ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume 66 15 Number 1, 2018
https://doi.org/10.11118/actaun201866010131

THE IN VITRO EFFECT OF TAURINE ON BOAR SPERMATOZOA QUALITY

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Abstract

PAÁL DUŠAN, STREJČEK FRANTIŠEK, TVRDÁ EVA, FORMICKI GRZEGORZ, KLEIN SABINE, RATH DETLEF, MASSANYI PETER. 2018. The in Vitro Effect of Taurine on Boar Spermatozoa Quality. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 66(1): 131–137.

The aim of this in vitro study was to evaluate the effects of taurine (TAU) supplementation on boar spermatozoa motility, viability, acrosome integrity and morphology. Eighteen boar semen samples were diluted with the Androhep Plus™ extender containing no TAU (control) or supplemented with 1.5 mM, 7 mM, 12.5 mM TAU and cultured at 4 °C for 18 days. Sperm motility was evaluated using the computer-aided sperm analysis (CASA) system. Furthermore, the samples were fixed and assessed for the occurrence of morphological abnormalities using phase contrast microscopy. Fluorescent dyes SYBR–14 and propidium iodide were used to determine the sperm viability. Acrosome integrity was examined using PNA–Alexa Fluor 647 and flow cytometry. A gradual decrease of the semen quality was detected in all experimental groups over the course of the study. CASA revealed no selective advantage of TAU supplementation on the spermatozoa motility ($p > 0.05$). TAU administration showed to be ineffective in preserving spermatozoa viability as well as acrosome integrity as measured by flow cytometry ($p > 0.05$). Under the conditions of this study, no significant positive effect of TAU was recorded following its administration to the Androhep Plus™ boar semen extender with respect to spermatozoa quality.

Keywords: taurine, boars, spermatozoa, Androhep Plus™, motility, viability, acrosome integrity, morphology

INTRODUCTION

Artificial insemination (AI) in pig breeding has been considerably growing over the past decades. In order to increase the insemination rate, numerous semen extenders have been developed as standard culture media for boar spermatozoa. Previous research has revealed that extended male gametes are more likely to survive the storage period before insemination into the female reproductive tract. The use of boar semen extenders has therefore become routine ever since the 1970s (Hirai *et al.*, 2001; Rath *et al.*, 2009). Although there are numerous brands of commercially available extenders, only few of them are extensively used in the insemination programmes, including Androhep Plus™. This culture medium is based on its predecessor, the Androhep™ extender consisting of glucose, sodium citrate, EDTA, sodium bicarbonate, HEPES and BSA (Gadea, 2003).

Despite a relatively high preservation capacity of such semen extenders, current research invests a substantial effort to the enhancement of their efficacy, particularly by their enrichment with

protective agents such as specific amino acids. These have been repeatedly demonstrated to act as potent antioxidants, with the ability to suppress oxidative stress inflicted upon spermatozoa cultured in vitro (Bucak *et al*., 2009; Dorado *et al*., 2014). Spermatozoa are of limited capacity to counteract the damaging effects of reactive oxygen species (ROS) (Bailey *et al*., 2000; Bucak *et al*., 2007). Although the presence of ROS is necessary to trigger several events crucial for fertilization, excessive lipid peroxidation (LPO) as a result of ROS overproduction impairs the structural integrity and functional activity of spermatozoa (Lenzi *et al*., 2002). During the terminal stages of differentiation mammalian spermatozoa discard most of the cytoplasm along with the key antioxidants essential to counteract the detrimental effects of ROS (Foote *et al*., 2002; Bucak *et al*., 2007). Therefore, such low antioxidant capacity would barely preserve the physiological functions of stored spermatozoa (Holmes *et al*., 1992; Aurich *et al*., 1997; Storey, 1997).

Taurine (2–aminoethanesulfonic acid; TAU) has been abundantly found in the mammalian body, including testes and spermatozoa (Hood *et al*., 1967; Meizel *et al*., 1980; Buff *et al*., 2001). Here, it serves a multitude of physiological roles such as bile salt formation (Sturman, 1993), calcium binding and transport (Lazarewicz *et al*., 1985), osmoregulation (Ozasa and Gould, 1982) or stabilisation of biological membranes (Huxtable and Bresler, 1973; Balkan *et al*., 2002). Moreover, TAU has been shown to be part of the antioxidant defence system of cells (Green *et al*., 1991). Studies in cats (Baran *et al*., 2009), rams (Ijaz and Ducharme, 1995), stallions (Jang *et al*., 2006) and donkeys (Dorado *et al*., 2014) report that TAU administration to mammalian semen prevents the loss of spermatozoa motility or viability in vitro.

This study was therefore designed to evaluate whether taurine could enhance the protective capacity of the Androhep Plus™ extender on boar spermatozoa motility, viability, acrosome integrity and morphology.

MATERIAL AND METHODS

Semen samples (n = 18) were obtained from two sexually mature and healthy German Landrace, Duroc and Yorkshire boars. The animals were carefully handled in accordance with the European Union Regulation 2010/63. Spermatozoa concentration was determined using NucleoCounter SP–100® (ChemoMetec A/S, Allerød, Denmark). Each sample was diluted in the Androhep Plus™ extender (Minitüb GmbH, Tiefenbach, Germany) containing no TAU (control), and the Androhep Plus™ extender supplemented with 1.5 mM, 7 mM and 12.5 mM TAU to a final concentration of 10^8 spermatozoa/mL. The samples were cultured at 4 °C instead of the recommended 15–20 °C for boar semen storage (Johnson *et al*., 2000) to evaluate the effect of taurine at very low storage temperatures. Furthermore, according to the manufacturer's technical report on Androhep Plus™, the extender contains a membrane protection ingredient of non-animal origin, which protects the highly sensitive membranes of boar spermatozoa when stored at temperatures as low as 5 °C (Wasilewska *et al*., 2016). All analyses were performed immediately upon treatment and subsequently in a three-four-day period until the total spermatozoa motility dropped below 30%.

Aliquots of samples were re-diluted in Androhep Plus™ to a final concentration of 10^7 spermatozoa/mL and then allowed to incubate for 10 min at 37 °C. Thereafter, 10 µl of the suspension was placed on a Makler Counting Chamber® (Sefi Medical Instruments, Germany) pre-warmed at 37 °C. A minimum of 200 cells were examined in triplicates for total motility using the computer-aided sperm analysis (CASA) system (HTM–IVOS®, Hamilton Thorne, Biosciences, Beverly, USA).

Aliquots of samples were fixed in 30 µl Hancock's solution, placed on slides and evaluated under a phase contrast microscope (Olympus BX60, Japan) using a 1000–fold magnification. A minimum of 200 spermatozoa from each sample were examined for the occurrence of morphological abnormalities.

Fluorescent dyes SYBR–14 and propidium iodide (PI) (Live/Dead® Sperm Viability Kit, Life Technologies, USA) were used to determine the spermatozoa viability. The acrosome integrity was examined using PNA–Alexa Fluor 647 (Life Technologies, USA) binding to damaged acrosomes. Briefly, 10 µl of suspension was pipetted to 480 µl Androhep Plus™ to reach the final concentration of 10^6 spermatozoa/mL. The mixture was stained with 5 µl SYBR–14 (1 µM) and 3 µl PI (240 µM) and subsequently incubated at 37 °C for 5 min. Afterwards, 5 µl of PNA–Alexa Fluor 647 (10 µg/mL) were added to the samples followed by an additional incubation at 37 °C for 10 min. A minimum of 20,000 spermatozoa were analysed using a flow cytometer (Gallios™, Beckman Coulter, Krefeld, Germany). SYBR–14–positive spermatozoa ($SYBR–14^+$) were considered to be live, while PI–positive (PI^+) spermatozoa were evaluated as dead. PNA–Alexa Fluor 647–positive spermatozoa (PNA^+) were classified as acrosome-damaged.

Differences between control and treatments were evaluated using One–Way ANOVA followed by the Tukey post-hoc test ($p = 0.05$). The calculations were performed using the SigmaPlot ver. 12.0. The data are expressed as mean ± standard error (SE).

RESULTS

The results of the CASA assessment of sperm movement are listed in Tab. I. Taurine did not maintain the total spermatozoa motility during cultivation at 4 °C for 18 days when compared with the control ($p > 0.05$). Furthermore, a moderate decrease in the motility was observed in all samples over the course of the experiment.

At the same time, Androhep Plus™ containing taurine did not confer any additional protection against spermatozoa morphology alterations when compared to the extender without any additional taurine ($p > 0.05$). The population of spermatozoa with altered morphology was increasing over the incubation period (Tab. II).

Microscopic examination of the samples did not confirm any improvement of the acrosome integrity of boar spermatozoa after being extended in taurine-enriched Androhep Plus™ for 18 days when compared to the control group ($p > 0.05$). However, a steep increase in the percentage of acrosome-altered spermatozoa was observed in all experimental groups following seven days upon semen dilution (Tab. III).

The results obtained by flow cytometry (Tab. IV) address no effect of taurine addition to Androhep Plus™ on the acrosome integrity of boar spermatozoa when compared to the samples extended without an additional taurine. In addition, measurements performed 11 days upon semen treatment revealed a moderate increase in the population of acrosome-damaged spermatozoa in all experimental groups.

Flow cytometric analyses of spermatozoa viability are shown in Tab. V and VI. No effect of taurine on spermatozoa viability was detected during the treatment. The population of live (SYBR - 14^+) or dead (PI^+) spermatozoa did not significantly differ from their respective controls ($p > 0.05$). However, a moderate decrease in spermatozoa viability was observed in all samples during 18 days of cultivation.

DISCUSSION

Over the past decades, great emphasis has been placed on the development of semen extenders, which could maintain the quality of semen during storage prior to insemination of females. Despite a great preservation capacity of the current boar semen extenders, there is still potential to increase their efficacy. Previous reports suggest that TAU added to semen extenders could provide an additional line of protection to stored spermatozoa.

A compelling evidence for TAU involvement in the ROS–quenching mechanisms in spermatozoa

I: *Total spermatozoa motility recorded upon taurine supplementation*

Taurine (mM)	%					
	Day 1	Day 4	Day 7	Day 11	Day 14	Day 18
Control	80.0 ± 1.8	76.8 ± 6.3	68.8 ± 5.1	64.0 ± 2.3	62.3 ± 7.4	63.0 ± 10.0
1.5	75.4 ± 2.5	74.7 ± 3.9	68.0 ± 4.7	63.8 ± 3.4	59.3 ± 5.0	64.0 ± 6.0
7	77.4 ± 3.8	73.5 ± 5.1	70.7 ± 5.8	67.5 ± 2.4	67.5 ± 5.4	62.0 ± 5.0
12.5	74.1 ± 5.1	73.7 ± 5.8	71.7 ± 5.8	69.8 ± 1.4	69.0 ± 5.6	58.0 ± 1.0

Total motility (in %) recorded for boar semen cultured with taurine during 18 days at 4 °C. Data are expressed as mean ± SE for eighteen treatments and control samples. No significant differences were detected between the treatments and the control group ($p > 0.05$).

II: *Occurrence of morphologically altered spermatozoa upon taurine supplementation*

Taurine (mM)	%					
	Day 1	Day 4	Day 7	Day 11	Day 14	Day 18
Control	23.1 ± 3.8	24.7 ± 4.8	26.8 ± 3.9	22.5 ± 2.4	28.6 ± 2.8	40.0 ± 7.0
1.5	24.7 ± 4.1	22.4 ± 4.1	24.3 ± 4.5	23.1 ± 1.7	28.3 ± 2.3	40.5 ± 8.0
7	22.2 ± 4.3	19.4 ± 4.2	26.3 ± 6.1	21.3 ± 3.0	25.0 ± 1.4	40.8 ± 2.8
12.5	21.0 ± 4.9	18.1 ± 4.3	23.2 ± 4.6	21.7 ± 1.8	20.0 ± 2.0	48.3 ± 1.3

Percentage of morphologically altered spermatozoa (in %) in boar semen cultured with taurine during 18 days at 4 °C. Data are expressed as mean ± SE for eighteen treatments and control samples. No statistical differences were detected between the treatments and the control group ($p > 0.05$)

III: *Occurrence of acrosome–altered spermatozoa upon taurine supplementation*

Taurine (mM)	%					
	Day 1	Day 4	Day 7	Day 11	Day 14	Day 18
Control	4.3 ± 1.0	8.3 ± 2.1	10.8 ± 1.9	14.4 ± 1.2	15.5 ± 3.4	15.2 ± 2.3
1.5	3.9 ± 1.0	7.6 ± 1.9	10.7 ± 4.0	14.5 ± 1.6	15.5 ± 2.7	18.5 ± 1.0
7	4.9 ± 1.0	5.8 ± 1.8	12.3 ± 5.2	12.4 ± 2.1	16.4 ± 2.3	19.8 ± 2.8
12.5	4.6 ± 1.4	5.4 ± 1.7	8.6 ± 2.1	12.2 ± 1.4	11.3 ± 1.5	19.5 ± 1.0

Percentage of acrosome–altered spermatozoa (in %) in boar semen cultured with taurine during 18 days at 4 °C. Data are expressed as mean ± SE for eighteen treatments and control samples. No significant differences were detected between the treatments and the control group ($p > 0.05$).

was provided by Alvarez and Storey (1983) who observed a significant decrease in the production of malondialdehyde in rabbit semen after being treated with TAU. A different study conducted by Fraser (1986) emphasized on the antioxidant effects of 0.1 mM TAU on the motility and reproductive capacity of murine spermatozoa. Furthermore, 25–50 mM TAU increased the preservation capacity of the Modena extender, resulting in an overall reduction of LPO in boar spermatozoa (Jang *et al.*, 2006). According to Perumal *et al.* (2013) 50 mM TAU promoted the activity of glutathione peroxidase, superoxide dismutase and catalase while concomitantly lowering the amount of malondialdehyde, hence preserving the viability and morphology of bovine spermatozoa. Similarly, Dorado *et al.* (2014) reported a significantly greater motility of donkey spermatozoa preserved in the GentA extender containing 20, 40 and 60 mM TAU when compared to the control group. However, the authors did not observe any improvement of spermatozoa morphology or plasma membrane integrity regardless of TAU concentration. Inversely, Martins-Bessa *et al.* (2007) observed no effect of TAU on the motility, viability, acrosome or mitochondrial integrity of canine spermatozoa. Intriguingly, a broad range (0.005–5 mM) of TAU did not affect the spermatozoa motility in fowls (Barna *et al.*, 1998). As such, the reports on the in vitro effects of TAU on spermatozoa differ significantly in mammals.

As for our results, we recorded no advantage of TAU administration to the Androhep Plus™ with respect to boar spermatozoa motility recorded over 18 days of treatment. In a similar way, our data address no improvement in the viability or morphological status of spermatozoa following TAU treatment. Hence, our study failed to demonstrate any benefit from TAU addition to Androhep Plus™ as this amino acid had no impact on any of the basic characteristics of sperm fertilization capacity in boars.

Over the course of evolution, mammalian spermatozoa developed an array of sophisticated molecular mechanisms to carefully regulate the generation of ROS. Taurine has been repeatedly shown to promote the activity of antioxidant enzymes such as thioredoxin reductase (Yildirim *et al.*, 2007), superoxide dismutase (Higuchi *et al.*, 2012),

IV: *Occurence of acrosome–damaged spermatozoa recorded upon taurine supplementation*

Taurine (mM)	%					
	1. day	4. day	7. day	11. day	14. day	18. day
Control	2.8 ± 0.5	2.6 ± 0.8	3.3 ± 0.8	3.4 ± 1.1	4.0 ± 0.8	4.6 ± 0.8
1.5	2.8 ± 0.5	2.4 ± 0.8	3.2 ± 0.8	3.2 ± 1.1	3.8 ± 0.8	4.6 ± 1.0
7	2.6 ± 0.5	2.6 ± 0.8	3.3 ± 0.8	3.2 ± 1.0	3.8 ± 0.6	4.9 ± 0.6
12.5	2.6 ± 0.4	2.8 ± 0.9	3.2 ± 0.8	3.4 ± 1.1	3.8 ± 0.6	4.7 ± 0.9

Percentage of acrosome–damaged spermatozoa (in %) in boar semen cultured with taurine during 18 days at 4 °C. Data are expressed as mean ± SE for eighteen treatments and control samples. No significant differences were detected between the treatments and the control group ($p > 0.05$).

V: *Occurence of live spermatozoa recorded upon taurine supplementation*

Taurine (mM)	%					
	Day 1	Day 4	Day 7	Day 11	Day 14	Day 18
Control	88.1 ± 1.1	86.7 ± 0.9	85.3 ± 1.0	82.5 ± 1.7	81.5 ± 2.0	81.0 ± 1.6
1.5	88.6 ± 1.0	87.3 ± 0.6	85.8 ± 1.0	82.9 ± 1.7	82.4 ± 1.4	81.2 ± 1.5
7	88.1 ± 1.0	87.2 ± 0.7	85.6 ± 0.9	82.8 ± 1.9	81.6 ± 1.4	80.6 ± 1.7
12.5	87.1 ± 1.2	86.5 ± 0.9	84.9 ± 0.9	81.4 ± 1.8	81.2 ± 1.3	79.2 ± 2.1

Percentage of live spermatozoa (%) in boar semen cultured with taurine during 18 days at 4 °C. Data are expressed as mean ± SE for eighteen treatments and control samples. No significant differences were detected between the treatments and the control group ($p > 0.05$).

VI: *Occurrence of dead spermatozoa recorded upon taurine supplementation*

Taurine (mM)	%					
	Day 1	Day 4	Day 7	Day 11	Day 14	Day 18
Control	8.4 ± 0.6	9.6 ± 0.6	10.3 ± 0.6	12.0 ±1.0	12.3 ± 0.7	14.1 ± 0.9
1.5	8.2 ± 0.7	9.2 ± 0.6	10.2 ± 0.8	11.9 ± 1.3	12.0 ± 0.6	14.1 ± 1.1
7	8.6 ± 0.7	9.5 ± 0.6	10.5 ± 0.6	12.3 ± 1.6	12.6 ± 0.8	14.6 ± 1.6
12.5	9.4 ± 0.9	9.9 ± 0.7	10.9 ± 0.7	13.4 ± 1.3	12.8 ± 0.4	15.3 ± 1.4

Percentage of dead spermatozoa (%) in boar semen cultured with taurine during 18 days at 4 °C. Data are expressed as mean ± SE for eighteen treatments and control samples. No significant differences were detected between the treatments and the control group ($p > 0.05$).

glutathione peroxidase (Nonaka *et al.*, 2001) and catalase (Perumal *et al.*, 2013). With the aid of these enzymes, spermatozoa have the ability to eliminate the excessive amount of intracellular ROS (Storey, 1997; Alvarez and Storey, 1983). Taken this into account, TAU needs to be first transported into spermatozoa in order to promote the activity of these enzymes. However, the lipophobic character of taurine does not allow its free diffusion through the phospholipid bilayer (Huxtable *et al.*, 1973), thus creating a significant concentration gradient across the plasma membrane of spermatozoa (Lambert *et al.*, 2015). Somatic cells possess a specific transport system, which facilitates TAU accumulation via the Na^+–dependent taurine transporter TauT and proton-coupled Na^+–independent β–amino acid transporter PAT1 (Lambert *et al.*, 2015). However, taurine transport mechanisms in spermatozoa are poorly documented. Aided by proteomic analyses, a weak expression of TauT has been detected in the spermatozoa of Japanese eel (Higuchi *et al.*, 2012). Furthermore, emerged data suggest the presence of at least one taurine transporter across the plasma membrane of mitochondria, as these organelles were observed to take up taurine from a culture medium (Suzuki *et al.*, 2002). Once in the mitochondrial matrix, TAU helps to stabilise the electron transport chain (Schaffer *et al.*, 2009; Jong *et al.*, 2012), and a role for TAU as a buffer in the mitochondrial matrix has been suggested as well (Alvarez and Storey, 1995; Hansen *et al.*, 2010). In addition, Ozasa and Gould (1982) suggested on the boar spermatozoa model an osmolality – dependent regulation mechanism of TAU transport, the attenuation of which occurs in an isosmotic extracellular environment. In our experimental setup, the osmolality of the culture media ranged from 310.5 to 322 mOsm/kg for the control group and for the media with 12.5 mM TAU, respectively. For Androhep™ it is generally recommended to extend boar semen in a slightly hypertonic medium with an osmolality of 309 mOsm/kg (Gadea, 2003). These relatively isoosmotic conditions of the culture media might have restricted TAU transport into the spermatozoa and suppressed any antioxidant effect of TAU. Furthermore, it is possible that one or more compounds present in the culture media interfered with the antioxidant actions of TAU. To our knowledge, there are no data confirming the absence of TAU in Androhep Plus™. However, Androhep™, the predecessor of the Androhep Plus™ extender contains BSA, a potent anti–lipoperoxide agent, which effectively binds free fatty acid hydroperoxides (Mrsny *et al.*, 1979; Alvarez and Storey, 1995). The possible presence of BSA in the culture medium might have masked any antioxidant action of TAU.

Summarizing all above mentioned observations as well as considering the variability among animal species or differences in the chemical composition and osmolality of the Androhep Plus™ culture media, no final conclusions can be drawn upon existing differences in TAU effects reported in our and previous studies.

CONCLUSIONS

In conclusion, no significant positive or negative effect of taurine administration to the Androhep Plus™ extender was recorded on the spermatozoa motion behaviour, viability, acrosome integrity or morphology. Furthermore, any possible in vitro antioxidant properties of taurine in male reproductive cells may be debatable. Further research is necessary elucidate the possible protective effect of taurine on the quality of boar semen stored under low temperatures. Additional experiments should be also performed to clarify the molecular underpinnings of taurine transport and antioxidant actions in spermatozoa.

Acknowledgments

We gratefully acknowledge the skilful laboratory assistance of Birgit Sieg, Rolf Poppenga, Meike Stünkel, Lothar Schindler and Ronald Wittig.

This work was funded by projects VEGA 1/0327/16, VEGA 1/0022/15, APVV-14-0001, APVV-15-0544, APVV-16-0289, UGA VIII/21/2015 and AgroBioTech Research Centre built in accordance with the project Building „AgroBioTech" Research Centre ITMS 26220220180.

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