transport and maturation [16,20].

74 The species-specific differences in sperm and their response to freeze-thaw process
75 are important determinants of fertility and need the development of successful freezing

76 protocol, identification of novel cryoprotectants and diluents [21]. Of all cryoprotectants
77 available, glycerol and dimethyleacetamide (DMA) are considered more adequate [14,22].
78 Glycerol is least toxic and more effective cryoprotectant for low fertility lines of poultry [14].
79 Nevertheless, due to its contraceptive properties removal of glycerol is needed prior to
80 artificial insemination that makes it difficult to use [7, 23-25]. Glycerol is also known to
81 interact with the metabolism of sperm, alter lipid-bilayer that possibly decreases the
82 efficiency of acrosome reaction [26, 27].

83 The DMA is a permeable cryoprotectant, is not contraceptive and does not need to be
84 removed prior to artificial insemination; thus may be helpful in reducing damage due to
85 cryoprotectant removal [14]. Furthermore, DMA may offer an alternative since high levels of
86 fertility have been obtained with this cryoprotectant in chicken [28,29]. DMA has the
87 capacity of penetration by increasing plasma membrane fluidity through the rearrangement of
88 lipids and proteins that cause reduction in formation of ice-crystallization at low temperature
89 [21,30]. DMA has been used as cryoprotectant for sperm in houbara bustards [31], fowls
90 [29,32], eagles, and peregrine falcons [33,34], ducks [35], landese ganders [36], sandhill
91 cranes and turkeys [37], Japanese quail [38], and emus [39]. However, the intensity of cryo-
92 induced changes are highly species-specific [30,40,41]. One major disadvantage of DMA is
93 the toxic effect associated with its concentration [34]. In a previous study, we showed that
94 glycerol could be an efficient cryoprotectant for red-jungle fowl sperm cryopreservation [9].
95 However, considering the importance of semen cryo-banking for conserving valuable genetic
96 resources of Indian red jungle fowl, and in order to simplify the cryopreservation process of
97 red-jungle fowl sperm, the present study was planned to evaluate the use of different
98 concentrations of DMA on quality and fertility of cryopreserved Indian red jungle fowl
99 sperm.

100

101 2. Materials and methods

102 All experimental procedures and animals used in this study were approved by the
103 ethical committee of the Department of Wildlife Management, PMAS-Arid Agriculture
104 University Rawalpindi, Pakistan.

105 2.1. Experimental animals

106 Eight sexually mature cocks (age: 5 years) of Indian red jungle fowl (*Gallus gallus*
107 *murghi*) were selected and raised at Avian Research Station, Pir Mehr Ali Shah Arid
108 Agriculture University Rawalpindi-Pakistan. The birds were housed in single sand floor pen
109 (8 m²) with roof cover and kept under natural photoperiod and temperature conditions. They
110 were fed commercial feed containing corn 61.0%, rice polish 4%, corn gluten 1%, canola
111 meal 5%, rapeseed meal 2%, soybean meal 13%, sunflower meal 4%, limestone 8%, DL-
112 Methionine 0.10%, soda bicarb 0.10%, salt 0.30%, vitamin and min. premix 0.40% and
113 MDCP 1%. Water was available over the experimental period *ad libitum*.

114 2.2. Experimental design

115 Semen was collected from eight mature cocks twice a week (October 2015-March
116 2016). A total of 40 ejaculates (5 ejaculates/cock) were processed for semen analysis.
117 Ejaculates (whole) having >60% motility were pooled and aliquoted for dilution in to five
118 experimental extenders. Experiment involved the addition of DMA at 4%, 6%, 8% and 10%
119 of extender and 20% glycerol as a control. Semen was cryopreserved and quality was
120 assessed at post-dilution, post-cooling, post-equilibration and post thawing. The whole
121 experiment was repeated/replicated for five times independently. Ten pools of semen with
122 6% DMA and 20% glycerol were used to inseminate 50 hens. A total of 80 ejaculates (10
123 ejaculates/cock) were frozen for each extender separately. Five hens per extender were
124 inseminated with each pool of semen and fertility was estimated through the fertilized and
125 unfertilized eggs collected up to 5 days following insemination.

126

127 2.3. *Semen collection and dilution with freezing extender*

128 Semen was collected twice a week as described by Burrows and Quinn [42] in a
129 graduated plastic tube using massage technique. Qualifying ejaculates from eight cocks were
130 pooled on each replicate. Each pool was immediately diluted 1:1 (v/v) using diluent
131 comprised of D-fructose (1.15g), sodium glutamate (2.1g), polyvinylpyrrolidone (0.6g),
132 glycine (0.2g) potassium acetate (0.5g) and distilled water (100mL) to final pH (7.0) and
133 osmolarity 380mOsm/kg [7]. Precaution of the temperature shock was taken, and thus the
134 tubes containing diluents were kept in water bath at 37°C. The extender was divided into 5
135 experimental extenders containing respectively 4%, 6%, 8%, 10% DMA, and 20% glycerol
136 (control). All diluents and media were prepared in the laboratory using analytical grade
137 chemicals purchased from Sigma-Aldrich, Co., 3050 Spruce street, St Louis, USA.

138 2.4. *Cryopreservation and thawing of semen*

139 The semen (mean concentration 1200×10^6 sperm/mL) diluted in freezing extenders
140 were immediately cooled to 4 °C in two hours ($0.275 \text{ }^\circ\text{C min}^{-1}$) and equilibrated for 10 min
141 at 4°C. Cooled semen was filled in 0.5 mL French straws (IMV, L'Aigle, France), kept over
142 liquid nitrogen vapors for 10 minutes and plunged into liquid nitrogen for storage. After 24
143 hours, the straws were thawed for 30 sec in water bath at 37 °C and held at 37 °C to assess
144 motility, plasma membrane integrity, viability, and acrosomal integrity. For fertility
145 measurements, semen frozen with 6% DMA and 20% glycerol were thawed as previously
146 described and used in artificial insemination. Glycerol was removed after thawing by
147 following stepwise dilution protocol as suggested by Purdy et al. [43].

148

149 2.5. *Semen quality assays*150 2.5.1. *Motility*

151 The percentage of motile sperm and the quality of sperm were assessed subjectively
152 as described by Santiago-Moreno et al. [16]. Semen sample was placed on a pre-warmed
153 (37°C) glass slide and observed under a phase contrast microscope (400x).

154 2.5.2. *Plasma membrane integrity*

155 Plasma membrane integrity at different stages of cryopreservation was assessed by
156 using hypo-osmotic swelling test (HOST) as described by Santiago-Moreno et al. [44]. The
157 HOS solution composed of sodium citrate (1g) and distilled water (100 mL). Previously
158 diluted 25 µl semen was mixed with 500 µl of a HOS solution (100 mOsm/kg) and incubated
159 at 37°C for 30 minutes. The slides were fixed in buffered 2% glutaraldehyde. The plasma
160 membrane integrity of sperm was scored on the basis of swollen heads, swollen and coiled
161 tails. The sperm that respond to HOS solution become swollen and coiled were classified as
162 normal sperm having intact plasma membrane. Nevertheless, sperm that did not respond to
163 HOS solution remained straight were classified as sperm with damaged plasma membrane. A
164 total of 200 sperm were counted at four separate fields under a phase-contrast microscope
165 (1000x with oil immersion).

166 2.5.3. *Sperm viability*

167 Viability (% Live / total sperm) at different stages of cryopreservation was examined
168 by adding eosin-nigrosin to the lake glutamate solution. Lake's glutamate solution [45] was
169 prepared by adding sodium glutamate (0.01735g), potassium citrate (0.00128g), sodium
170 acetate (0.0085g) and magnesium chloride (0.000676g) in 100 mL distilled water. Water
171 soluble nigrosin (5g) and water soluble Eosin-bluish (1g) were added into Lake's glutamate
172 solution. Twelve drops of stain were mixed with 1 drop of semen. A smear was made on a
173 glass slide, fixed and air dried. A total of 200 sperm were assessed per slide under a phase-
174 contrast microscope (1000x with oil immersion). The mixture provides a clear background in

175 the smear to enhance the contrast of white, unstained “live” sperm or the pinkish stained
176 “dead” sperm.

177 2.5.4. *Acrosomal integrity*

178 The percentage of sperm having intact acrosome was determined by phase contrast
179 microscopy, examining 200 Giemsa blue-stained cells under the magnification of 1000x as
180 described by Jianzhong and Zhang [46]. Giemsa staining involved spreading of 5µl of diluted
181 semen on to a glass slide, allowing them to dry and then fixed in neutral formal-saline (5%
182 formaldehyde) for 30 min followed by air-drying. Fixed slides were kept in Giemsa stain for
183 1.5 h and washed with distilled water and air-dried. Sperm with hooked, swollen, thinned,
184 unstained and missing acrosome were classified as sperm having damaged acrosome.
185 However, evenly stained spermatozoa were classified as normal sperm having intact
186 acrosome. A total of 200 sperm were observed at least in four separate fields. All variables
187 were analyzed by the same person.

188 2.6. *Recovery rate for semen quality parameters*

189 The recovery rate of sperm motility, plasma membrane integrity, viability and
190 acrosomal integrity rate were calculated through following formula [7,36];

$$\text{Recovery rate} = \frac{\text{Value of a given parameter in frozen semen}}{\text{Value of a given parameter in fresh semen}} \times 100$$

191

192 2.7. *Artificial insemination*

193 The fertilization capacity of sperm was estimated from the results of two consecutive (at
194 one day interval) intravaginal artificial inseminations involving 300 million sperm/female at
195 each insemination. A total of 50 hens of Indian red jungle fowl were inseminated using 6%
196 DMA and 20% glycerol. All inseminations were performed between 12:00 and 14:00 h as
197 described by Burrows and Quinn [47]. Eggs were collected from day 2 after the first

198 insemination up to 6 days, and there were a mean of 50 eggs/treatment. Fertility
199 (%fertile/incubated eggs) was determined by candling of eggs on day 7 of incubation.

200 2.8. *Statistical analysis*

201 The data were presented as means (\pm SEM). The effect of different stages of
202 cryopreservation and the cryoprotectant concentration were analyzed by two factor ANOVA
203 using (MSTAT-C® ; version 1.42, Michigan State University, East Lansing, MI, USA).
204 When F-ratio was found significant ($P<0.05$), post hoc comparison between the means was
205 done through Fisher's protected LSD test. Student t-distribution under the notion of
206 comparing two independent groups (6% DMA vs 20% glycerol) was used to test the
207 influence of cryoprotectant on fertility rate.

208

209 3. **Results**

210 The data on effect of DMA on motility, plasma membrane integrity, viability and
211 acrosome integrity of Indian red jungle fowl spermatozoa are presented in Figure 1. Sperm
212 motility, plasma membrane integrity, viability and acrosome integrity of Indian red jungle
213 fowl spermatozoa was recorded higher ($P<0.05$) in extender having 6% DMA compared to
214 control and other experimental extenders with 4%, 8%, 10% DMA at all stages of
215 cryopreservation. Nevertheless, cryopreservation have significantly ($P<0.05$) negative effect
216 on motility, plasma membrane integrity, viability and acrosome integrity of Indian red jungle
217 fowl spermatozoa irrespective of experimental extenders.

218 The data on recovery rates of motility, plasma membrane integrity, viability and
219 acrosome integrity of Indian red jungle fowl spermatozoa after cryopreservation are given in
220 Table 1. The recovery rates of all aforementioned parameters were recorded higher ($P<0.05$)
221 in extender having 6% DMA compared to control and other experimental extenders.

222 The results of fertility obtained after artificial insemination of frozen-thawed semen
223 with 6% DMA or 20% glycerol (Control) are given in Table 2. The highest ($P<0.05$) no. of
224 fertilized eggs, percent fertility, no. of hatched chicks, percent hatch and hatchability of
225 fertilized eggs were recorded after AI with 6% DMA compared to 20% glycerol.

226

227 **4. Discussion**

228 Gamete cryopreservation methodology has been developed for a wide range of
229 species over the last sixty-five years [47]. The success of sperm cryopreservation depends on
230 many factors including the type of cryoprotectant, packaging method (straws and pellet),
231 freezing and thawing rates for avian species [13,17, 29] that are responsible for the alteration
232 of structure and function of sperm [48]. Cryoprotectants help to prevent enzyme denaturation,
233 protein destabilization, and ice crystal formation during freeze-thaw process [49]. The most
234 commonly used cryoprotectant for avian sperm are glycerol [50] and DMA [51]. It has been
235 demonstrated that 20% glycerol can be used in cryopreservation of Indian red jungle fowl
236 sperm [7], while information on use of DMA is lacking in published literature. The previous
237 studies on chicken and turkey have reported that sperm cryopreservation with DMA/straws
238 produced less fertility compared to sperm cryopreserved with glycerol [51]. It is believed that
239 low fertility with DMA/straws based cryopreservation has been attributed to deleterious
240 interaction of plastic straws and the DMA. Moreover, this type of interaction is more
241 deleterious and spectacular to sperm viability in the species having low fertility. The sperm
242 cryopreservation with DMA is recommended for species having high fertility compared to
243 glycerol, as high technical skills are needed to remove glycerol before artificial insemination
244 [14]. The present study showed that 6% DMA efficiently protect Indian red jungle fowl
245 sperm during cryopreservation and yielded higher fertility rate compared to glycerol.

246 It is well known that stages of the cryopreservation (post-dilution, post-cooling, post-
247 equilibration, before freezing and after freezing, post-thawing) do affect sperm survival and
248 are highly dependable on the diluent and cryoprotectant used. In our study, damages to sperm
249 motility occurred first from post-dilution to post-cooling and were much higher with 10% and
250 4% DMA (10% decrease), intermediate with 8% DMA (6% decrease) and control 20 %
251 Glycerol (6%) and very less in extender having 6% DMA (3.7%). The second part of damage
252 occurred from post-cooling to post equilibrium stage of cryopreservation; very less at 6%
253 DMA (5%) compared to 13-21% damage at other concentrations of DMA. Finally, the
254 freezing-thawing itself was more deleterious as it cumulated the combined effects of the huge
255 temperature and osmotic stress of the freezing that induced membrane and metabolic injuries
256 at thawing such as potential re-crystallization of ice crystals imposed by the disruption of
257 proteins that may cause further cell damage [52,53]. However, in our study, the freezing-
258 thawing stages induced significantly less damage of sperm (22% for sperm motility with the
259 dose of 6% DMA) compared to control 20% glycerol (38%) and 4%, 8%, 10% DMA (23-
260 27%). The damage reported during cryopreservation was very high in the previous studies on
261 chicken [54,55] and duck [35]. It is relevant to mention that higher concentrations of DMA
262 are considered very toxic to rooster sperm [22].

263 It is relevant to discuss that Tselutin et al. [29] recommended the use of DMA in a
264 sperm cryopreservation process in pellets achieved by dropping diluted sperm samples
265 directly into liquid nitrogen. Rapid freezing is usually employed with DMA [56], while slow
266 freezing using nitrogen vapour in combination with packaging straws are usually used with
267 dimethyl sulphoxide [57] and glycerol [50]. A cryopreservation method with semen packaged
268 in straws was chosen in our study because the sperm freezing in nitrogen vapour was usually
269 used in our lab and other labs working with avian spermatozoa. Contrary to previous reports,

270 our findings revealed higher fertility rates than previous reports in chickens employing both
271 DMA in straws [43] and pellets [12].

272 As DMA is toxic to spermatozoa [15,22], the concentration used is therefore very
273 important. It should be noted that the sperm of native Mediterranean breed of chicken are not
274 very tolerant of DMA, and concentrations lower than 6% are recommended [12]. Although a
275 similar condition could be expected in the Indian red jungle fowl [12], the present results
276 showed that DMA 6% provided the greater cryoprotective activity. Moreover, DMA 6%
277 improved the fertility results regarding to previously used glycerol 20% protocol for Indian
278 red jungle fowl sperm.

279 For fertility, higher glycerol concentration was kept as control because in our previous
280 study, higher glycerol concentration gave better results [7]. Both glycerol and DMA are
281 permeable cryoprotectants that provide intra and extracellular protection, dehydrating the
282 sperm by an osmotically driven flow of water and thus avoid ice crystal formation during
283 cryopreservation process [23,32]. Permeable cryoprotectants improve membrane fluidity and
284 thus increase ability to survive post-thawing [21]. However, many permeable cryoprotectants
285 such as DMA may have toxic effect on sperm, that is directly related to cryoprotectant
286 concentration used and time to cell exposure [58,59]. Thus, the dose balances between a
287 positive cryoprotectant effect and a negative toxic effect is important. In the present study,
288 the optimum evolved concentration of DMA was 6%. The previous work on Houbara bustard
289 [31] and chicken [60] would confer our results that sperm cryopreserved in an extender
290 having 6% DMA can retain their viability, motility and fertility even after cryopreservation
291 and suggest that lower concentration (6%) of permeable cryoprotectant is more favorable to
292 sperm during cryopreservation process [12,29,35]. In our study, it has been observed that
293 mean fertility rates [56.4% (control), 66.8% (6% DMA)] were higher compared to previous
294 studies in ducks [35] and turkeys [54] using same cryoprotectant in different diluents.

295 It is concluded that the cryopreservation (pre-freeze steps and freezing-thawing)
296 process affects the quality of red jungle fowl sperm. Moreover, 6% DMA could be efficiently
297 used as cryoprotectant for the cryopreservation of Indian red jungle fowl sperm. To our
298 knowledge, this is the first report on the use of DMA as cryoprotectant for Indian red jungle
299 fowl sperm.

300

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Rakha, B., Ansari, M., Akhter, S., Zafar, Z., Naseer, A., Hussain, I., Santiago-Moreno, J., Blesbois, E. (2017). Dimethyleacetamide improves the cryosurvivability of Indian red jungle fowl (*Gallus gallus murghi*) sperm. *Theriogenology*, 103, 83-89. , DOI : 10.1016/j.theriogenology.2017.07.018

1 Table 1: Recovery rate (%) of semen quality parameters of Indian red jungle fowl semen after
 2 freezing-thawing (n = 40; 5 ejaculates /8 cocks).

Extender	Recovery rate (%)			
	Motility	Plasma membrane Integrity	viability	Acrosome integrity
20% Glycerol	51.8 ± 3.1 ^c	64.8 ± 1.6 ^c	61.9 ± 7.4 ^b	62.6 ± 5.2 ^b
4% DMA	71.2 ± 3.0 ^a	64.2 ± 1.5 ^c	67.7 ± 4.4 ^{ab}	68.0 ± 2.7 ^{ab}
6% DMA	75.0 ± 2.8 ^a	79.4 ± 1.9 ^a	79.6 ± 2.0 ^a	75.3 ± 2.4 ^a
8% DMA	62.0 ± 2.7 ^b	75.0 ± 3.8 ^{ab}	71.2 ± 4.2 ^{ab}	67.1 ± 5.9 ^{ab}
10% DMA	67.9 ± 4.3 ^{ab}	69.9 ± 4.1 ^{bc}	67.4 ± 3.5 ^b	67.3 ± 5.1 ^{ab}

3 The values (mean ± SE) with different superscript in a column differ significantly (P<0.05).

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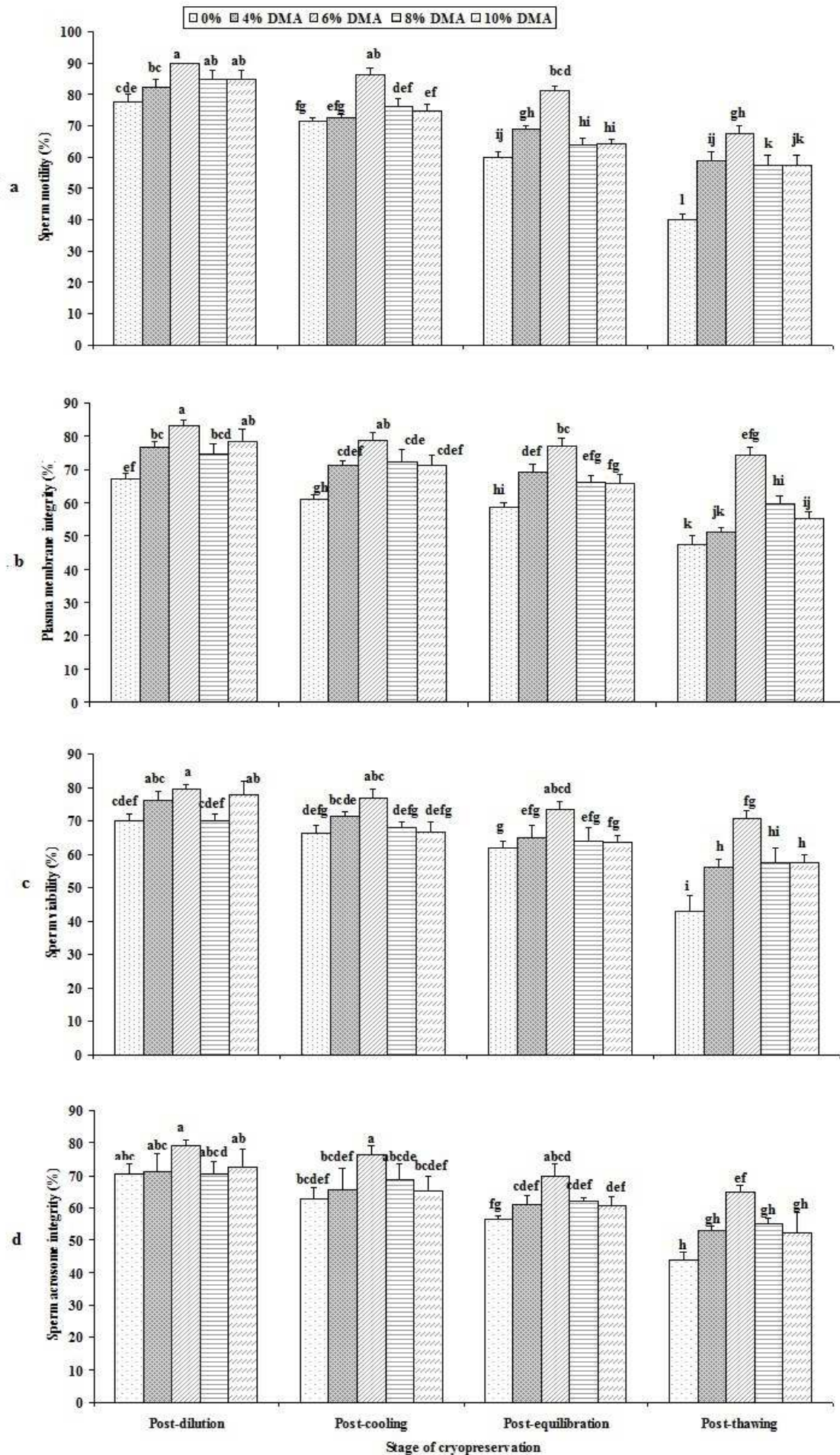
20 Table 2: Fertility and hatchability obtained with frozen-thawed sperm using as cryoprotectant
 21 glycerol 20% or DMA 6% (n = 80; 10 ejaculates/8 cocks).

22

Items	Cryoprotectant	Day after insemination					Mean \pm SE
		2	3	4	5	6	
Number of laid eggs	Glycerol 20%	50	45	47	48	45	45.0 \pm 1.4
	DMA 6%	45	47	48	40	45	47.0 \pm 0.9
Number of fertile eggs	Glycerol 20%	30	32	34	36	28	25.4 \pm 1.1 ^b
	DMA 6%	27	22	24	28	26	32.0 \pm 1.4 ^a
Fertility (%)	Glycerol 20%	60.0	46.8	50.0	70.0	57.8	56.9 \pm 4.1 ^b
	DMA 6%	60.0	71.1	72.3	75.0	62.2	68.1 \pm 3.0 ^a
Number of hatched chicks	Glycerol 20%	22	18	20	20	20	20.0 \pm 0.6 ^b
	DMA 6%	28	30	28	30	25	28.2 \pm 0.9 ^a
Hatch (%)	Glycerol 20%	48.9	38.3	41.7	50.0	44.4	44.7 \pm 2.2 ^b
	DMA 6%	56.0	66.7	59.6	62.5	60.1	60.1 \pm 2.1 ^a
Hatchability of fertile eggs (%)	Glycerol 20%	93.3	93.8	82.4	83.3	89.3	81.5 \pm 2.2 ^b
	DMA 6%	81.5	81.8	83.3	71.4	76.9	88.4 \pm 2.4 ^a

23 Different letters (a,b) between diluents, for each item, indicate significant differences
 24 (P<0.05).

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Figure 1: Effect of Dimethylacetamide (0%, 4%, 6%, 8% and 10%) on motility (a), plasma membrane integrity (b), viability (c) and acrosome integrity (d) of Indian red jungle fowl sperm at different stages of the freezing-thawing process. The results are expressed as mean \pm SEM, n= 40 ejaculates (5 ejaculates/8 cocks). Different superscripts indicate statistically significant differences ($P < 0.05$).

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Highlights

- Glycerol have contraceptive properties and must be replace with alternate cryoprotectant
- DMA was evaluated for cryopreservation of Indian red jungle fowl sperm
- DMA improve the sperm motility, PMI, viability and acrosome integrity
- DMA improve the fertility rate of frozen thawed Indian red jungle fowl semen

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